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INFLUENCE OF ENVIRONMENTAL CUES INTO THE QUORUM SENSING REGULATORY NETWORK IN THE OPPORTUNISTIC PATHOGEN *Pseudomonas aeruginosa*

By

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ABSTRACT

Opportunistic bacteria, as adaptable microorganisms, continually adjust to dynamic environments by fine-tuning gene expression at the transcriptional level. While transitioning from a saprophytic lifestyle to human infection, bacteria confront challenges like acclimating to elevated temperatures and securing attachment to host tissues for proliferation. A critical aspect of bacterial adaptation involves sensing fluctuations in cellular density, governed by quorum sensing (QS), an intercellular communication system. Within QS, bacteria produce signalling molecules whose concentrations increase with cellular density. Upon reaching a critical threshold, indicated by a specific concentration of these molecules, QS activates. This activation triggers transcriptional regulators, orchestrating coordinated responses essential for bacterial virulence. Given the rising threat of antibiotic resistance, there is growing interest in targeting QS to attenuate bacterial virulence. However, the success of this strategy hinges on a fundamental comprehension of QS function in bacteria. One of the most well-studied QS systems is from *Pseudomonas aeruginosa*, offering valuable insights into bacterial pathogenicity and potential therapeutic targets. *P. aeruginosa* QS comprises three intertwined systems: the *las*, *rhl*, and *pqs*. Each system has one transcriptional regulator (LasR, RhlR, and MvfR) and one main cognate autoinducer. Although QS function is interconnected, LasR-defective isolates persist within *P. aeruginosa* populations across clinical and non-clinical environments. The ecological relevance of these LasR-defective isolates was explored in the presented thesis, integrating their presence with often encountered environmental cues, namely surface sensing and temperature variations. The first research chapter delves into QS functions of surface-grown cells, mostly addressing the production of the cognate signal molecule from the *las* system, known as *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL). While dependent on the presence of LasR in its production in planktonic cells, this requirement is absent in surface-associated conditions. The second half of this document focuses on temperature variations and QS function. In LasR-defective isolates, RhlR is the regulator that sustains group responses in these isolates. RhlR function depends on the presence of QS-produced elements that stabilize it. Akin to these factors, a lower temperature also induces RhlR activity. Environmental-like temperature also impacts the QS function of LasR-defective isolates, exemplified by the characterization of the naturally evolved LasR A158P. Understanding these environmental-driven QS nuances is pivotal in deciphering the evolutionary trajectories of *P. aeruginosa* and guiding targeted therapeutic interventions.

RÉSUMÉ

Résumé de la thèse intitulée « Influence des signaux environnementaux sur le réseau de régulation de la communication intercellulaire *quorum sensing* chez l'agent pathogène opportuniste *Pseudomonas aeruginosa* »

Les bactéries opportunistes s'ajustent continuellement aux environnements dynamiques en affinant l'expression génique au niveau transcriptionnel. En passant d'un mode de vie saprophyte à une infection humaine, les bactéries sont confrontées à des défis tels que l'acclimatation à des températures élevées et la fixation aux tissus hôtes pour la prolifération. Un aspect critique de l'adaptation bactérienne implique la détection des fluctuations de la densité cellulaire, régulée par le système de communication intercellulaire appelé le *quorum sensing* (QS). Au sein du QS, les bactéries produisent des molécules de signalisation dont les concentrations augmentent avec la densité cellulaire. Lorsqu'un seuil critique est atteint, indiqué par une concentration spécifique de ces molécules, le QS s'active. Cette activation déclenche les régulateurs transcriptionnels, orchestrant des réponses coordonnées essentielles pour la virulence bactérienne. Face à la menace croissante de la résistance aux antibiotiques, il existe un intérêt croissant pour le ciblage du QS afin d'atténuer la virulence bactérienne. Cependant, le succès de cette stratégie repose sur une compréhension fondamentale de la fonction du QS chez les bactéries. L'un des systèmes de QS les plus étudiés provient de *Pseudomonas aeruginosa*, offrant des informations sur la pathogénicité bactérienne et les cibles thérapeutiques potentielles. Le QS de *P. aeruginosa* comprend trois systèmes entrelacés : *las*, *rhl* et *pqs*. Chaque système possède un régulateur transcriptionnel (LasR, RhlR et MvfR) et un auto-inducteur principal. Bien que la fonction du QS soit interconnectée, les isolats LasR-défectueux persistent au sein des populations de cette bactérie des environnements cliniques et non cliniques. La pertinence écologique de ces isolats LasR-défectueux a été explorée dans la thèse présentée, intégrant leur présence avec des signaux environnementaux souvent rencontrés, notamment la détection de surface et les variations de température. Le premier chapitre de recherche explore les fonctions du QS des cellules cultivées en surface, abordant principalement la production de la molécule de signal cognée du système *las*, le *N*-(3-oxododécanoyl)-L-homosérine lactone (3-oxo-C_{12}-HSL). Bien que dépendante de la présence de LasR dans sa production dans les cellules planctoniques, cette exigence est absente dans des conditions associées à la surface. La seconde moitié de ce document se concentre sur les variations de température et la fonction du QS. Chez les isolats LasR-défectueux, RhlR est le régulateur qui soutient les réponses de groupe. La fonction de RhlR dépend de la présence d'éléments produits par le QS qui le stabilisent. De manière similaire à ces facteurs, une température plus basse induit également l'activité de RhlR. Une température similaire à celle de l'environnement affecte également la fonction du QS des isolats LasR-défectueux, comme le montre la caractérisation du variant naturel LasR A158P. Comprendre ces nuances du QS entraînées par l'environnement est essentiel pour déchiffrer les trajectoires évolutives de *P. aeruginosa* et guider des interventions thérapeutiques ciblées.

SOMMAIRE RÉCAPITULATIF

Revue de littérature

Quorum sensing : Régulation génique au sein des communautés bactériennes

Les bactéries s'adaptent et prospèrent dans des environnements diversifiés. Ces microorganismes sont souvent présents au sein de diverses communautés, qu'ils soient attachés à des surfaces ou libres dans leur environnement. Pour faire face à ces modes de vie, les bactéries ont développé des stratégies uniques adaptées à leurs besoins.

Une stratégie remarquable est le *quorum sensing* (QS), un système de communication intercellulaire. Le QS permet aux bactéries individuelles de coordonner leurs activités en fonction de la densité de population. Il repose sur des molécules de signalisation, appelées auto-inducteurs, dont les concentrations augmentent avec le nombre croissant de bactéries. Une fois un seuil spécifique atteint, indiquant une population bactérienne suffisante, les voies du QS sont activées. Cette synchronisation permet aux bactéries de répondre collectivement aux signaux environnementaux, assurant leur survie.

Les bactéries à Gram positif et à Gram négatif utilisent le QS pour réguler diverses réponses biologiques, de la compétence génétique à la virulence. Notamment, ces deux groupes bactériens utilisent différents types de molécules de signalisation et de systèmes de régulation. Les bactéries à Gram positif utilisent des peptides auto-inducteurs (AIP) et des systèmes à deux composants, tandis que les bactéries à Gram négatif reposent sur des homosérine lactones acylées (AHL) et des régulateurs transcriptionnels de type LuxR.

Les AIP sont synthétisés de manière ribosomique et nécessitent des modifications post-traductionnelles avant d'être exportés. Ils interagissent avec des récepteurs histidine kinase liés à la membrane, déclenchant une réaction en chaîne conduisant à l'expression génique. D'autre part, les AHL diffusent librement dans les cellules bactériennes, interagissant avec les protéines LuxR pour réguler l'expression génique. Les structures spécifiques de ces molécules de signalisation déterminent leur affinité de liaison, assurant la spécificité du signal.

La plupart des AHL connues sont synthétisées par des protéines de type LuxI, et bien que divers précurseurs puissent être utilisés, ils dérivent généralement de la *S*-Adénosylméthionine et d'un acide gras conjugé à la protéine porteuse d'acyle. Certaines synthétases d'AHL, cependant, n'appartiennent pas à la famille LuxI, ce qui met en évidence la diversité des systèmes de QS.

Les protéines de type LuxR, les récepteurs des AHL, possèdent des domaines de liaison aux AHL et de liaison à l'ADN. Elles reconnaissent des séquences d'ADN conservées, appelées boîtes *lux*, permettant l'activation des gènes cibles.

Ces comportements sociaux observés chez les bactéries, y compris le QS, offrent des avantages significatifs tels que la résistance aux agents stressants et une colonisation efficace des surfaces. Cependant, ces comportements ont un coût métabolique, ce qui rend leur régulation cruciale. Dans le contexte de la pathogénicité bactérienne, le QS joue un rôle clé dans la régulation de l'expression des facteurs de virulence. La compréhension de ces systèmes de communication fournit des informations précieuses pour d'éventuelles interventions thérapeutiques.

Pseudomonas aeruginosa **et son système de communication intercellulaire**

Pseudomonas aeruginosa, une bactérie pathogène polyvalente, connue pour sa capacité à prospérer dans des environnements divers et à causer des infections opportunistes chez les humains, notamment ceux dont le système immunitaire est compromis. Elle représente une menace sérieuse, notamment dans les établissements de santé, en raison de sa résistance intrinsèque aux antimicrobiens et de sa capacité à former des biofilms. La souche PA14, connue pour sa virulence, est un modèle d'étude des déterminants génétiques de la pathogénicité de *P. aeruginosa*, incluant le QS.

P. aeruginosa utilise des systèmes de communication intracellulaires pour orchestrer l'expression de facteurs de virulence. Ces systèmes de QS, à savoir *las*, *rhl* et *pqs*, font usage de molécules de signalisation spécifiques telles que les AHL et les 4-hydroxy-2-alkylquinolines (HAQs). Collectivement, ces systèmes régissent une partie substantielle des gènes de *P. aeruginosa*.

Les systèmes *las* et *rhl* reposent sur une signalisation basée sur les AHL, chacun répondant à des autoinducteurs distincts : le 3-oxo-C_{12}-HSL pour le système *las* et le C_4-HSL pour le système *rhl*. Ces autoinducteurs sont synthétisés par des enzymes de type LuxI, désignées sous le nom de LasI pour le système *las* et de RhlI pour le système *rhl*. LasR contrôle la transcription dans le système *las*, tandis que RhlR assume le rôle régulateur dans le système *rhl*. En revanche, le système *pqs* utilise des autoinducteurs 4-hydroxy-2-alkylquinoline (HAQ), plus précisément le 4-hydroxy-2-heptylquinoline (HHQ) et le *Pseudomonas quinolone signal* (PQS ; 3,4-dihydroxy-2-heptylquinoline). MvfR est le régulateur du système *pqs*.

L'interconnexion des signaux de chaque circuit de communication cellulaire chez *P. aeruginosa* engendre un réseau complexe et sophistiqué. Les souches de référence, PAO1 et PA14, présentent une organisation hiérarchique dans leur réseau de régulation QS, avec le système *las*

en haut de la hiérarchie. La stabilité de cette organisation est soumise à l'influence des conditions environnementales et peut se révéler flexible dans certains contextes génétiques. LasR, en conjonction avec 3-oxo-C_{12}-HSL, orchestre l'activation des systèmes *rhl* et *pqs*. De plus, il est important de noter que le système *rhl* exerce une régulation négative sur le système *pqs*, bien que l'activation complète de RhlR dépende d'un élément essentiel provenant du système *pqs* : la protéine PqsE. La compréhension du fonctionnement de ce système de communication cellulaire est fondamentale pour appréhender la pathogénicité de *P. aeruginosa*. Cette connaissance pourrait également constituer la clé du développement de traitements novateurs ciblant spécifiquement les facteurs de virulence plutôt que les fonctions essentielles de cette bactérie.

Le réseau régulateur du QS chez *P. aeruginosa* est complexe et interconnecté, s'étendant au-delà des régulateurs centraux LasR, RhlR et MvfR. Divers composants cruciaux contribuent à l'orchestration des réponses bactériennes face aux changements de densité de population et aux indices environnementaux. Par exemple, en plus des régulateurs centraux LasR et RhlR, *P. aeruginosa* héberge une troisième protéine de type LuxR appelée QscR, classée comme une protéine de type LuxR orpheline. QscR ne possède pas de synthase apparentée et fonctionne en retardant l'expression de multiples exoproduits régulés par le QS grâce à la formation de dimères inactifs avec LasR et RhlR. Cet effet de retardement présenté par QscR est un exemple représentatif d'une catégorie plus large de protéines connues sous le nom d'anti-activateurs. Outre QscR, *P. aeruginosa* code également pour les anti-activateurs QteE et QslA, qui utilisent divers mécanismes pour moduler négativement l'activité des récepteurs LuxR LasR et RhlR, contribuant ainsi au réglage fin du réseau de QS.

Les indices environnementaux et les régulateurs périphériques tels que Vfr, VqsR, RsaL, CdpR, RpoS, RelA et DksA1 ajoutent de la profondeur à l'intrigue, permettant à *P. aeruginosa* de s'adapter à des conditions en constante évolution. Les petits ARN (sARN), régulateurs post-transcriptionnels, influencent également l'expression des gènes clés liés au QS, ajoutant ainsi une autre couche de complexité au système de communication de cette bactérie.

Contrairement à l’idée reçue, QscR possède la capacité de se lier et de répondre à la présence d'AHL à longue chaîne, comme la 3-oxo-C_{12}-HSL, la molécule de signal apparentée au système *las*. Cette capacité à répondre à de multiples AHL est appelée promiscuité des récepteurs, une caractéristique également observée dans une certaine mesure avec LasR mais pas avec RhlR. Il est important de noter que QscR et LasR n'interagissent pas avec des AHL à chaîne courte comme le C_4-HSL, soulignant ainsi que la promiscuité n'est pas une caractéristique pertinente

pour les monocultures de *P. aeruginosa*, mais revêt de l'importance dans les populations mixtes, couramment rencontrées dans les environnements naturels de cette bactérie.

Les isolats ayant un LasR défectueux sont prévalents au sein des populations de *P. aeruginosa*

Comme mentionné précédemment, *P. aeruginosa* est un agent pathogène opportuniste cliniquement significatif fréquemment isolé dans les infections chroniques chez les personnes atteintes de fibrose kystique (FK). Les populations isolées de ce contexte présentent souvent des adaptations, notamment une activité LasR altérée. Des observations récentes indiquent que l'émergence de souches présentant une activité LasR défectueuse s'étend au-delà des poumons des patients atteints de FK et est prévalente dans divers environnements, mettant en évidence leur importance adaptative. Ces souches sont souvent qualifiées de « tricheuses », mais leur coexistence avec des souches LasR-fonctionnel peut mutuellement bénéficier la population bactérienne.

Dans les souches prototypes, LasR occupe le sommet de la hiérarchie du QS, ce qui laisse supposer que la prévalence des isolats LasR-défectueux dans divers environnements implique la perte de phénotypes régulés par le QS. Cependant, des études sur certaines souches sans LasR ont remis en question cette hypothèse. Dans certains isolats sans LasR, RhlR reste actif ce que l'on appelle souches RAIL (RhlR actif indépendant de LasR). Ces isolats maintiennent le QS fonctionnel.

Adaptation aux conditions environnementales au-delà de la régulation du QS

La remarquable polyvalence et adaptabilité de *P. aeruginosa* trouvent leur origine dans son réseau complexe des systèmes de régulation. Avec un génome plus grand que celui des bactéries typiques, variant de 5,5 à 7 millions de paires de bases, *P. aeruginosa* possède une capacité régulatrice accrue grâce à son mode de vie libre. Cette capacité accrue lui permet de répondre aux environnements dynamiques, une caractéristique cruciale pour les agents pathogènes opportunistes tels que *P. aeruginosa* lorsqu'ils naviguent entre diverses niches environnementales.

Croissance sessile et adaptation à la surface

Dans son habitat naturel, *P. aeruginosa* adopte un mode de vie sessile, formant des biofilms qui sont des communautés polymicrobiennes structurées. Le processus de formation de biofilm implique des motifs distincts d'expression génique par rapport à son homologue planctonique. La détection de surface joue un rôle essentiel pour aider les bactéries à s'adapter aux surfaces

solides, initiant finalement le développement du biofilm. *P. aeruginosa* emploie des mécanismes complexes impliquant le flagelle et le pilus de type IV pour une détection rapide et prolongée de la surface. Ces mécanismes induisent des altérations dans les messagers intracellulaires, à savoir le c-di-GMP et le cAMP, entraînant des réponses physiologiques spécifiques. Ces messagers intracellulaires convergent pour moduler le comportement bactérien, favorisant la croissance sessile par rapport aux modes de vie planctoniques.

Contrôle thermique

P. aeruginosa présente une remarquable capacité à prospérer dans une large plage de températures, allant de 15 °C à 42 °C. Des températures élevées, telles que 37 °C, déclenchent l'expression de gènes de virulence, un processus distinct de la réponse typique au choc thermique. La bactérie emploie des thermomètres à ARN et d'autres mécanismes pour détecter et répondre efficacement aux fluctuations de température. Les thermomètres à ARN, présents au sein des transcriptions d'ARNm, facilitent ou inhibent la traduction des gènes en réponse aux variations de température.

Détection de surface et de température dans le réseau QS

L'attachement à la surface entraîne l'émergence de comportements sociaux chez *P. aeruginosa*, phénomène régulé en partie par le réseau de QS. Notamment, lorsque les cellules deviennent associées à la surface, les systèmes *las* et *rhl* manifestent des réponses distinctes, influençant leurs rôles au sein du circuit QS. Les efforts de recherche en cours visent à élucider les différences entre le circuit QS associé à la surface et le circuit QS planctonique, éclairant ainsi les mécanismes de régulation complexes en jeu.

Le système régulateur QS exerce une influence sur les facteurs de virulence, en particulier en réponse aux fluctuations de température. Le système *rhl*, en particulier, présente une réactivité accrue aux variations de température. Des boucles de rétroaction positives impliquant les thermomètres à ARN ont un impact significatif sur l'expression génique dans des conditions de température variables. L'étendue de la signification de la régulation thermique dans l'ensemble du réseau QS continue d'être un sujet d'étude en cours.

Énoncé du problème de recherche et hypothèse

Le ciblage des systèmes de communication bactérienne pour les thérapies anti-virulence représente une alternative prometteuse aux antimicrobiens traditionnels. Cependant, une compréhension complète de ces systèmes et de leur impact sur le comportement bactérien est cruciale pour une mise en œuvre efficace. Les circuits de QS de *P. aeruginosa* ont suscité une attention considérable en tant que modèle d'étude des systèmes de communication bactérienne et de la pathogenèse. Des études récentes ont affiné notre compréhension de ces circuits et de leurs interactions. La prévalence des isolats LasR-défectueux, autrefois considérés comme spécifiques aux poumons FK, suggère désormais une caractéristique d'adaptation plus large. RhlR joue un rôle central dans le maintien des comportements communaux via QS, même en l'absence d'un système *las* pleinement fonctionnel. Le modèle actuel de la pathogenèse de *P. aeruginosa* intègre le QS, la formation de biofilm et la température comme des facteurs clés de la régulation des traits de virulence. Cependant, l'interaction entre la détection de surface et de température avec le réseau QS reste mal comprise, ce qui nécessite des investigations supplémentaires.

Hypothèse

L'hypothèse postule que le réseau de régulation QS subit une modulation en réponse aux conditions environnementales, en particulier en ce qui concerne la détection de surface et les variations de température.

Objectifs généraux

Ce projet vise à répondre à l'hypothèse par le biais de deux objectifs principaux :

I) Examiner les disparités dans le réseau de régulation QS entre les cellules de *P. aeruginosa* en phase planctonique et celles en croissance en surface, en se concentrant sur l'impact de la croissance en surface sur la régulation QS.

II) Explorer l'impact de la variation de température sur le système de régulation QS, en particulier la modulation potentielle de l'activité du système *rhl*, compte tenu de sa dépendance unique à la formation de complexes protéiques.

Résultats

La section des résultats de cette thèse comprend trois manuscrits de recherche. Ci-dessous, une brève synthèse de chaque manuscrit est présentée, accompagnée d'un résumé étendu. Pour plus de détails, veuillez vous référer à la version en anglais de la thèse.

1. Titre de l'article : « La croissance en surface de *Pseudomonas aeruginosa* révèle un effet régulateur de la 3-oxo-C_{12}-homosérine lactone en l'absence de son récepteur cognitif, LasR »

Résume étendu :

La réussite de la colonisation de multiples niches écologiques par la bactérie *Pseudomonas aeruginosa* repose sur sa capacité à répondre aux concentrations de molécules de signal auto-produites, des auto-inducteurs. Ce système de communication intercellulaire, connu sous le nom de *quorum sensing* (QS), régule étroitement l'expression des déterminants de virulence et une diversité de fonctions de survie, y compris celles nécessaires aux comportements sociaux. Dans les cultures planctoniques de *P. aeruginosa*, le régulateur transcriptionnel LasR est généralement considéré comme étant au sommet de la hiérarchie du circuit QS ; son activation repose sur la liaison avec la 3-oxo-C_{12}-homosérine lactone (3-oxo-C_{12}-HSL), un produit de la synthase LasI. La transcription de *lasI* est activée par LasR, entraînant une boucle de rétroaction positive, une caractéristique classique de l'architecture des systèmes QS.

La perception des signaux chimiques, essentielle pour le QS, n'est pas la seule capacité sensorielle des bactéries. Elles répondent également aux signaux mécaniques, tels que ceux issus du contact physique entre les cellules et les surfaces ou entre les cellules elles-mêmes. Divers comportements, en particulier ceux associés à la vie en surface, sont régulés par le QS, notamment la motilité *swarming* et la formation de biofilm. Malgré le lien évident entre ces comportements, il reste un écart significatif dans la compréhension de la manière dont ces signaux s'influencent mutuellement. Étant donné que *P. aeruginosa* adopte un mode de croissance en biofilm en tant que communauté sur des surfaces dans son environnement naturel, la compréhension de la fonction de QS dans ce contexte est particulièrement pertinente.

Une autre caractéristique commune des populations de *P. aeruginosa* est la fréquence de l'apparition de la mutation de perte de fonction dans LasR. Ces mutants sont couramment isolés dans des sites environnementaux et cliniques. Par conséquent, dans les environnements naturels de *P. aeruginosa*, une fraction de la population est probablement composée d'isolats LasR-défectueux.

Dans cette étude, nous avons examiné l'effet de la croissance en surface chez les cellules LasR-défectueux. Alors que les cellules sauvages de PA14 produisent une quantité similaire de 3-oxo-C_{12}-HSL dans les conditions planctoniques et de surface, la souche Δ*lasR* présente une faible ou aucune production de cet AHL en culture planctonique. Cependant, en conditions de surface, les cellules *lasR* produisent inopinément du 3-oxo-C_{12}-HSL, bien que retardés par rapport à PA14, les niveaux correspondent à ceux des conditions où LasR est présent. Cette production nécessite la présence de l'activité LasI, comme le montrent les cellules cultivées en surface du mutant *lasI* et du double mutant *lasR lasI* qui ne présentent aucune trace de 3-oxo-C_{12}-HSL. De manière similaire, la transcription de *lasI* est maintenue dans les cellules cultivées en surface du mutant *lasR*, contrairement à celles de ce mutant en phase planctonique. Ainsi, la transcription de *lasI* est observée dans un mutant LasR-défectueux cultivé en surface. La fonction de RhlR est essentielle pour la production de 3-oxo-C_{12}-HSL dans cette condition. Cependant, RhlR n'agit pas sur la transcription de *lasI*; il agit plutôt sur sa traduction.

La synthèse inattendue de 3-oxo-C_{12}-HSL dans les cellules LasR-défectueux lors de la croissance en surface a d'abord été observée dans une souche génétiquement modifiée. Cependant, cette induction de la production de 3-oxo-C_{12}-HSL est également évidente dans les isolats naturellement LasR-défectueux trouvés dans des contextes environnementaux et cliniques. Ce phénomène répandu suggère que le 3-oxo-C_{12}-HSL pourrait jouer un rôle significatif dans la physiologie cellulaire, même en l'absence de LasR. En effet, le 3-oxo-C_{12}-HSL induit la production autologue de pyocyanine, un phénazine dépendant de RhlR, en l'absence de LasR. De plus, la production de 3-oxo-C_{12}-HSL par les cellules LasR-défectueux peut également être perçue par les cellules voisines LasR-fonctionnel, induisant l'expression de facteurs contrôlés par LasR dans des contextes de coculture. Notamment, dans un modèle d'infection chez la drosophile, la virulence de *P. aeruginosa* bénéficie de la contribution de 3-oxo-C_{12}-HSL produite par des souches LasR-défectueux, car la virulence de la population diminue lorsqu'une souche incapable de produire cette molécule remplace le mutant *lasR*.

Les résultats présentés ici révèlent un nouveau mécanisme sous-jacent à la différenciation entre les cellules planctoniques et les cellules associées à la surface. Il est particulièrement remarquable d'observer que les cellules LasR-défectueux retrouvent la capacité de produire du 3-oxo-C_{12}-HSL lors de leur association à la surface. Étant donné que la plupart des communautés bactériennes prospèrent dans des conditions de croissance en surface, cela suggère que les isolats de *P. aeruginosa* LasR-défectueux sont principalement trouvés en contact avec les surfaces dans leur environnement naturel. Ces mutants coexistent au sein de populations mixtes,

aux côtés de cellules LasR-fonctionnel, influençant ainsi le comportement de l'ensemble de la population par la production de molécules diffusibles. Cette étude offre une perspective complémentaire à la vision conventionnelle des isolats LasR-défectueux dans les populations de *P. aeruginosa*, qui se concentre généralement sur leur rôle dans la tricherie sociale. Au lieu de cela, nous apportons des preuves que ces mutants contribuent également à la production de molécules qui ont un impact sur l'ensemble de la population, potentiellement en conférant des avantages à la dynamique globale de la population.

2. Titre du manuscrit : « Contrôle de la virulence de *Pseudomonas aeruginosa* en fonction de la température par la stabilisation du régulateur de communication cellulaire RhlR »

Résumé étendu :

Pseudomonas aeruginosa, une bactérie Gram-négative polyvalente connue pour son adaptabilité et sa pathogénicité, prospère dans diverses niches écologiques, allant des sols contaminés et des réservoirs d'eau aux milieux cliniques, où elle représente une menace significative dans les infections nosocomiales, notamment chez les individus atteints de fibrose kystique. Son rayon d'infection s'étend au-delà des animaux à sang chaud pour inclure les reptiles, les insectes et les plantes. La régulation des déterminants de virulence chez *P. aeruginosa* opère principalement au niveau transcriptionnel, les fluctuations de température étant parmi les indices environnementaux cruciaux façonnant leur expression. À travers la détection de la densité cellulaire (*quorum sensing*, QS), médiée par des systèmes interconnectés tels comme *las*, *rhl* et *pqs*, *P. aeruginosa* orchestre des réponses coordonnées. Le régulateur QS RhlR, crucial pour contrôler de nombreux facteurs de virulence, présente une régulation complexe, dépendant non seulement de son autoinducteur cible C_4-HSL mais aussi de la chaperonne PqsE.

En effet, la détection de la température est essentielle à la régulation des facteurs de virulence, notamment lors de la colonisation humaine, où des températures distinctes pourraient signaler leur présence chez l'hôte. Des études antérieures mettent en évidence la thermorégulation de certains facteurs de virulence, y compris ceux sous contrôle de QS. En effet, un élément d'ARN sensible à la température, régulant la traduction de RhlR, entraîne des niveaux variables de protéines, affectant la régulation génique, y compris la synthèse de la pyocyanine. La production de pyocyanine implique des enzymes codées par les opérons *phzA1-G1* et *phzA2-G2*. Alors que certaines études indiquent une surexpression des opérons *phz* à 37°C par rapport aux

températures ambiantes, d'autres ne rapportent pas une telle régulation dépendante de la température.

Comme mentionné, l'activation complète de RhlR nécessite un élément stabilisateur supplémentaire, PqsE, aux côtés de son auto-inducteur C_4-HSL cognat, contrairement à d'autres régulateurs de type LuxR. Ce mécanisme de régulation a été élucidé grâce à des investigations sur la production de pyocyanine, en particulier la transcription de *phz1*, servant de signal pour l'activité de RhlR, et validé par des structures cristallines du complexe actif RhlR/C_4-HSL/PqsE.

L'activité et le rôle de RhlR revêtent une importance écologique. Ce système régule divers déterminants de survie dans des souches sauvages, mais il revêt une importance primordiale pour la fonction QS dans les isolats LasR-défectueux, une adaptation prévalente chez cette bactérie. L'activité QS soutenue garantit la production continue de molécules telles que la pyocyanine, qui contribue non seulement à la virulence, mais aide également à maintenir l'équilibre intracellulaire dans des conditions limitantes en oxygène.

Étant donné le rôle de QS dans le contrôle de l'expression des facteurs de virulence, nous avons émis l'hypothèse que la production de déterminants de virulence sous le contrôle de RhlR serait augmentée à 37°C, mimant les conditions d'infection humaine. Pour étudier l'interaction entre PqsE, C_4-HSL et l'activité de RhlR à 25°C et 37°C, nous avons évalué la transcription de *phz1*. Tout en confirmant la thermorégulation de RhlR, nous avons constaté de manière inattendue une activité accrue de RhlR à une température environnementale plutôt qu'à la température corporelle.

La régulation de la transcription de *phz1* n'est pas directement attribuable à l'expression de *rhlR* elle-même. Intéressant, de manière similaire à l'induction observée dans PA14, l'expression de *phz1* est significativement augmentée à 25°C chez les cellules dépourvues de C_4-HSL ou de PqsE. Cependant, en l'absence simultanée des deux facteurs, la transcription de *phz1* est complètement abolie même à une température semblable à l'environnement. Ces résultats suggèrent que la thermorégulation de la fonction de RhlR pourrait être étroitement liée à la composition du complexe actif impliquant RhlR, C_4-HSL et PqsE, avec la température agissant comme un troisième élément stabilisant pour RhlR. Selon cette hypothèse, une concentration équivalente de C_4-HSL induit une expression beaucoup plus forte de *phz1* dans les cellules incubées à 25°C par rapport à 37°C. Cette réponse est attribuée à l'induction de l'activité maximale de RhlR à 25°C, comparée à son niveau d'activation maximale à 37°C. De plus, dans les cellules dépourvues de PqsE, une température similaire à celle de l'environnement augmente l'affinité de RhlR pour son ligand C_4-HSL.

Le rôle de la température en tant que facteur stabilisant pour RhlR, affectant son activité, a été confirmé en manipulant les interactions connues pour moduler le complexe actif de RhlR. Cela incluait l'utilisation d'une variante fonctionnelle de RhlR (RhlR P61) qui reste stable même en l'absence à la fois de C_4-HSL et de PqsE, soulignant sa stabilité intrinsèque. Notamment, RhlR P61 ne présente aucune sensibilité à la température, ce qui souligne que la température influence RhlR de manière indirecte en renforçant sa stabilité, affectant ainsi son activité. La température influence notamment les traits clés de *P. aeruginosa*, en particulier sa virulence. Alors que les mutants *rhlI* et *pqsE* sont moins virulents que PA14 à 37°C, ils présentent une virulence comparable à la souche sauvage à 25°C, indiquant une activité accrue de RhlR dans ces conditions malgré l'absence de ces modulateurs.

3. Titre du manuscrit : « Exploration de la plasticité du *quorum sensing* chez *Pseudomonas aeruginosa* : Perspectives apportées par un variant naturellement évolué de LasR »

Résumé étendu :

Une caractéristique remarquable de *Pseudomonas aeruginosa* est sa capacité à s'adapter et à survivre dans diverses conditions. L'émergence d'une déficience de LasR, un composant essentiel de son système de détection de quorum (*quorum sensing*, QS), a été observée chez des souches provenant de différents sites d'isolement, indiquant sa prévalence généralisée en tant que caractéristique d'adaptation. Par exemple, des isolats défectueux en LasR émergent facilement dans l'évolution expérimentale en laboratoire. Il est à noter qu'une déficience du système *las* ne se traduit pas nécessairement par la perte des réponses régies par le système QS. Le système *rhl*, médié par le régulateur RhlR, peut encore réguler ces réponses.

La possibilité pour RhlR de prendre le relais en tant que régulateur principal en l'absence de LasR met en lumière l'adaptabilité au sein du système QS. La fonctionnalité de RhlR dépend de la présence à la fois de son autoinducteur cognat C_4-HSL et de PqsE. Par conséquent, il est généralement supposé que sans l'un de ces éléments, RhlR serait incapable de coordonner les fonctions QS, indépendamment de l'état d'activité du système LasR. On pourrait spéculer que dans des conditions inhibant l'activité de RhlR, l'activité de LasR persisterait pour maintenir la fonction QS dans les populations évoluées. Pour explorer ce concept, un mutant C_4-négatif (*rhlI*) a été utilisé. De manière surprenante, la fréquence d'isolats déficients en LasR dans ce contexte a dépassé même celle de PA14, suggérant qu'une fonction QS soutenue n'était pas favorisée. De plus, les populations évoluées de *rhlI* ont montré une expression plus forte des facteurs

dépendants de RhlR (comme la transcription de *phz1*) par rapport à la population parentale, indiquant une corrélation entre la fréquence des souches déficientes en LasR et l'activité de RhlR. Cette association a été confirmée par l'isolement d'une souche présentant des phénotypes associés à un dysfonctionnement du système *las* mais produisant des facteurs dépendants de RhlR, tels que la pyocyanine, malgré l'absence de production de C_4-HSL. Cette souche hébergeait un allèle *lasR* codant pour la variante LasR A158P, qui a ensuite été transférée à la souche sauvage PA14 et à ses mutants isogéniques. À 37°C, cette souche ne présentait aucune activité discernable. Cependant, cela a changé à 25°C, où la présence de la variante a induit la production de pyocyanine, et cette réponse liée à la température semble être en corrélation avec la fonction de la variante LasR A185P, donc active à 25°C mais pas à 37°C. De manière intrigante, la transcription des cibles contrôlées par LasR est restée insensible à la variante LasR A158P, même à l'état actif. Au lieu de cela, une cible contrôlée par RhlR, *phz1*, a montré une sensibilité à l'activité de LasR A158P. Ce phénomène a été lié à des niveaux altérés de C_4-HSL, sans affecter les autres cibles de RhlR testées. Les mécanismes par lesquels LasR A158P modifie la dynamique de QS restent à élucider, mais il fournit probablement un lien post-transcriptionnel entre les activités des systèmes *las* et *rhl*, remodelant potentiellement l'une des hypothèses fondamentales du système QS, à savoir la dépendance de leurs régulateurs à la présence de leurs autoinducteurs cognat.

Vue d'ensemble générale

Conclusion

Cette thèse visait à approfondir notre compréhension de l'impact de l'environnement sur le fonctionnement du réseau de régulation QS chez *P. aeruginosa*. En particulier, l'effet de la croissance en surface et l'influence de la température de croissance sur la modulation de l'activité de ce système de communication ont été étudiés. Ce travail a été entrepris en raison de l'importance de ces facteurs environnementaux dans les populations naturelles de *P. aeruginosa*, tant dans les environnements non cliniques que cliniques. Plus précisément, l'accent principal de cette thèse s'est centré sur l'élucidation des mécanismes moléculaires responsables de ces régulations sensibles à l'environnement.

Un autre facteur significatif influençant le point de vue adopté dans cette thèse est la prévalence des cellules LasR-défectueux parmi les isolats de *P. aeruginosa*. Lorsque nous avons observé que, contrairement aux cellules planctoniques, LasR était dispensable pour produire son autoinducteur cognat 3-oxo-C_{12}-HSL chez les cellules cultivées en surface, nous avons été intrigués par les implications de ce phénomène dans les populations naturelles composées de

cellules LasR-défectueux. L'impact démontré de la 3-oxo-C_{12}-HSL sur la population bactérienne peut fournir un aperçu partiel de la prévalence des isolats LasR-défectueux. La régulation différentielle entre les cellules planctoniques et celles cultivées en surface au sein du réseau de régulation QS souligne la nature limitée et spécifique aux conditions de notre compréhension globale du système QS. En effet, en utilisant un système qui représente plus étroitement l'environnement naturel de cette bactérie, nous avons mis en évidence la contribution d'un mécanisme régulé par RhlR qui contribue minoritairement à la production de 3-oxo-C_{12}-HSL dans les cellules planctoniques. De même, le système d'étude a le potentiel de révéler plusieurs autres voies spécifiques à la surface qui sont importantes pour les bactéries dans leur vie réelle.

Les autres questions abordées dans cette thèse, similaires à la réponse lors de l'association à la surface, étaient centrées sur les isolats LasR-défectueux chez *P. aeruginosa*. Sans LasR, RhlR devrait devenir le composant crucial maintenant la réactivité QS des cellules. L'investigation a porté sur les exigences uniques d'activation de RhlR, une protéine de type LuxR intrinsèquement instable, et sur la façon dont elle pourrait fonctionner en tant qu'élément régulateur. Il a été découvert que les températures de croissance ont un impact significatif sur la stabilité du complexe actif RhlR/C_4-HSL/PqsE. Contrairement aux idées reçues dans la littérature, les résultats indiquent que l'activité de RhlR est favorisée à des températures plus basses, semblables aux conditions environnementales, plutôt qu'aux températures plus élevées généralement associées aux conditions pendant l’infection chez l’humain.

L'étude de l'impact de la température de croissance sur le système *rhl* a été réalisée sur la souche sauvage PA14 et étendue aux isolats défectueux pour LasR. Nous avons cherché à établir un lien entre les réseaux de communication *las* et *rhl* en initiant un essai d'évolution expérimentale avec une souche incapable de produire du C_4-HSL. Cette démarche a conduit à l'émergence de la variante LasR A158P, associée à une forte induction de la production de pyocyanine dans des conditions où le C_4-HSL est absent. Cette variante s'est avérée particulièrement active à des températures ambiantes et dans des cellules cultivées en surface. Bien que les mécanismes exacts nécessitent encore des recherches, cette variante souligne l'importance d'étudier comment le réseau de régulation QS réagit aux signaux environnementaux. Curieusement, cet isolat résume la pertinence de comprendre l'influence des facteurs environnementaux sur les réponses bactériennes, car les deux entrées explorées dans cette thèse ont un impact significatif sur le comportement de cette variante LasR naturellement évoluée. De même, plusieurs autres variantes LasR sont membres de populations mixtes de *P. aeruginosa*, qui représentent tous les

environnements naturels de cette bactérie. La compréhension des réponses au niveau individuel pourrait aider à prédire les réponses au niveau de la population.

En conclusion, cette thèse souligne l'importance de poursuivre la recherche pour une compréhension approfondie du réseau QS dans des environnements naturels. Réévaluer le transcriptome et le phénome QS dans des contextes de croissance sur surfaces est crucial. De plus, il est essentiel d'étudier l'influence des températures différentes de celles du corps humain sur les réponses bactériennes. Les effets de ces facteurs environnementaux suggèrent l'importance d'autres éléments dans la régulation QS, soulignant ainsi la nécessité d'examiner chaque facteur individuellement pour une vue d'ensemble du réseau de régulation QS de *P. aeruginosa* dans diverses conditions.

Défis inhérents à cette étude et perspectives

Tout comme la section des résultats, les points abordés ici se concentreront sur l'un des trois manuscrits présentés dans ce document, numérotés de 1 à 3 selon leur ordre dans la section des résultats. Cette approche garantit une délimitation claire des idées présentées, car elles sont abordées séparément.

1. Les différences de comportement bactérien entre les cellules planctoniques et celles cultivées en surface soulignent la nécessité de mieux comprendre les mécanismes associés à la surface. Malgré les progrès réalisés, des questions fondamentales persistent concernant la manière dont les bactéries réagissent aux rencontres avec la surface et si ces réponses varient en fonction de la surface rencontrée. De plus, l'émission de signaux en réponse à ces rencontres reste peu claire. Le modèle de croissance associé à la surface utilisé dans cette étude présente des limites dans la mesure de la production de molécules de signalisation QS, principalement en raison de la nature hétérogène des populations cultivées en surface. Cette complexité inhérente rend difficile la discernabilité des contributions individuelles. Identifier le signal spécifique déclenchant la réponse observée est complexe, compte tenu de la présence de gradients d'oxygène, de nutriments et de déchets inhérents au système. Explorer les réponses au niveau des cellules individuelles ou localisées au sein de la population pourrait aider à identifier le signal et à déterminer si la réponse est déclenchée par le contact lui-même ou par une combinaison d'autres signaux présents dans le modèle.

La production de 3-oxo-C_{12}-HSL en l'absence de LasR et son rôle dans la modulation du comportement autologue sont intrigants. Comme LasR, QscR est un régulateur LuxR qui répond à 3-oxo-C_{12}-HSL. Intégrer sa fonction dans le modèle aidera à explorer les réponses contrôlées

par QS dans ce contexte, même si les résultats suggèrent que la réponse à 3-oxo-C_{12}-HSL est médiée par RhlR. Cette possibilité nécessite des investigations supplémentaires. Le séquençage d'ARN à haut débit offre un point de départ prometteur, en comparant des souches produisant ou ne produisant pas de 3-oxo-C_{12}-HSL en l'absence de LasR pour découvrir des gènes régulés de manière différentielle et éclairer les mécanismes régulateurs sous-jacents.

2. L'investigation explore la fonction de RhlR dans des conditions de température similaires à celles rencontrées par les bactéries environnementales vivant librement et celles infectant les humains. Elle met en évidence l'importance à la fois du chaperon PqsE et de l'autoinducteur C_4-HSL dans la stabilisation et l'activation de RhlR. De manière intéressante, des températures plus basses, similaires à celles des environnements naturels, renforcent encore l'activité de RhlR, suggérant que la température constitue un facteur de stabilisation supplémentaire.

Cependant, l'étude rencontre des défis pour valider ses conclusions en raison du manque de données biochimiques et des complexités de l'analyse structurale résultant des difficultés de purification de RhlR. Des questions méthodologiques se posent quant à savoir si la température stabilise uniquement RhlR ou indique des facteurs physiques préétablis, ce qui impacte les protocoles de purification des protéines.

Le modèle développé se concentre sur l'expression de *phz1* et la production de pyocyanine en tant qu'indicateurs de l'activité de RhlR, mais n'englobe pas tous les gènes régulés par RhlR. De plus, la dépendance de certains gènes à l'égard de PqsE et les mécanismes sous-jacents restent flous. Des investigations futures devraient explorer l'expression des gènes et l'interaction entre les différentes conformations structurelles de RhlR pour mieux comprendre les réseaux de régulation. À mesure que davantage de données s'accumulent, le modèle devrait évoluer pour capturer de manière exhaustive les subtilités de ces processus.

3. Le variant naturellement évolué LasR A158P confère une flexibilité au sein du système QS, permettant la production du facteur cible contrôlé par RhlR, la pyocyanine, en l'absence de son ligand C_4-HSL. Cependant, cette réponse n'est pas constitutive, mais induite par des températures plus basses. En conséquence, la production de 3-oxo-C_{12}-HSL, un facteur dépendant de LasR, n'est observée que lorsque cet isolat est cultivé à 25°C, mais pas à 37°C. Jusqu'à présent, cela indique qu'à une température environnementale, LasR A158P est actif, mais pas à des températures plus élevées. Cependant, d'autres cibles de LasR répondent de manière similaire au mutant *lasR* et à la variante LasR A158P, indépendamment de la température - et donc, du statut d'activation de cette protéine. Ce sont des résultats contradictoires, qui pourraient être abordés en réalisant une méthode de profilage phénotypique

basée sur la quantification de molécules extracellulaires dépendantes du QS, connue pour permettre de différencier les isolats LasR-fonctionnels et -défectueux. Si cette stratégie est réalisée à différentes températures, elle devrait permettre de vérifier si la température modifie effectivement la fonction de LasR A158P.

Les caractérisations présentées étaient centrées sur une souche sauvage portant ce variant. Bien que certaines différences soient observées par rapport à un mutant *lasR*, la plupart des résultats intéressants sont observés en l'absence concomitante de C_4-HSL, et il semble que ce soit également le cas pour PqsE. Par conséquent, les prochains efforts visant à comprendre la fonction de cette variante devraient utiliser ces souches. Cependant, il convient de noter que les contributions des systèmes *las* et *rhl* seraient présentes dans cette condition, ce qui présente un grand défi dans l'interprétation des résultats. Malgré les défis, les résultats sont prometteurs, car ils pourraient suggérer une interaction post-transcriptionnelle entre ces systèmes et peuvent être explorés pour évaluer ultérieurement la présence d'une telle régulation dans des contextes de type sauvage.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

2-AA	2-aminoacetophenone
2-ABA	2-aminobenzoylacetate
2-ABA-CoA	2-aminobenzoylacetyl-CoA
3-oxo-C_{12}-HSL	*N*-(3-oxododecanoyl)-L-homoserine lactone
5'UTR	5' untranslated region
ABC exporter	ATP-binding cassette exporter
ACP	Acyl carrier protein
AHL	*N*-acyl homoserine lactone
AIP	Autoinducing peptide
ATP	Adenosine triphosphate
bp	Base pair
c-di-GMP	Bis-(3′, 5′)-cyclic dimeric guanosine monophosphate
C_{10}-HSL	*N*-decanoyl-L-homoserine lactone
C_4-HSL	*N*-butanoyl-L-homoserine lactone
C_8-HSL	*N*-octanoyl-L-homoserine lactone
cAMP	Cyclic adenosine monophosphate
CF	Cystic fibrosis
CFU	Colony forming units
CoA	Coenzyme-A
CRP	cAMP receptor protein

DAP	Diaminopimelic acid
DHQ	2,4-dihydroxyquinoline
DNA	Deoxyribonucleic acid
e.g.	*Exempli gratia*, meaning "for example"
EPIC	Early *Pseudomonas* infection control
EPS	Exopolysaccharides
HAQ	4-hydroxy-2-alkylquinoline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHQ	4-hydroxy-2-heptylquinoline
HHQ-d4	Tetradeuterated 4-hydroxy-2-heptylquinoline
HPLC	High-performance liquid chromatography
HQNO	4-hydroxy-2-heptylquinolone-*N*-oxide
HTH	Helix-turn-helix
i.e.	*Id est*, meaning "that is"
LB	Lysogeny broth
LC/MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LPS	Lipopolysaccharide
mBTL	4-(3-bromophenoxy)-*N*-(2-oxotetrahydrothiophen-3-yl)butanamide (*meta*-bromo-thiolactone)
mRNA	Messenger RNA
MTA	5’-methylthioadenosine
OD	Optical density

PA14	UCBPP-PA14; wild-type *P. aeruginosa* strain isolated from human burn wound
PBS	Phosphate-buffered saline
PCA	Phenazine-1-carboxylic acid
PCR	Polymerase chain reaction
pH	Potential of hydrogen
ppm	Parts per million
PQS	*Pseudomonas* quinolone signal (3,4-dihydroxy-2-heptylquinoline)
QQ	Quorum quenching
QS	Quorum sensing
QSI	Quorum sensing inhibitor
RAIL	RhlR active independently of LasR
RFU	Relative fluorescence units
RLU	Relative light units
RNA	Ribonucleic acid
RNAP	RNA polymerase
ROSE elements	Repression of heat shock gene expression elements
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SAM	*S*-adenosylmethionine
SD sequence	Shine-Dalgarno sequence (also known as ribosomal binding site, RBS)
sRNA	small RNA
T1SS	Type I secretion system
T2SS	Type II secretion system

T3SS	Type III secretion system
T4P	Type IV pili
T5SS	Type V secretion system
T6SS	Type VI secretion system
TSB	Tryptic soy broth
TSS	Transcription start site
WHO	World Health Organization
WT	Wild-type
µM	Micromolar
α	Alpha
β	Beta
σ	Sigma (also a RNAP subunit)
χ	Chi

1 INTRODUCTION

In order to successfully colonize and thrive in diverse environments, bacteria must rapidly detect and adequately respond to a wide range of environmental cues. Individual, free-living planktonic bacteria do not consistently encounter these physicochemical cues in natural settings. Instead, bacteria often reside within complex polymicrobial communities typically associated with biotic or abiotic surfaces. Due to this dual mode of growth (free-living and attached), bacteria have developed strategies tailored to their needs, allowing them to adapt to their surroundings.

1.1 Quorum sensing: Gene regulation in bacterial communities

Regardless of the mode of bacterial growth, individual organisms seldom survive in isolation. Bacteria have, therefore, evolved a sophisticated intercellular communication strategy known as quorum sensing (QS), which relies on small and diffusible signalling molecules called autoinducers (Fuqua *et al.*, 1994b). During bacterial growth, the concentration of signalling molecules increases in the environment surrounding the producing bacteria. The QS regulatory pathways become active once a threshold of these molecules, indicative of an optimal bacterial cell density, or quorum, is reached. This activation leads to the synchronization expression of specific QS-regulated genes throughout the quorate bacterial community. In essence, QS ensures bacterial multicellularity, enabling bacteria to respond in a coordinated fashion to self and environmental cues (Whiteley *et al.*, 2017).

Relevant biological responses are regulated via QS in both Gram-positive and Gram-negative bacteria, including genetic competence (*Streptococcus pneumoniae*) and sporulation (*Bacillus subtilis*) in the former and bioluminescence (*Vibrio fischeri*), plasmid conjugal transfers (*Agrobacterium tumefaciens*), biosynthesis of antibiotics (*Erwinia carotovora*), as well as virulence (*Pseudomonas aeruginosa*) in the latter bacterial group (Fuqua *et al.*, 1994a; Håvarstein *et al.*, 1995; McGowan *et al.*, 1995; Miyashiro *et al.*, 2012; Passador *et al.*, 1993; Perego *et al.*, 1996). Notably, the chemical nature of the molecules mediating the cell-to-cell communication and the response modules diverge between the two bacterial groups. Gram-positive bacteria typically use processed oligopeptides to communicate and sense those signals using two-component systems. In contrast, Gram-negative bacteria often employ acylated homoserine lactones and cognate transcriptional regulators to respond to the signal (Miller *et al.*, 2001). Despite these differences in regulation, the fundamental principles of gene regulation by QS remain constant: they involve a module capable of producing a signalling molecule, the autoinducing signalling molecule itself,

and a cognate transcriptional regulator acting as a receptor of the signal. The latter links bacterial density, determined by the concentration of the signalling molecule, to the expression of target genes under QS control.

1.1.1 Peptides as QS molecules in Gram-positive bacteria

As mentioned above, Gram-positive bacteria utilize processed oligopeptides called autoinducing peptides (AIP) as signalling molecules to regulate QS (**Fig. 1.1.1**). The synthesis of AIPs is ribosomal, thus initiated in the cytoplasm as unprocessed precursor peptides. The processing of AIPs involves cleaving and, in many cases, post-translational modifications, resulting in an active and stable processed form (Kleerebezem *et al.*, 1997; Sturme *et al.*, 2002; Taga *et al.*, 2003). Exporting AIPs to the extracellular environment requires a dedicated ATP-binding cassette (ABC) exporter. The detection of AIPs, which accumulate in the extracellular milieu as bacterial density increases, is mediated by the interaction of AIPs with the external domains of membrane-bound two-component histidine kinase sensors. This interaction triggers the autophosphorylation of the sensor protein on a conserved histidine residue. Subsequently, the phosphoryl group is transferred to the cognate response regulator, the second element of two-component systems, on a conserved aspartate residue (**Fig. 1.1.1**) (Kleerebezem *et al.*, 1997; Sturme *et al.*, 2002; Taga *et al.*, 2003). The activated response regulator induces the expression of genes coding for AIPs, the histidine kinase sensor, and the response regulator itself. This regulation creates a positive feedback loop, typical of QS systems.

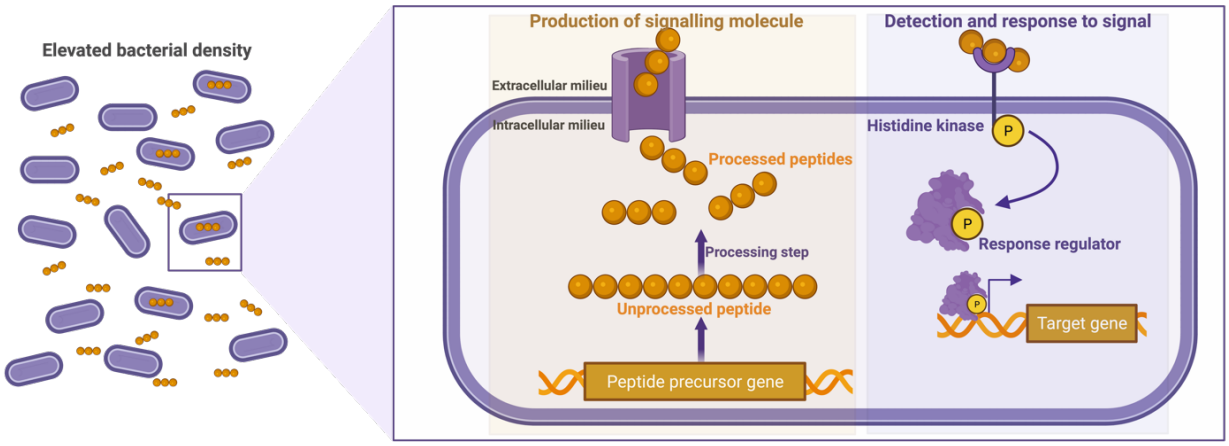

Figure 1.1.1 Canonical QS circuitry in Gram-positive bacteria.

Gram-positive bacteria use a communication system based on producing and detecting signalling peptides. The locus corresponding to the peptide precursor is translated into the precursor peptide (unprocessed peptide) and further converted to the processed and active form by cleaving (processed peptide). Active translocation to the extracellular milieu of the processed peptide during bacterial growth increases its concentration. The transmembrane sensor histidine kinase mediates the detection of the signalling molecule, inducing its autophosphorylation. The phosphoryl group is transferred to the response regulator, thereby activating transcriptional regulation of target genes. Inspired by (Sturme *et al.*, 2002; Taga *et al.*, 2003) and created using Biorender.

1.1.2 Gram-negative QS: Role of acylated homoserine lactones

In Gram-negative bacteria, the canonical QS circuitry closely resembles that initially described in the luminescent marine bacterium *V. fischeri* (Engebrecht et al., 1983). It comprises homologues of two proteins: LuxI and LuxR (**Fig. 1.1.2**) (Fuqua *et al.*, 1994b). The LuxI-type proteins are signal synthases, producing specific acylated homoserine lactones (AHLs), the autoinducer molecule used by this bacterial group. These AHL signals are released into the extracellular environment and, like AIPs in Gram-positive bacteria, accumulate in the extracellular environment as cell-population density increases. Detection and response rely upon LuxR-type transcriptional regulators, which bind specifically to their cognate AHL. The LuxR/autoinducer complex is transcriptionally active, regulating the expression of target genes by binding to specific DNA motifs in the regulatory sequence of QS-controlled genes, called *lux* boxes (**Fig. 1.1.2**) (Fuqua *et al.*, 2002). This process often forms a positive feedback loop, where LuxI protein genes are induced by the LuxR/autoinducer complex.

Concerning AHL transport, it is worth noting that these molecules are considered to diffuse freely into and out of the cell once produced, resulting in equivalent concentrations in both the intracellular and extracellular environments (Fuqua *et al.*, 2002). The conventional understanding

of AHL transport has faced challenges with reports describing the active efflux of the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) via the MexAB-OprM pump (Evans *et al.*, 1998; Pearson *et al.*, 1999). However, a recent study has contradicted these initial findings, suggesting that the cellular membrane is fully permeable to 3-oxo-C_{12}-HSL, similar to other AHLs (Alcalde-Rico *et al.*, 2020).

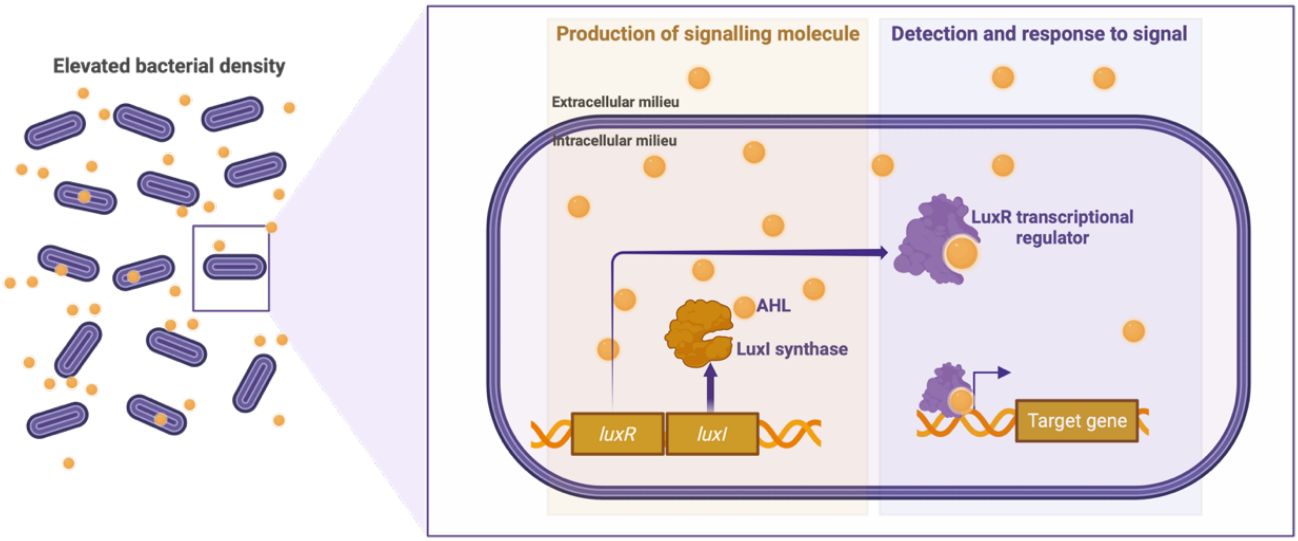

Figure 1.1.2 Typical Gram-negative QS circuitry.

The communication system comprises an AHL autoinducer synthase (LuxI-type protein) and a cognate transcriptional regulator (LuxR-type protein). The extra- and intracellular concentration of AHL depends on bacterial density. When a critical microbial quorum is present, the concentration of AHL molecules is sufficient to bind to and activate the transcriptional regulator. The active LuxR-AHL complex generally induces the expression of target genes, which typically includes the gene coding the LuxI synthase. Inspired by (Whitehead *et al.*, 2001) and created using Biorender.

1.1.2.1 AHL structures: The linguistic code of bacterial interactions

For effective communication, bacteria must differentiate their self-produced AHL signals from those generated by neighbouring bacterial species. Therefore, the proper functioning of QS systems inherently relies on specificity. Within Gram-negative bacteria, the structural components of AHLs are the base of this signal specificity. This class of molecules shares a common homoserine lactone moiety, differing solely in their acyl side chain. Most AHLs feature an aliphatic linear acyl side chain, ranging from 4 to 18 carbons, with diverse degrees of oxidation at position 3-C (i.e., fully oxidized carbonyl, bearing a hydroxyl group or fully reduced) (Fuqua *et al.*, 2002; Taga *et al.*, 2003; Whiteley *et al.*, 2017) (**Fig. 1.1.3**). Additionally, the presence of unsaturated or branched acyl chains, as well as aryl side chains have been reported (**Fig. 1.1.3**) (Ahlgren *et al.*, 2011; Gray *et al.*, 1996; Liao *et al.*, 2018; Lindemann *et al.*, 2011; Puskas *et al.*, 1997; Schaefer

et al., 2008). These structural differences arise due to the substrate specificity of LuxI-type proteins and are crucial for their specific binding to cognate LuxR-type transcriptional regulators. Collectively, these factors govern the specificity of QS signalling.

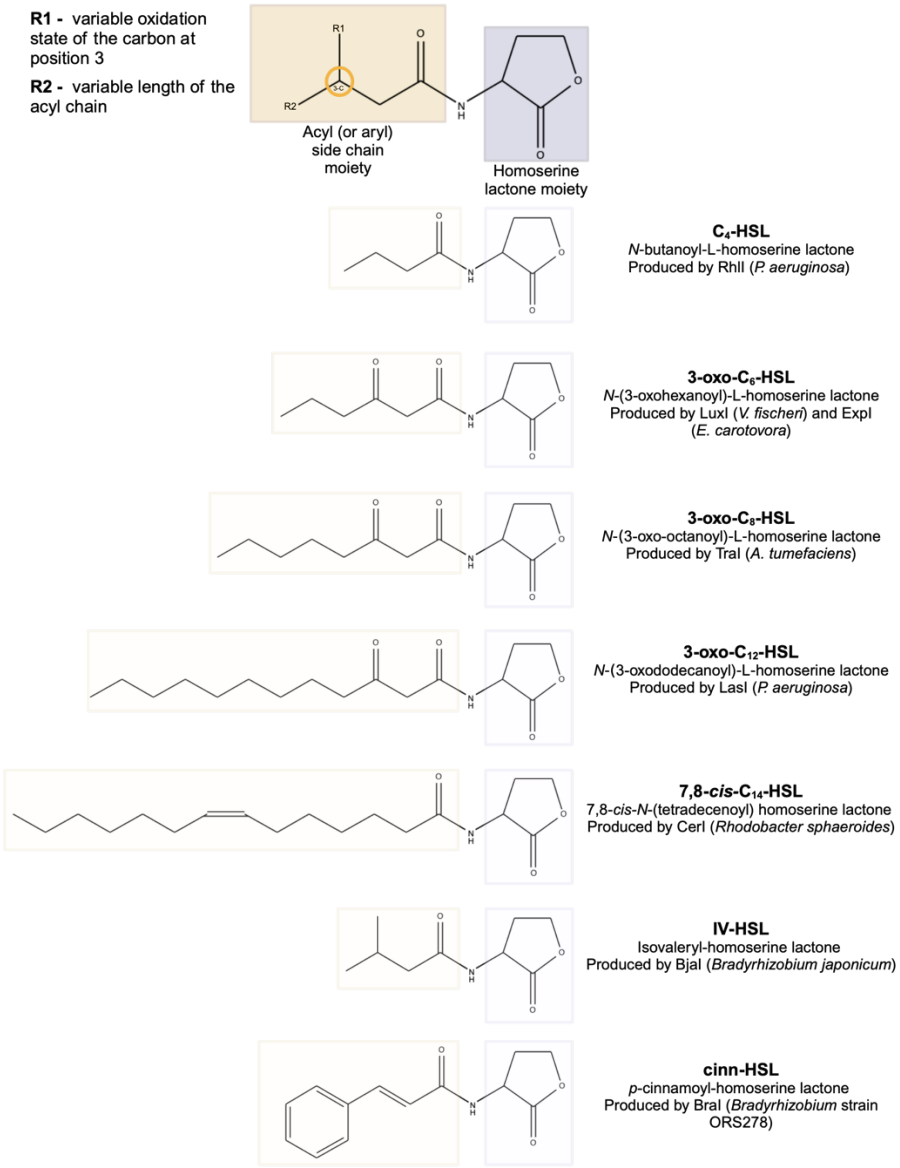

Figure 1.1.3. Structural diversity of AHL autoinducers.

The top portion of the figure illustrates the general chemical structure of AHL autoinducers. An amide bond connects the conserved homoserine lactone (highlighted in purple box) to the acyl/aryl side chain (highlighted in yellow box). The length of this acyl chain can vary, ranging from 4 to 18 carbons, and in some cases, it may be unsaturated or branched. The third carbon in the acyl chain exists in one of three states: as a fully oxidized carbonyl, bearing a hydroxyl group, or being fully reduced. This structural diversity is exemplified by various AHLs produced by different Gram-negative bacteria. Adapted from (Ahlgren *et al.*, 2011; Lindemann *et al.*, 2011; Puskas *et al.*, 1997; Taga *et al.*, 2003) and created using Biorender.

1.1.2.2 LuxI-type proteins dictate the bacterial language

The biosynthesis of AHLs is typically catalyzed by enzymes of the LuxI family. The conserved homoserine lactone component originates from *S*-adenosylmethionine (SAM), serving as the common precursor for these molecules (Moré *et al.*, 1996; Schaefer *et al.*, 1996). The second precursor required for this reaction is a fatty acid conjugated to the acyl carrier protein (acyl-ACP) or the coenzyme A (acyl-CoA) (Dong *et al.*, 2017; Lindemann *et al.*, 2011; Moré *et al.*, 1996; Schaefer *et al.*, 1996). Most studied LuxI-type enzymes utilize acyl-ACP and SAM, rather than acyl-CoA and SAM, to produce AHLs – the AHL biosynthetic pathway using acyl-ACP is depicted in **Fig. 1.1.4** (Moré *et al.*, 1996; Parsek *et al.*, 1999; Schaefer *et al.*, 1996). While ACP-dependent LuxI enzymes generally employ intermediates from fatty acid biosynthesis as the acyl chain, CoA-dependent ones are presumed to derive from the activity of cellular acyl-CoA ligases involved in organic acid catabolism (Liao *et al.*, 2018).

Numerous LuxI-type proteins have been identified, functionally distinguished by their utilization of either ACP or CoA. Significantly, the latter enzymes share close kinship, with approximately 50% of sequence identity, as they emerge from a single exaptation evolutionary event from the ACP-dependent LuxI proteins (Christensen *et al.*, 2014; Dong *et al.*, 2017). Additionally, CoA- and ACP-dependent LuxI enzymes exhibit a more distant relationship, with approximately 30% of sequence identity, a proportion also observed among ACP-dependent LuxI proteins (Christensen *et al.*, 2014; Dong *et al.*, 2017; Fuqua *et al.*, 2002).

The structural variations resulting from this diversity of LuxI-type proteins ensure the precision of AHLs production, as LuxI proteins specifically recognize carriers with a particular acyl chain moiety. While there is no direct correlation between the produced AHL and the sequence identity of LuxI homologues, the structure of their binding cavities has been linked to the length and derivatization of the acyl chain that they recognize (Fuqua *et al.*, 2002; Taga *et al.*, 2003).

In contrast, some AHL synthases do not belong to the LuxI family. For instance, the protein AinS from *V. fischeri* synthesizes *N*-octanoyl-L-homoserine lactone (C_8-HSL) using SAM and octanoyl-ACP as precursors, similar to LuxI-type proteins. However, in this case, an acyl conjugate with coenzyme-A can also serve as a synthesis precursor, replacing the acyl-ACP (Hanzelka *et al.*, 1999). This flexibility in precursor utilization is not observed in members of the LuxI family. Other examples of non-canonical AHL synthases include LuxM and VanM from *Vibrio harveyi* and *Vibrio anguillarum*, respectively, and HdtS from *Pseudomonas fluorescens* (Bassler *et al.*, 1994; Laue *et al.*, 2000; Milton *et al.*, 2001).

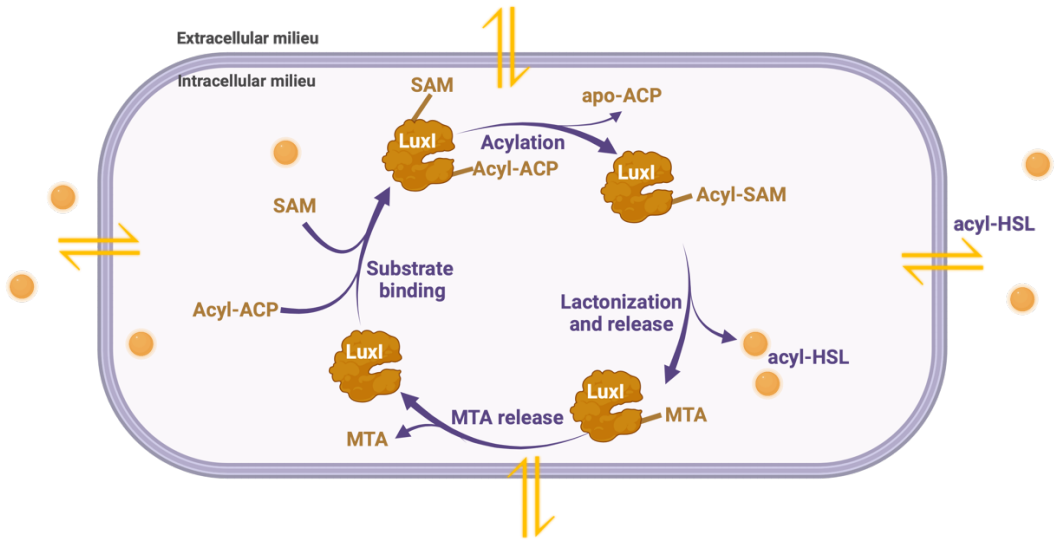

Figure 1.1.4. General features of AHLs biosynthesis by LuxI-type proteins.

A generic LuxI protein represents members of the LuxI family. SAM stands for *S*-adenosylmethionine, and ACP denotes the acyl carrier protein. The acylation of SAM occurs upon the binding of SAM and acyl-ACP to the LuxI synthase, leading to the release of apo-ACP. The lactonization of SAM promotes the formation of AHLs, depicted here as acyl-HSL. 5'-methylthioadenosine (MTA) is the by-product of this reaction. Double arrows indicate the permeable nature of bacterial membranes to AHLs. Adapted from (Fuqua *et al.*, 2002; Galloway *et al.*, 2011; Hanzelka *et al.*, 1999) and created using Biorender.

1.1.2.3 Deciphering the AHL language of bacteria: Role of LuxR transcriptional regulators

The LuxR protein family typically consists of AHL-responsive transcriptional activators. These transcriptional regulators selectively bind AHL and trigger a response to this signal. The biochemical structure of LuxR proteins aligns with these functions, featuring two domains: an AHL-binding domain in the amino-terminal portion and a helix-turn-helix domain (HTH) in the carboxy-terminal portion required for DNA binding (Choi *et al.*, 1991; Choi *et al.*, 1992; Hanzelka *et al.*, 1995). Motifs facilitating protein dimerization are also situated in the amino-terminal portion, with AHL binding typically inducing conformational changes and dimerization of the amino-terminal domain (Choi *et al.*, 1992).

The activated AHL-induced dimer recognizes conserved DNA sequences upstream of its target genes. These sequences often consist of perfect or imperfect inverted repeats – palindromic sequences – located proximal to the promoter of genes under QS regulation (Devine *et al.*, 1989; Egland *et al.*, 1999). The primary sequence of this DNA motif resembles the LuxR-recognized

sequence found upstream of target genes in *V. fischeri* and is thus referred to as a "*lux*-type box." These gene regulation boxes, approximately 20 base pairs in length, are situated about 40 base pairs upstream of the transcription start site (TSS) (**Fig. 1.1.5**) (Devine *et al.*, 1989; Egland *et al.*, 1999). Despite shared conserved features facilitating their recognition, each *lux* box sequence is unique. Consequently, in bacteria harbouring multiple LuxR-type proteins, genes under QS control may be preferentially regulated by a specific LuxR protein, potentially to the detriment of others, even if they both recognize a *lux* box.

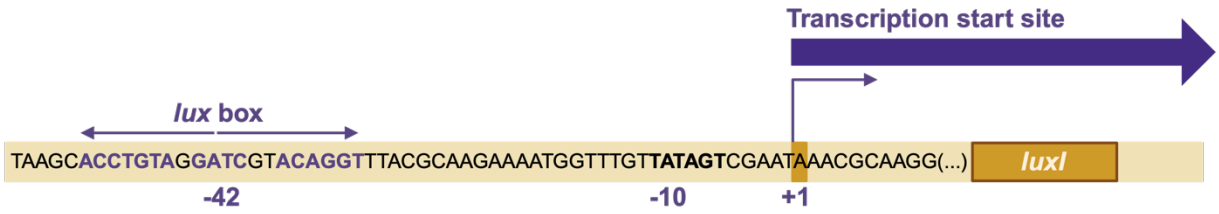

Figure 1.1.5. The *lux* box is located within the regulatory sequences of genes governed by QS.
The upstream DNA sequence of *luxI* in *V. fischeri* is used to depict the regulation of genes under QS transcriptional control. The *lux* box sequence is highlighted in purple on the left side of the image, with inverted arrows denoting the symmetry center of this sequence. Within the *lux* box, inverted repeats are emphasized in purple and bold. The sigma-binding consensus region, approximately 10 base pairs upstream of the transcription start site (TSS), is highlighted. The TSS is indicated by arrows at the right end of the image. The genomic organization of these regulatory sequences is conserved among Gram-negative bacteria. Inspired by (Egland *et al.*, 1999) and created using Biorender.

The binding of the dimeric LuxR-protein to the *lux* box sequence typically elicits transcriptional activation and positions this regulator directly upstream of another important regulatory sequence, known as the -35 element of the promoter region (see **Fig. 1.1.5**, also **Fig.1.1.6**). Such positioning facilitates the interaction of these regulators with the RNA polymerase (RNAP). LuxR-proteins are classified as ambidextrous activators because they interact with more than one surface of RNAP, specifically the carboxy-terminal domain and the σ subunit (Egland *et al.*, 1999; Finney *et al.*, 2002; Stevens *et al.*, 1999). These interactions are important and sufficient to induce transcriptional activation of target genes, as demonstrated by *in vitro* studies using purified RNAP and TraR, a LuxR-type protein from *A. tumefaciens* (Zhu *et al.*, 1999).

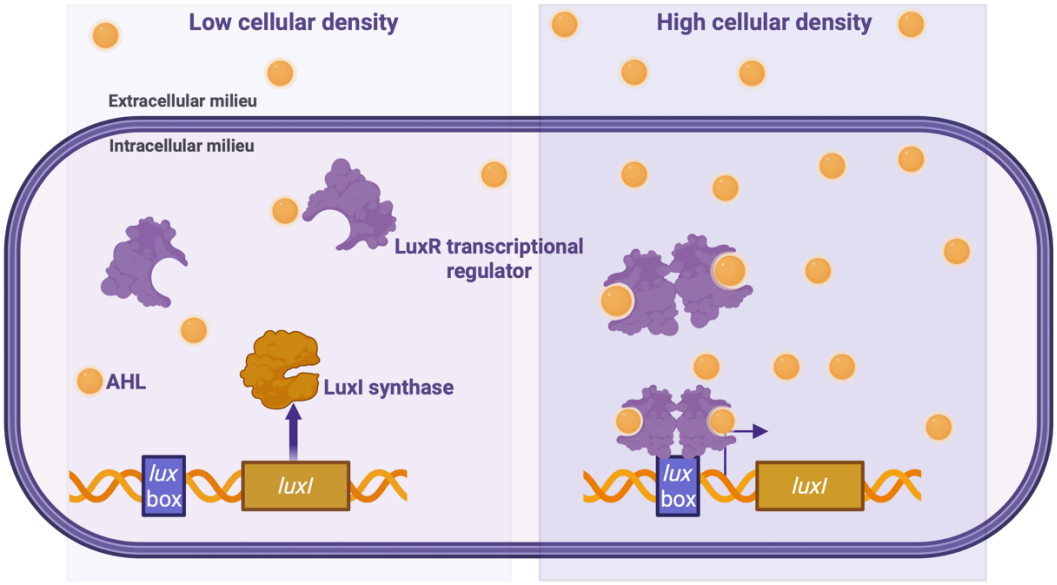

Figure 1.1.6. Dimers of Lux-type proteins bind to *lux* boxes in the regulatory regions of genes under their regulation.

Schematic representation of gene expression induction by LuxR-type proteins. In the left panel, when cellular densities are low, the concentration of AHLs produced by LuxI synthases is insufficient to trigger LuxR-type protein dimerization. Consequently, LuxR proteins remain in a monomeric state, lacking the ability to bind to DNA. In the right panel, increased AHL concentration facilitates the binding of AHL to LuxR proteins. Conformational changes within the LuxR monomer are induced by this binding, leading to its dimerization. The resulting complex represents the active form of the protein, capable of binding to *lux* boxes in the proximity of the promoters of genes regulated by LuxR, exemplified here by *luxI*. The binding recruits the RNA polymerase, thereby inducing transcription of the *luxI* gene. **Figure 1.1.5** complements this illustration, providing a more detailed view of the regulatory sequence upstream of *luxI*. Inspired by (Whiteley *et al.*, 2017) and created using Biorender.

The description provided so far outlines the mechanism of action of most LuxR-type proteins. However, this description does not encompass all the LuxR-proteins documented to date. Notably, a small subset of LuxR-proteins is active without their cognate AHLs, a state known as their apoprotein form (Tsai *et al.*, 2010). These atypical proteins are fully folded, binding to DNA independently of AHL presence. In most instances, the binding of the apo-LuxR protein to DNA represses the expression of the target genes, and in the presence of the cognate AHL, this repression is relieved (Tsai *et al.*, 2010). The binding of the cognate AHL, for these atypical LuxR-proteins, changes the protein conformation and induces its monomerization – thus disassociating the repressor from the DNA. Notably, the recognition boxes of these proteins to the DNA generally overlap the -10 promoter element (see **Fig. 1.1.5**), sterically blocking transcriptional activation by the RNAP. The first characterized LuxR-type protein functioning as a transcriptional repressor

was EsaR from *Pantoea stewartii* (von Bodman *et al.*, 1998). Other examples of such regulation are VirR (*Pectobacterium atrosepticum*) and SpnR (*Serratia marcescens*) (Horng *et al.*, 2002; Monson *et al.*, 2013). Comparing the primary sequence of these atypical LuxR-proteins with the canonical ones does not reveal any relevant differences (Fuqua *et al.*, 2002).

1.1.3 Are there advantages to social behaviours in bacteria?

Cell-to-cell communication among bacteria challenges the traditional notion that social behaviours are exclusive to multicellular organisms (Lyons *et al.*, 2015; West *et al.*, 2006). Participating in collective behaviours offers numerous advantages, such as resistance to physical and chemical stressors, an increased chance of survival during competitive interactions, and more efficient colonization of unexplored environments (Lyons *et al.*, 2015). Establishing group responses requires the expression of factors favouring this multicellular behaviour. Notably, the expression of these factors, which aid bacterial cooperation, is non-constitutive, partly due to the metabolic cost for individual cells. At high cellular densities, the benefits of these factors extend to the entire population (e.g., shared “public goods”), surpassing the negative metabolic impact on individual bacterial cells (West *et al.*, 2006).

1.1.4 QS and bacterial pathogenicity

Among the numerous traits regulated by QS is the expression of virulence by pathogenic bacteria, which was illustrated by *P. aeruginosa* (refer to **subsection 1.1**) (de Kievit *et al.*, 2000; Passador *et al.*, 1993; Rutherford *et al.*, 2012). This communication has broader implications for these bacteria, influencing their ability to form biofilms and their overall pathogenesis (Hooshangi *et al.*, 2008). Given the adverse health effects associated with a functioning QS system, understanding the intricacies of this communication system could offer alternatives to current antimicrobial therapies employed in clinical settings against pathogenic bacteria. Researchers worldwide have devoted considerable efforts to investigating QS regulation in bacterial pathogenesis. This exploration advances our understanding of bacterial communication and supports potential therapeutic interventions.

1.2 *P. aeruginosa* and its intercellular communication system

1.2.1 The bacterium *P. aeruginosa*

P. aeruginosa is a versatile rod-shaped gammaproteobacteria, and this characteristic trait is reflected in its ability to metabolize different sources of carbon and nitrogen and grow in different levels of oxygen availability (Stover *et al.*, 2000). This metabolic adaptability enables this bacterium to colonize a spectrum of environments closely related to human activities, ranging from free-living environments to host-associated settings (Crone *et al.*, 2020). In humans, *P. aeruginosa* primarily causes opportunistic infections, posing a significant threat to individuals with compromised natural barriers or weakened immune systems. Vulnerable populations include those undergoing chemotherapy, individuals with cystic fibrosis (CF), and those recovering from severe burns (Bodey *et al.*, 1983). Consequently, *P. aeruginosa* is notorious in the context of nosocomial infections. The frequency of those infections is heightened by this bacterium's propensity to adhere to various materials, including prostheses, catheters, and mechanical ventilators. Approximately 7% of healthcare-associated infections are estimated to be caused by this pathogen, and its prevalence drastically increases in intensive care units, representing up to 25% of patient infections (Magill *et al.*, 2014; Vincent *et al.*, 2020; Weiner *et al.*, 2016).

Notably, *P. aeruginosa* exhibits intrinsic high tolerance and resistance to numerous antimicrobials, presenting a significant challenge in effectively treating healthcare-associated infections and contributing to an elevated mortality rate associated with these infections (Poole, 2011). The primary factor underlying the intrinsic tolerance of this pathogen is the integrality and functionality of the outer membrane, which serves as an "impermeable" barrier to several antimicrobials. Additionally, efflux pumps actively expel antimicrobials that manage to transverse it (Poole, 2002). The development of multidrug resistance further exacerbates the pathogenesis of *P. aeruginosa*, and this process is frequently observed in healthcare settings. Chromosomal gene mutations mainly drive this phenotype, although horizontal gene transfer has also been documented (Colque *et al.*, 2020; Freschi *et al.*, 2019). Another critical element influencing the pathogenesis of *P. aeruginosa* is the tendency to form biofilms during chronic infections. Biofilms physically shield bacteria from antimicrobials and host defences, introducing an added layer of complexity to eradicating this pathogen (Ciofu *et al.*, 2019; Drenkard, 2003; Taylor *et al.*, 2014). Recognizing this challenge, the World Health Organization (WHO) has included carbapenem-resistant *P. aeruginosa* on its priority list for research and development efforts toward new effective drugs (Tacconelli *et al.*, 2018).

1.2.1.1 The strain UCBPP-PA14

The strain UCBPP-PA14 (Rahme *et al.*, 1995; Schroth *et al.*, 2018), hereafter referred to as PA14, is an isolate from human burn wounds and exhibits virulence across various infection models (Rahme *et al.*, 2000). The virulence of PA14 is heightened compared to the commonly used laboratory model PAO1, another *P. aeruginosa* prototypical strain (Rahme *et al.*, 2000; Rahme *et al.*, 1995). To unravel the genetic determinants influencing virulence, a comparative analysis of the genomes of these strains was conducted. The results revealed a high degree of conservation, with few divergent elements, which includes the presence of two pathogenic islands in PA14 (He *et al.*, 2004; Lee *et al.*, 2006a). Intriguingly, the unique genes of PA14 do not directly correlate with its increased virulence (Lee *et al.*, 2006a). This observation underscores the multifactorial nature of the pathogenesis of *P. aeruginosa*, including for the strain PA14.

1.2.2 Multiple factors contribute to the pathogenesis of *P. aeruginosa*

The development of *P. aeruginosa* infections results from the combined expression of cell-associated and secreted virulence factors (Morin *et al.*, 2021) (**Fig. 1.2.1**). The overall pathogenesis of this bacterium is tied to the coordinated expression of these factors as they orchestrate most of the crucial steps of bacterial colonization, from the initial attachment to the host to evading immune responses (Liao *et al.*, 2022). While the expression of most cell-associated factors is constitutive, the regulation of the majority of extracellular factors is contingent on cell density (de Kievit *et al.*, 2000; Morin *et al.*, 2021).

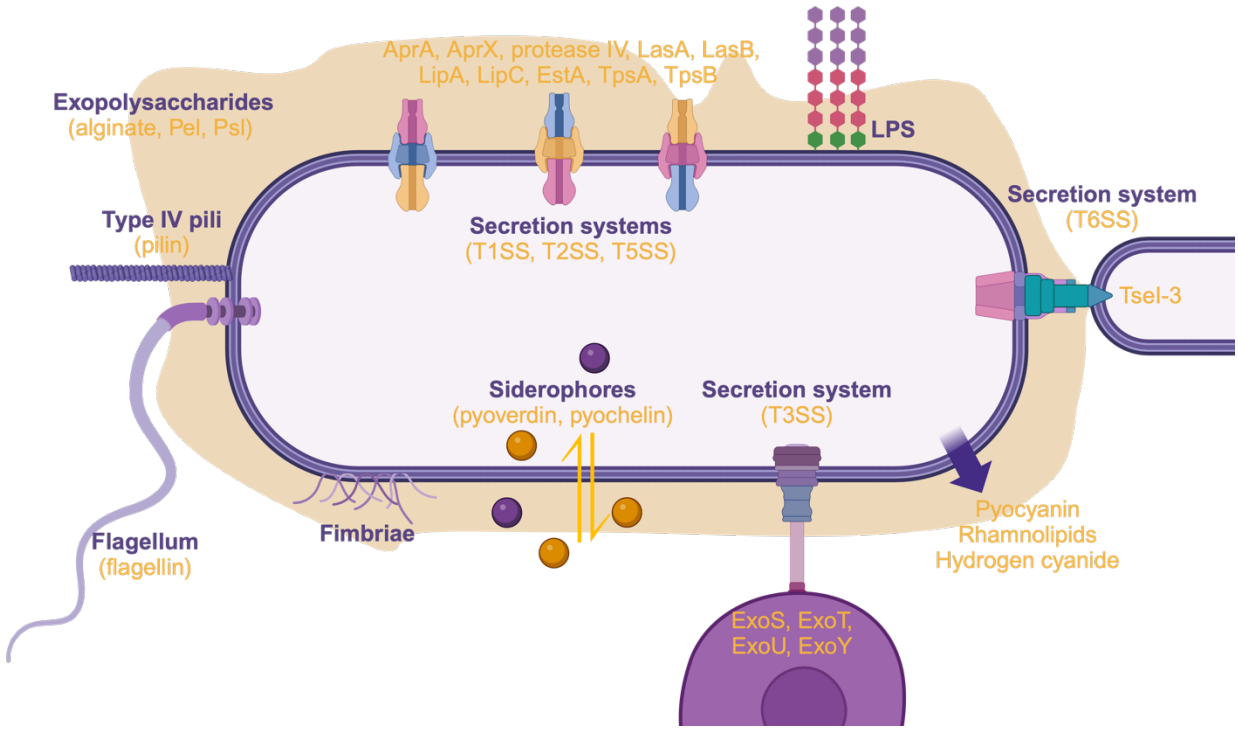

Figure 1.2.1. Virulence factors of *P. aeruginosa*.

Cell-associated and secreted extracellular factors contribute to the full virulence of this pathogen. While certain factors are illustrated here, Morin and collaborators provide a more comprehensive exploration of the virulence factors produced by this pathogen in the context of human infections (Morin *et al.*, 2021). Cell-associated factors include surface structures such as the flagellum, type IV pili (T4P), fimbriae, and LPS. Together, they favour colonization via motility and adhesion. Five secretion systems (T1SS, T2SS, T3SS, T5SS, and T6SS) contribute to the secretion of virulence factors into the extracellular milieu. Alternatively, they can inject virulence factors into bacterial (T6SS) and eukaryotic cells (T3SS). Notably, the pool of extracellular factors includes the redox-active pyocyanin, rhamnolipids, protease IV and elastase LasB, among others. Cell-to-cell communication regulates the production of a set of these factors. Exopolysaccharides (EPS) favour bacterial adhesion and maintain the structural integrity of *P. aeruginosa* biofilms. Adapted from (Liao *et al.*, 2022) and created using Biorender.

Therefore, QS in *P. aeruginosa* regulates the expression of numerous virulence factors, as highlighted in **Figure 1.2.1** [where QS governs the expression of LasA, LasB, pyocyanin, rhamnolipids, hydrogen cyanide, biofilm development, and T6SS (Morin *et al.*, 2021)]. The relationship between cellular density and virulence factor expression is important for bacterial behaviour. Coordinating expression patterns with growth enables the timely and appropriate production of virulence factors during different bacterial growth stages (de Kievit *et al.*, 2000), which has implications for infections. Indeed, this correlation has been validated through experiments utilizing diverse animal models (Azimi *et al.*, 2020; de Kievit *et al.*, 2000).

The evolving comprehension of *P. aeruginosa* virulence has prompted a novel theoretical approach to conventional antimicrobial treatments, typically based on bactericidal (or bacteriostatic) treatments. The challenge with classical approaches lies in their targeting of essential functions, exerting selective pressure that leads to the development of resistant variants through mutations. An alternative strategy proposes targeting (non-essential) virulence traits, alleviating this pressure and theoretically impeding the emergence of resistant variants. For example, non-essential structural components like the T3SS and flagellum have been explored as potential targets for tailored antivirulence strategies against *P. aeruginosa* (Dickey *et al.*, 2017; Liao *et al.*, 2022). These approaches have shown promise in animal models, and some are progressing to clinical trials (DiGiandomenico *et al.*, 2014; Jain *et al.*, 2018; Warrener *et al.*, 2014). Notably, targeting structures like the T3SS, which are shared among Gram-negative pathogens, could have broader implications for antimicrobial treatments against multiple human pathogens.

Tackling structural targets provides a focused approach by addressing specific virulence mechanisms. In contrast, regulatory systems such as QS coordinate the expression of multiple virulence factors, offering an attractive alternative to combat *P. aeruginosa*'s pathogenicity as they address several virulence factors simultaneously. The subsequent subsections will introduce the complex QS regulatory systems of *P. aeruginosa* as a building foundation and anti-QS strategies will be addressed in **subsection 1.2.3.10**.

1.2.3 Global framework of *P. aeruginosa* QS circuitry

In *P. aeruginosa,* cell-to-cell communication integrates three QS circuitries, each comprising one main autoinducer and its cognate transcriptional regulator, namely the *las*, *rhl*, and *pqs* systems. The *las* and *rhl* systems use AHLs as autoinducers, and the structure of their QS circuitry is homologous to the one previously presented in **section 1.1.2**. In contrast, the *pqs* system responds to molecules of the 4-hydroxy-2-alkylquinoline (HAQ) family.

These QS circuitries are intimately intertwined, collectively governing a significant portion of *P. aeruginosa* genes (5-10%) (Schuster *et al.*, 2003; Wagner *et al.*, 2003). This section will introduce each QS system and present their interconnections and particularities, culminating in the current model of the QS network within this pathogen.

1.2.3.1 The *las* system

The *las* QS system comprises the AHL synthase LasI, which synthesizes *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL; see **Fig. 1.1.3**) and the transcriptional regulator LasR. Activation of this system occurs when 3-oxo-C_{12}-HSL binds to LasR, inducing its dimerization, with each monomer containing a deeply buried ligand (Bottomley *et al.*, 2007; Schuster *et al.*, 2004b). Several virulence factors, including critical elements for tissue damage, such as the elastase LasB (*lasB*) and the endopeptidase LasA (*lasA*), are regulated by LasR (Gambello *et al.*, 1991; Schuster *et al.*, 2004b; Toder *et al.*, 1991). In addition to the expression of virulence genes, LasR also positively regulates the LasI synthase gene (*lasI*), creating a positive feedback loop classically associated with QS systems (Seed *et al.*, 1995). The concentration of 3-oxo-C_{12}-HSL does not increase continuously during growth, but instead peaks early on before rapidly decreasing (Déziel *et al.*, 2005). This regulation is partly attributed to the homeostatic control mechanism involving the negative transcriptional regulator RsaL, encoded by the *rsaL* gene located between the *lasR* and *lasI* genes, which LasR positively regulates (de Kievit *et al.*, 1999). Consequently, a negative feedback loop is formed, wherein RsaL, directly binding to the *lasI* promoter, represses its transcription (de Kievit *et al.*, 1999; Rampioni *et al.*, 2006). LasR and RsaL are the sole QS transcriptional regulators known to directly binding to the *lasI* promoter.

1.2.3.2 The *rhl* system

Similar to the *las* system, the *rhl* circuitry is based on AHL signalling. In this case, the LuxR/LuxI pair is represented by the autoinducer synthase RhlI producing *N*-butanoyl-L-homoserine lactone (C_4-HSL; see **Fig. 1.1.3**) and the transcriptional regulator RhlR (Latifi *et al.*, 1995; Pearson *et al.*, 1995). However, unlike most LuxR-type proteins, the activation of RhlR extends beyond the binding to its cognate signal, C_4-HSL, requiring a third element: the protein PqsE, regulated by the *pqs* system, as recently demonstrated (Borgert *et al.*, 2022; Feathers *et al.*, 2022). This activation mechanism is more complex and, to date, unique to the *rhl* system and is further presented in **subsection 1.2.3.4**.

The *rhl* system induces the expression of genes that encode enzymes producing extracellular factors such as rhamnolipids (*rhlAB* and *rhlC*), a surfactant released by *P. aeruginosa* essential to the collective translocation of cells, phenazines (operons *phzA1-G1* and *phzA2-G2*), and the poison hydrogen cyanide (*hcnABC*) (Brint *et al.*, 1995; Pessi *et al.*, 2000). Another distinctive feature of the *rhl* system is the absence of a conventional positive feedback loop activating the

expression of *rhlI*, encoding the RhlI synthase. Instead, LasR is the primary regulator of *rhlI* transcription, with RhlR solely taking over when LasR is unable to (Groleau *et al.*, 2020). This marks the first layer of QS intertwinement, elaborated further in **subsection 1.2.3.5**.

1.2.3.3 The *pqs* system

The principles of the *pqs* system are analogous to AHL-based circuitries, such as *las* and *rhl*. The transcription regulator MvfR, also known as PqsR, exhibits dual induction by both 4-hydroxy-2-heptylquinoline (HHQ) and the *Pseudomonas* quinolone signal (PQS; 3,4-dihydroxy-2-heptylquinoline) autoinducers (**Fig. 1.2.2**) (Xiao *et al.*, 2006a). The direct regulon of MvfR solely comprises the operons *phnAB* and *pqsABCDE*, encoding enzymes involved in synthesizing 4-hydroxy-2-alkylquinolines (HAQs) (Déziel *et al.*, 2005; Gallagher *et al.*, 2002; Rampioni *et al.*, 2016). HHQ and PQS belong to this chemical family (Déziel *et al.*, 2004), establishing a positive feedback loop that amplifies HAQ production. The final product of the synthetic pathway is HHQ, the PQS precursor (Déziel *et al.*, 2004) (**Fig. 1.2.3**). The monooxygenase PqsH, in aerobic environments, converts HHQ into PQS (Gallagher *et al.*, 2002; Schertzer *et al.*, 2010) (**Fig. 1.2.3**). In presence of oxygen, PQS is the main activator of MvfR, while HHQ is the ligand when oxygen is limiting (Schertzer *et al.*, 2010; Xiao *et al.*, 2006a). LasR regulates the transcription of the gene encoding PqsH (*pqsH*) (Gallagher *et al.*, 2002), providing another example of the interconnection between the QS systems (refer to **subsection 1.2.3.5**).

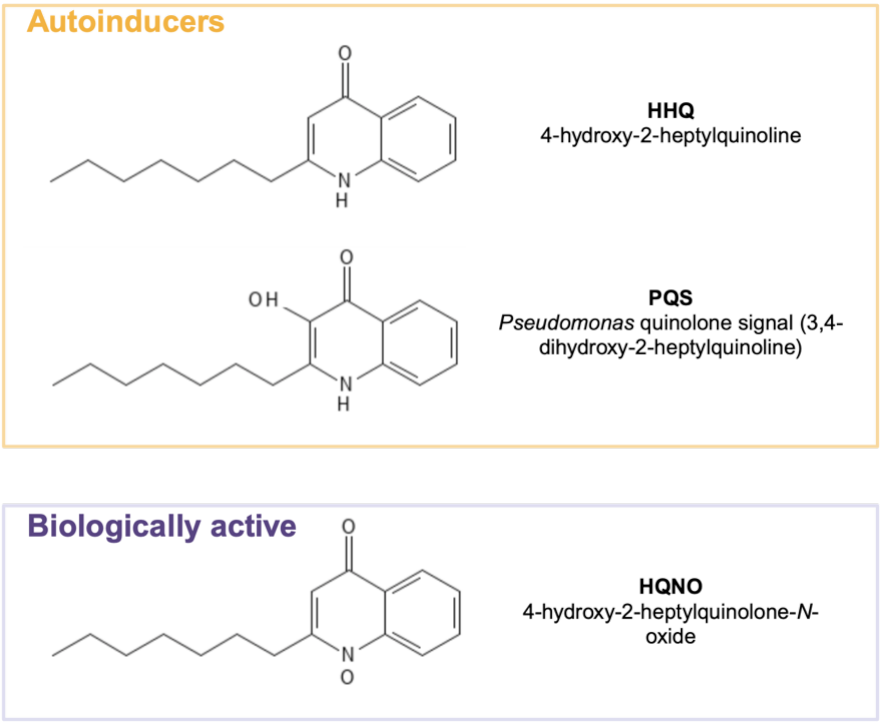

Figure 1.2.2. Structure of the fundamental HAQs of the *pqs* system.

The structures of the HHQ and PQS autoinducers are depicted within the yellow box. Among the HAQs synthesized by *P. aeruginosa*, these are the main activators of MvfR. In the purple box, HQNO is employed to illustrate a biologically active HAQ. HQNO, while not functioning as a signal molecule, is produced in substantial concentrations by *P. aeruginosa*. Created using Biorender.

Establishing a positive feedback loop amplifies HAQ production via MvfR, activating the *phnAB* and *pqsABCDE* operons. The *phnAB* operon encodes the anthranilate synthase PhnAB, converting chorismatic acid into anthranilic acid, the precursor molecule for the biosynthesis of HAQs (Palmer *et al.*, 2013) (**Fig. 1.2.3**). The enzymes encoded by *pqsABCDE*, PqsA-D, are instrumental in producing HAQs (Dulcey *et al.*, 2013), but the last encoded protein, PqsE, is not (Déziel *et al.*, 2004; Gallagher *et al.*, 2002) (**Fig. 1.2.3**). *P. aeruginosa* produces a diverse array of HAQs, including QS-active analogues like C9 congeners of HHQ and PQS, namely 3,4-dihydroxy-2-nonylquinoline (C9-PQS) and 4-hydroxy-2-nonylquinoline (HNQ) (Ilangovan *et al.*, 2013), along with other molecules exhibiting structural similarities to quinolones (Déziel *et al.*, 2004; Lépine *et al.*, 2004). Quinolines are known for their antimicrobial properties, suggesting potential biological activities for non-QS-active HAQs (Heeb *et al.*, 2011). Notably, HAQ *N*-oxide production requires the PqsL enzyme's activity (encoded by *pqsL*) (Déziel *et al.*, 2004; Lépine *et al.*, 2004). This set of molecules includes 4-hydroxy-2-heptylquinolone-*N*-oxide (HQNO), a

molecule produced in substantial concentrations by *P. aeruginosa* (Lépine *et al.*, 2004) (**Fig. 1.2.3**). Serving as a potent cytochrome inhibitor, HQNO contributes to the competitive advantage of this bacterium against other microorganisms and eukaryotic cells (Heeb *et al.*, 2011). HQNO also plays a crucial role in bacterial programmed cell death, acting as a self-poisoning agent that triggers bacterial autolysis and DNA release (Hazan *et al.*, 2016). Importantly, evidence of adaptation against HQNO-mediated autotoxicity has also been identified (Raba *et al.*, 2018), allowing controlled DNA release that contributes to biofilm formation, thus benefiting the bacterial population. Through a distinct mechanism, HQNO has recently been proposed to protect *P. aeruginosa* against prophage-mediated autolysis (Giallonardi *et al.*, 2023).

As depicted in **Figure 1.2.3**, PqsE has a thioesterase activity but is dispensable for HAQ biosynthesis (Déziel *et al.*, 2004; Gallagher *et al.*, 2002), possibly due to the functional complementation by alternative thioesterases in its absence (Drees *et al.*, 2015). This dispensability suggests that PqsE may have limited significance in QS-controlled responses in *P. aeruginosa*. However, in contrast, bacterial behaviours regulated by the *pqs* system heavily depend on PqsE, and the absence of MvfR can be restored by overexpressing PqsE; in other words, QS-controlled behaviours depend on PqsE in a HAQ-independent manner (Déziel *et al.*, 2005; Farrow *et al.*, 2008). Moreover, these PqsE-dependent behaviours are independent of the enzymatic activity of PqsE, as indicated by a catalytically inactive PqsE variant (PqsE D73A) (Simanek *et al.*, 2022; Taylor *et al.*, 2021). From this, two significant insights emerge regarding the *pqs* system and its regulation: firstly, the production of HAQs, including HHQ and PQS, is not the primary driver of MvfR-dependent QS control, and secondly, the feedback loop formed may primarily be used to amplify the concentration of PqsE, not the autoinducers *per se*. The mechanisms by which PqsE modulates QS-controlled responses will be discussed in **subsection 1.2.3.4**, as it concerns the interconnection of the *pqs* and *rhl* systems.

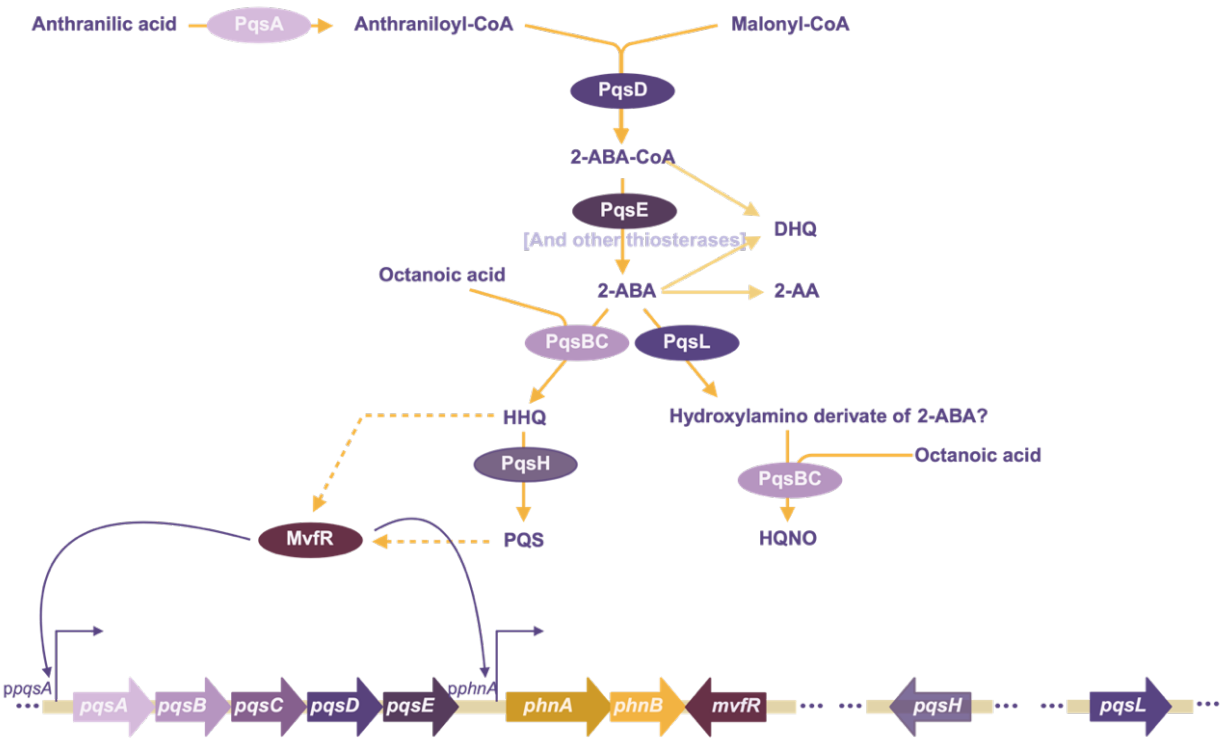

Figure 1.2.3. Biosynthesis of the autoinducers from the *pqs* system, HHQ and PQS.

The biosynthesis of HAQs is initiated with anthranilic acid as the precursor molecule. The *phnAB* operon, depicted in yellow, encodes the anthranilate synthase PhnAB that converts chorismic acid into anthranilic acid, therefore preceding the biosynthetic pathway presented. The genes for HAQ synthesis are predominantly located within the *pqsABCDE* operon, with additional essential elements found in the accessory and genomic distant *pqsH* and *pqsL* genes, crucial for producing PQS and HQNO, respectively. The enzymatic conversion of each molecule is depicted alongside the corresponding mediating enzyme. Notably, PqsE is included in the biosynthetic pathway, although alternative enzymes can functionally complement this conversion without PqsE. 2-ABA-CoA stands for 2-aminobenzoylacetyl-CoA; 2-ABA for 2-aminobenzoylacetate; 2-AA for 2-aminoacetophenone; DHQ for 2,4-dihydroxyquinoline. The fundamental components of the *pqs* system include MvfR, its autoinducers HHQ and PQS, as well as PqsE and HQNO. Solid yellow lines represent biosynthetic steps, while dotted lines signify activation of MvfR. Solid purple lines illustrate transcriptional activation. Adapted from (Rampioni *et al.*, 2016) and created using Biorender.

1.2.3.4 The full activity of the *rhl* system requires PqsE

One intriguing aspect of the *P. aeruginosa* QS regulatory network is the connection between the *rhl* and *pqs* systems. The initial evidence for this link emerged from the observation that this bacterium failed to synthesize the phenazine pyocyanin and hydrogen cyanide – exoproducts regulated by RhlR – in the absence of PqsE (Diggle *et al.*, 2003; Gallagher *et al.*, 2002). This connection was later reinforced when the MvfR regulon was determined, revealing a set of RhlR-dependent genes requiring PqsE (Déziel *et al.*, 2005) and by the PqsE regulon itself (Hazan *et al.*, 2010; Rampioni *et al.*, 2016; Rampioni *et al.*, 2010). The mechanisms by which PqsE exerts control over genes under RhlR regulation remained elusive until recently.

Comprehension of the PqsE structure was required to elucidate its mechanism of action. The three-dimensional structure of PqsE conforms to the typical architecture of proteins within the metallo-hydrolases superfamily. It lacks a canonical DNA binding domain (Yu *et al.*, 2009), suggesting that an unidentified enzymatic activity is associated with the regulatory role of PqsE. Subsequent investigations identified a natural substrate for this protein as 2-aminobenzoylacetyl-CoA (2-ABA-CoA), whose conversion into 2-aminobenzoylacetate (2-ABA) is catalyzed by PqsE, thereby placing it within the biosynthetic pathway of HAQs (see **Fig. 1.2.3**) (Drees *et al.*, 2015). Notably, PqsE acts independently of HAQ production, as previously stated, suggesting that this protein has dual functions: one characterized by an enzymatic activity, specifically a functionally redundant enzymatic activity, and another involving QS induction with a mechanism yet unknown (Déziel *et al.*, 2005; Gallagher *et al.*, 2002). The autonomy of these functions was reinforced by introducing point mutations in the enzymatic active site and employing enzymatic inhibitors (Taylor *et al.*, 2021; Valastyan *et al.*, 2020; Zender *et al.*, 2016). Neither approach significantly impacted the PqsE-controlled QS extracellular factors, with pyocyanin often serving as a marker of PqsE activity due to its easily identifiable nature. Together, these structural-based studies of PqsE failed to pinpoint how this protein elicits the expression of RhlR-controlled genes.

In parallel with the structural studies of PqsE, a complementary genetic approach was taken to determine the interaction of the *rhl* and *pqs* systems via RhlR and PqsE. A couple of propositions have been attributed to PqsE, such as the potential to enhance the affinity of RhlR for C_4-HSL (Farrow *et al.*, 2008) or even to increase the protein levels of RhlR (García-Reyes *et al.*, 2021). PqsE has also been proposed to synthesize an alternative diffusible ligand for RhlR (Mukherjee *et al.*, 2018). The production of an alternative signal molecule for RhlR, synthesized by PqsE, would require its enzymatic activity and re-associate the enzymatic function to the regulatory one, which was unlikely as it does not align with known structural data. Unsurprisingly, the existence

of this putative PqsE-produced ligand was contradicted, as the culture supernatant of strains that do not produce C_4-HSL, the primary ligand of RhlR, failed to induce its transcriptional activity (Groleau *et al.*, 2020). Even if these studies cannot thoroughly explain the role of PqsE in RhlR regulation, they confirm the importance of PqsE to the mentioned regulation. Notably, this requirement is evidenced in the absence of a functional *las* system, a context in which PqsE drives RhlR-dependent QS alongside C_4-HSL (Groleau *et al.*, 2020).

The existing molecular model of the interaction between RhlR and PqsE required the structural study of RhlR. LuxR-type proteins, as previously discussed, adopt a folded and stable protein structure when bound to their cognate autoinducers. Consequently, the purification of these transcriptional regulators can be easily performed in their autoinducer-stabilized form (Schuster *et al.*, 2004b; Urbanowski *et al.*, 2004; Venturi *et al.*, 2018; Weingart *et al.*, 2005; Zhu *et al.*, 1999). In contrast to other LuxR-type proteins, RhlR is inherently insoluble, and even in the presence of C_4-HSL, RhlR is unstable, posing challenges for biochemical and structural studies. Methods to generate a soluble form of RhlR include the use of the synthetic ligand 4-(3-bromophenoxy)-*N*-(2-oxotetrahydrothiophen-3-yl)butanamide (*meta*-bromo-thiolactone, mBTL) or the selection of stabilizing variants of RhlR through mutant screenings (Borgert *et al.*, 2022; Feathers *et al.*, 2022; McCready *et al.*, 2019; O'Loughlin *et al.*, 2013; Taylor *et al.*, 2021). These stabilizing strategies, primarily the utilization of mBTL, have been instrumental in the elucidation of the molecular mechanism underlying the relationship between RhlR and PqsE: these proteins form a protein complex, and the direct interaction of PqsE with RhlR further stabilizes RhlR/C_4-HSL, thereby inducing the activity of this transcriptional regulator (Borgert *et al.*, 2022; Feathers *et al.*, 2022; Taylor *et al.*, 2021) (**Fig. 1.2.4**).

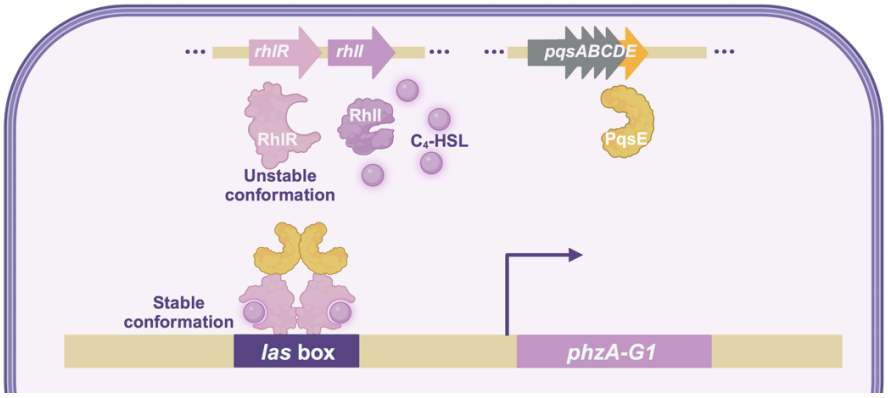

Figure 1.2.4. Regulatory dynamics of RhlR activity within the *rhl* system.

The stability of RhlR is influenced by two key elements, C_4-HSL and PqsE. In its unbound state, RhlR is inherently unstable. The binding of its cognate autoinducer, C_4-HSL, serves as an initial stabilizing factor for this transcriptional regulator. However, complete induction and sustained stability are achieved through the additional role of PqsE. The interaction occurs between a homodimer of PqsE and a homodimer of RhlR. Each monomer of RhlR, in turn, binds to a C_4-HSL molecule, culminating in the formation of an active RhlR complex. This model of regulatory dynamics was described for pyocyanin production and the expression of *phz1*. The active RhlR complex binds to the *las* box within the regulatory sequence of *phz1* (Whiteley *et al.*, 2001). Both LasR and RhlR can recognize *las* boxes, but RhlR primarily drives the *phz1* transcription (Groleau *et al.*, 2020). Adapted from (Borgert *et al.*, 2022) and created using Biorender.

This model contributes to understanding the RhlR activity and finally sheds light on studies conducted over the last two decades. However, certain behaviours defy integration into this model, indicating a greater complexity than currently considered. Through an elegant experimental design employing a strain expressing *rhlR* and *pqsE* in a QS-independent manner to untangle the regulatory networks intrinsic to the QS circuitry (refer to **subsection 1.2.3.5**), the regulon of RhlR and PqsE has been identified (Letizia *et al.*, 2022). This study emphasizes the negligible impact of PqsE on gene regulation in the absence of RhlR, reinforcing that the influence of PqsE in QS control is mediated through this transcriptional regulator. However, the reciprocal relationship is not true, as RhlR also has a PqsE-independent regulon. PqsE expands the RhlR regulon and differentially modulates distinct subsets of these genes (Letizia *et al.*, 2022). Notably, the genes involved in pyocyanin production (*phz1* and *phz2* operons) belong to the subset of genes activated in the concomitant presence of RhlR and PqsE (Letizia *et al.*, 2022). Consequently, these genes may only represent this RhlR-controlled subregulon, not the complete set of genes under RhlR control. Therefore, the model requires refinement to incorporate this PqsE-driven differential regulation, acknowledging that if the model depicted in **Figure 1.2.4**

represented the overall activity of RhlR, all the genes regulated by this transcriptional regulator should also depend on PqsE.

1.2.3.5 The QS systems of *P. aeruginosa* interconnect intricately

Integrating signals from each QS circuitry necessitates a complex intertwinement, resulting in a sophisticated and interconnected network. As with other regulatory systems, a comprehensive understanding of these interconnections demands implementing a suitable model. In the context of *P. aeruginosa* QS regulatory network, the prototypical strains PAO1 and PA14 are by far the best studied. Under standard laboratory conditions, the QS regulatory network in these strains exhibits a hierarchical organization (Latifi *et al.*, 1996; McKnight *et al.*, 2000; Pesci *et al.*, 1997; Wade *et al.*, 2005). Atop this hierarchy is the *las* system, exerting positive control over the activation of the *rhl* and *pqs* systems, as previously mentioned (Pesci *et al.*, 1997; Wade *et al.*, 2005; Xiao *et al.*, 2006b). The maintenance of this hierarchy depends on environmental conditions (Duan *et al.*, 2007; Soto-Aceves *et al.*, 2021), and in some genetic contexts, it has been shown to be flexible (Dekimpe *et al.*, 2009).

The sequential activation of the QS circuitries is assured by the LasR/3-oxo-C_{12}-HSL complex, inducing the transcription of both core elements of the *rhl* system: *rhlR* and *rhlI* (de Kievit *et al.*, 2002; Groleau *et al.*, 2020; Pesci *et al.*, 1997). LasR is the primary regulator of *rhlI* transcription (Groleau *et al.*, 2020). This regulatory mechanism deviates from the classical positive feedback loop commonly observed in QS circuitries within the *rhl* system. Additionally, the *las* system may exert posttranslational regulation on RhlR (Pesci *et al.*, 1997), based on the interaction of RhlR homodimers with 3-oxo-C_{12}-HSL, a non-cognate autoinducer (Pesci *et al.*, 1997; Ventre *et al.*, 2003). This putative interaction would downregulate RhlR activity by disassociating its homodimers (Ventre *et al.*, 2003), strategically coupling the timing of RhlR activation with the depletion of 3-oxo-C_{12}-HSL.

The LasR/3-oxo-C_{12}-HSL complex also induces the activity of the *pqs* system. LasR directly activates the transcription of *mvfR*, the gene encoding the LysR-type transcriptional regulator of the *pqs* system (Gilbert *et al.*, 2009; Xiao *et al.*, 2006b). Consequently, LasR serves as an indirect regulator of *pqsABCDE* expression. Additionally, LasR directly activates the transcription of *pqsH*, the monooxygenase involved in the conversion of HHQ to PQS (Déziel *et al.*, 2004; Gallagher *et al.*, 2002; Gilbert *et al.*, 2009; Whiteley *et al.*, 1999) (refer to **Fig. 1.2.3**). In essence, the *las* system exerts a dual influence on the *pqs* system: it activates the expression of the regulator of the *pqs*

system and directs the conversion of HHQ into PQS, a more potent activator of MvfR compared to its precursor HHQ (Xiao *et al.*, 2006a).

While the expression of *lasI*, *lasR*, *rhlI*, and *rhlR* remain unaffected by the *pqs* system (Déziel *et al.*, 2005), the latter is subject to negative regulation by the *rhl* system. Indeed, the RhlR/C_4-HSL/PqsE complex represses the expression of the *pqsABCDE* operon (Hazan *et al.*, 2010; McGrath *et al.*, 2004; Wade *et al.*, 2005; Xiao *et al.*, 2006b). However, RhlR is solely known as a transcriptional activator, and the molecular regulation leading to this repression was later characterized. RhlR induces an alternative transcript of *pqsABCDE*, wherein translation is repressed by blocking the access of the ribosome to the Shine-Dalgarno (SD) sequence (Brouwer *et al.*, 2014). This regulatory interplay likely controls RhlR activation homeostasis, in which the activity of RhlR represses the expression of one of its activators, PqsE. The complex regulatory interactions introduced here are illustrated in **Figure 1.2.5**, with additional regulators to be introduced in the next subsection.

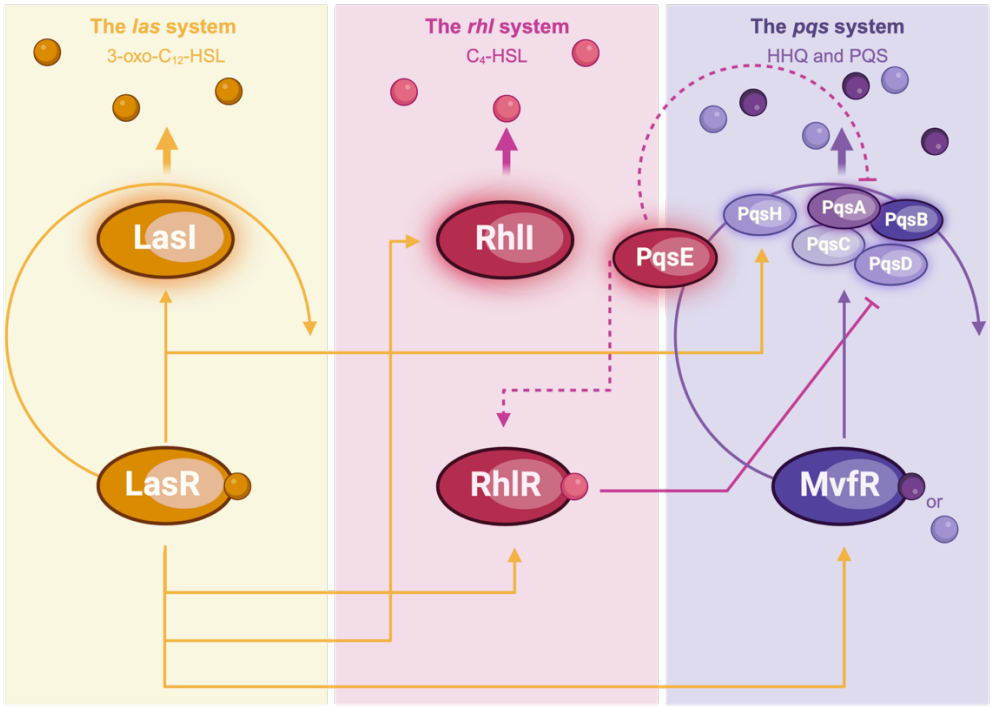

Figure 1.2.5. The three QS systems of *P. aeruginosa* are interconnected.

Under standard laboratory conditions, the *las* system induces the expression of the *rhl* and *pqs* systems. These systems are visually represented in yellow, pink, and purple. Arrows signify the transcriptional activation of the genes encoding the indicated proteins, with each colour denoting the corresponding QS circuitry. A similar colour-coded logic is applied to indicate inhibition, which, in this case, represents a post-transcriptional regulation (flat-headed arrow). PqsE, a by-product of the *pqs* system, directly interacts with RhlR, influencing the activity of the *rhl* system and creating an indirect negative feedback loop towards the *pqs* system. A dotted line visually illustrates RhlR/PqsE interaction. Circular arrows are used to denote the existence of a positive feedback loop within the regulatory mechanisms. Modified from (Groleau *et al.*, 2020) and created using Biorender.

1.2.3.6 The QS regulatory network of *P. aeruginosa* involves other regulators, including a solo LuxR-protein

The complexity within the QS system heavily lies on the global gene regulation network, comprising not only the regulators that compose each system (e.g., LasR, RhlR, and MvfR) but also other regulators that act at the transcriptional and post-transcriptional levels. This regulation mechanism allows the integration of QS into other regulatory systems, permitting bacterial adaptation to ever-changing environmental conditions. Most known interactions regulating the activity of the QS effectors are summarized in **Figures. 1.2.6** and **1.2.7**.

In addition to LasR and RhlR, *P. aeruginosa* encodes one additional LuxR-type protein, QscR. This protein is referred to as an “orphan” as no cognate synthase has been associated with it.

Like canonical LuxR-proteins, QscR consists of an amino-terminal autoinducer binding domain and a carboxy-terminal DNA binding domain. QscR delays the expression of various QS-regulated exoproducts, such as pyocyanin and hydrogen cyanide, by forming inactive heterodimers with LasR and RhlR (Chugani *et al.*, 2001; Ledgham *et al.*, 2003). These heterodimers are established in the absence of AHLs, thereby inhibiting the activities of both LasR and RhlR. Consequently, this leads to the repression of genes encoding the AHL synthases, *lasI* and *rhlI*, during the early stages of growth (Chugani *et al.*, 2001). As previously mentioned, QscR possesses an amino-terminal autoinducer domain and binds to long-chain AHL, including 3-oxo-C_{12}-HSL (Lequette *et al.*, 2006; Wellington *et al.*, 2019). Notably, the QscR/3-oxo-C_{12}-HSL complex exclusively controls the expression of the nearby single locus *PA14_40020-PA14_39990* in PA14 (Ding *et al.*, 2018). The mechanism governing global regulation in this context remains unknown. The regulatory sequence of *qscR* contains a *las* box, and RhlR induces its transcription but not LasR (Ledgham *et al.*, 2003; Letizia *et al.*, 2022).

P. aeruginosa additionally encodes a protein known as VqsR, often categorized as a LuxR-type protein. However, VqsR lacks an autoinducer binding domain, rendering it incapable of binding to any autoinducer, whether self-produced or from external sources (Patankar *et al.*, 2009; Schuster *et al.*, 2006). VqsR positively regulates AHLs production and QS-controlled traits; in its absence, none of these molecules is produced (Juhas *et al.*, 2004). VqsR directly represses the expression of *qscR* and is activated by LasR (Gilbert *et al.*, 2009; Li *et al.*, 2007; Liang *et al.*, 2012). By repressing QscR, VqsR indirectly activates the expression of the core elements of the QS regulatory network.

Like QscR, other regulators in *P. aeruginosa* negatively modulate the activity of LuxR receptors (LasR and RhlR) by direct binding, raising the activation threshold inherent to the QS circuitry. These proteins are called anti-activators (Asfahl *et al.*, 2017; Smith *et al.*, 2022). In addition to QscR, *P. aeruginosa* encodes two more anti-activators: QteE and QslA. The consequence of the direct interaction of these proteins with LuxR receptors varies. As mentioned, QscR forms inactive heterodimers with LasR and RhlR (Chugani *et al.*, 2001; Ledgham *et al.*, 2003), while interaction with QteE prevents autoinducer binding, destabilizing LasR and RhlR (Siehnel *et al.*, 2010). QslA is known to obstruct the dimerization interface of LasR (Fan *et al.*, 2013), hindering its transcriptional activity. This mechanism enables the dissociation of active dimers bound to the DNA (Seet *et al.*, 2011).

Environmental cues, such as the nutriment conditions and the bacterial mode of growth, are integrated into the QS regulatory network through the activity of peripheral regulators, as

previously mentioned. A segment of this regulatory process is visually represented in **Figure 1.2.6**. For instance, Vfr is the sole regulator known to activate LasR expression directly (Albus *et al.*, 1997). A homolog of *Escherichia coli*'s cyclic adenosine monophosphate (cAMP) receptor protein (CRP), the activity of Vfr is coactivated by cAMP (West *et al.*, 1994a). However, the activation of LasR deviates from the conventional cAMP requirement, as cAMP is not necessary for this Vfr-driven activation (Fuchs *et al.*, 2010). As mentioned earlier, LasR activates the expression of numerous genes, including the one coding RsaL, assuring the homeostasis of the 3-oxo-C_{12}-HSL autoinducer. RsaL exerts positive control over the *pqs* system directly through *pqsH* and indirectly through *cpdR* (Kang et al., 2017). Like RsaL, CdpR induces transcription of *pqsH* (Zhao *et al.*, 2016), favouring the conversion of HHQ to PQS.

Peripheral regulators play an important role in cell-to-cell communication, as illustrated by RpoS, a sigma factor associated with broad stress responses (Jørgensen *et al.*, 1999; Suh *et al.*, 1999). The RpoS-dependent regulon significantly overlaps with that of QS (Schuster *et al.*, 2004a). However, the precise mechanism governing this regulation remains unknown, as the transcription of the genes coding the regulator genes, LasR and RhlR, undergoes only mild modulation by RpoS (Schuster *et al.*, 2004a). Stringent response regulators – RelA and DksA1, involved in bacterial strategies to survive challenging environmental conditions, have also been linked to the modulation of QS responses (Jude *et al.*, 2003; van Delden *et al.*, 2001). RelA modulates QS responses in an RpoS-dependent and -independent manner, the latter regulation controlling the expression timing of core QS elements – comprising LasR, RhlR, and their cognate autoinducers (van Delden *et al.*, 2001). In contrast, DskA1 does not alter the transcriptional expression of genes under QS control; instead, it impacts their translation (Jude *et al.*, 2003).

Gaps exist in understanding mechanisms for some other regulators involved in QS-controlled responses. For example, the overexpression of QsrO drastically represses QS responses governed by the three QS systems. Nevertheless, the underlying mechanism remains elusive (Köhler *et al.*, 2014).

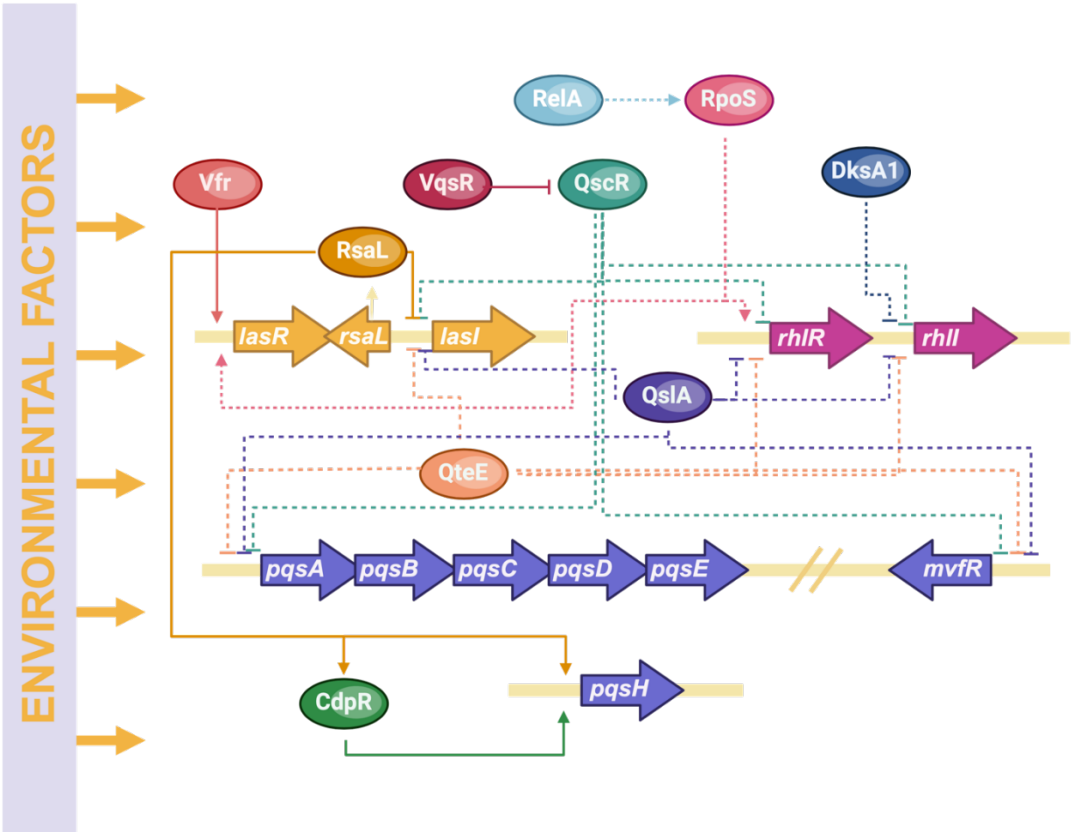

Figure 1.2.6. Additional regulators widen the QS regulatory network.

The interconnection within the QS regulatory network presented in **Figure 1.2.5** is not illustrated here. Vfr directly activates the transcription of *lasR*, initiating a cascade where active LasR induces the transcription of *rsaL* and *lasI*, along with other genes. RsaL is essential for the homeostasis of 3-oxo-C_{12}-HSL and also activates the *pqs* system, favouring the conversion of HHQ to PQS directly and indirectly, via CdpR. A significant proportion of genes under QS regulation is dependent on RpoS. However, RpoS is a mild modulator of *lasR* and *rhlR* transcription. The stringent response regulator RelA induces the activity of RpoS, indirectly regulating QS responses, and also alters the timing of QS activation in a RpoS-independent manner. DksA1 translationally represses RhlI. The impact of anti-activators (QscR, QteE, and QslA) is depicted as indirect repression, negatively affecting genes under LasR- (for QscR, QteE, and QslA) and RhlR-control (for QscR and QteE). VqsR indirectly influences the QS regulatory network by directly repressing QscR (Liang *et al.*, 2012). Positive regulation is represented by pointed arrows and negative by flat-headed arrows. Direct regulation is illustrated by solid lines and indirect ones, by dashed lines. Adapted from (Schuster *et al.*, 2006; Venturi, 2006; Williams *et al.*, 2009) and created using Biorender.

In this subsection, the highlighted regulatory mechanisms have predominantly focused on the transcriptional modulation of genes under QS regulation – either directly or indirectly. Nevertheless, there is a growing recognition of the significant role of small RNAs (sRNAs) in shaping QS responses in *P. aeruginosa* (**Fig. 1.2.7**). These regulatory RNAs, conserved across

bacterial species, act as post-transcriptional regulators by interacting with proteins or mRNA (Dutta *et al.*, 2018; Gottesman *et al.*, 2011; Storz *et al.*, 2011).

In Gram-negative bacteria, the RNA binding protein Hfq is typically required for sRNAs' proper function and stability (Storz *et al.*, 2011). The first characterized sRNA associated with QS in *P. aeruginosa* is PhrS (Sonnleitner *et al.*, 2011). Active in oxygen-limiting conditions (as ANR regulates it), PhrS promotes the translation of MvfR in an Hfq-dependent manner (Sonnleitner *et al.*, 2008). Subsequent discoveries have revealed additional examples of sRNA-mediated regulation. For instance, the sRNA ReaL serves as a linker of the *las* and *pqs* systems in *P. aeruginosa*, inducing the translation of PqsC, thereby positively affecting MvfR, and is repressed by LasR (Carloni *et al.*, 2017). ReaL also regulates other QS responses by negatively impacting the translation of RpoS (Thi Bach Nguyen *et al.*, 2018), a crucial peripheral regulator discussed earlier.

Furthermore, additional sRNAs linking the activity of one QS to another have been identified. LasR, for instance, represses AmiL, while this sRNA induces RhlI (Pu *et al.*, 2022). Similarly, RhlR represses PrrH, and in turn, this sRNA represses LasI (Lu *et al.*, 2019). sRNA P27 represses the translation of RhlI (Chen *et al.*, 2019b), whereas RhlS induces its expression (Thomason *et al.*, 2019). We summarize these interactions in **Figure 1.2.7**.

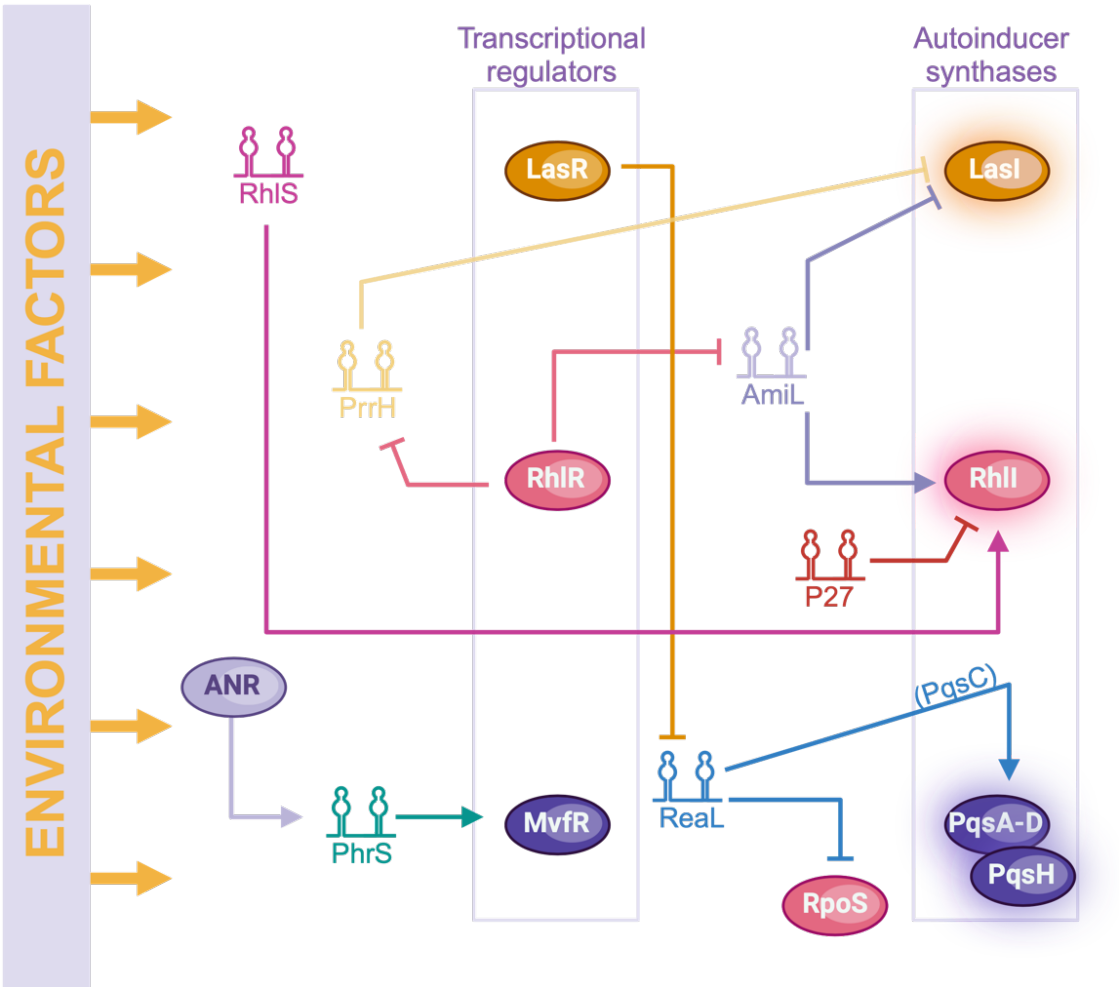

Figure 1.2.7. Noncoding small RNAs further widen the QS regulatory network.

The interconnection within the QS regulatory network presented in **Figures 1.2.5** and **1.2.6** are not illustrated here. The only sRNA presented here capable of directly inducing the expression of a QS transcriptional regulator is PhrS. By base pairing, PhrS induces the translation of MvfR. In contrast, modulating the synthesis of autoinducers is preferred among the presented sRNAs. PrrH and AmiL reduce the expression of LasI, hampering the activity of the *las* system. RhlI is induced by RhlS and AmiL but repressed by P27. ReaL induces PqsC, inducing the production of HHQ and PQS. ReaL also inhibits the translation of RpoS (refer to **Fig. 1.2.6**). The expression of some of these regulatory elements is driven by QS regulators (LasR inhibits ReaL, while RhlR inhibits PrrH and AmiL). Based on (Carloni *et al.*, 2017; Chen *et al.*, 2019b; Lu *et al.*, 2019; Pu *et al.*, 2022; Sonnleitner *et al.*, 2011; Sonnleitner *et al.*, 2008; Thi Bach Nguyen *et al.*, 2018; Thomason *et al.*, 2019) and created using Biorender.

1.2.3.7 Transcriptional regulator promiscuity in *P. aeruginosa*: Another factor to consider in the interconnection of QS systems?

When introducing canonical QS systems, it was emphasized that LuxR-type proteins specifically recognize their cognate autoinducer, thus ensuring precise and effective communication (refer to **subsection 1.1.2.1**). While specificity is a fundamental trait of the QS circuitry, the term "promiscuous" describes a set of LuxR-type proteins that undergo dimerization and activation upon interaction with non-cognate autoinducers. This concept is particularly relevant within the *P. aeruginosa* QS regulatory network, which comprises two complete AHL circuits – LasR/LasI and RhlR/RhlI, and the orphan LuxR-type protein, QscR. Consequently, 3-oxo-C_{12}-HSL and C_4-HSL, two distinct autoinducers, concurrently exist within the bacterium, potentially leading to crosstalk.

Although the maximal activity of LasR is induced by its cognate autoinducer 3-oxo-C_{12}-HSL, LasR exhibits promiscuity towards AHLs with a medium- and long-length acyl chain, particularly those in which the third carbon in the acyl chain is fully oxidized (Gerdt *et al.*, 2017; Kylilis *et al.*, 2018; Wellington *et al.*, 2019). QscR shares a similar specificity with LasR but is activated by a broader range of AHL structures. An important distinction between these LuxR-type proteins is that QscR maximal activity was elicited by *N*-decanoyl-L-homoserine lactone (C_{10}-HSL), an autoinducer not produced by *P. aeruginosa* (Wellington *et al.*, 2019). Neither LasR nor QscR responds to AHLs with short-length acyl chains, such as C_4-HSL, suggesting limited interaction between these molecules.

In contrast to LasR and QscR, the activity of RhlR is specifically driven by its cognate autoinducer and is unresponsive to the presence of 3-oxo-C_{12}-HSL (Kylilis *et al.*, 2018; Wellington *et al.*, 2019). Hence, crosstalk is not an integral feature of *P. aeruginosa* QS circuitry, as this bacterium produces two AHLs structurally very distinct from one another. However, the promiscuity within QS systems may be relevant to the physiology of this bacterium in its natural environment and will be further explored in **subsection 1.3.1**.

1.2.3.8 Strains with impaired LasR activity are a common feature of *P. aeruginosa* populations

As mentioned earlier, individuals with CF face an increased susceptibility to chronic colonization by *P. aeruginosa*. Over time, the microbial communities in their lungs undergo evolution, resulting in the selection of adaptive traits tailored to this unique environment. Guided by this rationale, the consistent identification of *P. aeruginosa* strains with impaired LasR activity within the CF lung prompted the inference that these strains were systematically selected over time and were well-adapted to the CF lung environment (D'Argenio *et al.*, 2007; Hoffman *et al.*, 2009; Smith *et al.*, 2006). The selection of strains lacking LasR activity in chronic infection was, at first, counterintuitive, given that several virulence factors are regulated by the QS circuitry (refer to **subsection 1.2.2**), and LasR is generally considered the master regulator of this regulatory network (refer to **subsection 1.2.3.5**). However, the presence of LasR-defective strains is associated with heightened markers of inflammation, such as proinflammatory cytokines and neutrophil recruitment, worsening the pulmonary function of CF individuals (LaFayette *et al.*, 2015).

Importantly, we now know that the prevalence of *P. aeruginosa* LasR-deficient strains extends beyond the chronic colonization of CF lungs. A high prevalence of strains with impaired LasR is also observed in acute infections and non-clinical environments, including sinks, contaminated soils, and animal products (Groleau *et al.*, 2022; O'Connor *et al.*, 2022; Trottier *et al.*, 2024). The widespread emergence of these strains suggests that it is an adaptive feature within populations of *P. aeruginosa*, not confined to CF lungs but common to all sampled environments. Several factors driving this emergence have been proposed, and we have recently published a perspective paper on that subject (Trottier *et al.*, 2024). LasR-deficient cells exclusively emerge in populations also comprising cells with a functional *las* system, and one recurrent hypothesis proposes they act as “cheaters,” alleviating their metabolic burden by exploiting exoproducts produced by wildtype, cooperative cells (Diggle *et al.*, 2007; Köhler *et al.*, 2009; Sandoz *et al.*, 2007; West *et al.*, 2006). Cheating only occurs when exploitable exoproducts (“public goods”) are required for growth; however, LasR-defective strains also arise in conditions outside of this scope (Mould *et al.*, 2022).

In contrast to considering LasR-defective strains as mere opportunists, their presence can contribute to the overall fitness of the population. For instance, in an experiment employing directed evolution focused on social cell translocation, known as swarming motility, LasR-deficient variants are readily selected. The frequency of these variants rapidly rises to and stabilizes at

50%, and the swarmer colonies of the evolved, mixed population exhibit greater fitness than those comprised solely of WT or LasR-defective cells (Robitaille *et al.*, 2020). This concept gains support from the observation that WT and LasR-defective variants can engage in reciprocal cross-feeding, exchanging molecules that alter QS signalling and function (Mould *et al.*, 2020). Consequently, the coexistence of LasR-functional and LasR-defective strains within the *P. aeruginosa* population likely mutually benefits both subpopulations, whether through the direct interchange of QS-controlled exoproducts (i.e., cheating) and other molecules or functions, ultimately resulting in a more robust and adaptable population (Trottier *et al.*, 2024).

1.2.3.9 QS-controlled factors can be expressed in the absence of a functional LasR

Given that LasR activates both *rhl* and *pqs* systems in the prototypical strains PAO1 and PA14, there has been a prevailing assumption that *P. aeruginosa* LasR-defective strains would be inherently unable to regulate their behaviour through QS mechanisms. Contrary to this long-held notion, RhlR can serve as a surrogate activator of LasR-controlled genes in strains lacking LasR (i.e., *lasR* mutants) (Dekimpe *et al.*, 2009). This alternative regulation was demonstrated in the well-established PA14 QS regulatory background, indicating that the QS system is flexible, even when considering a heavily studied QS circuitry. Furthermore, similar behaviours are observed in naturally occurring LasR-deficient strains, where RhlR functions independently of LasR (Chen *et al.*, 2019a; Cruz *et al.*, 2020; Feltner *et al.*, 2016; Kostylev *et al.*, 2019; Morales *et al.*, 2017); these are referred to as RhlR-active independently of LasR strains (RAIL) (Groleau *et al.*, 2022). Such strains do not have a specific ecological niche, and they have been isolated alongside wildtype and LasR-defective strains from both environmental and clinical settings, including the CF lungs (Feltner *et al.*, 2016; Groleau *et al.*, 2022; Trottier *et al.*, 2024).

The mechanisms underlying the existence of RAIL strains among LasR-defective variants remain unclear, and they are often associated with the reprogramming of the QS regulatory network. However, considering the architectural diversity within the QS circuitry, associating the QS circuitry of RAIL strains with a “reorganization” should be questioned. Are they reorganized, or is this perspective merely a reflection of what is known about prototypical strains? While the answers to these questions are yet unknown, it is important to consider the possibility of an alternative organization of the QS regulatory network independent of LasR, wherein RhlR acts as the QS master regulator. In addition to this, evidence indicates that the sustained RhlR activity of RAIL strains depends on the functioning of the *pqs* system, thanks to the activity of PqsE, suggesting

similarity to the canonical QS circuitry (refer to subsection **1.2.3.4**) (Chen *et al.*, 2019a). Furthermore, although not all RAIL strains necessarily represent the reprogramming of QS responses, this system can be reprogrammed in the prototypical strain PAO1. In this strain, RhlR activity can be rapidly uncoupled from LasR regulation in a *lasR* background when QS-responsiveness is required for growth under laboratory conditions (Kostylev *et al.*, 2019).

1.2.3.10 Targeting quorum sensing as an anti-*Pseudomonas* strategy

The fundamental aspects of the QS regulatory network in *P. aeruginosa* have been introduced, highlighting that this communication system regulates the expression of several virulence factors produced by this bacterium simultaneously (from **subsection 1.2.3.1** to **1.2.3.9**). QS disruption, termed quorum quenching (QQ), is an attractive antivirulence strategy against *P. aeruginosa* (Elfaky, 2024; Grandclément *et al.*, 2016; Zhu *et al.*, 2013). Enzymes and chemical compounds have been reported to mediate QS disruption, often by cleaving signalling molecules or acting as competitive inhibitors. QQ enzymes deactivate QS signals, while chemicals disrupting QS pathways are QS inhibitors (QSIs) (Grandclément *et al.*, 2016). This diversity allows various approaches to disturb communication systems, often using naturally occurring compounds from prokaryotic or eukaryotic origins.

Bacteria frequently employ mechanisms to degrade QS signals, recycling their self-produced molecules or interfering with those produced by nearby competing bacterial cells. Lactonases and acylases can cleave AHLs, hindering their activity, and these molecules can also be modified by AHLs by AHL oxidoreductases (LaSarre *et al.*, 2013). Examples of the three types of QQ enzymes have been reported to affect AHL-based communication in *P. aeruginosa* (Hemmati *et al.*, 2020). Lactonases target the homoserine lactone ring of AHL, a common feature of this class of signalling molecules (refer to **Fig. 1.1.3**), conferring an overall broad AHL specificity to these enzymes (Liu *et al.*, 2008). The mechanism of action of lactonases hinders the development of targeted anti-*Pseudomonas* approaches based on their activity, even if some have been shown to reduce virulence traits of this bacterium (Dong *et al.*, 2018; Reimmann *et al.*, 2002; Tang *et al.*, 2015).

Unlike lactonases, the acyl chain length and substitutions at the 3-C position drive substrate selectivity in AHL acylases (refer to **Fig. 1.1.3**) (Bokhove *et al.*, 2010). These enzymes hydrolyze the acyl-amide bond between the acyl chain and the homoserine lactone ring, releasing a fatty acid chain and a homoserine lactone moiety. *P. aeruginosa* encodes four acylases homologues (i.e., PvdQ, HacB, QuiP, and PA14_40040 – an uncharacterized protein to date) (Bokhove *et al.*, 2010; Hemmati *et al.*, 2020; Huang *et al.*, 2006; Wahjudi *et al.*, 2011). While these characterized

acylases degrade the AHL 3-oxo-C_{12}-HSL, their impact on virulence is not comparable. For instance, the exogenous addition or the overexpression of PvdQ attenuates the virulence of *P. aeruginosa* towards *Caenorhabditis elegans*, but the impact of HacB in this behaviour is limited (Papaioannou *et al.*, 2009; Wahjudi *et al.*, 2011). AiiD, an acylase isolated from *Ralstonia*, has a similar effect to PvdQ on *P. aeruginosa* virulence (Lin *et al.*, 2003).

The AHL oxidoreductases do not degrade these signalling molecules, instead modifying the structure of the acyl side chain. The oxidation or reduction of this moiety renders the molecule inactive. Data on these enzymes are not yet abundant, but their QQ potential has also been reported for *P. aeruginosa* (Bijtenhoorn *et al.*, 2011).

The functionality of QQ enzymes, which deactivate signalling molecules to quench the communication system, can be explored in other ways to develop antivirulence therapies. For example, antibodies can also target autoinducers, leading to their degradation. A chimeric antibody aimed at 3-oxo-C_{12}-HSL demonstrated cross-reactivity with C_4-HSL, the two AHL signals produced by *P. aeruginosa*. Administration of the antibody notably increased survival rates in both *C. elegans* and mouse infection models in response to *P. aeruginosa* infections (Palliyil *et al.*, 2014).

The strategies discussed thus far focus on diminishing the activity of signalling molecules, but not all QSIs function this way. There are alternative mechanisms within QSIs offering different approaches to disrupt QS systems. For example, blocking the perception of QS signals is an attractive approach (LaSarre *et al.*, 2013). However, identifying compounds that effectively block QS communication poses a challenge. One approach is to investigate plant-derived compounds, leveraging the long-established medical properties of plants. Several plant extracts target the communal behaviours of *P. aeruginosa*, as outlined in (Hemmati *et al.*, 2020), underscoring the potential of this approach. Additionally, screening antivirulence compounds using a library of synthetic compounds has shown promise against *P. aeruginosa* (Borlee *et al.*, 2010; Hossain *et al.*, 2020; Müh *et al.*, 2006; Smith *et al.*, 2003; Starkey *et al.*, 2014). Upon identifying an active compound, understanding its structure-activity relationship enables activity optimization (Fong *et al.*, 2017; Singh *et al.*, 2022; Starkey *et al.*, 2014).

Challenges inevitably arise when targeting *P. aeruginosa* virulence through its communication system, regardless of the chosen strategy. Achieving specificity becomes a primary concern, given the potential to preserve patient microbiota during treatment (Elfaky, 2024). Specificity depends on selecting the right target. The *las* system has been a common choice in many studies due to its central role in the QS regulatory network of prototypical strains. However, as

emphasized in **subsection 1.2.3.8**, a significant portion of the population comprises LasR-deficient strains, making targeting this system less effective across the entire *P. aeruginosa* population. In contrast, mutations in the *rhl* system are rare in both environmental and clinical isolates of this opportunistic pathogen, suggesting that targeting the *rhl* system could be a highly effective antivirulence approach against *P. aeruginosa* infections (Trottier *et al.*, 2024). Additionally, the requirement of PqsE for RhlR activity (as discussed in **subsection 1.2.3.4**) offers an advantage in selecting this system, given its high specificity to *P. aeruginosa* (Trottier *et al.*, 2024). Nonetheless, developing effective strategies necessitates a comprehensive understanding of this bacterium's QS system, underscoring the importance of ongoing research in this field.

1.3 Beyond QS regulation: How *P. aeruginosa* adapts to environmental conditions

As previously mentioned, *P. aeruginosa* exhibits remarkable versatility and adaptability (refer to **subsection 1.2.1**), a trait intricately linked to a complex network of sensory, metabolic, and regulatory proteins. Regulation factors occupy a significantly larger proportion of large bacterial genomes (Cases *et al.*, 2003). By comparison, the "typical" bacterial genome consists of approximately 5 million base pairs, while the genome of the prototypical *P. aeruginosa* PA14 strain has 6.5 million base pairs – ranging from 5.5 to 7 million base pairs in other *P. aeruginosa* isolates (Land *et al.*, 2015; Lee *et al.*, 2006a; Schmidt *et al.*, 1996). This large genome aligns with its free-living lifestyle. In essence, free-living organisms encounter dynamic environments, and the absence of environmental stability is reflected in an enriched set of regulatory proteins (Cases *et al.*, 2003). This expanded regulatory capacity enables them to effectively respond and integrate various environmental signals. This adaptability is especially relevant for opportunistic pathogens, like *P. aeruginosa*, which face drastic environmental changes. *P. aeruginosa* is a saprophyte that transitions to a pathogenic state when it encounters a suitable host with compromised natural barriers. The characteristics of these niches differ significantly, necessitating physiological adjustments for a successful transition between them.

This section will focus on how *P. aeruginosa* perceives surfaces and temperature changes – environmental cues routinely encountered by this bacterium. Notably, these factors are fundamental to the chronic infection of the CF lungs. In this context, *P. aeruginosa* establishes surface-attached communities and thrives at mammalian body temperature, which significantly differs from environmental temperatures. Following the introduction of how these factors are sensed, their known interconnection with the QS regulatory network will be explored.

1.3.1 Understanding sessile growth: Biofilms and the contrast with the planktonic lifestyle

In their natural environment, bacteria predominantly adopt a non-motile, sessile lifestyle within structures known as biofilms (Costerton *et al.*, 1987; Costerton *et al.*, 1999; Flemming *et al.*, 2019). Biotic and abiotic surfaces are susceptible to colonization, leading to the development of mature biofilms – a structured polymicrobial community encased in a self-produced polymeric matrix (Rumbaugh *et al.*, 2020). This matrix structure facilitates exchanges within the microbial community, including the sharing of molecules that mediate the QS regulatory network. As mentioned earlier, signal promiscuity is observed in LasR and the orphan LuxR-protein QscR, allowing them to respond to non-self autoinducers. This promiscuity might be a significant modulator of bacterial physiology, enabling the regulation of genes under QS control to be responsive to the autoinducers produced by neighbouring cells.

In *P. aeruginosa*, biofilm formation is a finely regulated process encompassing the transition from planktonic cells (which is referred to as planktonic lifestyle or planktonic mode of growth) to a surface-associated lifestyle (Rumbaugh *et al.*, 2020). Notably, the planktonic and sessile growth modes exhibit fundamental distinctions, each associated with a unique gene expression profile (Rumbaugh *et al.*, 2020). Sessility induces the production of the biofilm matrix components, including exopolysaccharides, proteins, DNA, and RNA (Flemming *et al.*, 2010; Mugunthan *et al.*, 2023). This intricate structure provides mechanical stability and overall cohesion to the bacterial community, interconnecting and immobilizing individual cells within it, and various other population-wise benefits (Flemming *et al.*, 2010).

Beyond structural differences, the divergence between these lifestyles encompasses an array of variations. For instance, the colonization of a surface entails not only the mechanical perception of the surface itself but also an intrinsic heterogeneity within the bacterial population. The spatial arrangement of individual cells determines the availability of factors instrumental for life maintenance, such as nutrients and oxygen (Davey *et al.*, 2000). This spatial distribution is directly related to the social, multicellular aspect of sessility, facilitating community-level responses over individual ones. While planktonic individuals can also cooperate, this collaboration is not an intrinsic determinant of their lifestyle.

1.3.1.1 Surface sensing: Bacteria and their sensitivity to touch

As mentioned earlier, sessility is the predominant bacterial lifestyle. The ecological success of this growth mode relies on the capacity of bacteria to sense physicochemical stimuli upon encountering a solid substratum and appropriately respond to these cues, adapting both physiology and behaviour (Laventie *et al.*, 2020). An illustration of this adaptive behaviour is evident in the way individuals move. Planktonic cells, not adapted to life on surfaces, utilize their flagellum for displacement. In contrast, on semi-solid and solid surfaces, bacteria employ either a combination of the flagellum with a wetting agent or the type IV pilus (T4P), resulting in behaviours known as swimming, swarming, and twitching, respectively (Wadhwa *et al.*, 2022).

Mechanosensation, or surface sensing, denotes the mechanisms that translate a physical signal (such as encountering a surface) into biochemical outputs. The perception of initial attachment to surfaces enables individuals to adapt to this newly colonized environment, a process known as surface adaptation, culminating in the formation of bacterial biofilms (Laventie *et al.*, 2020) (**Fig. 1.3.1**). As illustrated, this multicellular structure formation is sequential, initiating with the adhesion of free-floating individuals to a solid substratum. Upon encounter, bacteria adhere reversibly to the substratum, facilitated by their flagellum (Tuson *et al.*, 2013). In *P. aeruginosa*, rapid surface sensing is triggered by the flagellar motor (via MotAB), promptly elevating the intracellular concentration of the second messenger bis-(3′, 5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (Schniederberend *et al.*, 2019). Within minutes after contact, T4P-mediated surface sensing becomes active, initiating both cAMP- and c-di-GMP-dependent signalling pathways (Luo *et al.*, 2015; Persat *et al.*, 2015). Thus, the timescale determines the predominant mechanism of surface sensing in this bacterium: rapid (seconds) responses are driven by the flagellar motor-dependent surface sensing, whereas a prolonged response (minutes to hours) relies on T4P-mediated surface sensing. Notably, in *P. aeruginosa*, the flagellum and T4P are localized within the cell pole(s) (Bouteiller *et al.*, 2021; Jain *et al.*, 2012), inducing the local production of c-di-GMP or both c-di-GMP and cAMP.

1.3.1.2 Surface adaptation: How do second messenger signalling pathways modulate bacterial behaviour?

Surface sensing activates the c-di-GMP and cAMP signalling pathways. Bacterial adaptation in response to these second messengers will be discussed individually, and **Figure 1.3.1** offers a comprehensive overview of their roles in bacterial behaviour.

Upon surface sensing, the flagellar motor-dependent system rapidly produces c-di-GMP in response to its reduced rotational speed (Tuson *et al.*, 2013). These planktonic cells, recently attached to the surface, are not conditioned to the use of their pilus machinery, which is exemplified by their relatively low activity (O'Toole *et al.*, 2016). c-di-GMP allosterically induces T4P biogenesis and activity, thereby enabling the function of the T4P-dependent surface sensing system (Jain *et al.*, 2017). This system comprises at least two mechanosensors, PilA (the major pilin subunit) and PilY1. While PilA, upon surface sensing, mediates the elevation of cAMP concentration, PilY1 is linked to the modulation of c-di-GMP. PilY1, functioning as an adhesin associated with T4P, independently influences c-di-GMP production in response to surface sensing (Heiniger *et al.*, 2010; Luo *et al.*, 2015). This protein resides on the outer leaf of the outer membrane, and although the mechanism remains uncharacterized, this localization is important for inducing c-di-GMP production via the inner membrane-associated diguanylate cyclase SadC (Kuchma *et al.*, 2010; Luo *et al.*, 2015). This cyclic second messenger is a global inducer of sessility, reducing flagellar rotation and promoting the synthesis of several adhesins and EPS through FleQ (Matsuyama *et al.*, 2016).

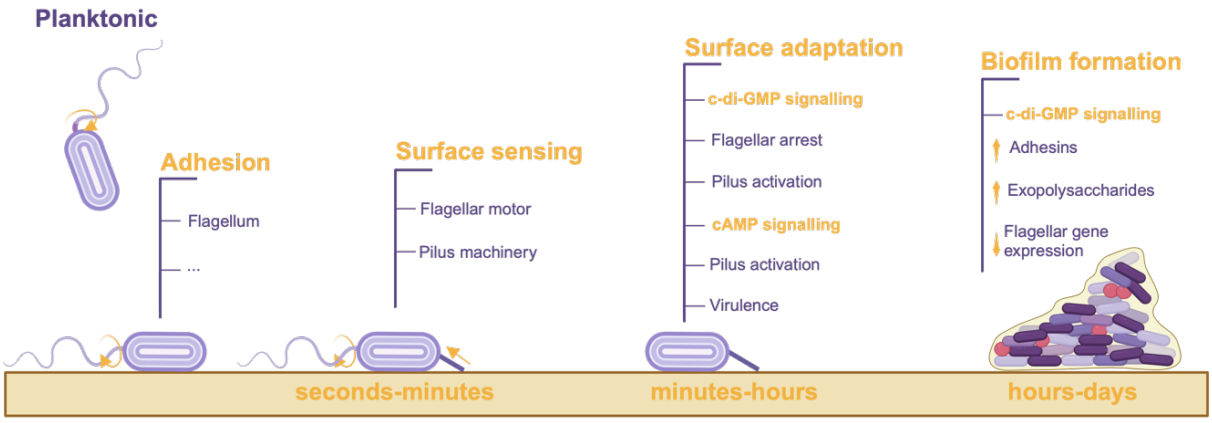

Figure 1.3.1. Biofilm formation unfolds in a series of steps, beginning with the adhesion of bacteria to a surface. The flagellar motility facilitates the encounter of planktonic *P. aeruginosa* individuals to the substratum. The attachment reduces the rotational speed of the flagellar motor, enabling bacteria to sense the substratum; within seconds, this response is perceived, culminating in the production of c-di-GMP. Within a longer timescale (minutes to hours), the surface sensing driven by the T4P also alters the intracellular production of c-di-GMP and cAMP. The T4P also coordinates the mechanosensor PilY1 that, once secreted, activates the production of c-di-GMP. This second messenger is involved in the positive regulation of surface adhesins and in arresting flagellar activity, thus inducing cellular sessility. cAMP positively modulates the expression of virulence factors through the cAMP-dependent transcriptional factor Vfr, and the mechanosensor PilY1. Together, these signalling pathways favour biofilm formation by inducing the presence of adhesins and EPS while inhibiting the activity of the flagellum. Importantly, cAMP signalling is not active in established sessile communities as its levels drop to basal planktonic levels approximately 4h post-adhesion. Adapted from (Laventie *et al.*, 2020) and created using Biorender.

As mentioned, the intracellular concentration of cAMP increases in a PilA-dependent manner. Under tension, PilA adopts a specific stretched conformation that signals surface sensing, inducing cAMP production via CyaB (Fulcher *et al.*, 2010; Persat *et al.*, 2015). CyaB mediates the production of this cyclic nucleotide derived from ATP through the Chp system, and cycles of extension and retraction of T4P amplify the cAMP concentration (Luo *et al.*, 2015; Persat *et al.*, 2015). The genes involved in the biogenesis of T4P are induced by Vfr-cAMP (Wolfgang *et al.*, 2003), creating a positive feedback loop. Several virulence factors, as well as secretion systems, such as T2SS, T3SS, exotoxins, and proteases, are also induced by Vfr-cAMP (refer to **subsection 1.2.2** and **Figure 1.2.1** for an overall view of *P. aeruginosa* virulence factors). Notably, this transcriptional complex indirectly induces the expression of PilY1 via the two-component system FimS/AlgR (Belete *et al.*, 2008; Luo *et al.*, 2015). This cAMP-dependent signalling regulates the transition of growth modes, as the concentration of cAMP gradually

increases in response to adhesion but returns to basal planktonic levels after (time window starts from minutes to 4h post-adhesion) (Luo *et al.*, 2015; Persat *et al.*, 2015).

The interaction between the second messengers c-di-GMP and cAMP during surface sensing is intricate, and there are several unanswered questions in *P. aeruginosa* mechanosensing. Significantly, this interplay unravels the distinct gene expression profiles associated with planktonic and sessile growth modes, emphasizing caution when comparing gene expression patterns between these lifestyles.

1.3.2 Thermal control of bacterial behaviour: *P. aeruginosa* grows in a wide range of temperatures

Some environmental cues, such as temperature, are inherently variable. Organisms must sense these variations and integrate them, enabling appropriate responses. In the case of opportunist pathogens infecting warm-blooded hosts, the sense of elevated temperatures serves as a signal of their presence within hosts, consequently inducing the expression of virulence genes (Shapiro *et al.*, 2012). However, the optimal range for growth might encompass more significant variations. This is the case of *P. aeruginosa*, which grows in temperatures ranging from 15 to 42°C (Tribelli *et al.*, 2022).

The impact of transitioning from the environment to the human body on the gene expression profile of *P. aeruginosa* has been investigated. Studies compared the transcriptional profiles of cells grown at a lower temperature (representing environmental conditions at 28°C and 22°C) to those grown at 37°C, reflecting the human body temperature (Barbier *et al.*, 2014; Wurtzel *et al.*, 2012). The temperature shift regulates a significant fraction of this bacterium's genes, accounting for approximately 6.5% (Barbier *et al.*, 2014; Wurtzel *et al.*, 2012). Notably, genes associated with energy metabolism are downregulated at environmental temperatures, while those linked to virulence are upregulated at 37°C. Importantly, this transition is not perceived as a heat shock, as neither heat-shock proteins nor chaperones exhibit differential regulation (Barbier *et al.*, 2014; Wurtzel *et al.*, 2012).

1.3.2.1 Thermo-adaptation requires sensing signals: How is temperature sensed?

Bacteria commonly employ a regulatory mechanism based on RNA thermometers to modulate gene expression in response to temperature changes (Tribelli *et al.*, 2022). RNA thermometers are RNA control elements positioned in the 5' untranslated region (5'UTR) of mRNA transcripts. Gene translation relies on ribosome binding to the SD sequence near the AUG start codon. At lower temperatures, the RNA thermometer forms a hairpin structure involving both the SD and the AUG start codon, hindering gene translation. As temperature rises, the stability of this structure decreases, enabling translation by releasing the SD sequence (Shapiro *et al.*, 2012). Importantly, RNA thermometers respond readily to temperature variations, and the gradual temperature increase promotes gene expression by altering the proportion of mRNAs with a destabilized hairpin. The most prevalent class of RNA thermometers is represented by the repression of heat shock gene expression (ROSE) elements (Shapiro *et al.*, 2012; Tribelli *et al.*, 2022).

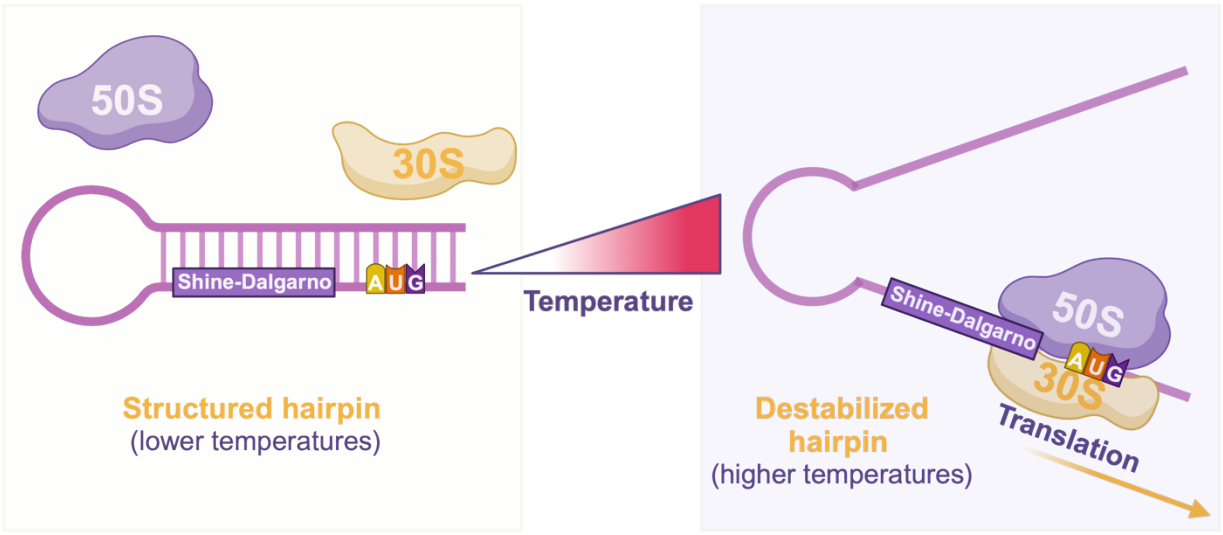

Figure 1.3.2. Principles of temperature-responsive RNA elements.

RNA thermometers are located in the 5' untranslated region (5'UTR) of mRNA molecules. In the left panel, at lower temperatures, the Shine-Dalgarno (SD) sequence and the AUG codon start are paired, forming a structured hairpin that includes these sequences. The ribosomal subunits 50S and 30S remain disassociated, as the access to the mRNA molecule is physically blocked, preventing ribosomal binding. In the right panel, at higher temperatures, the strength of base paring diminishes, leading to the destabilization of the hairpin structure. Once the structure is compromised, the ribosomal binding site (SD) sequence becomes accessible, enabling the binding of both ribosomal subunits. Translation initiates at the start codon. Adapted from (Shapiro *et al.*, 2012) and created using Biorender.

Other macromolecules can also detect temperature fluctuations. For instance, the topology of DNA and the fluidity of the membrane function as mechanisms to sense and respond to these variations. Compared to RNA thermometers, more pronounced temperature fluctuations are necessary to activate these systems. These significant variations would also trigger the expression of chaperones and heat-shock proteins. As mentioned earlier, the transition from the environment to a warm-blooded host does not prompt a protein-based temperature response in *P. aeruginosa* (Shapiro *et al.*, 2012; Tribelli *et al.*, 2022), indicating that the tested temperature variations might not be substantial enough to induce such responses. Importantly, these responses require downstream processes, such as transcription and translation, and the bacterial responses are not readily observed in response to the stimulus.

1.3.3 Surface sensing: Known interconnections with the QS regulatory network

Cell adhesion to surfaces induces multicellular, social behaviours. In earlier sections of this document, these social aspects were illustrated through examples such as biofilm formation and the coordinated movement of cells on semi-solid surfaces (swarming). Notably, the QS regulatory network is among the regulatory mechanisms that govern these behaviours. For instance, cell-to-cell communication is required for biofilm development, and also for the production of the wetting agent that supports swarming motility. However, despite the QS circuitry of *P. aeruginosa* being extensively studied, most of these investigations were conducted in batch liquid cultures. Consequently, the QS regulatory network outlined in **subsection 1.2** represents the organization of this system under planktonic culture conditions, without an understanding of its relevance in surface-associated cells. Still, limited current evidence strongly suggests that the *las* and *rhl* systems exhibit distinct responses upon surface association compared to their planktonic counterparts.

Surface association alters the responsiveness of the *las* system to its cognate signal 3-oxo-C_{12}-HSL by promoting the expression of *lasR* (Chuang et al., 2019). Consequently, the same concentration of this molecule elicits a more robust response in surface-associated cells compared to planktonic ones (Chuang *et al.*, 2019). Similarly, the *rhl* system exhibits distinct behaviour under these growth conditions. In planktonic cells, the RhlI regulon governs nearly 60% of the genes regulated by RhlR, whereas, in surface-associated conditions, this proportion drops significantly to 13% (Mukherjee *et al.*, 2017). The number of genes under RhlR regulation is comparable between the growth conditions, suggesting that surface association somehow

diminishes the necessity of RhlI for RhlR activity (Mukherjee *et al.*, 2017). A parallel observation was reported regarding the modulatory role of PqsE in the activity of an RhlR variant (RhlR*): while PqsE is necessary to activate RhlR* in planktonic cells, on surfaces, PqsE becomes dispensable for pyocyanin production in cells expressing RhlR* (McCready *et al.*, 2019).

These findings provide specific examples suggesting that the QS regulatory network is context-specific. The extent of similarity between this circuitry in surface-grown cells and planktonic ones remains undetermined. Subsequent research endeavours will contribute to clarifying whether the surface-associated QS circuitry necessitates substantial rewriting or if it broadly aligns with the planktonic configuration.

1.3.4 Temperature perception and its interplay with the QS regulatory network

As discussed in **subsection 1.2.1**, the QS regulatory system governs a significant set of virulence factors in *P. aeruginosa*. When growing at temperatures mimicking human infection conditions, the expression of virulence determinants, including those under QS control, is induced (Barbier *et al.*, 2014; Wurtzel *et al.*, 2012). This behaviour aligns with the evolutionary adaptation of opportunistic pathogens (Brown *et al.*, 2012). Nevertheless, the precise molecular mechanisms through which temperature cues integrate with the QS circuitry remain incompletely understood.

Among the QS circuits, the *rhl* system exhibits an enhanced responsiveness to temperature changes. RhlR directly regulates the expression of the *rhlAB* operon, situated upstream and near *rhlR*. In *P. aeruginosa*, a condition-specific polycistronic mRNA encompassing *rhlAB-rhlR* has been identified, establishing a positive feedback loop facilitated by RhlR (Croda-García *et al.*, 2011). An RNA thermometer located in the 5'UTR of the *rhlAB-rhlR* transcript induces the translation of RhlAB and RhlR at 37°C (Grosso-Becerra *et al.*, 2014). At this elevated temperature, the heightened concentration of this transcriptional regulator leads to increased gene expression under RhlR control compared to cells grown at lower temperatures (Grosso-Becerra *et al.*, 2014). In this regulation, two critical aspects deserve emphasis: the experimental confirmation of the theoretical positive feedback loop and the determination of the presence of this polycistronic transcript under various experimental conditions in *P. aeruginosa* cultures.

Located in the 5'UTR of the *lasI* transcript, another RNA thermometer has been proposed, prompting the expression of LasI at 37°C. However, the impact of growth temperatures on 3-oxo-C_{12}-HSL is relatively modest (Grosso-Becerra *et al.*, 2014), raising doubts about the significance of this regulation in the overall functioning of the *P. aeruginosa* QS regulatory network.

2 STATEMENT OF THE RESEARCH PROBLEM AND HYPOTHESIS

Targeting bacterial communication systems for anti-virulence therapies presents a promising alternative to traditional antimicrobials. However, effectively implementing such strategies demands a comprehensive understanding of these systems and their influence on bacterial behaviours. This explains the sustained interest of the scientific community in the QS circuitries of the opportunistic pathogen *P. aeruginosa*, serving as a model for studying bacterial communication systems and their impact on pathogenesis.

Numerous studies have enabled the schematization of *P. aeruginosa*'s three QS circuits – *las*, *rhl*, and *pqs* – and their intricate interactions. Ongoing research constantly refines our understanding of this regulatory system. For instance, the prevalence of LasR-defective strains was initially considered an adaptation to the chronically infected CF lungs. However, this notion has evolved, indicating a widespread adaptation feature of *P. aeruginosa*, with the underlying factors yet to be characterized. Significantly, this adaptation highlights the central role of RhlR in sustaining the responsiveness to communal behaviours via QS, even in the absence of a completely functioning *las* system.

The current model of the pathogenesis of *P. aeruginosa* incorporates factors under QS control and other communal behaviours, such as forming biofilms – surface-associated communities linked to a resistant profile. Within warm-blooded hosts, such as humans, these communities are additionally influenced by elevated temperatures. All these factors, namely QS, surface- and temperature-sensing, collectively drive the expression of virulence traits.

Despite evidence from various laboratories (detailed in **subsections 1.3.3** and **1.3.4**) suggesting an interconnection between surface and temperature sensing with the QS regulatory network, how these factors influence each other still needs to be better understood. This knowledge gap underscores the need for further exploration to unravel the interplay of surface sensing, temperature variations, and QS in shaping the virulence profile of *P. aeruginosa*.

2.1 Hypothesis

The working hypothesis posits that the QS regulatory network undergoes modulation in response to environmental conditions, specifically in relation to surface sensing and temperature variations.

2.2 General objectives

This project outlines two general objectives to address the proposed hypothesis:

I) Investigate the disparities in the QS regulatory network between planktonic and surface-grown bacterial cells. While this communication system regulates social behaviours in bacteria, its characterization has essentially focused on planktonic cells. This objective aims to understand how surface growth affects QS regulation in *P. aeruginosa*.

II) Explore the impact of temperature variation on the QS regulatory system. Environmental temperatures determine numerous physiological responses, and at the molecular level, they influence the stability of macromolecules such as proteins. Notably, the QS system of *P. aeruginosa* features a unique aspect, with the activity of the *rhl* system relying on the formation of a protein complex. This objective seeks to elucidate whether temperature variations can modulate the *rhl* system's activity.

3 SURFACE-DRIVEN REGULATION OF QS

3.1 Article: "Surface growth of *Pseudomonas aeruginosa* reveals a regulatory effect of 3-oxo-C_{12}-homoserine lactone in the absence of its cognate receptor, LasR"

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3.1.1 Abstract

Successful colonization of a multitude of ecological niches by the bacterium *Pseudomonas aeruginosa* relies on its ability to respond to concentrations of self-produced signal molecules. This intercellular communication system known as quorum sensing (QS) tightly regulates the expression of virulence determinants and a diversity of survival functions, including those required for social behaviours. In planktonic cultures of *P. aeruginosa*, the transcriptional regulator LasR is generally considered on top of the QS circuitry hierarchy; its activation relies on binding to 3-oxo-C_{12}-homoserine lactone (3-oxo-C_{12}-HSL), a product of the LasI synthase. Transcription of *lasI* is activated by LasR, resulting in a positive feedback loop. Few studies have looked at the function of QS during surface growth, even though *P. aeruginosa* typically lives in biofilm-like communities under natural conditions. Here, we show that surface-grown *P. aeruginosa*, including the prototypical strain PA14, produces 3-oxo-C_{12}-HSL in the absence of LasR. This phenotype is commonly observed upon surface association in naturally occurring environmental and clinical LasR-defective isolates, suggesting a conserved alternative function for the signal. Notably, in surface-grown cultures, 3-oxo-C12-HSL reaches higher levels compared to planktonic cells, and there is a delayed timing of its production. Accordingly, 3-oxo-C_{12}-HSL upregulates the autologous expression of pyocyanin and LasR-controlled virulence determinants in neighbouring cells, even in the absence of the cognate regulator LasR. This highlights a possible role for 3-oxo-C_{12}-HSL in shaping community responses and provides a possible evolutive benefit for mixed populations to carry LasR-defective cells, a common feature of natural populations of *P. aeruginosa*.

3.1.2 Importance

The bacterium *Pseudomonas aeruginosa* colonizes and thrives in many environments, in which it is typically found in surface-associated polymicrobial communities known as biofilms. Adaptation to this social behaviour is aided by quorum sensing (QS), an intercellular communication system pivotal in the expression of social traits. Regardless of its importance in QS regulation, the loss of function of the master regulator LasR is now considered a conserved adaptation of *P. aeruginosa*, irrespective of the origin of the strains. By investigating the QS circuitry in surface-grown cells, we found an accumulation of QS signal 3-oxo-C_{12}-HSL in the absence of its cognate receptor and activator, LasR. The current understanding of the QS circuit, mostly based on planktonic growing cells, is challenged by investigating the QS circuitry of surface-grown cells. This provides a new perspective on the beneficial aspects that underline the frequency of LasR-deficient isolates.

3.1.3 Introduction

Bacteria are social organisms that often respond to environmental cues in coordination. *Pseudomonas aeruginosa* is a highly adaptable Gram-negative bacterium that colonizes diverse ecological niches. The flexibility of this opportunist human pathogen is aided by several regulatory networks, assuring proper responses to changing environmental conditions. Quorum sensing (QS) is a gene expression regulation mechanism based on the production, release, detection and response to diffusible signalling molecules that synchronize the transcription of target genes in a population density-dependent manner (Fuqua *et al.*, 1994b). In *P. aeruginosa*, three interlinked QS systems regulate the expression of hundreds of genes – including several encoding virulence determinants (Azimi *et al.*, 2020). In this bacterium, QS regulation is structured as a hierarchical network composed of two *N*-acyl homoserine lactone (AHL)-based circuits (*las* and *rhl*) and the *pqs* system, that relies on signalling molecules of the 4-hydroxy-2-alkylquinoline (HAQ) family. The *las* and *rhl* systems comprise an AHL synthase (LasI and RhlI) responsible for the syntheses of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), respectively (Pearson *et al.*, 1994; Pearson *et al.*, 1995). These autoinducers activate their cognate LuxR-type transcriptional regulators – LasR and RhlR, which, in turn, can induce the transcription of target QS-regulated genes. Under standard laboratory conditions, the *las* system is generally considered to be atop the regulatory hierarchy. Once activated by the binding with its cognate autoinducer, LasR regulates several virulence traits, such as the elastase LasB (*lasB*) (Gambello *et al.*, 1991; Schuster *et al.*, 2004b). LasR also induces the transcription of the LasI synthase coding gene, creating a positive feedback loop (Seed *et al.*, 1995). The *pqs* system relies on the LysR-type transcriptional regulator MvfR (also known as PqsR) (Cao *et al.*, 2001; Gallagher *et al.*, 2002). The latter directly activates the operons *pqsABCDE* and *phnAB*, both required for HAQ biosynthesis and indirectly regulates the expression of many other QS-regulated genes via PqsE (Cao *et al.*, 2001; Déziel *et al.*, 2005; Déziel *et al.*, 2004; Farrow *et al.*, 2008; Groleau *et al.*, 2020; Letizia *et al.*, 2022). MvfR has dual ligands as it can be induced by 4-hydroxy-2-heptylquinoline (HHQ) and the *Pseudomonas* quinolone signal (PQS; 3,4-dihydroxy-2-alkylquinoline), both members of the HAQ family (Wade *et al.*, 2005; Xiao *et al.*, 2006a). The *rhl* and *pqs* circuits are directly and positively regulated by LasR, which induces the transcription of *rhlR* and *rhlI* as well as *mvfR* (de Kievit *et al.*, 2002; Groleau *et al.*, 2020; Pesci *et al.*, 1997; Wade *et al.*, 2005).

In addition to sensing the surrounding chemical environment, bacteria are also responsive to mechanical signals, such as those involved in the physical encounter of the cell with surfaces or

with each other. Indeed, several behaviours are specific to life on surfaces, including movement on semi-solid (swarming motility) and solid surfaces (twitching motility) as well as biofilm formation (Kearns, 2010; Mattick, 2002; O'Toole *et al.*, 2016). Not surprisingly, virulence is also induced by surface attachment, as many infection strategies require contact with the host (Galán *et al.*, 1999; Persat *et al.*, 2015; Siryaporn *et al.*, 2014). Even though QS and surface-sensing regulate many of the same social behaviours, little is known about how these different regulatory cues converge to modulate bacterial responses. Exploring the link between surface-sensing and QS is particularly relevant as *P. aeruginosa* readily adopts a surface-attached mode of growth as biofilms in its natural habitats. Biofilms are organized communities encased in a self-produced exopolymeric matrix. In the context of infections, biofilms contribute to host immune evasion and delay antibiotic penetration (Alhede *et al.*, 2014; Williams *et al.*, 2010). In fact, *P. aeruginosa* persists as biofilms in the lungs of people with cystic fibrosis, a genetic disease (Moreau-Marquis *et al.*, 2008).

While the emergence of LasR-defective mutants has long been associated with adaptation to the CF lung environment (D'Argenio *et al.*, 2007; Feltner *et al.*, 2016; Hoffman *et al.*, 2009; Smith *et al.*, 2006), it is actually a common feature of *P. aeruginosa* from diverse environments (Groleau *et al.*, 2022; O'Connor *et al.*, 2022). Interestingly, some LasR-defective isolates, known as RhlR active independently of LasR (RAIL), retain a functional RhlR regulator (Asfahl *et al.*, 2022; Chen *et al.*, 2019a; Cruz *et al.*, 2020; Feltner *et al.*, 2016; Groleau *et al.*, 2022; Kostylev *et al.*, 2019). Their sustained QS responses are in line with our previous report showing that in the presence of a nonfunctional LasR, RhlR acts as a surrogate activator for a set of LasR-regulated genes (Dekimpe *et al.*, 2009). It is noteworthy that in the wild-type *P. aeruginosa* strain PA14 background, surface-sensing upregulates *lasR* and that surface-grown cells induce LasR targets more strongly than their planktonic counterpart (Chuang *et al.*, 2019). Thus, surface-sensing appears to sensitize cells to the cognate autoinducer 3-oxo-C_{12}-HSL. Considering the prevalence of LasR-defective mutants, which neither produce nor respond to 3-oxo-C_{12}-HSL, we wondered how *P. aeruginosa* would respond to surface attachment, as biofilm formation is essential to bacterial physiology and pathology.

In this study, we investigated the effect of surface sensing on QS responses of LasR-defective strains. We found that upon surface attachment, LasR becomes dispensable to the production of 3-oxo-C_{12}-HSL. This response is conserved among naturally occurring environmental and clinical LasR-defective isolates. Production of 3-oxo-C_{12}-HSL modulates the production of virulence factors at the individual (LasR-defective background) and community levels (mixed with LasR-

responsive cells). We propose that the production of 3-oxo-C_{12}-HSL by LasR-negative cells, modulating biological bacterial responses on diverse levels, has a positive role in shaping community responses of the population.

3.1.4 Materials and Methods

3.1.4.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in **Table 3.1.1** and **Table 3.1.2**, respectively. The oligonucleotides used are listed in **Table 3.1.3**. Bacteria were routinely grown in tryptic soy broth (TSB; BD Difco, Canada) at 37°C in a TC-7 roller drum (NB, Canada) at 240 rpm or on Lysogeny broth (LB; BD Difco, Canada) agar plates. For quantification of QS-signalling molecules and related data, King's A broth (planktonic growth) or King's A agar (surface-associated growth) supplemented with 100 µM $FeCl_3$ was used (King *et al.*, 1954). For the latter, sterile King's A agar was poured into each well of a 96-well plate (200 µl per well) and allowed to solidify at the center of a biosafety cabinet. When needed, the following concentrations of antibiotics were included: for *Escherichia coli* 100 µg/ml carbenicillin, 15 µg/ml gentamicin, and 15 µg/ml tetracycline were used; diaminopimelic acid (DAP) was added to cultures of the auxotroph *E. coli* χ 7213 at 62.5 µg/ml; Irgasan (20 µg/ml) was used as a counter-selection agent against *E. coli*. For *P. aeruginosa*, 300 µg/ml carbenicillin, 30 µg/ml gentamicin, and tetracycline at 125 µg/ml (solid) or 75 µg/ml (liquid).

Table 3.1.1. Strains used in this study

Strain	Lab ID #	Relevant genotype or description	Reference
P. aeruginosa			
PA14	ED14	Clinical isolate from a human burn patient UCBPP-PA14	(Rahme *et al.*, 1995)
PA14 Δ*lasR*	ED4409	PA14 derivate; unmarked in-frame *lasR* deletion	This study
PA14 Δ*lasI*	ED4539	PA14 derivate; unmarked in-frame *lasI* deletion	(Hogan *et al.*, 2004)
PA14 Δ*rhlR*	ED4406	PA14 derivate; unmarked in-frame *rhlR* deletion	This study
PA14 *lasR*$^-$ *rhlR*$^-$	ED266	PA14 derivate; marked deletion of *lasR* (*lasR*::Gm) and *rhlR* (*rhlR*::Tc)	(Dekimpe *et al.*, 2009)

PA14 Δ*lasR* Δ*rhlI*	ED4541	PA14 derivate; unmarked in-frame double *lasR* and *rhlI* deletion	This study
PA14 *lasR*⁻ Δ*pqsE*	ED247	PA14 derivate; marked deletion of *lasR* (*lasR*::Gm) and an unmarked *pqsE* deletion	(Groleau *et al.*, 2020)
PA14 Δ*lasR* Δ*lasI*	ED4540	PA14 derivate; unmarked in-frame double *lasR* and *lasI* deletion	This study
PA14 Δ*lasR* Δ*rhlR*	ED4545	PA14 derivate; unmarked in-frame double *lasR* and *rhlR* deletion	This study
PA14 Δ*lasR* Δ*lasI* Δ*rhlI* *attB*::CTX *phzA1-lux*	ED4544	PA14 derivate; unmarked in-frame triple *lasR, lasI* and *rhlI* deletion carrying the chromosomal *phzA1-lux* reporter	This study
PA14 Δ*lasR* Δ*lasI* *attB*::CTX *phzA1-lux*	ED4591	PA14 derivate; ED4540 carrying the chromosomal *phzA1-lux* reporter	This study
PA14 Δ*lasI* *attB*::CTX *lasB-lux*	ED4543	PA14 derivate; ED4539 carrying the chromosomal *lasB-lux* reporter	This study
PA14 Δ*lasR* *attB*::CTX *lasI-lux*	ED4542	PA14 derivate; ED4409 carrying the chromosomal *lasI-lux* reporter	This study
PA14 Δ*lasR* Δ*pilT*	ED4556	PA14 derivate; unmarked in-frame double *lasR* and *pilT* deletion	This study
PA14 Δ*lasR* *pilU*	ED4557	PA14 derivate; unmarked in-frame *lasR* and marked *pilU* mutant (*pilU::*MrT7)	This study
18G	ED4592	Oil-contaminated soil	(Déziel *et al.*, 1996)
32R	ED4593	Oil-contaminated soil	(Déziel *et al.*, 1996)
78RV	ED4590	Oil-contaminated soil	(Déziel *et al.*, 1996)
E41	ED4160	Cystic fibrosis isolate	(Feltner *et al.*, 2016)
E113	ED4144	Cystic fibrosis isolate	(Feltner *et al.*, 2016)

E167	ED4152	Cystic fibrosis isolate	(Feltner *et al.*, 2016)
E. coli			
SM10(λ*pir*)	ED222	*thi thr leu tonA lacY supE recA*::*RP4-2*-Tc::*Mu* Km λ*pir*	Lab collection
χ 7213	ED743	*thr-1 leuB6 fhuA21 lacY1 glnV44 recA1* Δ*asdA4* Δ(*zhf-2*::Tn*10*) *thi-1 RP4-2*-Tc::*Mu*[λ *pir*]	Lab collection

Table 3.1.2. Plasmids used in this study

Plasmid	Description	Reference or source
pTOP01	pEX18Ap-Δ*lasR*; gene replacement vector for the in-frame deletion of *lasR* by allelic recombination, Carb^r	This study
pTOP02	pEX18Ap-Δ*rhlR;* gene replacement vector for the in-frame deletion of *rhlR* by allelic recombination, Carb^r	This study
pTOP03	pEX18Ap-Δ*rhlI*; gene replacement vector for the in-frame deletion of *rhlI* by allelic recombination, Carb^r	This study
pTOP04	pEX18Ap-Δ*pilT*; gene replacement vector for the in-frame deletion of *pilT* by allelic recombination, Carb^r	This study
pEX18Gm-Δ*lasI*	Gene replacement vector for the in-frame deletion of *lasI* by allelic recombination, Gm^r	(Hogan *et al.*, 2004)
pTOP05	Promoter of *lasI* in mini-CTX-*lux*, Tet^r	This study
pCDS101	Promoter of *phz1* in mini-CTX-*lux*, Tet^r	(Sibley *et al.*, 2008)
pCTX-1-P$_{lasB}$-*lux*	Promoter of *lasB* in mini-CTX-*lux*, Tet^r	(Cabeen, 2014)
pME3853	*lasI'–'lacZ* translational fusion, Tet^r	(Pessi *et al.*, 2001)

Table 3.1.3. Oligonucleotides used in this study

Name	Sequence (5'-3')	Description
TO_lasR_up_fwd	taaaacgacggccagtgccaAACTGGAAAAGTGGCTATG	Designed to construct in-frame *lasR* deletion by cloning PCR-amplified fragments into previously digested pEX18Ap vector (HindIII and BamHI)
TO_lasR_up_rev	gccgaatattCATAGCGCTACGTTCTTC	
TO_lasR_down_fwd	tagcgctatgAATATTCGGCGGAAGTTCG	
TO_lasR_down_rev	attcgagctcggtacccgggAACTTGTGCATCTCGCCC	
rhlR_up_RE_fwd	taaaacgacggccagtgccaGGTACACCCCAAGTTCAAC	Designed to construct in-frame *rhlR* deletion by cloning PCR-amplified fragments into previously digested pEX18Ap vector (HindIII and BamHI)
rhlR_up_RE_rev	cttcttctggCATTGCAGTAAGCCCTGATC	
rhlR_down_RE	tactgcaatgCCAGAAGAAGTTCGACGCG	
rhlR_down_RE	attcgagctcggtacccgggCTGGACGAGATGGCGGAATG	
RE_rhlI_up_fwd	taaaacgacggccagtgccaTGATTTTGCCGTATCGGC	Designed to construct in-frame *rhlI* deletion by cloning PCR-amplified fragments into previously digested pEX18Ap vector (HindIII and BamHI)
RE_rhlI_up_rev	gcagccattcCATGACCAAGTCCCCGTG	
RE_rhlI_down_fwd	cttggtcatgGAATGGCTGCAGGGTGTAC	

RE_rhlI_down_rev	attcgagctcggtacccgggGTACTACTTGCCGTGCGC	
pilT_up_fwd	taaaacgacggccagtgccaGATTCCTTCAAGAAGCCG	Designed to construct in-frame *pilT* deletion by cloning PCR-amplified fragments into previously digested pEX18Ap vector (HindIII and BamHI)
pilT_up_rev	gccaggatcaCATGGGACTCCCCAATTAC	
pilT_down_fwd	gagtcccatgTGATCCTGGCGCCGATCC	
pilT_down_rev	attcgagctcggtacccgggCACACCTGGCCATGACGG	
TO_lasI_fwd_miniCTX-lux	ctatagggcgaattgggtacGAGCTGGCGATCGGTAATTTG	Designed to have the promotor region of *lasI* as a driver of luminescence production. PCR fragment cloned into previously digested mini-CTX-*lux* vector (EcoRI and KpnI)
TO_lasI_rev_miniCTX-lux	gggatcccccgggctgcaggCTTCACTTCCTCCAAATAGGAAG	

3.1.4.2 Construction of in-frame deletion mutants

An allelic exchange technique based on the use of a suicide vector was used to construct gene knockout deletions (Hmelo *et al.*, 2015). Mutant alleles, flanked by regions of homology to the recipient chromosome, were synthesized *in vitro* by PCR from PA14 genomic DNA and then cloned into the allelic exchange vector pEX18Ap (yielding pTOP01, pTOP02, pTOP03, and pTOP04). Plasmids were assembled from purified PCR products and restriction enzyme-cleaved plasmid backbone by employing a seamless strategy of ligation-independent cloning (pEASY® - Uni Seamless Cloning and Assembly Kit, TransGen Biotech Co.). These suicide vectors were transferred into *P. aeruginosa* by conjugation with *E. coli* donor strain (SM10). Carbenicillin was used to select recipient merodiploid cells, and *E. coli* donor cells were counter-selected using Irgasan. Double-crossover mutants were isolated by sucrose counter-selection and confirmed by PCR.

3.1.4.3 Inactivation of *pilU* gene

Transfer of transposon insertion (::MrT7) from the PA14 non-redundant transposon insertion mutant library was used (Liberati *et al.*, 2006) to inactivate *pilU*. Genomic DNA from *pilU*::MrT7 (mutant ID # 53607) was extracted and transformed into the recipient PA14 Δ*lasR* background. Gentamicin (15 µg/ml) was used to select transformants.

3.1.4.4 Construction of reporter strains

The promoter region of *lasI* was PCR-amplified from PA14 genomic DNA. pTOP05 (mini-CTX-*lasI-lux*) was constructed by the assembly of the purified PCR product and the enzyme-cleaved mini-CTX-lux backbone (Becher *et al.*, 2000). pTOP05, pCTX-1-P_{lasB}-*lux* and pCDS101 were integrated into the *attB* chromosomal site of PA14 and isogenic mutants by conjugation on LB agar plates. Selection was performed on LB agar plates containing tetracycline. The non-integrative plasmid pME3853 carrying the *lasI'–'lacZ* translational fusion was transformed into electrocompetent *P. aeruginosa* cells and selected with tetracycline (Choi *et al.*, 2006).

3.1.4.5 Gene expression reporter measurements

For *lux* reporter readings, luminescence was measured using a Cytation 3 multimode plate reader (BioTek Instruments, USA). Relative light units (RLU) were normalized by colony-forming units per millilitre (reported in RLU CFU^{-1}). The activity of *lacZ* reporters was determined by β-galactosidase activity and was normalized by CFU (reported in Miller units per cell) (Miller, 1972). When mentioned, AHLs were added to a final concentration of 1.5 µM of C_4-HSL and 3 µM of 3-oxo-C_{12}-HSL from stocks prepared in high-performance liquid chromatography (HPLC)-grade acetonitrile. Acetonitrile only was added in controls.

3.1.4.6 Quantification of QS signalling molecules

The concentration of 3-oxo-C_{12}-HSL was measured for bacteria grown in liquid King's A (planktonic growth) and on King's A agar (surface growth) using HPLC/tandem mass spectrometry (LC/MS/MS), with modifications of the previously described protocol (Lépine *et al.*, 2018). Quantification was performed at indicated times post-inoculation in both growth conditions. For planktonic growth, overnight cultures grown on TSB were diluted to OD_{600} 0.1 in fresh King's A medium. At the given time points, cultures were mixed with acetonitrile containing the internal standard tetradeuterated 4-hydroxy-2-heptylquinoline (HHQ-d_4), in a 4:1 ratio of culture to solvent

(HHQ-d_4 final concentration of 3 ppm). Bacterial suspensions were vortexed and centrifuged at 12,000 x *g* for 10 min in order to pellet bacterial cells. The resulting mixture was transferred into vials for LC/MS/MS analyses. Alternatively, for cells grown on agar surfaces, overnight cultures on TSB were diluted to OD_{600} 0.05 in TSB medium. Cultures were grown until an OD_{600} of 1, and agar plugs were inoculated with 5 µl of bacterial suspension. Plates were incubated at 37°C, and samples were collected at the indicated time points. Each sample was composed of two agar plugs mixed with 1 mL of acetonitrile containing the internal standard. This mixture was incubated at 4°C for 16h under gentle agitation, optimizing the diffusion of signalling molecules from the agar to the solvent. After incubation, the mixture was centrifuged at maximum speed for 10 min, and the resulting supernatant was transferred into an LC/MS vial. All samples were injected using an HPLC Waters 2795 (Mississauga, ON, Canada) on a Kinetex C8 column (Phenomenex) with an acetonitrile-water gradient containing 1% acetic acid. The detector was a tandem quadrupole mass spectrometer (Quattro premier XE; Waters) equipped with a Z-spray interface using electrospray ionization in positive mode (ESI+). Nitrogen was used as a nebulizing and drying gas at flow rates of 15 and 100 ml · min^{-1}, respectively. Concentration was normalized by CFUs per mL^{-1} and reported in ng CFU^{-1}. All experiments were performed in triplicates and repeated at least twice independently.

3.1.4.7 Pyocyanin quantification

Quantification of pyocyanin produced by surface-grown cells was performed similarly to that described in a previous study (Mould *et al.*, 2020). Overnight cultures were diluted and grown in TSB until an OD_{600} of 1. At this point, 5 µL were used to inoculate agar plugs from a 96-well plate containing King's A agar supplemented with $FeCl_3$ (200 µL per well). Plates were incubated at 37°C for 24h. Pyocyanin was extracted in 500 µL of chloroform from two agar plugs (by replicate). Tubes were vortexed and centrifuged for 3 min at 12,000 x *g*. Then, 200 µL of the organic phase was recovered in a new tube, and a second chloroform extraction was performed on the plugs. The organic phase (400 µL) was acidified with 500 µL 0.2 N HCl and vortexed. The samples were centrifuged for 3 min at 12,000 x *g,* and the absorbance of the pink aqueous phase was read at $OD_{520\ nm}$. Blank was performed by pyocyanin extraction from uninoculated agar plugs. Values were corrected by colony-forming units per millilitre from samples prepared in the same conditions.

3.1.4.8 *Drosophila melanogaster* feeding assay

Fruit flies (*D. melanogaster*) were infected orally in a feeding assay model (Apidianakis *et al.*, 2009). Male flies (4 to 6 days old) were anesthetized under a gentle stream of carbon dioxide and separated into vials, each containing 10 males. Each strain (or condition) tested was composed of three independent vials, totalizing 30 flies. Vials were prepared with 5 mL of a solution of sucrose agar (5% of sucrose and 1.5% agar). Once solidified, a sterile filter disk was placed on the surface. Prior to infection, bacteria were grown in 6 mL of TSB until an OD_{600} of 3. At this point, the bacterial suspension was centrifuged for 3 min at 12,000 x g, and the pellet was resuspended in 100 µL of sterile 5% sucrose and dispensed on the filter papers. Sterile 5% sucrose alone was used as a control. Males were starved 6-8h prior to the infection. Flies were kept at 25°C and about 50% humidity. They were subjected to 12h light/dark cycles. Mortality was monitored daily for 8 days. The experiment was performed twice, each time in triplicate.

3.1.5 Results

3.1.5.1 Surface growth induces production of 3-oxo-C_{12}-HSL in the absence of LasR

In *P. aeruginosa* prototypical strains such as PA14**,** the quorum sensing regulatory cascade is considered to be primarily activated by the *las* system. LasR, once activated by the binding of 3-oxo-C_{12}-HSL, regulates the transcription of target genes, including the gene coding the LasI synthase. This process induces the production of more 3-oxo-C_{12}-HSL, resulting in a positive feedback loop. In standard laboratory liquid cultures of *P. aeruginosa*, production of 3-oxo-C_{12}-HSL peaks early and decreases over time ((Déziel *et al.*, 2005); **Fig. 3.1.1A**). We note the same pattern of production in wild-type *P. aeruginosa* PA14 (WT) cells grown on an agar surface (**Fig. 3.1.1A**). Surprisingly, in a LasR-negative background, the production pattern of 3-oxo-C_{12}-HSL is influenced by suspended vs surface culture conditions (**Fig. 3.1.1**). As expected, production of the LasR ligand is barely detectable at the stationary phase of a Δ*lasR* mutant in broth cultures. However, its concentration is elevated during surface growth (**Figs. 3.1.1A and B**). In WT culture, the peak concentration is observed during the exponential growth phase, while it shifts to late stationary phase in the Δ*lasR* mutant, solely when growing on the surface. This shift might indicate a role for other regulators in the activation of *lasI* transcription in the absence of LasR.

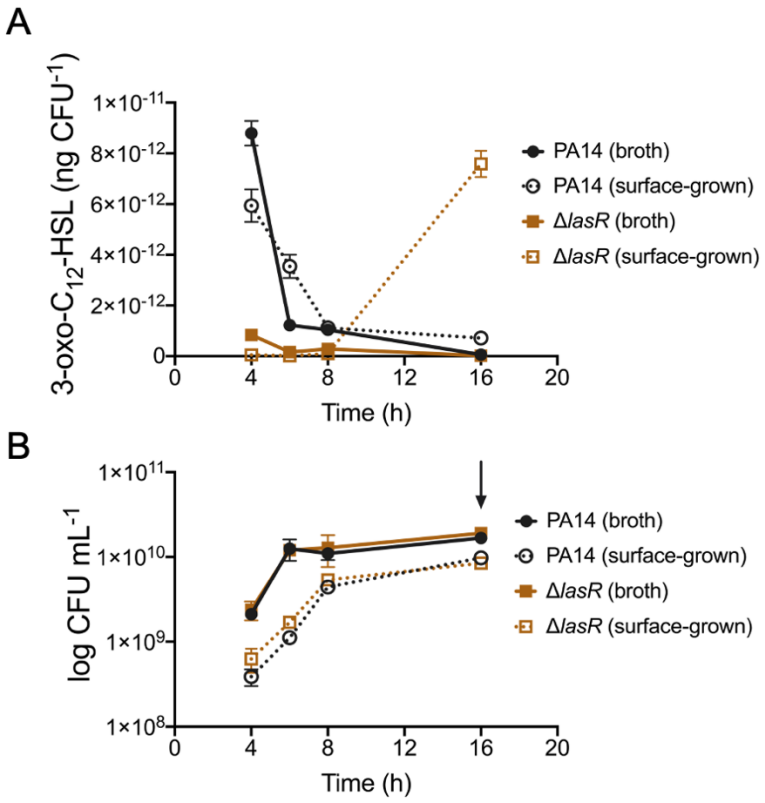

Figure 3.1.1. Surface growth induces 3-oxo-C_{12}-HSL production in a PA14 LasR-null strain.
(**A**) 3-oxo-C_{12}-HSL concentration was measured in PA14 and the isogenic Δ*lasR* mutant (PA14 Δ*lasR*) at different time points during planktonic (broth culture) and surface growth (surface of agar-solidified culture media) by liquid chromatography/mass spectrometry. Values were normalized by the viable cell counts and shown in nanograms per CFU (**B**). Growth in broth and surface conditions was determined by the count of viable cells per millilitre (CFU mL^{-1}). The arrow indicates the time point at which 3-oxo-C_{12}-HSL is induced in a Δ*lasR* mutant in (**A**). The values are means ± standard deviation (error bars) from three replicates.

3.1.5.2 Production of 3-oxo-C_{12}-HSL and expression of *lasI* are RhlR dependent in LasR-negative backgrounds

Expression of the gene coding the LasI synthase, responsible for the synthesis of 3-oxo-C_{12}-HSL, is typically considered to be regulated by LasR. Therefore, little to no production of this AHL is expected in LasR-defective strains, which is what is observed in planktonic cultures. However, upon surface growth, 3-oxo-C_{12}-HSL is produced in the absence of LasR. To make sure the production of 3-oxo-C_{12}-HSL in this condition still requires LasI activity, we measured concentrations of this AHL in a Δ*lasI* mutant grown under the same surface-associated conditions.

As expected, 3-oxo-C_{12}-HSL is not detectable in a Δ*lasI* mutant, irrespective of the growth phase (**Fig. 3.1.2A**). The concentrations of this signal molecule were also assessed in the Δ*lasR*Δ*lasI* double mutant, and similarly to the Δ*lasI* mutant, we detected no 3-oxo-C_{12}-HSL (data not shown). These results suggest that transcription of *lasI* can occur in the absence of LasR upon surface growth. To further investigate this, we measured the activity of a chromosomal *lasI-lux* reporter in a Δ*lasR* background in both planktonic and surface-grown cells. In agreement with the production of 3-oxo-C_{12}-HSL, transcription of *lasI* was observed in LasR-negative background grown on a surface (**Fig. 3.1.2B**).

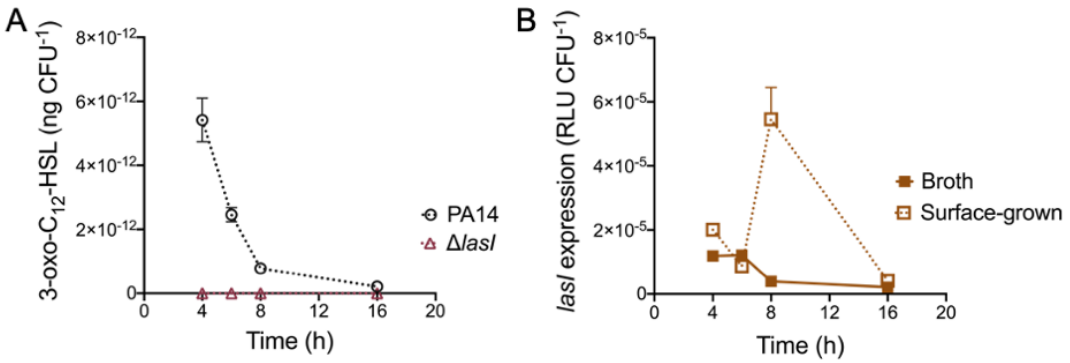

Figure 3.1.2. Transcription of *lasI* can occur in the absence of LasR in cells growing on a surface.
(**A**) 3-oxo-C_{12}-HSL was measured in PA14 and its isogenic Δ*lasI* mutant at different time points during surface growth by liquid chromatography/mass spectrometry. (**B**) Transcription activity from the chromosomal *lasI-lux* reporter in a Δ*lasR* background.

We have previously reported indications that RhlR can act as a surrogate regulator of LasR-dependent factors in the absence of LasR (Dekimpe *et al.*, 2009). In *P. aeruginosa* planktonic cultures, this activation is seen by the production of 3-oxo-C_{12}-HSL at late stationary phase in LasR-negative backgrounds. However, as shown in **Fig. 3.1.1A**, the concentration of this AHL in a Δ*lasR* mutant in broth cultures remains extremely low early on. In contrast, surface growth readily induces production and the corresponding upregulation of *lasI* transcription in a Δ*lasR* mutant (**Figs. 3.1.1A and 3.1.2B**). To verify if RhlR is responsible for this upregulation, we measured concentrations of 3-oxo-C_{12}-HSL in a Δ*rhlR* and a double *lasR rhlR* mutant (**Figs. 3.1.3 and 3.1.4**) upon surface growth. The production profile of 3-oxo-C_{12}-HSL is similar between the WT and a Δ*rhlR* mutant, peaking at exponential growth phase and decaying overtime (**Fig. 3.1.4**). On the other hand, the concomitant inactivation of *lasR* and *rhlR* abrogates 3-oxo-C_{12}-HSL

production, which concurs with our previous finding of RhlR being the alternative activator of LasI in LasR-negative backgrounds (**Figs. 3.1.3 and 3.1.4**). This result suggested that RhlR in surface-grown cells mediates the transcription of *lasI*. To verify the potential transcriptional regulatory activity of RhlR on the expression of *lasI*, we introduced the transcriptional *lasI-lux* reporter construct in the double *lasR rhlR* mutant. Surprisingly, the transcriptional profile of *lasI* in surface-grown cells of this double mutant is similar to the 3-oxo-C_{12}-HSL-producing Δ*lasR* mutant, despite the absence of production of this signal. To further establish a connection between the RhlR-dependent production of 3-oxo-C_{12}-HSL and *lasI* expression during surface growth, we assessed the translation of *lasI* using a reporter fusion in the double Δ*lasR*Δ*rhlR* mutant and Δ*lasR*. Similar to the transcription findings for *lasI* (**Fig. 3.1.2B**), the translation also correlates with the production of 3-oxo-C_{12}-HSL in the Δ*lasR* mutant (**Fig. 3.1.5B**). However, in the absence of both LasR and RhlR, no translation of *lasI* is detected, thus explaining the lack of 3-oxo-C_{12}-HSL production by the Δ*lasR*Δ*rhlR* mutant (**Fig. 3.1.5B**).

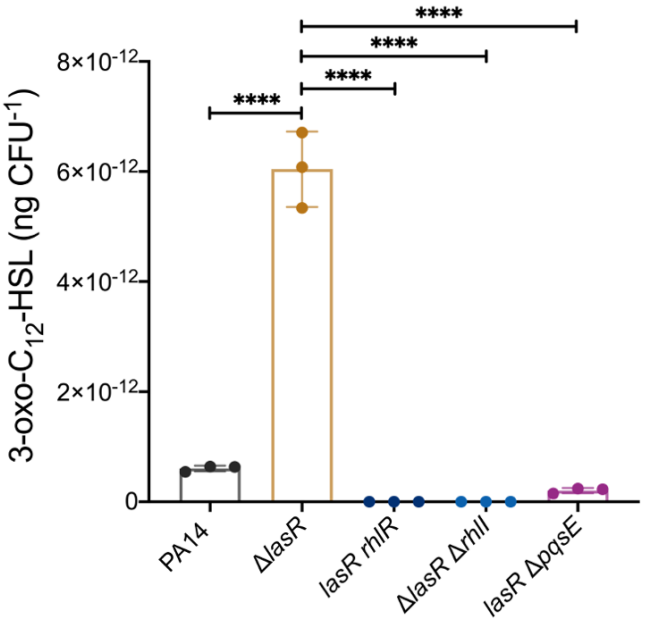

Figure 3.1.3. Activity of the Rhl system is required to induce the production of 3-oxo-C12-HSL upon surface growth.

3-oxo-C_{12}-HSL was measured in PA14, isogenic single-mutants Δ*lasR* and Δ*rhlR*, and the double-mutants *lasR-rhlR*-, Δ*lasR*Δ*rhlI*, and *lasR*-Δ*pqsE* at 16 h of surface growth by LC/MS. Concentration was normalized by the viable cell count. The values are means ± standard deviation (error bars) from three replicates. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons posttest were used to quantify statistical significance. ****$P \leq 0.0001$.

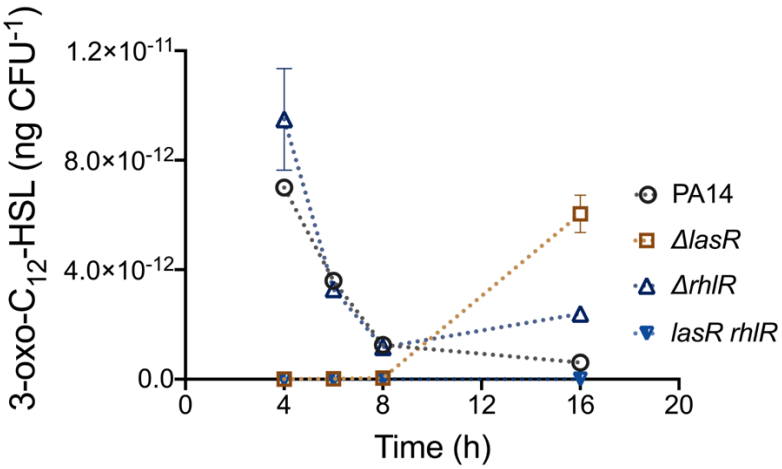

Figure 3.1.4. Production of 3-oxo-C_{12}-HSL does not require RhlR in LasR-inactive cells.

Concentration of 3-oxo-C_{12}-HSL was measured at different time points during surface growth by LC/MS. Values were normalized by the viable cell counts and shown in ng CFU^{-1}. Values are means ± standard deviation (error bars) from three replicates.

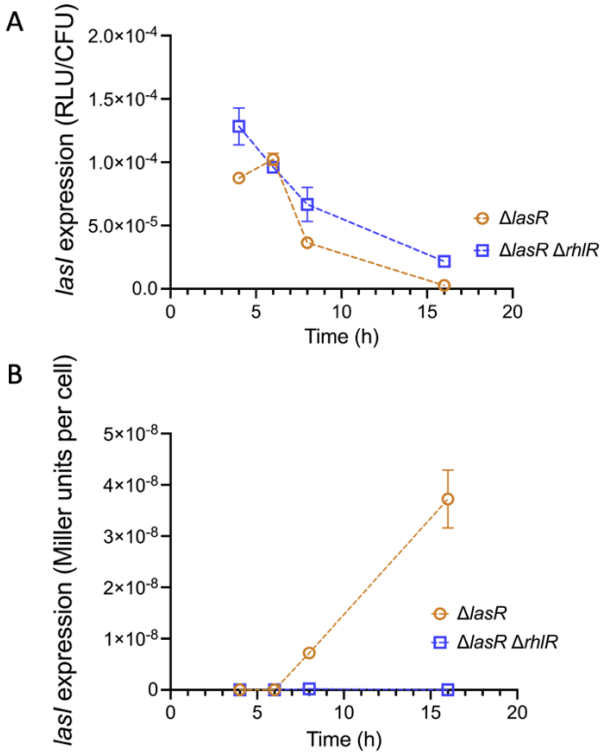

Figure 3.1.5. RhlR controls the translational expression of *lasI* in surface-grown cells.

(**A**) Transcription activation from the chromosomal *lasI-lux* reporter in the backgrounds Δ*lasR* and Δ*lasR* Δ*rhlR* at different time points during surface growth. (**B**) The translational activity of *lasI* was measured in the same conditions via β-galactosidase activity of a *lasI'-'lacZ* translational fusion (pME3853). The values are means ± standard deviation (error bars) from three replicates.

Thus, in the absence of LasR, surface-grown cells rely on the activity of the *rhl* system to control QS-regulated factors, including the production of 3-oxo-C_{12}-HSL. Since the full activity of RhlR depends on both C_4-HSL and PqsE (Groleau *et al.*, 2020), we measured the concentration of 3-oxo-C_{12}-HSL in the double mutants Δ*lasR*Δ*rhlI* and *lasR* Δ*pqsE* in order to further elucidate the role of the Rhl system in this mechanism. As expected, inactivating *rhlI* or *pqsE* in a *lasR* background severely affects the production of 3-oxo-C_{12}-HSL (**Fig. 3.1.3**) and confirms that the production of 3-oxo-C_{12}-HSL by LasR-null cells growing on a surface is dependent on the RhlR-mediated regulation of *lasI*.

3.1.5.3 Induction of the production of 3-oxo-C_{12}-HSL upon surface growth is a widespread response among *P. aeruginosa* strains

Conserved regulation pathways strongly suggest the importance of bacterial responses to their fitness (Jordan *et al.*, 2002). We have observed that surface growth induces production of 3-oxo-C_{12}-HSL in an engineered *lasR* deletion mutant of *P. aeruginosa* PA14. To verify if this response is restricted to this prototypical strain, we measured concentrations of this AHL in six naturally occurring LasR-defective *P. aeruginosa* isolates: three strains we recently identified among a collection of environmental isolates (Groleau *et al.*, 2022), and the other three are LasR-defective CF clinical isolates (E41, E113, and E167) from the Early *Pseudomonas* Infection Control (EPIC) study (Asfahl *et al.*, 2022; Feltner *et al.*, 2016). The timing of sampling was chosen based on the 3-oxo-C_{12}-HSL production profile of PA14 Δ*lasR*, which peaks at late exponential phase (**Fig. 3.1.1**). Considering that growth curves can differ greatly between *P. aeruginosa* strains, we also decided to include a 24h time point. Environmental and clinical LasR-negative strains behave similarly to the engineered PA14 Δ*lasR* mutant, with production of 3-oxo-C_{12}-HSL being augmented upon surface growth when compared to planktonic (**Fig. 3.1.6**). The production profile varies among the LasR-negative backgrounds: strain 18G steadily produces 3-oxo-C_{12}-HSL during surface growth. At 24h, there is sixfold more 3-oxo-C_{12}-HSL from surface grown samples than those from planktonic growth conditions. The environmental strain 32R and the clinical strain E113 have production profiles similar to PA14 Δ*lasR*, and the concentration of 3-oxo-C_{12}-HSL peaks at the late exponential phase (**Figs. 3.1.6 and 3.1.7**). Production is advanced (compared with PA14 Δ*lasR*) in strains 78RV and E167. In these strains, AHL production peaks at early exponential phase (**Figs. 3.1.6 and 3.1.7**). Finally, upregulation of 3-oxo-C_{12}-HSL production upon surface growth was not observed for the clinical strain E41 under our test conditions. Taken together, these results confirm that the absence of a functional LasR generally induces the production of 3-oxo-C_{12}-HSL in response to growth in association with surfaces, despite the general requirement of LasR to produce this AHL in standard laboratory planktonic culture conditions.

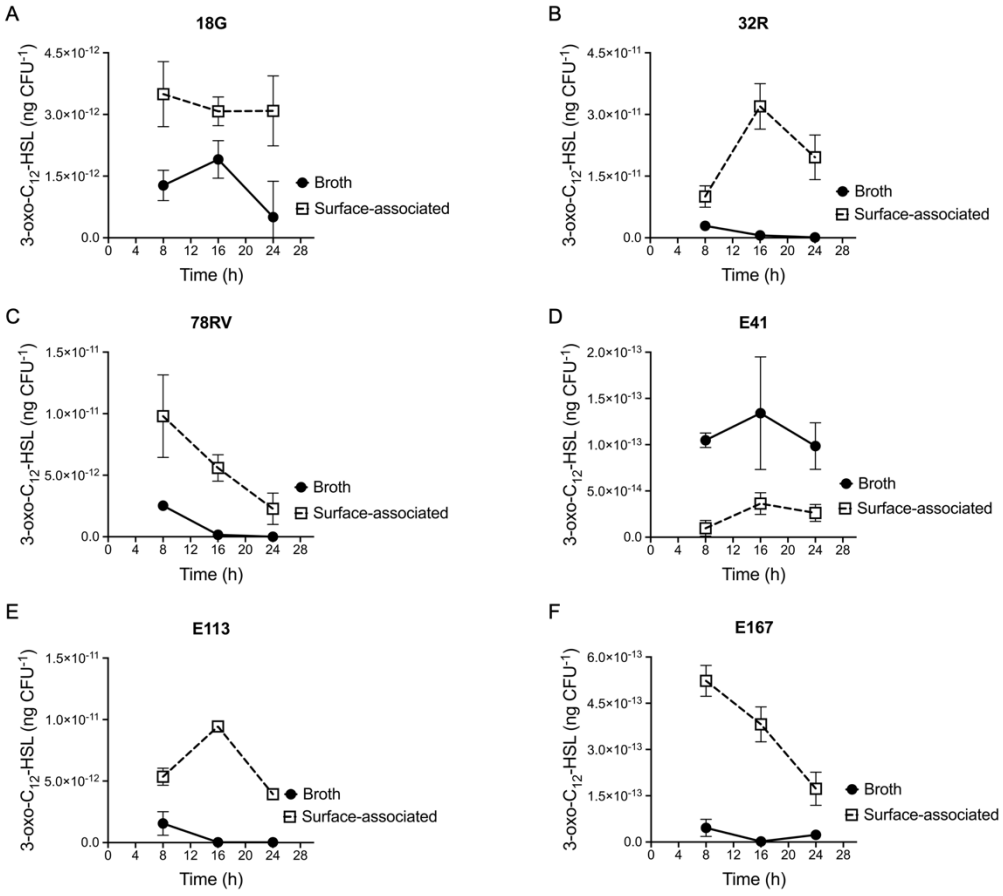

Figure 3.1.6. Production of 3-oxo-C_{12}-HSL is a widespread feature among LasR-defective strains growing on a surface.

3-oxo-C_{12}-HSL was measured at different time points during planktonic and surface growth by LC/MS of naturally evolved LasR-defective strains. (**A**) 18G. (**B**) 32R. (**C**) 78RV. (**D**) E41. (**E**) E113. (**F**) E167. Concentration was normalized by viable cell count and is shown in nanograms per CFU. The values are means ± standard deviation (error bars) from three replicates.

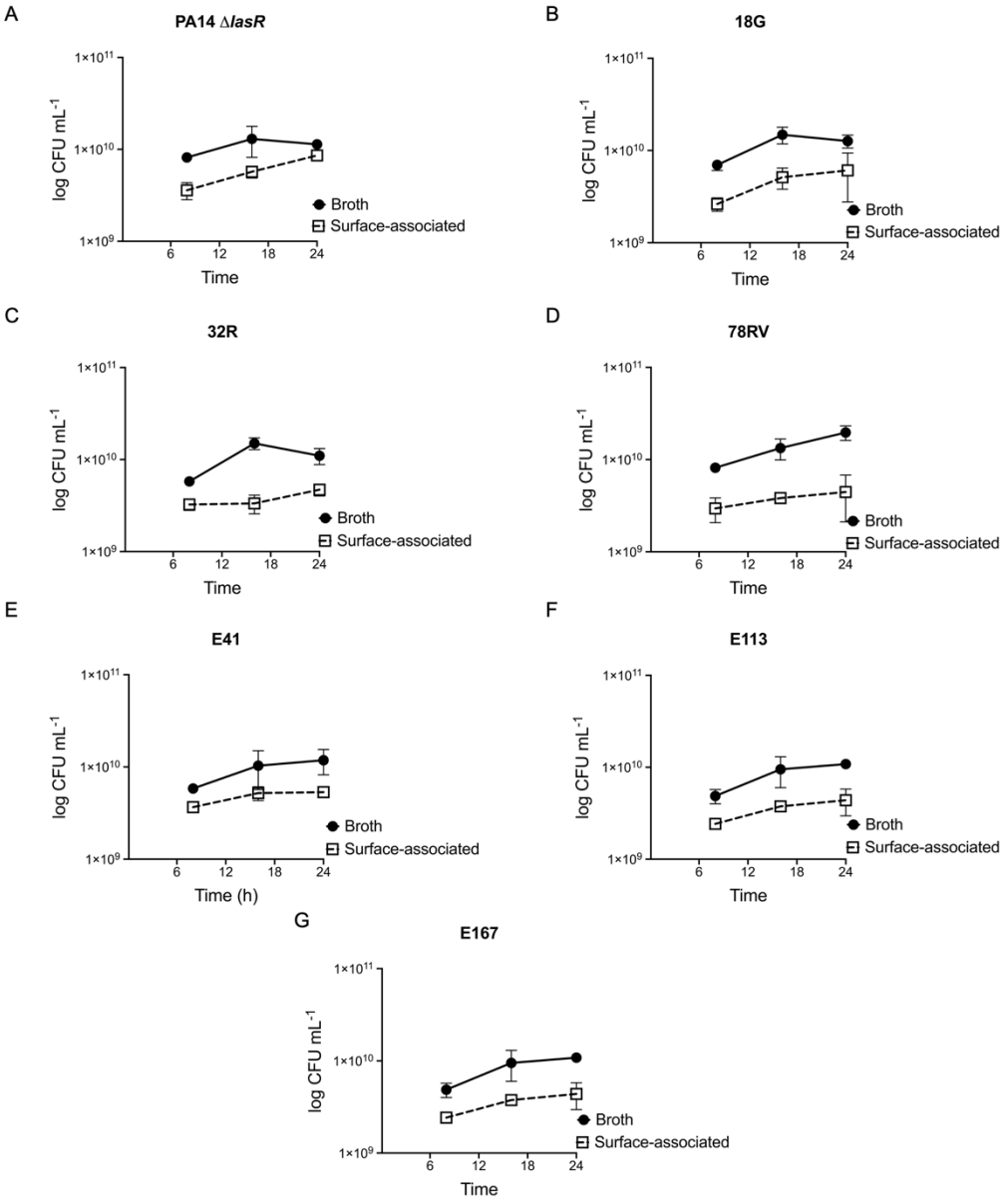

Figure 3.1.7. Growth profile of natural occurring LasR-defective isolates.

Growth in broth and surface conditions was determined by the count of viable cells per millilitre (CFU mL^{-1}). This data is complementary to the one shown in Figure **4.1.6**. The values are means ± standard deviation (error bars) from three replicates.

3.1.5.4 3-oxo-C_{12}-HSL induces the expression of pyocyanin in the absence of LasR

The conservation of surface-primed induction of 3-oxo-C_{12}-HSL production in LasR-defective isolates strongly suggests that this signalling molecule mediates significant biological responses in this context. Because 3-oxo-C_{12}-HSL is only/essentially known as the autoinducing ligand of LasR, in a LasR-defective background, its production could be considered as a waste of resources. Thus, a plausible explanation for the conservation is that, in the absence of a functional LasR, 3-oxo-C_{12}-HSL remains beneficial when *P. aeruginosa* is growing on a surface. Pyocyanin production relies on the expression of the redundant operons *phzA1B1C1D1E1F1G1* (*phz1*) and *phzA2B2C2D2E2F2G2* (*phz2*) – culminating in the synthesis of phenazine-1-carboxylic acid (PCA). PCA is converted to several phenazines, including pyocyanin, the blue pigment characteristic of *P. aeruginosa* cultures (Mavrodi *et al.*, 2001). Transcription of the *phz1* operon relies on RhlR (Groleau *et al.*, 2020; Whiteley *et al.*, 2001). To verify if 3-oxo-C12-HSL could be implicated in RhlR-dependant QS, we evaluated the level of transcription from the *phz1* promoter during surface growth, using a chromosomal *phzA1-lux* fusion reporter, in an AHL- and LasR-negative triple mutant (Δ*lasR*Δ*lasI*Δ*rhlI*). As expected, no transcription is seen in the control condition or when only 3-oxo-C_{12}-HSL is provided, and upon addition of exogenous C_4-HSL, *phz1* transcription is induced, consistent with the requirement of C_4-HSL for RhlR activity (**Fig. 3.1.8A**). However, unexpectedly, the combined addition of C_4-HSL and 3-oxo-C_{12}-HSL further induces the expression of *phz1* (**Fig. 3.1.8A**). The synergetic activation of these signal molecules is also seen for pyocyanin production (**Fig 3.1.8B**). The concomitant addition of C_4-HSL and 3-oxo-C_{12}-HSL induces almost threefold the production of this redox-active molecule compared to the addition of C_4-HSL alone. Similar to the observed *phz1* expression, 3-oxo-C_{12}-HSL alone is not sufficient to induce pyocyanin production. These results clearly demonstrate that 3-oxo-C_{12}-HSL modulates QS-regulated responses even in the absence of its cognate response regulator, LasR. This activity depends on the presence of C_4-HSL, thus likely on the function of RhlR.

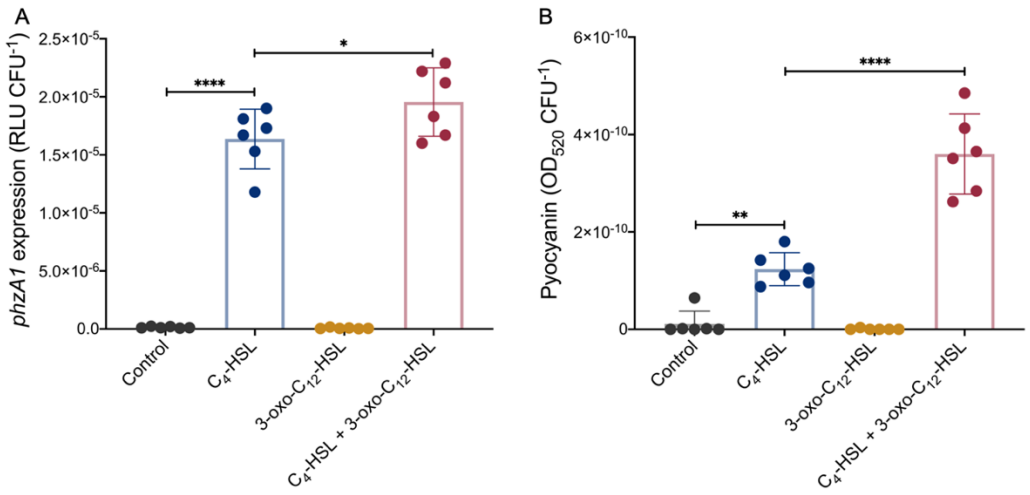

Figure 3.1.8. Exogenous 3-oxo-C_{12}-HSL induces transcription of the operon *phz1* and pyocyanin production in a *lasR* negative background.

(**A**) Luminescence of the *phzA1-lux* chromosomal reporter was measured in the AHL-negative LasR-defective background (Δ*lasR*Δ*lasI*Δ*rhlI*) after the addition of 1.5 μM of C_4-HSL, 3 μM of 3-oxo-C_{12}-HSL, or both molecules at late stationary phase (24h). Acetonitrile alone was used as a control. Relative light units were normalized by viable cell counts and shown in RLU CFU^{-1}. (**B**) Pyocyanin produced by Δ*lasR*Δ*lasI*Δ*rhlI* in response to exogenous AHLs was chloroform-extracted at 24h. Production was normalized by cell viable counts and shown in OD_{520} CFU^{-1}. The values are means ± standard deviations (error bars) from six replicates. Statistical analyses were performed using ANOVA and Tukey's multiple comparisons posttest with * $P \leq 0.05$; ** $P \leq 0.01$ and **** $P \leq 0.0001$.

3.1.5.5 3-oxo-C_{12}-HSL produced by LasR-negative strains positively regulates the LasB virulence determinant in cocultures

AHLs are conserved extracellular intraspecies signalling molecules. Based on this characteristic, we wondered if 3-oxo-C_{12}-HSL produced by LasR-defective isolates could be used by surrounding LasR-active cells to induce LasR-dependent factors. These factors include several exoproducts, such as proteases (e.g. LasA and LasB), that can be used by the whole population ("public goods"). To verify this, we measured the activity of the chromosomal *lasB-lux* reporter inserted in Δ*lasI* mutant (Δ*lasI*::CTX *lasB-lux* background) in a surface-associated coculture with a Δ*lasR* mutant. Because the Δ*lasI* mutant is unable to produce 3-oxo-C_{12}-HSL, the *las* system cannot be activated in this background; however, this strain is LasR-active and prone to induction by exogenous 3-oxo-C_{12}-HSL. As expected, *lasB* transcription is at basal levels in Δ*lasI* mutant monoculture (**Fig. 3.1.9**). Coculture with Δ*lasR*, which produces 3-oxo-C_{12}-HSL under these surface culture conditions, induces the transcription of the *lasB*-lux reporter by more than fourfold

at late stationary phase in which the concentration of LasR-inducing 3-oxo-C_{12}-HSL is at its peak. This upregulation depends solely on the production of 3-oxo-C_{12}-HSL by the Δ*lasR* mutant, as it is not seen in cocultures with the double mutant Δ*lasR*Δ*lasI*. Thus, 3-oxo-C_{12}-HSL produced by LasR-negative strains can be used by surrounding LasR-active cells, modulating the expression of the QS-regulated genes at the communal level.

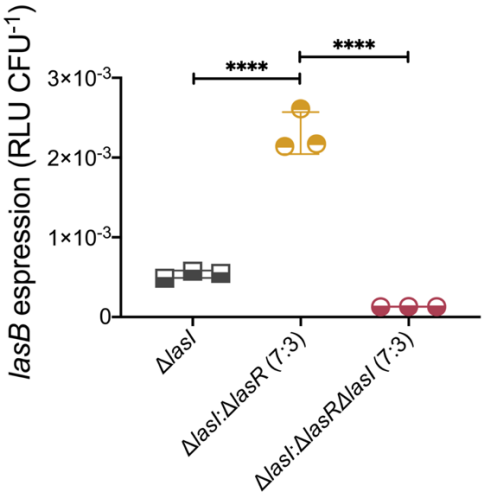

Figure 3.1.9. Surface-grown LasR-active cells utilize 3-oxo-C_{12}-HSL produced by surrounding LasR-defective mutants, inducing *lasB* expression.

Luminescence reading of a *lasB-lux* chromosomal reporter inserted in a LasR-active Δ*lasI* mutant (Δ*lasI*::CTX *lasB-lux*). Monoculture of Δ*lasI* was used as control (gray). Coculture Δ*lasI* and Δ*lasR* with 7:3 Δ*lasI*-to-Δ*lasR* cell initial ratio (yellow). Coculture of Δ*lasI* and Δ*lasR* Δ*lasI* with 7:3 Δ*lasI*-to-Δ*lasR*Δ*lasI* cell initial ratio (red). Relative light unit was normalized by viable cell count of Δ*lasI*::CTX *lasB-lux* strain at 16h and is shown in RLU CFU^{-1}. The values are means ± standard deviations (error bars) from three replicates. Statistical significance was calculated by ANOVA and Tukey's multiple comparisons posttest with **** $P \leq 0.0001$.

3.1.5.6 Virulence of *P. aeruginosa* in coinfection settings is partially dependent on the enrichment of 3-oxo-C_{12}-HSL provided by LasR-defective cells.

Even in the absence of a functional LasR or endogenous production of its cognate autoinducer, virulence traits are positively regulated by 3-oxo-C_{12}-HSL at the individual and community levels (**Figs. 3.1.8 and 3.1.9**). Thus, we postulated that a coinfection with a mixture of LasR-responsive and LasR-defective strains would be more virulent than a separate infection with the respective strains. To test this, we used the fruit fly *Drosophila melanogaster* as an infection host – in which *P. aeruginosa* causes a disease and mortality (Apidianakis *et al.*, 2009). We fed fruit flies with *P.*

aeruginosa cells and monitored the survival of the flies for 8 days post-infection. Feeding assay mimics a chronic infection (Purdy *et al.*, 2011). The virulence of WT PA14 (LasR-active) and Δ*lasR* mutant (LasR-defective) was evaluated individually, as well as in a coinfection setting with a 7:3 ratio, respectively **(Figs. 3.1.10 and 3.1.11)**. Additionally, the virulence of the double mutant Δ*lasR*Δ*lasI* was assessed in both individual and coinfection settings to elucidate the role of 3-oxo-C_{12}-HSL in this response **(Figs. 3.1.10 and 3.1.11).** Under our conditions, the survival rate of the coinfection with PA14 and Δ*lasR* was comparable to that of the infection with PA14 only throughout the duration of the experiment. In contrast, the coinfection with PA14 and the Δ*lasR*Δ*lasI* double mutant induced less fly mortality. These observations underscore that virulence in coinfection settings with LasR-negative cells is partially dependent on the production of 3-oxo-C_{12}-HSL by the latter as it is significantly reduced when this molecule cannot be produced (i.e. double mutant Δ*lasR*Δ*lasI*).

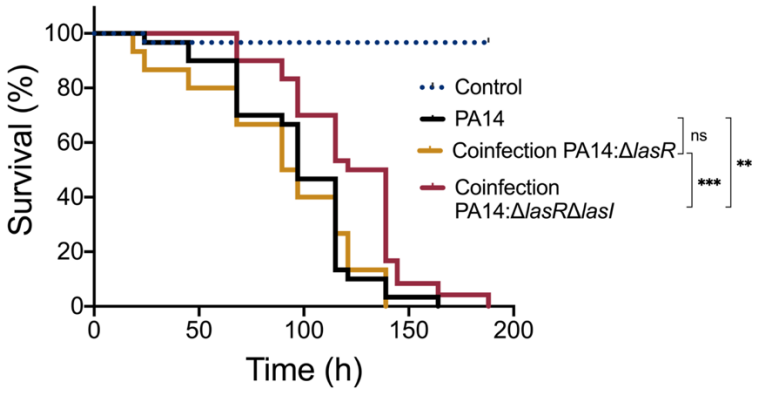

Figure 3.1.10. In coinfection settings, full virulence of *P. aeruginosa* toward *D. melanogaster* depends on the provision of 3-oxo-C_{12}-HSL produced by Δ*lasR*.

Fruit flies were infected with suspended cells in 5% sucrose. Fly survival was monitored over time. n = 30 flies per group for each experiment. Experiment was performed independently twice. Statistical significance was determined using Mantel-Cox survival analysis. ns, nonsignificant, ** $P \leq 0.01$, and *** $P \leq 0.001$.

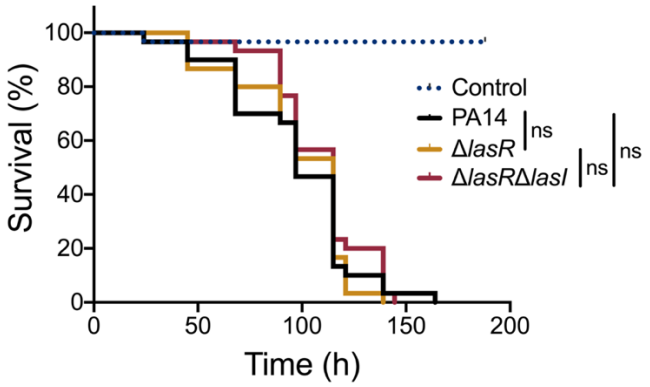

Figure 3.1.11. Functionality of the *las* system is not required for *P. aeruginosa* virulence toward *D. melanogaster.*

Fruit flies were infected with suspended cells in 5% sucrose. Fly survival was monitored over time. *n* = 30 flies per group for each experiment. The experiment was performed independently twice. Statistical significance was determined using the Kaplan-Meier survival analysis. ns, nonsignificant.

3.1.1 Discussion

The characteristics and behaviours displayed by bacteria within biofilms have been extensively investigated over the years. These surface-associated communities exhibit features that clearly distinguish them from their free-living counterpart. This is due to a sequential and highly regulated process that mediates the transition from planktonic to a sessile lifestyle (Rumbaugh *et al.*, 2020). Although QS regulates social behaviours, often also modulated by aspects related to the sessile way of life, it has been essentially characterized genetically and biochemically in cells grown in broth. In the present study, we show that surface association is sufficient to induce the LasR-independent expression of *lasI* in *P. aeruginosa* and that 3-oxo-C_{12}-HSL modulates the expression of virulence determinants even in the absence of the cognate transcriptional regulator LasR (**Fig. 3.1.12**).

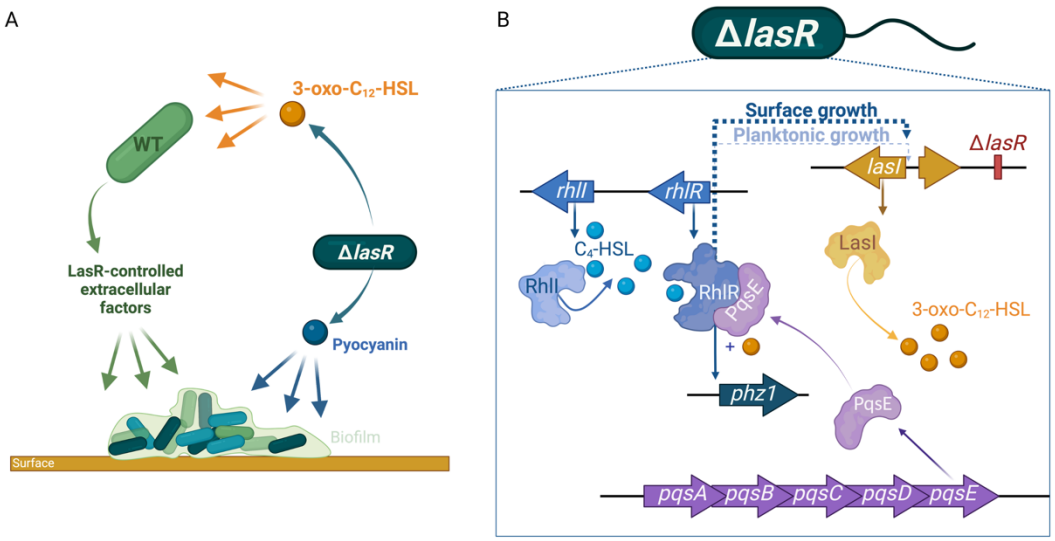

Figure 3.1.12. Schematic overview of the investigated QS pathways.

(**A**) Interactions in a surface-grown mixed population of *P. aeruginosa*. LasR-negative cells enrich the populational 3-oxo-C_{12}-HSL pool, further inducing LasR-controlled extracellular factors and autologous pyocyanin production, which benefits the population. (**B**) QS regulation in a LasR-negative cell of *P. aeruginosa* PA14. In the absence of LasR, 3-oxo-C_{12}-HSL production is regulated by the active RhlR protein (i.e. in complex with C_4-HSL and PqsE). This indirect regulation is induced in surface-grown cells (ticker arrow), compared to the planktonic lifestyle (tinner arrow). The presence of 3-oxo-C_{12}-HSL appears to modulate the active RhlR complex, inducing the expression of *phz1* and the production of pyocyanin.

Surface sensing has been previously linked to differential bacterial responses. For instance, we have shown that regulation of the small RNAs RsmY/RsmZ is modulated differently in broth versus surface-grown cells, probably aiding bacterial adaptation to growth conditions (Jean-Pierre *et al.*, 2017). Similarly, the expression of *lasR* increases in a surface-dependent manner, culminating in a surface-primed QS activation, due to the sensitization of surface-grown cells to the cognate AHL 3-oxo-C_{12}-HSL (Chuang *et al.*, 2019). Therefore, the QS of *P. aeruginosa* responds differently to the same concentration of 3-oxo-C_{12}-HSL: weaker QS activation is seen in planktonic cultures, in contrast to high QS activation in surface-associated cells. This mechanism is reported to rely on type IV (T4P) pili retraction, as surface-primed *lasR* upregulation is lost in the absence of the motors PilT and PilU (Chuang *et al.*, 2019). Thus, a relationship between QS and surface sensing is established, but its complexity remains to be clearly defined.

LasR-defective *P. aeruginosa* isolates have been generally related to human chronic infections, in which this bacterium persists in the lungs of people with CF as a biofilm. Recently, the generally high occurrence of such isolates challenged this long-held notion (Groleau *et al.*, 2022; O'Connor *et al.*, 2022). Loss of LasR function appears to be a widespread adaptation feature of this bacterium (Groleau *et al.*, 2022). Our results support a model in which surface attachment, a growth condition often encountered by *P. aeruginosa*, induces RhlR-dependent production of 3-oxo-C_{12}-HSL in LasR-defective background – sustaining QS-responsiveness in this condition. The mechanism by which RhlR induces *lasI* expression remains elusive, as in our settings, the transcription of *lasI* was not coupled with the production of 3-oxo-C_{12}-HSL. RhlR, as other LuxR-type proteins, is known as a transcriptional activator, and yet, our results suggest an RhlR-dependent (most likely indirect) posttranscriptional regulation of *lasI*. To our knowledge, the only report of such level of regulation on *lasI* relies on the RNA thermometer and, therefore, cannot explain the regulation observed here (Grosso-Becerra *et al.*, 2014). The most plausible explanation for the RhlR-dependent production of 3-oxo-C_{12}-HSL is that this regulator activates a putative translational regulator required for *lasI* expression; in the absence of RhlR – and consequently, of this RhlR-dependent translational regulator, the translation of *lasI* is blocked and no 3-oxo-C_{12}-HSL is produced. And other questions related to this regulation still remain. For instance, why is RhlR-dependent expression of LasI observed in a LasR-deficient background more prominent in sessile cells? Compared to planktonic growth, both sessile LasR-active and LasR-defective cells produce more C_4-HSL (**Fig. 3.1.13**), which could lead to a stronger activation of the *rhl* system, culminating in the upregulation of RhlR-dependent factors. However, the RhlR-dependent 3-oxo-C_{12}-HSL overproduction in sessile cells is seen only in LasR-defective backgrounds (**Fig. 3.1.12**).

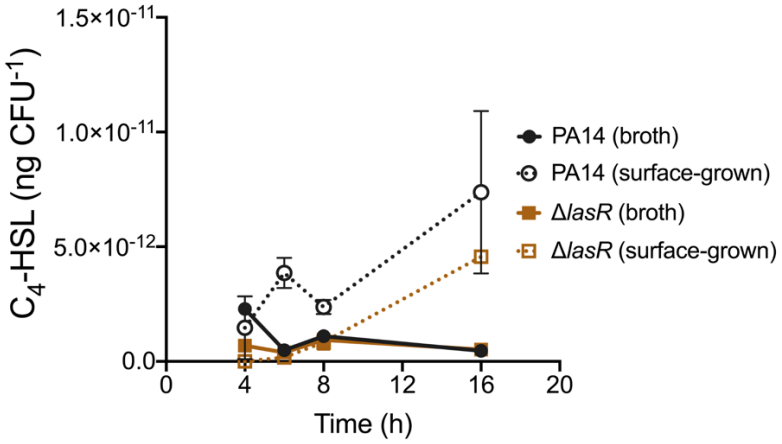

Figure 3.1.13. Surface growth induces C_4-HSL production in both PA14 and its isogenic *lasR* mutant.
C_4-HSL concentration was measured in PA14 and the isogenic *lasR* mutant at different time points during planktonic (broth culture) and surface growth (surface of agar-solidified culture media) by LC/MS. Values were normalized by the viable cell counts and shown in ng CFU^{-1}. The values are means ± standard deviation (error bars) from three replicates.

Irrespective of the mechanism, the production of 3-oxo-C_{12}-HSL appears to have important biological implications. As mentioned before, surface association upregulates LasR, thus sensitizing cells to 3-oxo-C_{12}-HSL (Chuang *et al.*, 2019). Upregulation of 3-oxo-C_{12}-HSL in the LasR-defective background does not appear dependent on the same mechanism, as the T4P retraction motors PilT and PilU are not required for this response (**Fig. 3.1.14**).

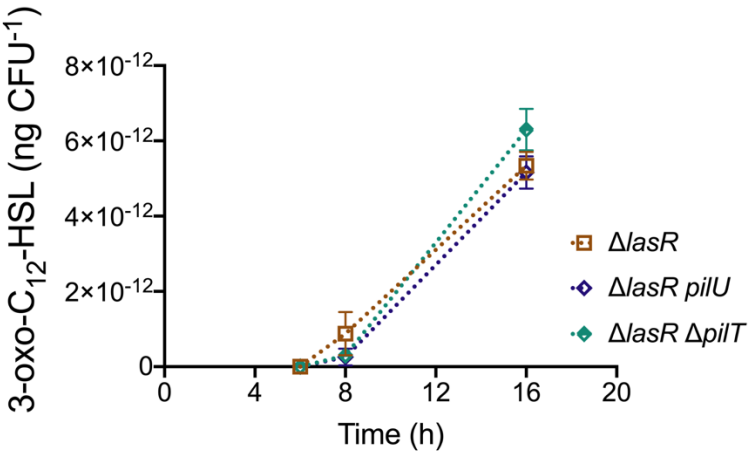

Figure 3.1.14. Type IV pili motors PilU and PilT are not responsible for surface-primed 3-oxo-C_{12}-HSL induction. 3-oxo-C_{12}-HSL concentration was measured in PA14 Δ*lasR* and the double mutants Δ*lasR pilU* and Δ*lasR* Δ*pilT* at different time points during surface growth by LC/MS. Values were normalized by the viable cell counts and shown in ng CFU^{-1}. The values are means ± standard deviation (error bars) from three replicates.

The conservation of surface-primed induction of 3-oxo-C_{12}-HSL in naturally occurring LasR-defective isolates of *P. aeruginosa* is an indicator of its importance. We observed this response in naturally evolved LasR-defective isolates from both clinical and environmental origins (Déziel *et al.*, 1996; Groleau *et al.*, 2022). The environmental isolates used here, namely 18G, 32R and 78RV, were recently characterized as LasR-defective strains based on their inability to perform LasR-dependent activities in liquid cultures (Groleau *et al.*, 2022). Of note, due to the ability to mediate RhlR-regulated QS, LasR-defective 78RV was characterized as a RAIL strain (Groleau *et al.*, 2022), like the CF isolates E113 and E167, which also have functional RhlR-dependent QS responses (Asfahl *et al.*, 2022). In the absence of a functional LasR, surface association induces the production of the 3-oxo-C_{12}-HSL signal irrespective of the QS-responsiveness mediated by LasR-independent RhlR. This response is prevalent but not universal. Isolate E41 produces trace concentrations of 3-oxo-C_{12}-HSL, and its production was not induced by surface association when compared with planktonic cells. Response variability is not surprising considering the diversity of *P. aeruginosa* isolates, but our results highlight that LasR-deficient *P. aeruginosa* isolated from both clinical and environmental settings are often proficient in the production of 3-oxo-C_{12}-HSL when adopting an attached growth mode. Thus, this ability appears to be an intrinsic and beneficial feature of this species.

Mutations in the cognate synthase gene, *lasI*, are much less frequently detected than those found in the *lasR* gene (O'Connor *et al.*, 2022). The most accepted explanation for this discrepancy is social cheating. Cheaters are individuals that benefit from a shared beneficial product or function ("public good") while contributing less than average to the metabolic cost. Inactivation of LasI would not prevent response to 3-oxo-C_{12}-HSL produced by neighbouring WT cells and thus activate a functional LasR. LasR-defective isolates emerge even in experimental conditions that do not apparently require QS-induced products (and thus cheating) (Mould *et al.*, 2022). An alternative explanation for a lower frequency of *lasI*-null isolates is that 3-oxo-C_{12}-HSL might contribute an alternative function beyond LasR activation. This interpretation is supported by our results, where LasR-defective strains retain the ability to respond to the presence of 3-oxo-C_{12}-HSL. Indeed, the expression of *phz1*, a QS-regulated operon required for pyocyanin production, is controlled by RhlR and its cognate ligand C_4-HSL. Concomitant addition of 3-oxo-C_{12}-HSL further induces *phz1* transcription and positively regulates pyocyanin production, suggesting a response to this non-cognate AHL (**Fig. 3.1.8**). The induction of RhlR-controlled *phz1* expression by 3-oxo-C_{12}-HSL is also seen in the double mutant Δ*lasR*Δ*lasI* (**Fig. 3.1.15**). Basal expression of *phz1* is due to the self-produced C_4-HSL. The addition of 3-oxo-C_{12}-HSL further enhances *phz1* transcription activity, and the highest expression is seen when C_4-HSL is added with 3-oxo-C_{12}-HSL. The requirement of C_4-HSL to induce the transcription of *phz1* by 3-oxo-C_{12}-HSL indicates that this response is RhlR-dependent, as proposed in **Figure 3.1.12**. However, it is possible that other regulatory factors also contribute to this regulation. *P. aeruginosa* possesses a third LuxR-type regulator named QscR (Chugani *et al.*, 2001). Unlike LasR and RhlR, QscR does not have a cognate synthase (Chugani *et al.*, 2001). Interestingly, QscR is a promiscuous receptor capable of binding to various long-chain AHLs, including 3-oxo-C_{12}-HSL (Lee *et al.*, 2006b; Lintz *et al.*, 2011). In a wild-type strain, QscR suppresses pyocyanin production (Chugani *et al.*, 2001). However, in the absence of LasR, the dynamics of QS regulation are reconfigured, and the contribution of QscR to pyocyanin production is conceivable. Alternatively, 3-oxo-C_{12}-HSL could partially induce a LuxR-independent response. Indeed, such regulation has been described in *P. aeruginosa* (Chugani *et al.*, 2010). The addition of exogenous AHLs, both self and non-self-produced, elicited a response in a LuxR-null background (i.e. in the absence of LasR, RhlR and QscR). However, LuxR-independent responses did not modulate the expression of genes associated with pyocyanin production (Chugani *et al.*, 2010). This observation reduces the likelihood of 3-oxo-C_{12}-HSL inducing pyocyanin production through this particular regulatory pathway.

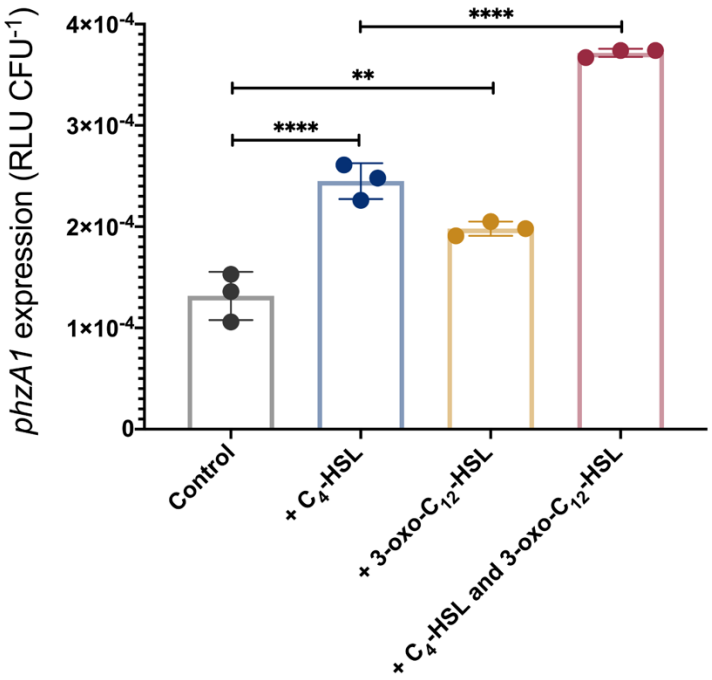

Figure 3.1.15. Induction of *phz1* operon by 3-oxo-C_{12}-HSL is also seen with endogenous C_4-HSL.
Luminescence of the *phzA1-lux* chromosomal reporter was measured in a *las* system negative background (Δ*lasR*Δ*lasI*) after the addition of 1.5 μM of C_4-HSL, 3 μM of 3-oxo-C_{12}-HSL or both molecules at 8h. Solvent alone was used as control. Relative light units were normalized by viable cell count and are shown in RLU CFU^{-1}. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons posttest with ** $P \leq 0.01$ and **** $P \leq 0.0001$.

Producing 3-oxo-C_{12}-HSL in the absence of LasR can also have a positive community outcome. Because it is exported, we have shown that this AHL can have exogenous effects in surrounding cells in a surface-associated setting (**Fig. 3.1.12**). Thus, localized production of 3-oxo-C_{12}-HSL by LasR-negative clusters could induce the expression of QS-regulated virulence factors in LasR-active cells, with minimal metabolic cost to the LasR-negative producers. Moreover, the production profile is delayed in LasR-defective strains when compared to the WT. Therefore, the mixed population composed of both LasR-active and LasR-defective cells would be subjected to steady levels of 3-oxo-C_{12}-HSL.

Furthermore, in natural habitats, *P. aeruginosa* is typically part of complex polymicrobial communities. Microbes within these communities can actively respond to one another, and these interactions range from cooperation to competition (Mitri *et al.*, 2013). For example, in mixed populations consisting of wild-type and LasR-negative cells, QS-controlled molecules are

positively regulated. This regulation relies on reciprocal cross-feeding between the populations. The release of the siderophore pyochelin by LasR-negative cells induces the production of citrate by the wild-type counterpart. In turn, citrate positively regulates RhlR activity in LasR-negative cells, leading to the induction of QS responses (Mould *et al.*, 2020). Similarly, the continuous production of 3-oxo-C_{12}-HSL by *P. aeruginosa* may play a significant role in shaping the biological activities of the population. These examples emphasize the importance of the exchange of molecules within mixed populations, which modulates population dynamics. Furthermore, apart from influencing dynamics within *P. aeruginosa* populations, 3-oxo-C_{12}-HSL may also impact interspecies communication.

In this context, LuxR homologues BtaR1 and BtaR2 from *Burkholderia thailandensis*, are promiscuous and can be activated by 3-oxo-C_{12}-HSL (Wellington *et al.*, 2019). It is worth noting that both *P. aeruginosa* and *B. thailandensis* are soil saprophytes that can inhabit similar environmental niches (Zhao *et al.*, 2012). The ecological importance of perceiving signals produced by neighbouring cells becomes apparent with the presence of an orphan LuxR homologue (SdiA) in *Salmonella enterica* serovar Typhimurium, a bacterium unable to produce AHLs (Dyszel *et al.*, 2010). This ability to “eavesdrop” on AHL signals produced by other bacteria is likely not exclusive to this bacterium and may influence interactions between different species. It provides a rationale for the sustained production of 3-oxo-C_{12}-HSL in *P. aeruginosa*. Further support for this idea comes from *Ruegeria* sp., a bacterium associated with marine sponges that possesses a solo LuxI homologue and cannot employ this molecule in a conventional QS-regulated pathway (Zan *et al.*, 2015).

QS signals also play a pivotal role in host-pathogen interactions. QS-regulated molecules can act as interkingdom QS signals, thus responsible for the communication of bacteria with mammalian cells and the modulation of host immune systems. Indeed, this was reported for 3-oxo-C_{12}-HSL (recently reviewed in reference (Fan *et al.*, 2022)). Due to the long acyl chain of this autoinducer, the molecule has lipophilic properties and, by directly interacting with biological membranes, can enter mammalian cells and interact with intracellular molecules (Ritchie *et al.*, 2007). The presence of 3-oxo-C_{12}-HSL induces apoptosis of hematopoietic cells and cytotoxicity of non-hematopoietic cells, including those of the airway epithelium (Kravchenko *et al.*, 2006; Li *et al.*, 2009; Schwarzer *et al.*, 2012; Shiner *et al.*, 2006; Tateda *et al.*, 2003). The host immune responses are also suppressed by 3-oxo-C_{12}-HSL, negatively impacting cytokines production, T-cell differentiation, and the function of antigen-presenting cells (Li *et al.*, 2015; Ritchie *et al.*, 2005; Telford *et al.*, 1998). Thus, this signal molecule is central to the virulence and pathogenesis of *P.*

aeruginosa, and the sustained production of 3-oxo-C_{12}-HSL by biofilm-growing LasR-deficient isolates in infected hosts might account for worse clinical outcomes. In infected hosts, could the immunomodulatory activity of 3-oxo-C_{12}-HSL, rather than its role as a quorum sensing signal, justify the regulatory bypass in the absence of LasR?

Sustained production of 3-oxo-C_{12}-HSL in the absence of LasR in response to surface growth, the most common lifestyle adopted by *P. aeruginosa* in its natural environments, appears to be beneficial to the colonization of many environmental niches. Combined with the widespread feature underlying the emergence of LasR-defective isolates, it raises an important question: do these isolates emerge solely to benefit from the cooperating individuals or could they play a positive role in shaping the bacterial community responses?

3.1.2 Acknowledgments

We thank George A. O'Toole (Dartmouth) and Matthew T. Cabeen (Oklahoma State University) for gifts of strains and plasmids used in this work. TOP was the recipient of PhD scholarships from the Fondation Armand-Frappier. This research was supported by the Canadian Institutes of Health Research (CIHR) operating grant MOP-142466.

3.2 Supplemental data concerning QS regulation in surface-grown cells

3.2.1 Contextualization

The data in the article entitled “Surface growth of *Pseudomonas aeruginosa* reveals a regulatory effect of 3-oxo-C_{12}-homoserine lactone in the absence of its cognate receptor, LasR,” primarily focuses on the wild-type PA14 strain and its isogenic *lasR* mutant, with a specific emphasis on the autoinducer 3-oxo-C_{12}-HSL. The present section will present further data on other autoinducers and various QS-deficient backgrounds during surface growth. Specifically, an overall view of autoinducers in the wild-type background in surface-grown cells will be provided.

3.2.2 Material and Methods

Table 3.2.1. Strains employed in this supplementary investigation

Strain	ED #	Description	Reference
PA14 Δ*pqsE*	ED36	Strain PA14 derivate; unmarked in-frame *pqsE* deletion	(Déziel *et al.*, 2004)
E113 Δ*rhlR*	ED4145	Strain E113 derivate carrying an unmarked deletion in the *rhlR* gene	(Asfahl *et al.*, 2022)
E167 Δ*rhlR*	ED4153	Strain E167 derivate carrying an unmarked deletion in the *rhlR* gene	(Asfahl *et al.*, 2022)

3.2.3 Results

3.2.3.1 RhlR is crucial for 3-oxo-C_{12}-HSL production in PA14, but is not universally required in all LasR-defective backgrounds

The production of 3-oxo-C_{12}-HSL is induced in surface-grown cells of naturally occurring LasR-deficient isolates, as shown in **Figure 3.1.6**. In the prototypical strain PA14, this response requires RhlR (**Fig. 3.1.3**). Therefore, one may infer that the RhlR-induced 3-oxo-C_{12}-HSL production in surface-grown cells is likely conserved in other LasR-deficient backgrounds. To verify this hypothesis, the concentrations of 3-oxo-C_{12}-HSL in surface-grown cells of the LasR-deficient isolates E113 and E167 were monitored during growth on King’s A agar, a condition in which the concentration of this autoinducer is induced compared to its planktonic counterpart (**Fig. 3.1.6**).

The production profile of 3-oxo-C_{12}-HSL was compared with that of their isogenic *rhlR* mutants (i.e., E113 Δ*rhlR* and E147 Δ*rhlR*). In contrast to the dependency on RhlR demonstrated for PA14, these strains presented comparable production profiles of 3-oxo-C_{12}-HSL (**Fig. 3.2.1**). This indicates that even though the surface-induced production of 3-oxo-C_{12}-HSL is conserved among *P. aeruginosa* LasR-deficient isolates, the mechanism mediating this response is not.

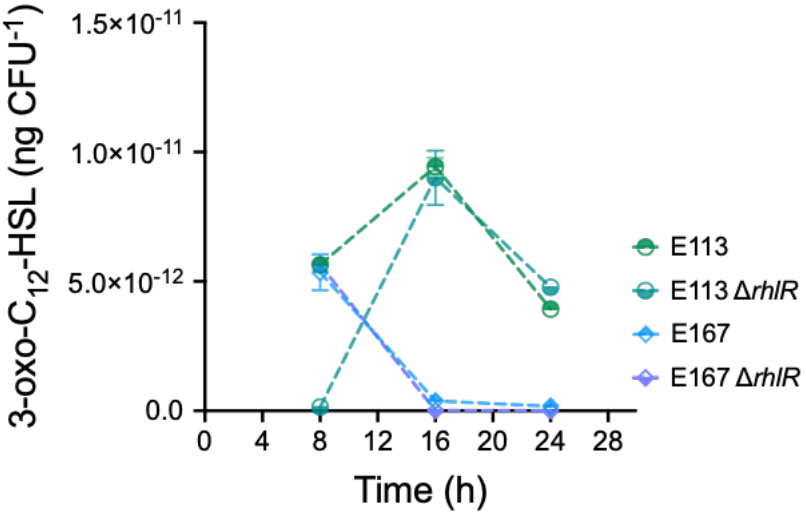

Figure 3.2.1. RhlR is not universally required for 3-oxo-C_{12}-HSL production in LasR-defective isolates.
3-oxo-C_{12}-HSL concentration was measured in the LasR-deficient isolates E113 and E167, and their isogenic mutants E113 Δ*rhlR* and E167 Δ*rhlR* at different time points during surface growth by LC/MS. Values were normalized by the viable cell counts and shown in ng CFU^{-1}. The values are means ± standard deviation (error bars) from three replicates.

3.2.3.2 Profiles of autoinducer production between surface-grown and planktonic cells

In addition to quantifying 3-oxo-C_{12}-HSL and C_4-HSL, as presented in **Figures 3.1.1** and **3.1.13**, the levels of other autoinducers in the QS circuitry – specifically HHQ and PQS – and the molecule HQNO were measured in the surface-grown *P. aeruginosa* wild-type strain PA14. The goal was to compare the production patterns of these signalling molecules to those in planktonic cells and discern potential differences in their production profiles under these distinct growth conditions. As previously shown, the production profile of 3-oxo-C_{12}-HSL remains unaffected by the bacterial growth mode (**Fig. 3.1.1**). Surface growth induces the production of C_4-HSL (**Fig. 3.1.13**), but a clear effect is not observed for HHQ or PQS (**Fig. 3.2.2**). Notably, in planktonic cells, the concentration of HHQ and HQNO steadily decreases throughout growth (refer to **Fig. 3.1.1B** for a growth curve of PA14 in planktonic and surface-associated conditions). This variation in concentration is not observed in surface-grown cells, where HHQ and HQNO concentration

remains relatively stable (**Fig. 3.2.2A** and **3.2.2C**). Conversely, the production profile of PQS is similar between the tested conditions (**Fig. 3.2.2B**).

As previously presented, alongside the production of the signalling molecules HHQ and PQS, the *pqs* system also generates significant amounts of HQNO (refer to **Fig. 1.2.3**); together, these molecules represent the overall activity of this signalling circuitry and, indirectly, of MvfR activity. Therefore, the total HAQ produced by PA14 under planktonic and surface-associated conditions was compared (**Fig. 3.2.1D**). The comparison of the production profile between these conditions is similar to that of HHQ and HQNO, in which the concentration of HAQs is much more variable during planktonic growth and somewhat steady during growth in surface-associated cells. The concentration of these molecules is reduced in the latter condition, indicating the decreased activity of the *pqs* system in surface-grown cells.

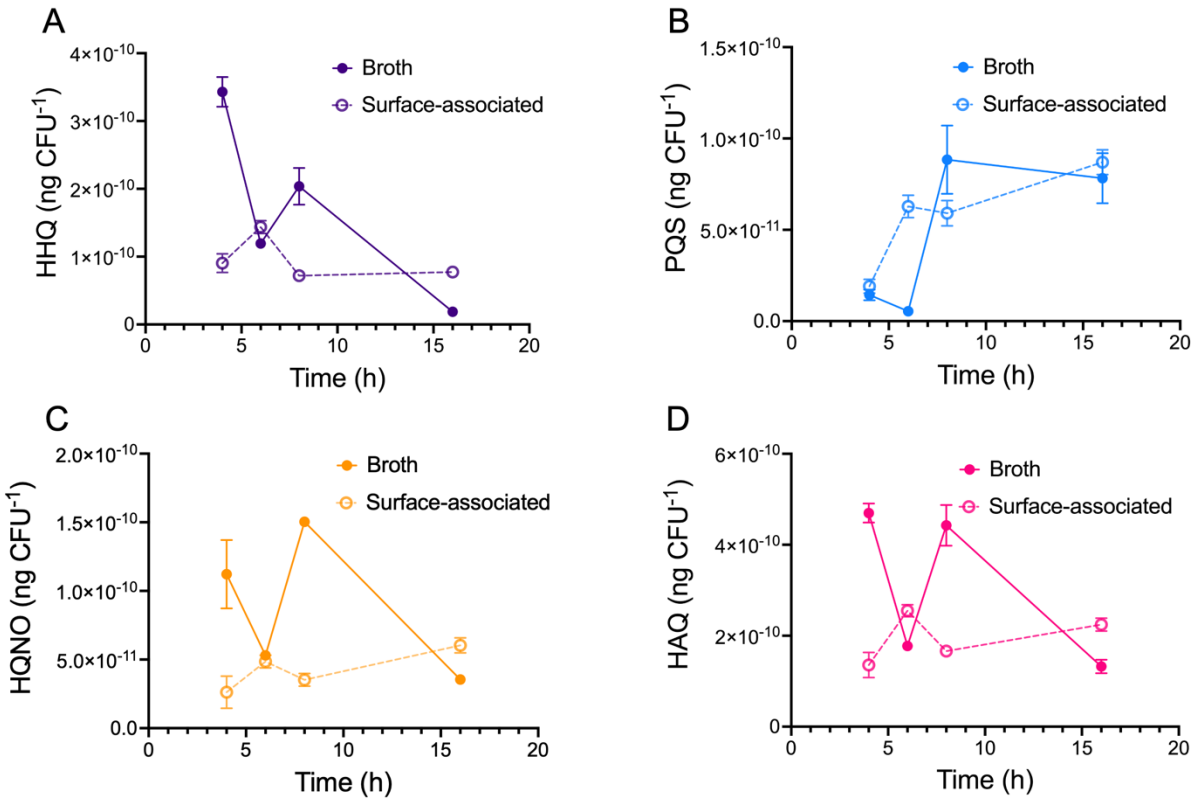

Figure 3.2.2. Mode of growth modulates the production of HHQ, but not PQS in the wild-type PA14 background. **(A)** HHQ, **(B)** PQS, and **(C)** total HAQ concentration was measured in PA14 at different time points during planktonic (broth culture) and surface growth (surface of agar-solidified culture media) by liquid chromatography/mass spectrometry. Total HAQ comprises the production of the signalling molecules of the *pqs* system, HHQ and PQS, and HQNO. Values were normalized by the viable cell counts and shown in nanograms per CFU. The values are means ± standard deviation (error bars) from three replicates.

3.2.3.3 Inducing C_4-HSL in surface-grown cells: what triggers the production of this molecule?

As shown above, the bacterial lifestyle affects the production of C_4-HSL and HAQs in the PA14 background. The surface association promotes an increase in C_4-HSL production but concurrently suppresses the accumulation of HHQ and overall HAQs. Given these findings, this investigation aimed to understand how surface association affects these molecules, particularly the dynamics of C_4-HSL production in this subsection.

As previously mentioned, the production of C_4-HSL in planktonic cells is primarily governed by LasR through *rhlI* transcription, with some contribution from RhlR. To examine whether this regulatory mechanism also occurs in sessile cells, C_4-HSL was measured in both *lasR* and *rhlR* mutants. **Figure 3.2.3A** shows that the production profile of this signalling molecule in surface-grown cells mirrors that observed in planktonic cells. In the absence of LasR, there is a delayed detection of C_4-HSL, while the reduction is only marginal in the absence of RhlR compared to the WT PA14 strain. This suggests that LasR primarily regulates C_4-HSL production in surface-grown cells as well, but RhlR can take on this role when LasR is inactive. This is corroborated by the decreased production of C_4-HSL in the double *lasR pqsE* mutant compared to the single *lasR* mutant (**Fig. 3.2.3A**), as PqsE is required to activate RhlR, and this requirement is evidenced in the absence of LasR (Groleau *et al.*, 2020). Notably, the production of C_4-HSL is abolished in the double *lasR rhlR* mutant, emphasizing the essential role of these regulators (**Fig. 3.2.3A**).

While the described regulation aligns with the production profile of C_4-HSL in planktonic cultures, there is an exception for the stationary phase time point, corresponding here to 16 hours of incubation (**Fig. 3.2.3A**). The concentration at this time point is presented separately for clarity (**Fig. 3.2.3B**). Surprisingly, in the stationary phase, the *lasR* and *rhlR* mutants exhibit higher C_4-HSL levels than the WT strain. This raises questions about the potential repressive role of these transcriptional regulators on C_4-HSL production. However, the simultaneous absence of these regulators, as evident in the *lasR rhlR* double mutant, results in the complete loss of C_4-HSL production (**Fig. 3.2.3B**). Curiously, in the *lasR pqsE* double mutant, there is a mild reduction in C_4-HSL production compared to the *lasR* mutant, but its production profile does not mirror the *rhlR* mutant (**Fig. 3.2.3B**). In the *lasR* background, the surrogate activity of RhlR relies heavily on PqsE, impacting C_4-HSL production in planktonic cells (Groleau *et al.*, 2020) and 3-oxo-C_{12}-HSL production in surface-grown cells (**Fig. 3.1.3**). This observation suggests that *rhlI* transcription might be induced by different conformations of RhlR – including one that is PqsE-independent,

resulting in a concentration similar to the WT in the *lasR pqsE* double mutant. Notably, this regulation is not surface-driven, as it is not observed for 3-oxo-C_{12}-HSL (**Fig. 3.1.3**).

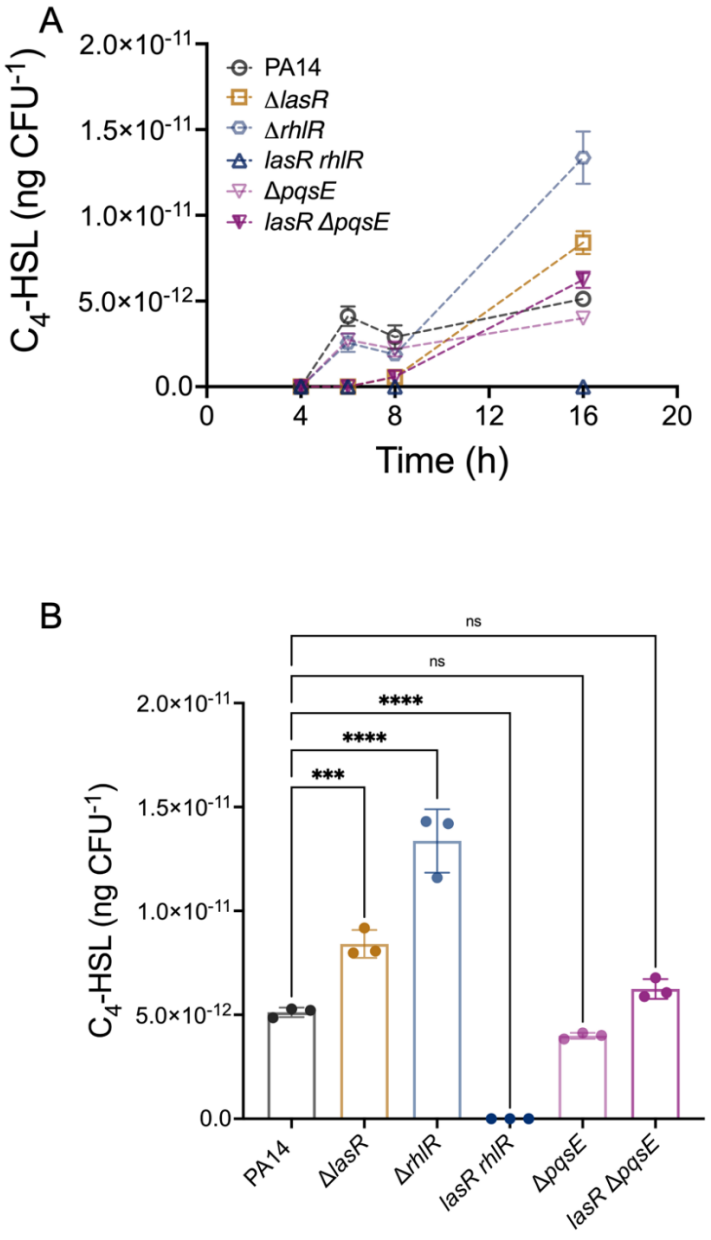

Figure 3.2.3. LasR is the main regulator of C_4-HSL production in surface-grown cells.

(A) C4-HSL concentration was measured in PA14 and isogenic mutants Δ*lasR*, Δ*rhlR*, *lasR*- *rhlR*-, Δ*pqsE*, and *lasR* Δ*pqsE* at different time points during surface growth (surface of agar-solidified culture media) by liquid chromatography/mass spectrometry. **(B)** C_4-HSL concentration at 16h from (A). Values were normalized by the viable cell counts and shown in nanograms per CFU. The values are means ± standard deviation (error bars) from three replicates. One-way analysis of variance (ANOVA) and Dunnett's multiple comparisons post-test were used to quantify statistical significance. ns, nonsignificant; *** $P \leq 0.0005$; **** $P \leq 0.0001$.

3.2.3.4 Surface-associated growth represses HAQ production: which QS system is involved in this response?

In the canonical organization of the QS regulatory network, the activity of the *pqs* circuitry is positively modulated by the *las* system and negatively by the *rhl* system. As mentioned earlier, the concentration of total HAQ represents the activity of the *pqs* system as a whole. In this scenario, it indicates repression or reduced activation of this system in surface-grown cells. Consequently, two alternative explanations for this behaviour were proposed: either the *las* system is less active in sessile cells, resulting in reduced expression of the primary regulator of the *pqs* system, *mvfR*, or the *rhl* system is more active, directly repressing the expression of the *pqsABCDE* operon.

As frequently addressed in this section, the primary target of the *las* system is the gene coding its cognate synthase LasI. In WT PA14 surface-grown cells, the production profile of 3-oxo-C_{12}-HSL (molecule synthesized by LasI) is comparable to the one observed in planktonic cells (**Fig. 3.1.1A**), indicating the activity of the *las* system is not regulated by growth conditions. Therefore, the surface-dependent response impacting the *pqs* system should be regulated by the *rhl* system.

To assess the impact of the *rhl* system on the activity of the *pqs* system, measurements of HAQ levels were conducted in the *rhlR* mutant under both planktonic and surface-grown conditions. The working hypothesis was predicated on the idea that if the *rhl* QS system exhibited greater activity in surface-associated conditions, a pronounced repression of the *pqs* system should be observed in surface-grown cells of the PA14 background compared to their planktonic counterparts. Furthermore, it was anticipated that this repression would be alleviated in the absence of RhlR. For quantification, the total HAQ concentration in planktonic cells was compared to that of surface-grown cells at various time points during growth for both PA14 and Δ*rhlR* strains, and these ratios are presented in **Figure 3.2.4**.

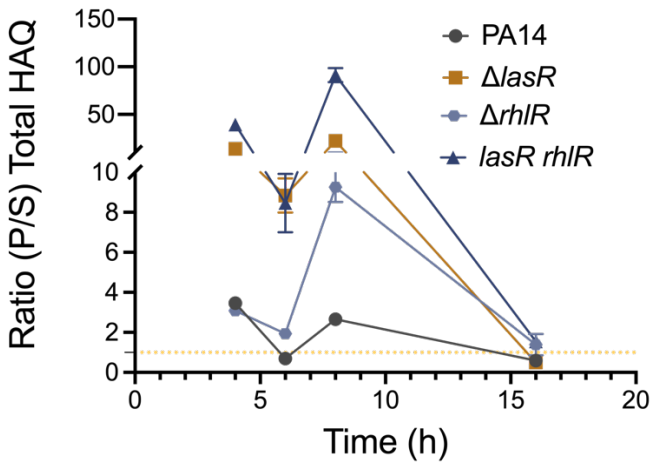

Figure 3.2.4. The *rhl* system does not preferentially repress the production of HAQs in surface-grown cells. HAQ concentration (representing HHQ, PQS and HQNO) was measured in the PA14 strain and the isogenic Δ*lasR,* Δ*rhlR*, and *lasR rhlR* mutants at different time points during planktonic and surface growth (surface of agar-solidified culture media) by liquid chromatography/mass spectrometry. Values were normalized by the viable cell counts. The ratios presented depict the normalized concentration in planktonic (P) per surface-associated (S) conditions. A dotted yellow line is traced at 1. Points above this line indicate a higher concentration of HAQs in planktonic than in surface-grown cells, whereas points below the line signify a higher concentration in surface-grown cells.

It is important to acknowledge that the chosen representation for HAQ production in **Fig. 3.2.4** possesses inherent bias. This representation, which relies on the ratios of HAQ production during planktonic growth compared to surface-associated conditions, does not allow for the differentiation of whether HAQ production is induced during planktonic conditions or repressed during surface growth. However, it does enable inferences to be drawn from the ratios observed during growth in the wild-type PA14 strain, suggesting HAQ production is favoured in planktonic growth. This observation aligns with the concept of repression of the *pqs* system in the surface-grown cells.

Nevertheless, the ratios measured in the Δ*rhlR* mutant do not indicate relief from the predicted repression in surface-associated conditions, confirming that RhlR activity does not account for the reduced HAQ production in this growth condition. Nonetheless, these findings imply that HAQ production in planktonic cells is favoured, akin to the pattern observed in the wild-type PA14 strain, albeit with more pronounced differences (**Fig. 3.2.4**).

Interestingly, the HAQ ratio is further accentuated in the *lasR* mutant, indicating similar responses despite their contrasting roles in activating the *pqs* system (**Fig. 3.2.4**). This response is significantly amplified in the double *lasR rhlR* mutant. As mentioned previously, the inherent bias

in this representation hampers a precise analysis of the presented results. Therefore, the decision was made to employ the HAQs production profile of the wild-type PA14 background as a reference for comparison and to assess production in the aforementioned isogenic mutants separately for each growth condition and time point.

By conducting these comparisons, the positive regulation (mediated by the *las* system; **Fig. 3.2.5A**) and the negative regulation (mediated by the *rhl* system; **Fig. 3.2.5B**) in both planktonic and surface-grown cells were elucidated. Interestingly, despite LasR being recognized for inducing *mvfR* transcription in planktonic cells, a significantly stronger response of the *pqs* system to LasR is observed under surface-grown conditions. In planktonic cells, the ratio of HAQ production in the wild-type PA14 strain was approximately fivefold higher than in the *lasR* mutant (**Fig. 3.2.5A**), confirming that LasR induces HAQ production in this condition. This response diminishes rapidly during growth, indicating that the positive impact of LasR on HAQ production is limited to earlier growth phases (**Fig. 3.2.5A**). Conversely, the absence of LasR exerts a stronger impact on HAQ production in surface-grown cells. In this lifestyle, the ratio of HAQ production in the wild-type PA14 strain was approximately 21-fold higher than in the *lasR* mutant during earlier growth, and the positive effect of this regulator persists until the stationary phase (**Fig. 3.2.5A**). In contrast to the initial assumption of a weaker contribution of the *las* system, resulting in lower HAQ levels in surface growth conditions, the conducted comparisons yielded an unexpected result. The data suggests that during surface growth, cells experience a more robust positive regulation of the *las* system.

In contrast to the positive regulation mediated by LasR that diminishes during growth, planktonic cells experience a gradual increase in the RhlR-mediated negative regulation (**Fig. 3.2.5B**). In contrast, surface-grown cells exhibit HAQ production ratios in the PA14 strain close to 1 during earlier growth, indicating that the concentration of HAQs produced by these strains is similar. Therefore, RhlR does not significantly act as a repressor in the PA14 background until the stationary phase, where a substantial effect is observed, reaching an approximately ninefold higher concentration in the *rhlR* mutant compared to the wild-type PA14 strain (0.11 on the graphic; **Fig. 3.2.5B**). These findings suggest that the reduced levels of HAQ in surface-grown cells, specifically HHQ and HQNO, are not due to more significant repression of the *rhl* system, consistent with **Fig. 3.2.4**. Instead, the strength of repression seems to be in function of the overall concentration of HAQs produced per CFU which is much higher in planktonic cells, leading to a much more robust repression. Importantly, LasR is not the primary regulator of HAQ production, as the concentration of these molecules does not align with the inputs induced by this regulator.

This could indicate the search for homeostasis for both positive (LasR) and negative (RhlR) inputs. This would explain the differences between the activation of these systems in planktonic and surface-grown cells but does not elucidate the mechanism driving the differential production of the *pqs* system in these lifestyles.

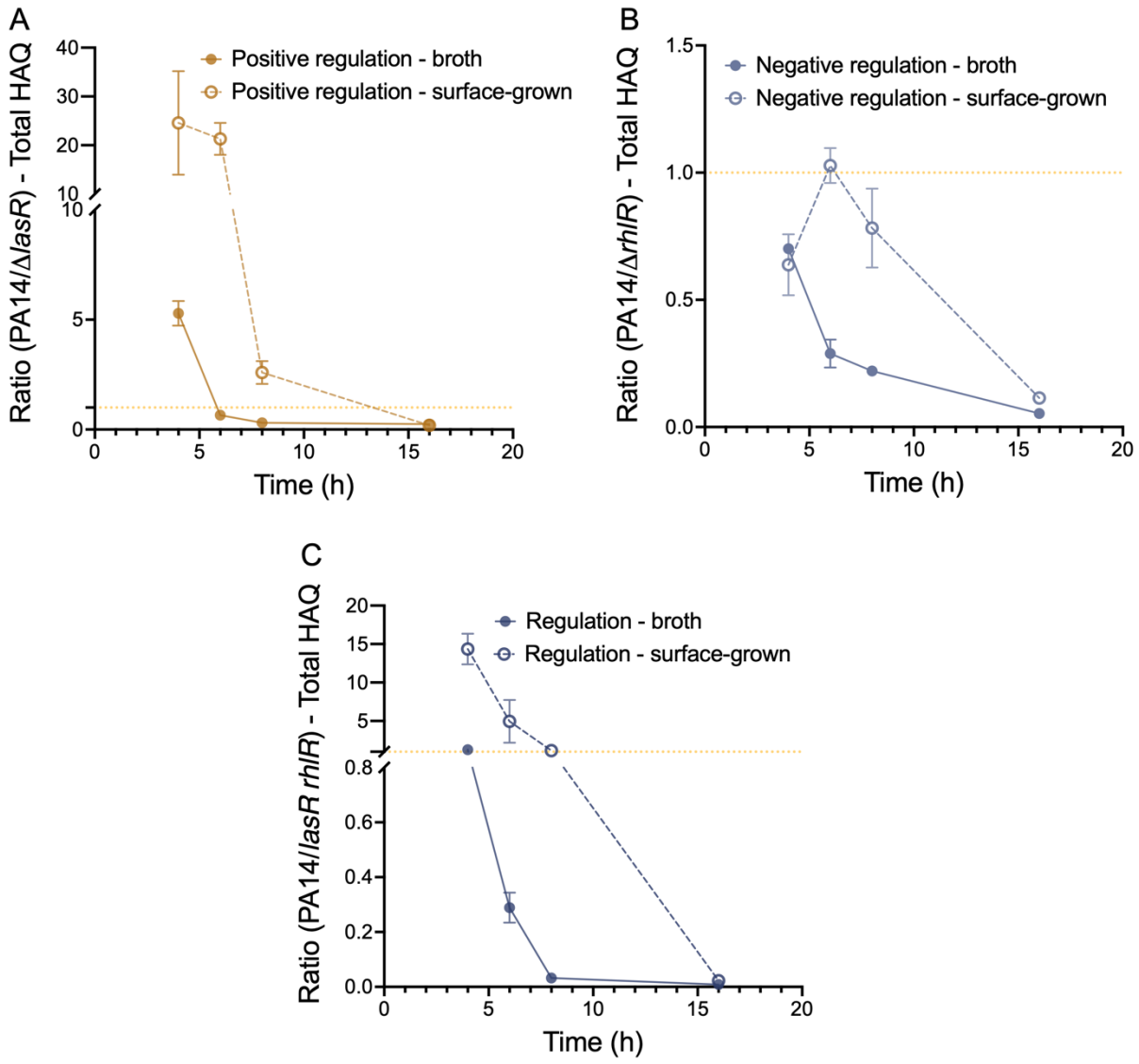

Figure 3.2.5. The regulation of the *pqs* system in planktonic cells differs from that in surface-grown cells. HAQ concentration (representing HHQ, PQS and HQNO) was measured in the PA14 strain and the isogenic Δ*lasR,* Δ*rhlR*, and *lasR rhlR* mutants at different time points during planktonic and surface growth (surface of agar-solidified culture media) by liquid chromatography/mass spectrometry. Values were normalized by the viable cell counts. The ratios presented depict the normalized concentration in the PA14 strain per that in **(A)** Δ*lasR*, **(B)** Δ*rhlR*, and **(C)** *lasR rhlR* mutants. A dotted yellow line is traced at 1. Points above this line indicate a higher concentration of HAQs in planktonic than in surface-grown cells, whereas points below the line signify a higher concentration in surface-grown cells. The planktonic lifestyle is represented by solid lines (broth) and sessility by dotted ones (surface-grown).

Consistent with the idea that other regulators influence the activity of the *pqs* system, both positive and negative regulations are observed in the absence of both *lasR* and *rhlR* (i.e., double *lasR rhlR* mutant). In planktonic cells, the HAQs ratios indicate a significant imbalance between the production profile of the PA14 strain and the *lasR rhlR* mutant, indicating the loss of a strong repressor when comparing these strains (**Fig. 3.2.5C**). Notably, these ratios are much more substantial than when only *rhlR* is absent, suggesting the involvement of other regulators in this response. During surface growth, the opposite scenario is present during the earlier stages of growth. The absence of both *lasR* and *rhlR* in this condition results in ratios of HAQ production in the PA14 strain approximately 14-fold higher than in the double mutant, indicating the loss of an activator (**Fig. 3.2.5C**). The positive regulation gradually diminishes during growth and shifts to a negative one at the stationary phase.

3.2.4 Discussion

Various multicellular behaviours are influenced both by the QS regulatory circuitry and the bacterial lifestyle. In natural environments, the predominant growth mode for bacteria, including for the bacterium *P. aeruginosa*, is sessility, characterized by growth within an encased polymeric matrix (biofilms). Notably, biofilm formation and the induction of virulence – prevalent features in sessile polymicrobial structures, are recognized factors subject to QS regulation. However, the existing literature offers limited examples demonstrating differential regulation of QS-controlled responses between planktonic and surface-associated cells. Indeed, the majority of our understanding of QS system functionality, ranging from biochemical studies to comprehensive evolutionary experiments, predominantly focuses on the planktonic lifestyle.

Despite the limited available information regarding the relationship between surface growth and QS regulation, evidence of this connection can be found in the literature. In this chapter, contributions were made to its understanding by investigating QS regulation in surface-grown cell, centered on the wild-type PA14 background and its isogenic *lasR* mutant. Indeed, the impact of bacterial lifestyle on QS extends beyond the modulation of 3-oxo-C_{12}-HSL production, significantly altering the conventional interplay of the QS system as depicted in **Figure 1.2.5**. As QS systems rely on the production of their respective autoinducers, these molecules were quantified during surface growth to enhance our comprehension of the QS system during this growth condition. These QS autoinducers, as well as molecules produced in dependence on QS, were measured at various time points during growth, as the activity of this communication system is inherently intertwined with bacterial growth.

Examining the production profiles of AHL autoinducers, specifically 3-oxo-C_{12}-HSL and C_4-HSL (**Fig. 3.1.1** and **3.1.13**, respectively), as well as HHQ, PQS, and HQNO (**Fig. 3.2.2**) in surface-grown cells has provided an initial, albeit incomplete, framework for understanding the QS regulatory network in this growth mode. A proposed surface QS regulatory network is depicted in **Figure 3.2.6**.

Notably, while the production profiles of 3-oxo-C_{12}-HSL and PQS had minimal differences compared to their planktonic counterparts, distinct variations were observed in the production profiles of C_4-HSL and HHQ. When considering the 3-oxo-C_{12}-HSL in isolation, one might assume that the activity of the *las* system remains consistent between these growth modes. However, assumptions drawn from the presented data suggest that other factors under LasR control, such as MvfR and RhlI, undergo differential regulation in response to surface growth conditions. These assumptions must be directly assessed to understand the contribution of LasR during surface growth.

Understanding the inherent complexity of QS poses a considerable challenge. This intricate communication system involves several integrated regulators, as depicted in **Figure 1.2.6**. Quantitative analyses have underscored the presence of these regulators, influencing the activity of the *pqs* system, presumably through both MvfR and PqsH (**Fig. 3.2.6**). Elevated levels of HAQs have been demonstrated in planktonic cells compared to surface-grown counterparts. Despite efforts to explore this response, it remains challenging to definitively establish whether the *pqs* system is robustly induced in planktonic cells or significantly repressed in surface-grown cells. This recurring question often emerges when comparing different conditions, necessitating the establishment of an arbitrary baseline for meaningful comparisons. One potential approach to mitigate this inherent bias involves identifying the putative condition-specific activator or repressor and analyzing the production profile without the involvement of this regulatory element. However, it is important to acknowledge that the existing literature does not provide clear guidance on identifying this putative regulator, making it necessary to determine experimentally.

This complexity is also observed in the effort to comprehend the activity of RhlI. The production profile of the signalling molecule it synthesizes, C_4-HSL, mirrors that of planktonic cells, with LasR being its primary regulator. However, a divergence occurs at the stationary phase, where the production of C_4-HSL surpasses that of the PA14 strain in both *lasR* and *rhlR* mutants. This peculiarity suggests a potential repression of C_4-HSL production in these mutants during this stage in PA14 (**Fig. 3.2.6**). This unexpected observation challenges conventional understanding, as both LasR and RhlR have been exclusively characterized as transcriptional activators to date.

Further investigation is required to unravel the intricacies of this response. For instance, measuring the transcriptional activation of pivotal genes for both *rhl* and *pqs* systems – including *rhlR*, *rhlI*, *mvfR*, the *pqsABCDE*, and *pqsH* – would provide valuable insights into the regulatory dynamics of these systems in surface-associated conditions. Conducting this parallel exploration is essential as the "surface-associated" QS system presented here relies on an indirect perspective. Additionally, it is crucial to keep interpretations open until further confirmation is obtained, especially considering that surface association has been previously observed to induce unexpected responses, as exemplified by the production of 3-oxo-C_{12}-HSL in the absence of LasR.

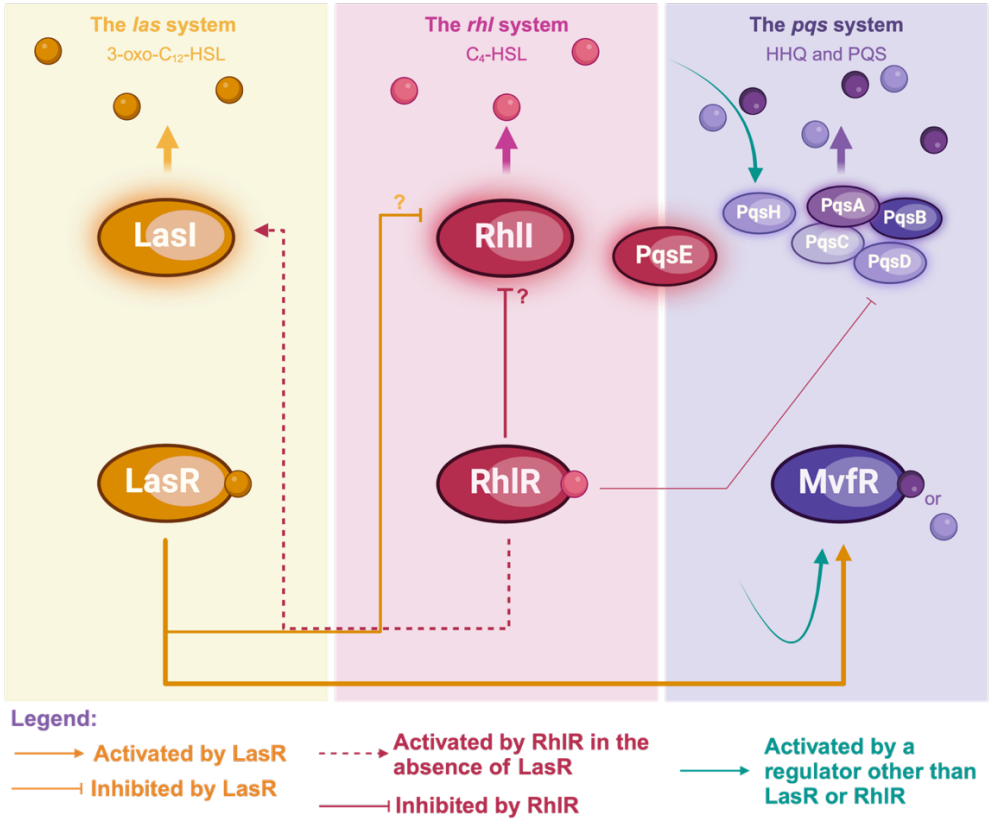

Figure 3.2.6. Surface alters the canonical interconnections of the QS regulatory network.
The three QS systems are visually distinguished by colour: yellow for the *las*, pink for the *rhl*, and purple for the *pqs* system. The depicted connections were established based on the quantification of autoinducers during surface growth, excluding connections previously described in planktonic cells (refer to **Fig.1.2.5**). The width of the line corresponds to the strength of the represented regulation. An interrogation mark symbolizes unexpected interactions, demanding further confirmation.

4 ENVIRONMENTAL TEMPERATURE AS A MODULATOR OF QS

This chapter consists of two manuscripts focusing on the influence of growth temperature on QS-dependent responses in *P. aeruginosa*.

Section 4.1 introduces the first article, titled “Temperature-responsive control of *Pseudomonas aeruginosa* virulence determinants through the stabilization of quorum sensing transcriptional regulator RhlR,” followed by **section 4.2**, which provides complementary results to enhance the understanding of the presented data.

The second article, titled “Unravelling the plasticity of quorum sensing in *Pseudomonas aeruginosa*: Insights from a naturally evolved LasR variant,” is presented in **section 4.3**, followed by another section (**section 4.4**), presenting complementary results.

To conclude, a concise discussion will unify the data presented throughout this chapter. It is presented in **section 4.5**.

4.1 Article: "Temperature-responsive control of *Pseudomonas aeruginosa* virulence determinants through the stabilization of quorum sensing transcriptional regulator RhlR"

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Laboratory experiments done by: TOP

Data analysis and interpretation: TOP, MCG, ND, and *ED*

Manuscript writing: TOP, MCG and ED.

4.1.1 Abstract

The versatile bacterium *Pseudomonas aeruginosa* thrives in diverse environments and is notably recognized for its role as an opportunistic pathogen. In line with its adaptability, *P. aeruginosa* produces various exoproducts crucial to survival and virulence, several of which regulated through quorum sensing (QS). These factors are also regulated in response to environmental cues, such as temperature changes. As a pathogen, *P. aeruginosa* is generally thought to activate its virulence factors at temperatures akin to warm-blooded hosts rather than environmental temperatures. Recent studies elucidated the functional structure of the QS transcriptional regulator RhlR, which depends on the stabilizing effects of its cognate autoinducing ligand, *N*-butanoyl-L-homoserine lactone (C_4-HSL), and of the moonlighting chaperone PqsE. Given the influence of temperature on biomolecular dynamics, we investigated how it affects RhlR activity using the RhlR-regulated *phzA1* promoter, as a proxy. Unexpectedly, we found that RhlR activity is higher at 25°C than at 37°C. This temperature-dependent regulation likely stems from altered RhlR turnover, with the presence of PqsE extending RhlR activity tenfold from its basal level at 37°C to that observed at 25°C. This lower, environmental-like temperature promotes increased affinity between RhlR and C_4-HSL, a trait significantly compromised in the absence of PqsE. These results suggest that this response depends on the structural integrity of the complex, indicating that temperature functions as an additional regulating and stabilizing factor of RhlR function. Accordingly, lower growth temperature fails to increase the activity of a structurally stabilized version of RhlR. The thermoregulation aspect of RhlR activity and signalling impacts the virulence profile of a mutant unable to produce C_4-HSL, underscoring its significance in bacterial behaviours and potentially conferring an evolutionary advantage.

4.1.2 Author Summary

Pseudomonas aeruginosa is recognized for its capacity to colonize vastly different environments, thereby encountering a range of temperatures. The bacterium's ability to adapt to these settings necessitates finely regulated gene expression. Within this regulatory framework lies quorum sensing (QS), the intercellular communication system used by *P. aeruginosa* to orchestrate the expression of genes responsible for producing diverse exoproducts, including the blue phenazine pyocyanin. RhlR primarily governs the expression of genes required for pyocyanin production, including the *phz1* operon. Unlike other QS regulators, RhlR possesses a distinctive characteristic – in addition to its cognate signalling ligand C_4-HSL, it depends on the presence of the chaperone-like protein PqsE for stability and activity. This intrinsic instability implies that RhlR may be

susceptible to external influences that can modulate its function. Indeed, a lower culture temperature, akin to an environmental-like condition, induces the transcription of the *phz1* operon, used as a proxy for RhlR activity. Using a combination of genetic approaches, we present evidence that this thermoregulation is due to an impact on the stability of the RhlR/C_4-HSL/PqsE complex. We further show the biological effect of this regulation mechanism in an infection setting, which could underscore a relevant role for other bacterial behaviours.

4.1.3 Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* exhibits a remarkable adaptability attributed to its extensive genomic repertoire, enabling its survival in various ecological niches. Found in natural habitats, environmental isolates of *P. aeruginosa* are mostly distributed in niches closely related to human activity, including contaminated soils and water reservoirs (Crone *et al.*, 2020; Green *et al.*, 1974; Mena *et al.*, 2009). In addition to its frequent isolation from environmental sites, *P. aeruginosa* is a clinically important opportunistic pathogen, frequently involved in nosocomial infections and posing a particular threat to people with cystic fibrosis (CF), leading to severe morbidity and mortality (Reynolds *et al.*, 2021). Interestingly, *P. aeruginosa* infections extend beyond warm-blooded hosts, encompassing reptiles, insects, and plants (Apidianakis *et al.*, 2009; Starkey *et al.*, 2009; Tan *et al.*, 2000; Xiong *et al.*, 2022). Virulence determinants are highly conserved across *P. aeruginosa* isolates regardless of their origin (environmental or clinical), underscoring the importance of these factors in diverse natural environments (Wolfgang *et al.*, 2003).

The production of virulence determinants in *P. aeruginosa* are primarily regulated at the transcriptional level, ensuring appropriate responses to environmental cues. Several regulatory systems are involved in this regulation, including quorum sensing (QS). QS relies on the production of signalling molecules called autoinducers, which, upon entry ito the cells, interact with cognate transcriptional regulators, enabling coordinated responses across the population based on cell density (Diggle *et al.*, 2007; Waters *et al.*, 2005). In *P. aeruginosa*, a network of three interlinked systems traditionally represents the QS circuitry. The *las* system comprises the LasR transcriptional regulator and the cognate acyl-homoserine lactone (AHL) synthase LasI. The latter produces the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL), which binds to LasR and activates its transcriptional function (Pearson *et al.*, 1994). Activation of LasR leads to the transcription of several target genes, including coding for virulence determinants and other QS regulatory elements (Gilbert *et al.*, 2009; Schuster *et al.*, 2003;

Whiteley *et al.*, 1999). The *rhl* system is composed of the transcriptional regulator RhlR and the associated synthase RhlI, responsible for synthesizing signal *N*-butanoyl-L-homoserine lactone (C_4-HSL) (Pearson *et al.*, 1995). Activated by LasR, the *rhl* system controls the expression of genes related to the production of numerous virulence determinants, including the redox-active blue phenazine pyocyanin (Brint *et al.*, 1995; Gilbert *et al.*, 2009; Latifi *et al.*, 1996; Pearson *et al.*, 1997; Pesci *et al.*, 1997; Whiteley *et al.*, 1999).

In addition to the AHL-based QS systems *las* and *rhl*, the QS regulatory network of *P. aeruginosa* also includes the *pqs* system. Unlike the AHL-based systems, the *pqs* system relies on the production of autoinducers belonging to the 4-hydroxy-2-alkylquinolines family (HAQs). The synthesis of HAQs is activated by the QS transcriptional regulator MvfR (also known as PqsR) (Cao *et al.*, 2001; Gallagher *et al.*, 2002). Among the enzymes required for the HAQs synthesis are PqsA-D, encoded by the *pqsABCDE* operon (Déziel *et al.*, 2004; Dulcey *et al.*, 2013; Gallagher *et al.*, 2002). Notably, the PqsE protein, encoded by the last gene in this operon, plays a crucial role in fully activating the *rhl* system. Despite the suggestion of an interaction between the *rhl* and *pqs* systems for two decades, it was only recently elucidated (Borgert *et al.*, 2022; Déziel *et al.*, 2005; Diggle *et al.*, 2003; Farrow *et al.*, 2008; Feathers *et al.*, 2022; Groleau *et al.*, 2020; Letizia *et al.*, 2022). PqsE moonlights as a chaperone, enhancing the stability of RhlR and, in conjunction with the cognate signal C_4-HSL, mediating the complete activation of the *rhl* system (Borgert *et al.*, 2022; Feathers *et al.*, 2022).

For opportunistic pathogens such as *P. aeruginosa*, regulating virulence determinants also involves the capacity to sense and adapt to temperature variations. When this bacterium colonizes the human body, it encounters a temperature environment distinct from aquatic and soil settings. Previous studies have documented the thermoregulation of specific virulence factors in *P. aeruginosa*, including those under the QS control (Barbier *et al.*, 2014; Bisht *et al.*, 2022; Bisht *et al.*, 2021; Wu *et al.*, 2011; Wurtzel *et al.*, 2012). Genes under RhlR control, including those implicated in pyocyanin production, are thermoregulated as the protein level of RhlR varies within the cell according to growth temperatures (Grosso-Becerra *et al.*, 2014). The synthesis of pyocyanin involves enzymes encoded by two paralogous operons, *phzA1-G1* (*phz1*) and *phzA2-G2* (*phz2*) (Mavrodi *et al.*, 2001). These enzymes produce phenazine-1-carboxylic acid (PCA), which is further converted into phenazines such as pyocyanin (Mavrodi *et al.*, 2001). While some high-throughput gene expression profiling comparing the effects of temperature variations indicates the upregulation of the expression of the *phz* operons at 37°C compared to

environmental temperatures (Wu *et al.*, 2011; Wurtzel *et al.*, 2012), some report no such regulation (Barbier *et al.*, 2014; Bisht *et al.*, 2021).

The dynamics of RhlR activity and the subsequent expression of RhlR-dependent virulence determinants are intricate and extend beyond a mere dependence on RhlR protein concentrations. The distinctive activation of this regulator was uncovered using the transcription of *phz1* as a proxy for RhlR activity (Borgert *et al.*, 2022; Feathers *et al.*, 2022; Groleau *et al.*, 2020), and the expression data concurred with crystal structures of the active RhlR/C_4-HSL/PqsE complex (Borgert *et al.*, 2022).

The intricacies of regulating the *rhl* system likely hold ecological significance for an adaptable bacterium like *P. aeruginosa*. Indeed, the emergence of LasR-defective isolates is a common adaptation of this bacterium in both environmental and clinical contexts (Groleau *et al.*, 2022; O'Connor *et al.*, 2022; Trottier *et al.*, 2024). In these strains, QS-responsiveness persists due to the activity of RhlR and, consequently, of the *rhl* system as a whole (Asfahl *et al.*, 2022; Chen *et al.*, 2019a; Cruz *et al.*, 2020; Feltner *et al.*, 2016; Groleau *et al.*, 2022). Notably, the production of QS-controlled products, such as pyocyanin, has a positive impact on *P. aeruginosa*'s population dynamics (Hall *et al.*, 2016; Lau *et al.*, 2004; O'Malley *et al.*, 2004; Price-Whelan *et al.*, 2006; Saunders *et al.*, 2020).

Given that QS controls the expression of several virulence factors, we hypothesized that the production of virulence determinants under RhlR control would be favoured at 37°C, a temperature mimicking human infection conditions. We used phenazine production as a temperature-sensitive determinant to explore the intricate interplay between PqsE, C_4-HSL, and the expression of RhlR-dependent regulation at selected environmental (25°C) and mammalian body (37°C) temperatures. While our experiments confirmed that RhlR activity is subject to thermoregulation, we unexpectedly observed indications that RhlR activity is higher at an environmental temperature rather than at the mammalian body temperature. Our findings suggest that temperature acts as a third stabilizing factor for RhlR, working synergistically with C_4-HSL and PqsE to optimally regulate the activity of this transcriptional regulator.

4.1.4 Material and Methods

4.1.4.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in **Tables 4.1.1** and **4.1.2**, respectively. Bacterial cultures were routinely grown in tryptic soy broth (TSB; BD Difco, Canada) at 37°C in a TC-7 roller drum (New Brunswick, Canada) at 240 rpm or on lysogeny broth (LB; BD Difco, Canada) agar plates. Cultures for gene expression experiments were grown in TSB at 37°C or 25°C in a Multitron Pro incubator (INFORS HT, Switzerland) at 240 rpm. The following concentrations of antibiotics were added when required: for *P. aeruginosa* PA14 tetracycline at 125 µg/ml (solid) or 75 µg/ml (broth), 300 µg/ml carbenicillin, and 100 µg/ml gentamicin. For *Escherichia coli*, 15 µg/ml tetracycline, 100 µg/ml carbenicillin, and 15 µg/ml gentamicin. Irgasan (20 µg/ml) was used as a counterselection agent against *E. coli*.

Table 4.1.1. Strains used in this study

Strain	**Lab ID #**	**Relevant genotype or description**	**Reference**
P. aeruginosa			
PA14	ED14	Clinical isolate from a human burn patient UCBPP-PA14	(Rahme *et al.*, 1995)
PA14 Δ*rhlR*	ED4406	PA14 derivate; unmarked in-frame *rhlR* deletion	(de Oliveira Pereira *et al.*, 2023)
PA14 Δ*rhlI*	ED4407	PA14 derivate; unmarked in-frame *rhlI* deletion	This study
PA14 Δ*pqsE*	ED36	PA14 derivate; unmarked in-frame *rhlI* deletion	(Déziel *et al.*, 2004)
PA14 Δ*rhlI* Δ*pqsE*	ED4408	PA14 derivate; unmarked in-frame double *rhlI* and *pqsE* deletion	This study

PA14 Δ*rhlI* Δ*pqsE* Δ*rhlR*	ED4700	PA14 derivate; unmarked in-frame triple *rhlI*, *pqsE*, and *rhlR* deletion	This study
E. coli			
SM10(λ*pir*)	ED222	*thi thr leu tonA lacY supE recA*::*RP4-2*-Tc::*Mu* Km λ*pir*	Lab collection

Table 4.1.2. Plasmids used in this study

Plasmid	**Description**	**Reference or source**
pTOP03	pEX18Ap-*rhlI*; gene replacement vector for the in-frame deletion of *rhlI* by allelic recombination; $Carb^R$	(de Oliveira Pereira *et al.*, 2023)
pTOP02	pEX18Ap-*rhlR;* gene replacement vector for the in-frame deletion of *rhlR* by allelic recombination, $Carb^R$	(de Oliveira Pereira *et al.*, 2023)
pCDS101	Promoter of *phz1* in mini-CTX-*lux*; Tet^R	(Sibley *et al.*, 2008)
pTOP08 (pUCP24m p*rhlR* RhlR WT)	pUCP24m encoding the wild-type sequence of RhlR and 500 bp of the region upstream of *rhlR*; Gm^R	This study
pUCP24m p*rhlR* RhlR P61	pUCP24m encoding the RhlR-P61 (stable version of RhlR) sequence and 500 bp of the region upstream of *rhlR*; Gm^R	(Borgert *et al.*, 2022)

4.1.4.2 Construction of plasmids

pTOP08 (pUCP24m p*rhlR* RhlR WT) was constructed based on pUCP24m p*rhlR* RhlR P61 (Borgert *et al.*, 2022). The latter was linearized by restriction enzymes *Bam*HI and *Hind*III. The

p*rhlR* RhlR WT, comprising 500 bp upstream *rhlR* and its coding sequence, was amplified by PCR from PA14 genomic DNA and then cloned into the linearized pUCP24m backbone (**Table 4.1.3** for oligonucleotide sequences). pTOP08 was assembled from the purified PCR product and linear vector backbone by employing a seamless strategy of ligation-independent cloning (pEASY® -Uni Seamless Cloning and Assembly Kit, TransGen Biotech Co.).

Table 4.1.3. Oligonucleotides used in this study.

Name	**Sequence (5'-3')**	**Description**
TO_WTrhlR_pUCP24m_fwd	attcgagctcggtacccgggGCAGCGCGCCTACGCGCC	Designed to clone the upstream (500 bp) and coding sequence of *rhlR* into the previously digested (*Hind*III and *Bam*HI) pUCP24m vector
TO_WTrhlR_pUCP24m_rev	taaaacgacggccagtgccaTCAGATGAGGCCCAGCGCCG	
MCG_RhlRqRT-F	CTGGGCTTCGATTACTACGC	RT-qPCR of *rhlR*
MCG_RhlRqRT-F	CCCGTAGTTCTGCATCTGGT	
nadBq_fwd	CTACCTGGACATCAGCCACA	RT-qPCR of *nadB*
nadBq_rev	GGTAATGTCGATGCCGAAGT	
SLG_qRTphzA1_F	AACCACTTCTGGGTCGAGTG	RT-qPCR of *phzA(1-2)*
SLG_qRTphzA1_R	TCGAGTTCGAAGGAATGGAT	

4.1.4.3 Construction of in-frame deletion mutants

Suicide vectors (pTOP02 and pTOP03) were transferred into recipient *P. aeruginosa* strains by conjugation with donor *E. coli* SM10. The recipient merodiploid cells were selected with carbenicillin, and *E. coli* donor cells were counter-selected using Irgasan. Double crossover mutants were isolated by sucrose counter-selection, and PCR confirmed the presence of a mutated allele.

4.1.4.4 Construction of chromosomal reporter strains

The *phzA1-lux* reporter from vector pCDS101 was integrated into the *attB* chromosomal site of PA14 and isogenic mutants by conjugation on LB agar plates. Tetracycline was used for selection, and Irgasan was used as a counterselection agent against *E. coli*.

4.1.4.5 Luminescence reporter readings

Luminescence was measured using a Cytation 3 multimode plate reader (BioTek Instruments, USA) at several time points during bacterial growth in TSB. The integration time of each reading was one second, a parameter that was used to determine the rate of RhlR activity (V_{max}). Relative light units (RLU) were normalized by cell density (OD_{600}); the latter was measured using a NanoDrop ND100 spectrophotometer (Thermo Fisher Scientific, Canada). When mentioned, C_4-HSL was added to the indicated concentrations from a stock prepared in high-performance liquid chromatography (HPLC)-grade acetonitrile. Solvent only was added in controls.

4.1.4.6 RNA extraction and quantitative reverse transcription-PCR experiments (RT-qPCR)

Total RNA was extracted from PA14 cultures grown in TSB at either 37°C or 25°C using *TransZol* RNA extraction reagent (TransGen Biotech Co.). Cells from three independent cultures were harvested at an OD_{600} of 1.8. After extraction, total RNA underwent two treatments with the TURBO DNA-Free kit (Ambion Life Technologies), following the manufacturer's guidelines. cDNA was synthesized using iScript reverse transcription supermix (Bio-Rad Laboratories) and used as a template for amplification cycles conducted on a Corbett Life Science Rotor-Gene® 6000 thermal cycler with SsoAdvanced universal SYBR green supermix (Bio-Rad Laboratories). The reference gene was *nadB* (Ghosh *et al.*, 2011), and the specific oligonucleotide sequences can be found in **Table 4.1.3**. The $2^{-\Delta\Delta CT}$ formula was applied to determine the relative gene expression (Livak *et al.*, 2001).

4.1.4.7 *Galleria mellonella* larvae infections

Greater wax moth (*G. mellonella*) larvae were infected by direct injection of *P. aeruginosa* into the last abdominal proleg, with minor adjustments to a previously published protocol (Koch *et al.*, 2014). Briefly, bacterial overnight cultures were diluted in fresh TSB to an OD_{600} of 0.05. The cultures were then incubated at 37°C until they reached an OD_{600} of 0.6-0.8. Cultures were then

rapidly cooled on ice for 5 min. The bacterial density was then adjusted a cellular density equivalent to $5x10^3$ CFU mL^{-1} in 10 mM $MgSO_4$ solution and maintained on ice until the time of infection. For the infection process, larvae were anesthetized under a gentle carbon dioxide stream and infected by injecting 10 µL of the ice-cold bacterial suspension. Each experimental condition comprised five larvae, and this was replicated with three independent infection groups for each condition, resulting in a total of 15 individuals tested per condition. The data was compared against an infection control group, which received only sterile 10 mM $MgSO_4$ solution. Larvae were then incubated at 37°C or 25°C, and morbidity and mortality were monitored for 46 hours to assess the infection score. The entire experiment was conducted twice.

4.1.5 Results and Discussion

4.1.5.1 Growth temperature modulates the expression of RhlR-controlled survival determinants

Quorum sensing regulates the transcription of several extracellular products, typically referred to as virulence factors or determinants (Rutherford *et al.*, 2012). These factors are crucial in host infections and bacterial colonization across diverse environments. Therefore, we will adopt the term “survival determinants” to better encompass their broader functions. In *P. aeruginosa*, these survival determinants are predominantly regulated by the one of the QS systems, but sometimes by multiple ones, as is the case for *hcnABC* and *lasB* (Gilbert *et al.*, 2009; Letizia *et al.*, 2022; Pessi *et al.*, 2000). The production of the redox-active phenazine pyocyanin depends on the expression of the *phz1* and *phz2* operons, which are primarily controlled by the *rhl* QS system (Groleau *et al.*, 2020); its easily detectable accumulation in cultures has served as a reliable proxy for assessing RhlR activity, alongside the expression of the *phz1* operon (Borgert *et al.*, 2022; Groleau *et al.*, 2020). The regulatory influence of RhlR on *phz1* is attributed to a *las-rhl* box motif within its promoter region (Whiteley *et al.*, 2001). Importantly, optimal activation by RhlR depends on the involvement of both PqsE and C_4-HSL (Borgert *et al.*, 2022; Feathers *et al.*, 2022). Given these considerations, we used *phz1* transcription as a proxy to assess the activity status of RhlR under two different temperature conditions.

To investigate the influence of temperature on the function of RhlR, we measured the activity of a chromosomal *phzA1-luxCDABE* reporter in the wild-type (WT) *P. aeruginosa* strain PA14. These measurements were carried out at two distinct temperatures: 37°C, mimicking conditions within mammalian hosts, and 25°C, representing an environmental-like temperature. The

transcription profile of *phz1* was similar under these conditions, and the expression peak was achieved at an equivalent cell density at both temperatures (i.e., OD_{600} of 1.8) (**Fig. 4.1.1**). Remarkably, a threefold increased expression level was measured at 25°C compared to 37°C, indicating that *phz1* transcription is specifically higher at a lower temperature.

Noteworthy, previous studies have employed the bioluminescence *luxCDABE* reporter to investigate the impact of temperature on gene regulation (Bresolin *et al.*, 2006a; Bresolin *et al.*, 2006b; Bresolin *et al.*, 2008; Liang *et al.*, 2016). A 2.5-fold decrease in light production was revealed in bacterial cells grown at 25°C compared to 37°C due to reduced enzymatic activity (Bresolin *et al.*, 2006a). Importantly, the relative light unit (RLU) values presented in this study do not account for this 2.5-fold reduction. Consequently, the observed higher *phz1* transcription at 25°C may even be an underestimation. To confirm the role of RhlR in this temperature-responsive behaviour, we conducted the same measurements with an isogenic *rhlR* deletion mutant (Δ*rhlR*). As expected, the transcription of *phz1* was abolished at both temperatures in the absence of RhlR (**Fig. 4.1.1**) (Cabeen, 2014; Dekimpe *et al.*, 2009; Groleau *et al.*, 2020).This confirmed that the thermoregulation of *phz1* transcription relies on RhlR and suggested that an environmental-like temperature may enhance RhlR expression or its activity.

Based on previous literature that reported an induction of pyocyanin expression at human body temperature (Barbier *et al.*, 2014; Grosso-Becerra *et al.*, 2014; Wurtzel *et al.*, 2012), we anticipated a similar response. However, our reported findings diverged from this initial hypothesis. To address this unexpected result, we validated the transcriptional *phz1* reporter by measuring the mRNA levels of *phzA* using RT-qPCR. The expression of *phzA* confirmed the results obtained with the transcriptional reporter, providing additional evidence that *phz1* transcription is induced at 25°C (see **Fig. 4.1.2**). This validation underscores the reliability of the *phzA1-lux* reporter to measure the transcription of the *phz1* operon and again established it as a valuable tool for investigating the transcriptional thermoregulation of this RhlR-controlled survival determinant.

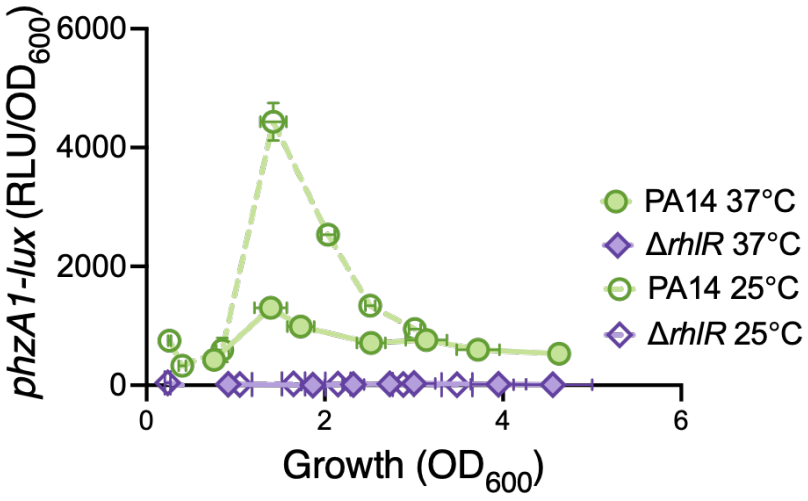

Figure 4.1.1. The transcription of *phz1* requires RhlR and is higher at an environmental-like temperature than at body temperature

Luminescence readings were used to measure the expression of the chromosomal reporter *phzA1-lux* during growth in TSB of the wild-type PA14 strain and its isogenic *rhlR* mutant (Δ*rhlR*). The cultures were incubated at 37°C (solid symbols and solid lines) and 25°C (open symbols and dotted lines). The values are means ± standard deviation (error bars) from three replicates.

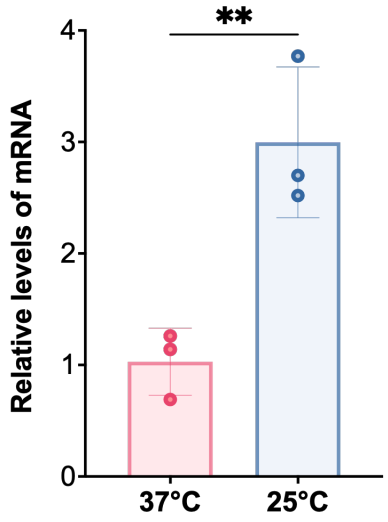

Figure 4.1.2. Thermoregulation of *phzA* transcription

Gene expression was assessed through RT-qPCR, utilizing total RNA isolated from wild-type PA14 strain cultures grown in TSB at 37°C (red) and 25°C (blue). Cultures were harvested at an OD_{600} of 1.8 based on the transcriptional profile of *phzA1* (see Figure 4.1.1). The results are presented as relative quantification compared to PA14 grown at 37°C, which was set as 1. *phzA1* and *phzA2* have nearly identical sequences and cannot be differentiated. The values are means ± standard deviation (error bars) from three biological replicates. An independent t-test was used to quantify statistical significance. ** $P \leq 0.01$.

One plausible reason for the different transcriptional profile of *phz1* between both temperature conditions is a potential variation in RhlR. To explore this possibility, we performed RT-qPCR analyses to assess the transcription levels of *rhlR*. This is necessary because this gene includes four promoters located directly upstream of it and one responsible for driving the expression of a large transcriptional unit containing *rhlA*, *rhlB*, and *rhlR* (Croda-García *et al.*, 2011; Medina *et al.*, 2003). No significant differences in *rhlR* transcription mRNA levels were measured between cells grown at 37°C and 25°C (**Fig. 4.1.3A**), aligning with previous research comparing *rhlR* transcription from the four proximal promoters under similar conditions (Grosso-Becerra *et al.*, 2014). A previously described thermoregulation of RhlR expression relies upon the large transcript, and its presence was characterized under phosphate limitation (Grosso-Becerra *et al.*, 2014). Notably, the transcription initiated from *rhlR*'s promoters is condition-specific (Croda-García *et al.*, 2011). This alternative transcript has not been described in *P. aeruginosa* cultures grown in rich media, such as TSB. Compared to the previous study, our results suggest the thermoregulation of *phz1* expression does not occur in response to altered transcriptional levels of *rhlR* and is thus independent of the large transcriptional unit (Grosso-Becerra *et al.*, 2014). Further investigation into alternative mechanisms, including the potential thermoregulation of RhlR activity, is necessary to understand the *phz1* transcriptional profile under different temperatures fully.

4.1.5.2 C_4-HSL and PqsE are not both required for RhlR-mediated thermoregulation of gene expression

As full RhlR activity depends on both its autoinducer C_4-HSL and the presence of PqsE (Borgert *et al.*, 2022; Feathers *et al.*, 2022; Groleau *et al.*, 2020; Letizia *et al.*, 2022), we measured *phz1* expression in a *rhlI* mutant, which cannot produce C_4-HSL, and a *pqsE* mutant, to investigate their impact on thermoregulation of RhlR activity. At 37°C, *phz1* transcription remained significant even when either *rhlI* or *pqsE* were inactive (**Fig. 4.1.3B**), consistent with prior studies (Groleau *et al.*, 2020). Moreover, neither mutant strain produced pyocyanin under these conditions, corroborating previous findings and our results (see **Fig. 4.1.4A**) (Brint *et al.*, 1995; Gallagher *et al.*, 2002; Mukherjee *et al.*, 2017). Interestingly, similar to the the wild-type strain, transcription from the *phz1* promoter was higher at 25°C than at 37°C in both mutant backgrounds (**Fig. 4.1.3B**). This upregulation even restored pyocyanin production in the Δ*rhlI* mutant but not in the Δ*pqsE* mutant (see **Fig. 4.1.4B**) , potentially due to the greater dependence of *phz1* transcription on PqsE than C_4-HSL during the stationary phase. We also note that the absence of *rhlI* led to higher

transcription levels at both temperatures, consistent with a greater contribution of PqsE than C_4-HSL on RhlR function in this system. Despite the differing transcription profiles resulting from the absence of PqsE or C_4-HSL, these results indicate that the RhlR-driven thermoregulation of *phz1* expression does not necessitate the simultaneous presence of both factors.

To further investigate the role of RhlR's activity in its thermoregulation, we explored conditions where RhlR function would be lost, presumably in the simultaneous absence of C_4-HSL and PqsE. Hence, we utilized the Δ*rhlI* Δ*pqsE* mutant background to assess the connection between the thermoregulation of RhlR and its activity. Interestingly, *phz1* transcription was completely abolished in this strain at both temperatures, strengthening the idea that temperature influences RhlR function through these factors.

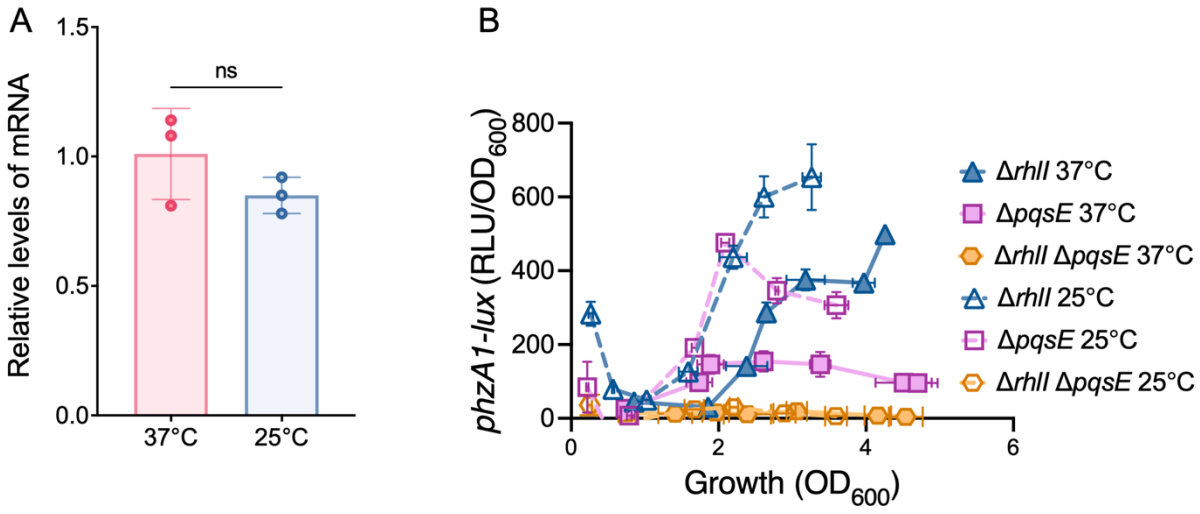

Figure 4.1.3. RhlR activity, rather than its expression, mediates the thermoregulation of *phz1* transcription
(A) Expression of *rhlR* was assessed by RT-qPCR from total RNA isolated from wild-type PA14 strain grown in TSB at 37°C (red) and 25°C (blue). The results are presented as relative quantification compared to PA14 grown at 37°C, which was set as 1. The values are means ± standard deviation (error bars) from three biological replicates. An independent t-test was used to quantify statistical significance. ns, nonsignificant. **(B)** Activity of the chromosomal reporter *phzA1-lux* was measured during growth in TSB in the strains Δ*rhlI*, Δ*pqsE* and the double mutant Δ*rhlI* Δ*pqsE* incubated at 37°C (solid symbols and solid lines) and 25°C (open symbols and dotted lines). The values are means ± standard deviation (error bars) from three replicates.

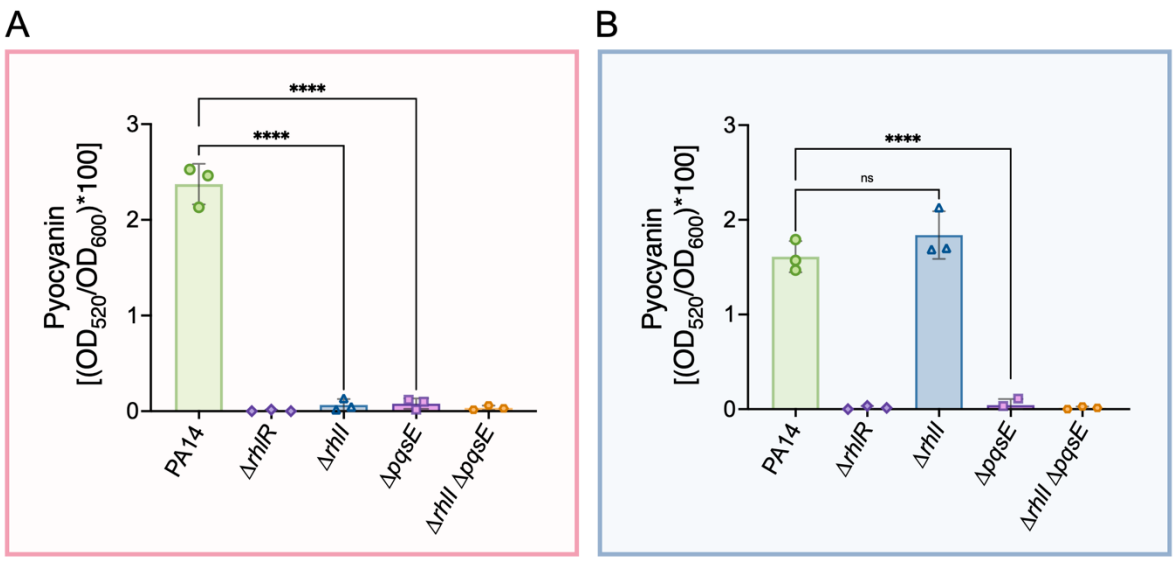

Figure 4.1.4. Thermoregulation of pyocyanin production.

Pyocyanin was chloroform extracted and quantified in the PA14 strain and those with impaired *rhl* system: Δ*rhlR*, Δ*rhlI*, Δ*pqsE*, and the double mutant Δ*rhlI* Δ*pqsE*. These strains were cultivated in TSB and incubated at either 37°C (red, left panel) or 25°C (blue, right panel) for 24. The values are means ± standard deviation (error bars) from three biological replicates. An independent t-test was used to quantify statistical significance. **** $P \leq 0.001$; ns, nonsignificant.

4.1.5.3 Environmental temperature enhances RhlR activity

The requirement for either C_4-HSL or PqsE in the thermoregulation of RhlR activity on *phz1* transcription indicates that this effect is likely going through the functional structure of RhlR. Indeed, the RhlR homodimer exhibits an inherently unstable folding (Borgert *et al.*, 2022; McCready *et al.*, 2019; O'Loughlin *et al.*, 2013). This characteristic is common to most LuxR-type proteins (Churchill *et al.*, 2011). Typically, these proteins adopt stable three-dimensional structures when bound to their cognate autoinducers. However, in contrast to typical LuxR-type proteins, RhlR stability requires the presence of both its ligand C_4-HSL and chaperone-like PqsE (Borgert *et al.*, 2022). Based on the thermoregulation's profile, we hypothesized that lower temperatures resembling environmental conditions would contribute to an increase in the stability of the active RhlR complex compared with 37°C animal temperature. In other words, we assumed temperature variations influence the actual stability of the RhlR complex, putatively mitigating the absence of C_4-HSL and PqsE under certain environmental conditions.

To investigate the predicted dynamic behaviour of the active RhlR complex formation under different temperature conditions, we used a strain incapable of producing C_4-HSL (i.e., Δ*rhlI*

mutant). We then introduced a defined concentration of exogenous C_4-HSL an evaluated *phz1* transcription in cultures grown at 37°C or 25°C. Interestingly, when an equivalent concentration of C_4-HSL was added to cultures grown at 25°C, *phz1* transcription levels were up to tenfold higher than those observed in cells grown at 37°C (**Fig. 4.1.5A**). One possible explanation for this transcriptional behaviour could be that temperature variations modulate the affinity of RhlR towards C_4-HSL. Increased temperatures could also negatively impact the stability of the active RhlR/C_4-HSL/PqsE complex, and thus effective concentrations in the cell.

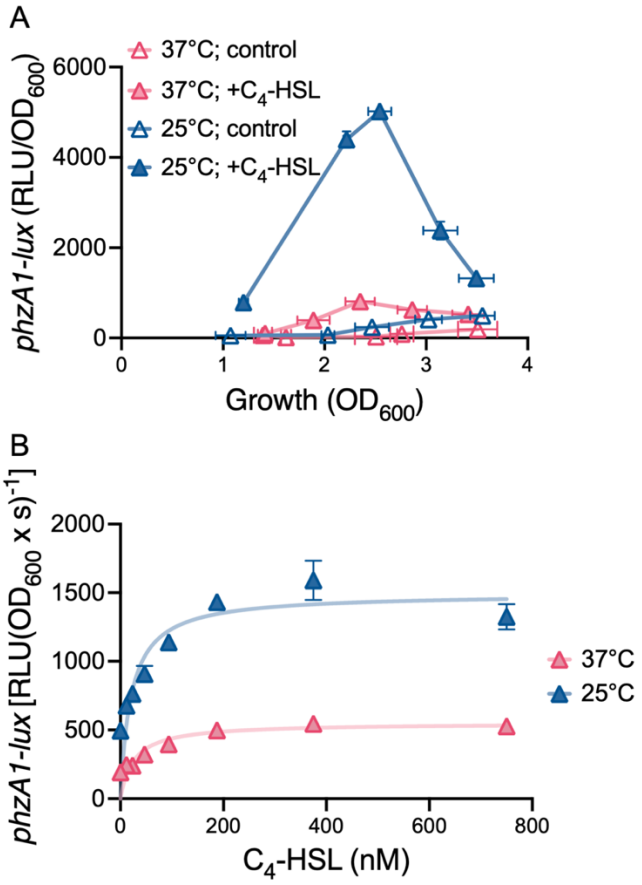

Figure 4.1.5. At an environmental temperature (25°C), the maximum activity of RhlR is extended from its basal level at 37°C.

(A) Transcription of the chromosomal reporter *phzA1-lux* was measured in the Δ*rhlI* mutant grown in TSB at 37°C (red) and 25°C (blue) in response to supplemented 750 nM C_4-HSL. Solid symbols represent C_4-HSL conditions, while open symbols denote control conditions. The values are means ± standard deviation (error bars) from three replicates. **(B)** Increasing concentrations of C_4-HSL (up to 750 nM) were used to assess their impact on *phzA1-lux* transcription during the stationary growth phase (OD_{600} of 3.4-3.5) at 37°C and 25°C in the Δ*rhlI* background. Data analysis was performed using non-linear regression resembling enzymatic kinetics in the Michaelis-Menten model (solid lines).

Next, we investigated whether temperature variations could affect the profile of *phz1* transcription. According to our hypothesis, lower concentrations of C_4-HSL would be required to elicit an equivalent response in cell growth at 25°C relative to 37°C. To investigate this, we assessed *phz1* transcription in response to a series of C_4-HSL concentrations under defined temperature conditions. The induction profile of *phz1* transcription in response to C_4-HSL varied during growth phases (**Fig. 4.1.5A**). Given this varying response, we compared the transcriptional profiles of cells in equivalent growth phases at 37°C and 25°C. Interestingly, the dose-dependent effect induced by increasing concentrations of C_4-HSL resulted in a profile akin to steady-state Michaelis-Menten enzyme kinetics, which describes the relationship between substrate concentration and the rate of the corresponding enzymatic reaction (Johnson *et al.*, 2011), predominantly during a stationary growth phase (**Figs. 4.1.5B** and **4.1.6**). Although RhlR is not an enzyme and C_4-HSL is not a catalytic substrate *per se*, the outcome in measured reaction rate (i.e., luminescence as an indicator of transcriptional activity) still follows steady-state kinetics behaviour inherently linked with the enzymatic activity of the corresponding RNA polymerase (Ball *et al.*, 2019). This indirect connection between RhlR and RNA polymerase activity could explain the observed pattern, wherein varying concentrations of the C_4-HSL ligand elicit a corresponding response. Thus, we can draw a parallel between Michaelis-Menten kinetics and our model, whereby the RNA polymerase enzymatic reaction follows RhlR activity in response to C_4-HSL binding at equilibrium.

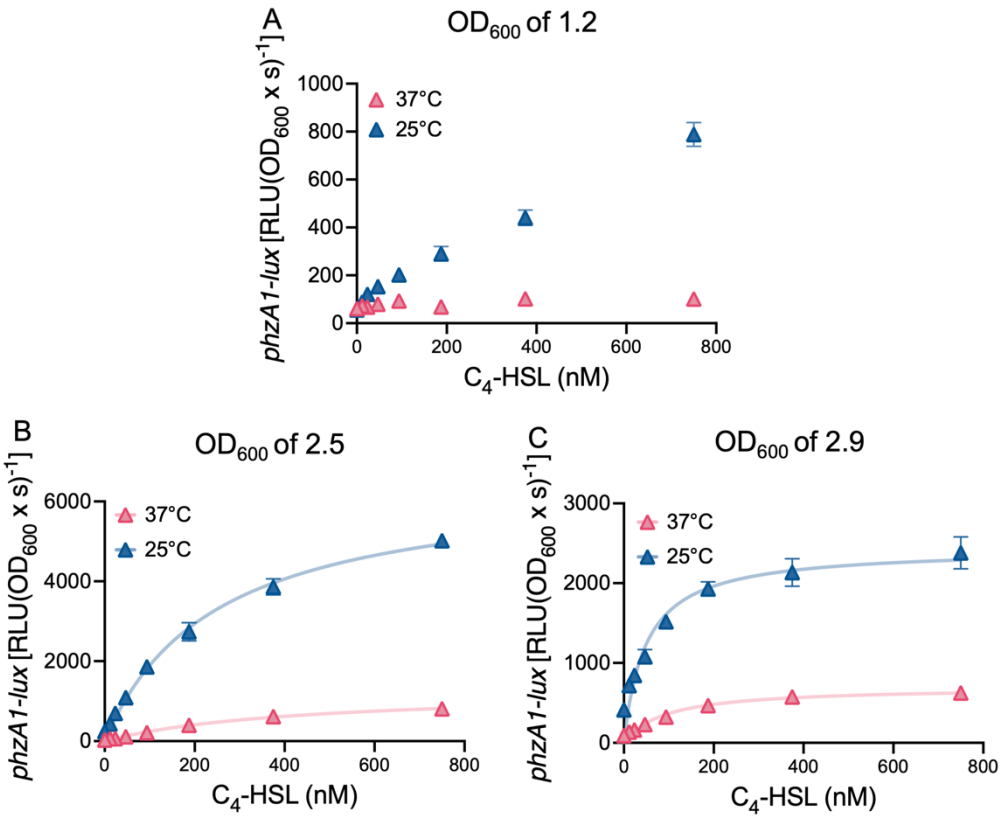

Figure 4.1.6. The transcriptional profile of *phz1* in response to C_4-HSL varies with population density and is consistently higher at 25°C compared to 37°C.

Increasing concentrations of C_4-HSL (up to 750 nM) were used to assess their impact on *phzA1-lux* transcription during bacterial growth in TSB in the Δ*rhlI* mutant. Response profiles were compared at equivalent cell densities, corresponding to growth at 37°C (red) and 25°C (blue). Data analysis was performed using non-linear regression resembling the Michaelis-Menten enzyme kinetics model (solid lines), which requires the saturation of the response and could not be estimated for (A). The response profile varied with the growth phase, with maximum RhlR activity (V_{max}) tending to be higher at 25°C than at 37°C. **(A)** Optical density (OD_{600}) of 1.2, with not determined V_{max} and K_m for both tested temperatures. **(B)** OD_{600} of 2.5 and V_{max}= 1283 ± 65 RLU $(DO_{600} \times s)^{-1}$ and K_m= 427.6 ± 43 nM at 37°C, and V_{max}= 6554 ± 225 RLU $(DO_{600} \times s)^{-1}$ and K_m= 246 ± 20 nM at 25°C. **(C)** OD_{600} of 2.9 and V_{max}= 699 ± 30.5 RLU $(DO_{600} \times s)^{-1}$ and K_m= 90.4 ± 12.5 nM at 37°C, and V_{max}= 2437 ± 108.2 RLU $(DO_{600} \times s)^{-1}$ and K_m= 48.4 ± 8 nM at 25°C. The accurate determination of V_{max} (maximum RhlR activity) and K_m (RhlR affinity for C_4-HSL) requires a saturated profile. The response profile tends to become increasingly saturated throughout growth, with full saturation observed only during the stationary phase, as depicted in **Figure 4.1.5B** at an OD_{600} of 3.4-3.5. Thus, the curves presented here solely exhibit a behavioural tendency of RhlR activity and its affinity in response to the tested temperatures.

From this observation, two relevant kinetic constants were extracted and applied to our model analysis. In a Michaelis-Menten relationship, reaction velocity increases with substrate concentration until it reaches a maximum rate (V_{max}), indicating that the enzyme is fully saturated by the substrate. Additionally, the affinity of an enzyme for its substrate can be determined by extracting the Michaelis constant (K_m), which represents the substrate concentration at which the enzyme achieves half of its maximum reaction velocity ($0.5V_{max}$) (Johnson *et al.*, 2011; Srinivasan, 2022). A lower K_m value indicates higher affinity since the enzyme can achieve half of its maximum velocity at a lower substrate concentration. Conversely, a higher K_m value suggests lower substrate affinity. Applying this to our biological system, V_{max} represents maximal RhlR activity induced by a specific concentration of C_4-HSL, which in turn allows us to determine the affinity of RhlR for C_4-HSL (i.e., K_m) at both 37°C and 25°C (**Figs. 4.1.5B** and **4.1.6**). Our results show that RhlR affinity for C_4-HSL remains constant across the temperatures tested, contrary to our hypothesis that lower temperatures would increase the affinity of RhlR for C_4-HSL (K_m at 37°C is 20.8 ± 5.8 nM and at 25°C, 25.6 ± 7.2 nM). In contrast, temperature primarily affects the maximum activity of RhlR (i.e., V_{max}). At 25°C, RhlR exhibits higher activity [V_{max} = 1494 ± 89.3 RLU (DO_{600} x s)$^{-1}$] relative to the basal level observed at 37°C [V_{max} = 550.5 ± 35.2 RLU (DO_{600} x s)$^{-1}$] (**Fig. 4.1.5B**). In simpler terms, regardless of its affinity for C_4-HSL, the activity of RhlR increases at 25°C and remains unchanged under these temperature conditions. This response is observed throughout bacterial growth phases (**Fig. 4.1.6**).

In the context of *in vitro* systems, enzyme concentration is a critical factor influencing V_{max} (Srinivasan, 2022). Thus, the regulation of RhlR concentration under the temperature conditions tested could explain the increased activity of this regulator at 25°C. As mentioned earlier, we did not observe a direct impact of temperature on *rhlR* transcription (**Fig. 4.1.3A**). However, it remains unclear whether it affects the post-transcriptional regulation of this gene. Indeed, others have observed a link between these factors, with enhanced RhlR concentration at 37°C compared to a lower temperature (Grosso-Becerra *et al.*, 2014). This profile challenges our data, which instead suggests a higher RhlR concentration at 25°C. Nevertheless, it hints at the potential modulation of RhlR concentration by temperature variations. Another factor that could contribute to the increased activity of RhlR at 25°C is an altered turnover number of RhlR under temperature conditions investigated. This change in catalytic rate would result from varying stability of the RhlR/C_4-HSL/PqsE ternary complex under these conditions. According to this hypothesis, lower temperatures would favour more stable complex formation, allowing it to persist longer at 25°C than at 37°C. Current observations do not provide enough evidence to determine the most probable mechanism for the thermoregulation of RhlR activity.

4.1.5.4 In the absence of PqsE, environmental temperature increases the affinity of RhlR for C_4-HSL

To further understand how temperature influences RhlR activity, we examined *phz1* transcription induced by increasing concentrations of C_4-HSL in the Δ*rhlI* Δ*pqsE* genetic background. The interaction between the RhlR homodimer and PqsE within the active RhlR complex is critical for stabilizing RhlR (Borgert *et al.*, 2022; Feathers *et al.*, 2022). We discussed two alternative explanations for the increased RhlR activity at 25°C and hypothesized that disrupting the ability of RhlR to maintain a stable conformation by the absence of PqsE would help us differentiate between these hypotheses. Notably, akin to the profile obtained with Δ*rhlI*, the exogenous addition of an equivalent concentration of C_4-HSL resulted in enhanced *phz1* transcription in cultures grown at 25°C compared to those grown at 37°C (**Fig. 4.1.7A**). As expected, this response was significantly lower than that elicited by the equivalent concentration of C_4-HSL in the presence of PqsE, reinforcing the importance of the latter in RhlR activity (Borgert *et al.*, 2022; Groleau *et al.*, 2020; Letizia *et al.*, 2022). Due to this partial dependence on PqsE for activation, the concentration of C_4-HSL required to saturate RhlR activity in the absence of PqsE was considerably higher compared to the *rhlI* mutant at both tested temperatures (for instance, K_m value shifted from 25.6 ± 7.2 nM to 6.4 ± 0.8 μM C_4-HSL at 25°C and 20.8 ± 5.8 nM to 33.1 ± 2 μM C_4-HSL at 37°C) (**Figs. 4.1.7B** and **4.1.8**). The relationship between PqsE and the affinity of RhlR for C_4-HSL has been previously observed in a heterologous system (Farrow *et al.*, 2008), which reinforces our results and underscores the role of PqsE in modulating RhlR activity in response to C_4-HSL.

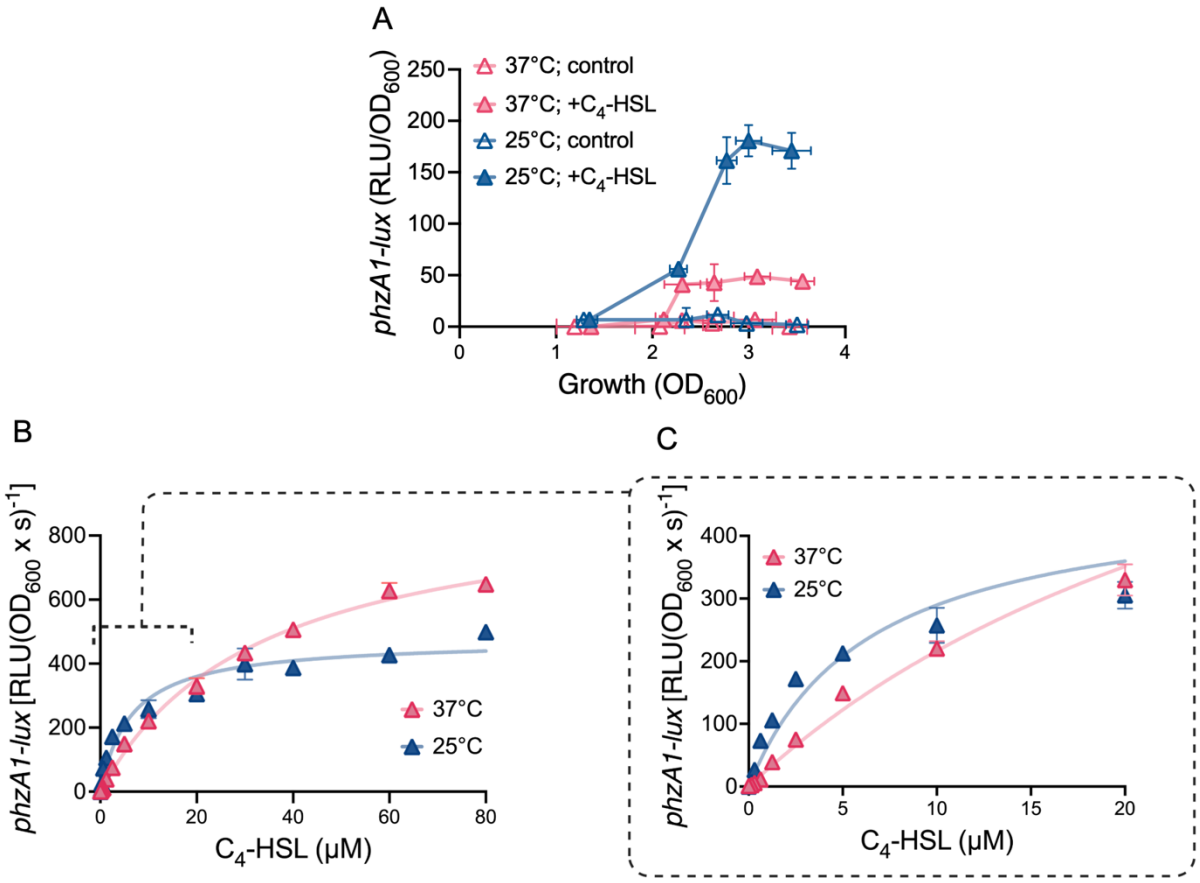

Figure 4.1.7. Lower concentrations of C_4-HSL are required to elicit comparable RhlR activity at environmental temperature in the absence of PqsE.

(A) Transcription of the chromosomal reporter *phzA1-lux* was measured in the double mutant Δ*rhlI* Δ*pqsE* grown in TSB at 37°C (red) and 25°C (blue) in response to 750 nM of C_4-HSL. Solid symbols represent C_4-HSL conditions, while open symbols denote control conditions. The values are means ± standard deviation (error bars) from three replicates. **(B)** Increasing concentrations of C_4-HSL (up to 80 μM) were used to assess the impact on *phzA1-lux* transcription during the stationary growth phase (OD_{600} of 3.4-3.5) at 37°C and 25°C in the Δ*rhlI* Δ*pqsE* background. Data analysis was performed using non-linear regression resembling the Michaelis-Menten enzyme kinetics model (solid lines). Panel **(C)** shows an inset of panel B for C_4-HSL concentrations ranging from 0-20 μM.

By analyzing the saturation profile of *phz1* transcription, we determined the maximum activity of RhlR at both 37°C and 25°C. Interestingly, in the absence of PqsE, we observed that lower temperature did not result in increased activation relative to 37°C, and the maximum activity of RhlR remained relatively stable across growth phases [V_{max} = 932.7 ± 25.4 RLU $(OD_{600} \times s)^{-1}$ at 37°C and 474.6 ± 15.3 RLU $(OD_{600} \times s)^{-1}$ at 25°C]. This thermoregulatory pattern contrasts with that observed in the presence of PqsE (**Figs. 4.1.5** and **4.1.6**), despite employing the same

environmental conditions (**Figs. 4.1.7B** and **4.1.8**). We reasoned that if the concentration of stable RhlR was the primary driver of the thermoregulated response, as one explanation suggests, we would expect a lower temperature to extend RhlR activity compared to 37°C, similar to the profile of the *rhlI* mutant. However, our data did not support this hypothesis.

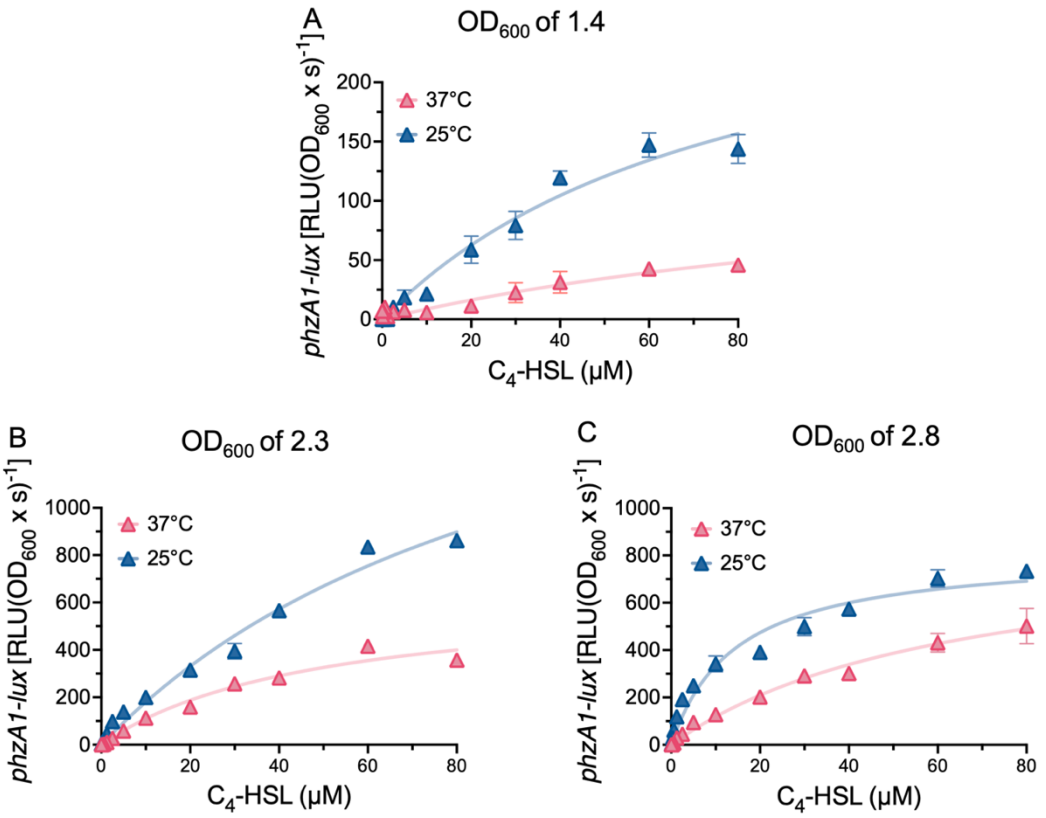

Figure 4.1.8. The affinity of RhlR for C_4-HSL is temperature-dependent in the absence of PqsE.
Increasing concentrations of C_4-HSL (up to 80 μM) were used to assess their impact on *phzA1-lux* transcription during bacterial growth in TSB in the Δ*rhlI* Δ*pqsE* genetic background. Response profiles were compared at equivalent cell densities, corresponding to growth at 37°C (red) and 25°C (blue). Data analysis was performed using non-linear regression resembling the Michaelis-Menten enzyme kinetics model (solid lines). **(A)** Optical density (OD_{600}) of 1.4 and V_{max}= 138.7 ± 61 RLU (DO_{600} x s)$^{-1}$ and K_m= 150.8 ± 90.9 μM at 37°C, and V_{max}= 313.5 ± 40.4 RLU (DO_{600} x s)$^{-1}$ and K_m= 80 ± 16.9 μM at 25°C. **(B)** OD_{600} of 2.3 and V_{max}= 641.5 ± 53.3 RLU (DO_{600} x s)$^{-1}$ and K_m= 48.4 ± 7.9 μM at 37°C, and V_{max}= 2093 ± 236.2 RLU (DO_{600} x s)$^{-1}$ and K_m= 106.5 ± 18 μM at 25°C. **(C)** OD_{600} of 2.8 and V_{max}= 888.1 ± 78.9 RLU (DO_{600} x s)$^{-1}$ and K_m= 64.9 ± 10.1 μM at 37°C, and V_{max}= 816.4 ± 34.8 RLU (DO_{600} x s)$^{-1}$ and K_m= 14.5 ± 1.9 μM at 25°C. The accurate determination of V_{max} (maximum RhlR activity) and K_m (RhlR affinity for C_4-HSL) requires a saturated profile. The response profile tends to become increasingly saturated throughout growth, with full saturation observed only during the stationary phase, as depicted in **Figure 4.1.7B** at an OD_{600} of 3.4-3.5. Thus, the curves presented here solely exhibit a behavioural tendency of RhlR activity and its affinity in response to the tested temperatures.

Considering these findings, we considered the alternate hypothesis that temperature affects RhlR turnover, possibly through the stability of the complex. The idea is that when the active structure is compromised (e.g., in the absence of PqsE), the stabilizing effect of lower temperature is insufficient to overcome this structural limitation, leading to similar maximal levels of RhlR activity between the temperature conditions tested. Indeed, temperature acts as a stabilizing factor regulating the function of various biological systems. For instance, in prokaryotes such as *Listeria monocytogenes*, a foodborne facultative intracellular pathogen, the formation of a protein complex between MorG and GmaR, triggered at environmental temperatures, modulates the activity of the transcriptional repressor MorG, thereby regulating the transcription of flagellar motility genes (Kamp *et al.*, 2011). Similar thermoregulatory mechanisms are observed in eukaryotes, as illustrated by *Arabidopsis*. In this plant model, flowering is controlled by a thermoregulated mechanism that stabilizes the RING-finger E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) protein at lower temperatures (Jang *et al.*, 2015). Consistent with these examples, our results suggest that lower temperatures correlate with increased RhlR complex stability in *P. aeruginosa*.

Although temperature does not modulate maximal RhlR activity in the absence of PqsE, it does influence a crucial aspect related to the activity of this regulator. In this genetic context, we observed that RhlR exhibits higher affinity for C_4-HSL at 25°C (K_m = 6.4 ± 0.8 µM) than at 37°C (K_m = 33.1 ± 2 µM) (**Fig. 4.1.7B** and **4.1.7C**). This indicates that a lower C_4-HSL concentration is required to achieve maximal RhlR activity at 25°C. Notably, at this temperature, half of the maximum velocity of RhlR is reached with just 6.4 ± 0.8 µM of C_4-HSL, a concentration within the physiological concentration range of this signalling molecule (ranging from 3.5 µM to 10 µM) (Déziel *et al.*, 2005; Pearson *et al.*, 1995). In contrast, the concentration of C_4-HSL required to elicit an equivalent response increases to 33.1 ± 2 µM at 37°C, which falls outside the physiological concentration range of this molecule in *P. aeruginosa*.

4.1.5.5 Artificial RhlR stability also disrupts thermoregulation

To validate our model thats temperature influences RhlR activity by altering the stability of the active complex, we used a RhlR variant known for its increased intrinsic stability. This variant, RhlR-P61, exhibits 61 amino acid residue substitutions compared to the WT (Borgert *et al.*, 2022). Importantly, the core regulatory function of RhlR remains unaltered since this variant retains a WT DNA-binding domain (Borgert *et al.*, 2022). The RhlR-P61 variant activates pyocyanin production independently of C_4-HSL and PqsE (Borgert *et al.*, 2022). The involvement of RhlR intrinsic stability in thermally induced responses was investigated by inserting a plasmid encoding either RhlR-P61 or RhlR-WT in a genetic background lacking *rhlI*, *pqsE*, and *rhlR* (triple mutant Δ*rhlI* Δ*pqsE* Δ*rhlR*) and measuring the transcription of the *phzA1-lux* reporter with and without exogenous C_4-HSL supplementation at both 37°C and 25°C. As expected, in the presence of RhlR-WT, *phz1* transcription remains minimal regardless of the incubation temperature as the strain is deficient in the stabilizing elements C_4-HSL and PqsE (**Fig. 4.1.9A**). The addition of C_4-HSL induces more robust expression at 25°C, reaffirming the trends observed thus far. In contrast, in the presence of the stabilized RhlR-P61 variant, transcription of *phz1* is restored despite the absence of C_4-HSL (**Fig. 4.1.9B**). This confirms that *phz1* transcription driven by this variant is independent of the presence of C_4-HSL and PqsE. Notably, the change in incubation temperature did not affect this basal transcription (**Fig. 4.1.9B**), indicating that the inherent stability of the RhlR variant remains unaltered by temperature variations. The addition of C_4-HSL does not result in a significant induction of *phz1* under these conditions, whether at 37°C or 25°C (**Fig. 4.1.9**). These findings unequivocally show that the induction of *phz1* expression in the presence of RhlR WT is responsive to stabilizing factors, such as C_4-HSL and temperature. The combined presence of both stabilizing factors maximizes the response, and the presence of PqsE further enhances it.

It is important to note that the sequence cloned into the plasmid used to complement RhlR consists of only 500 nucleotides upstream of the coding region of this regulator. The identification of thermoregulation in the presence of this plasmid reaffirms that this response is independent of the previously described RNA thermometer (Grosso-Becerra *et al.*, 2014). This RNA control element, situated upstream of *rhlA*, is not present in the constructed system we employed. In essence, this demonstrates that the thermoregulated responses of the *rhl* system are also influenced by the stability of the active RhlR complex.

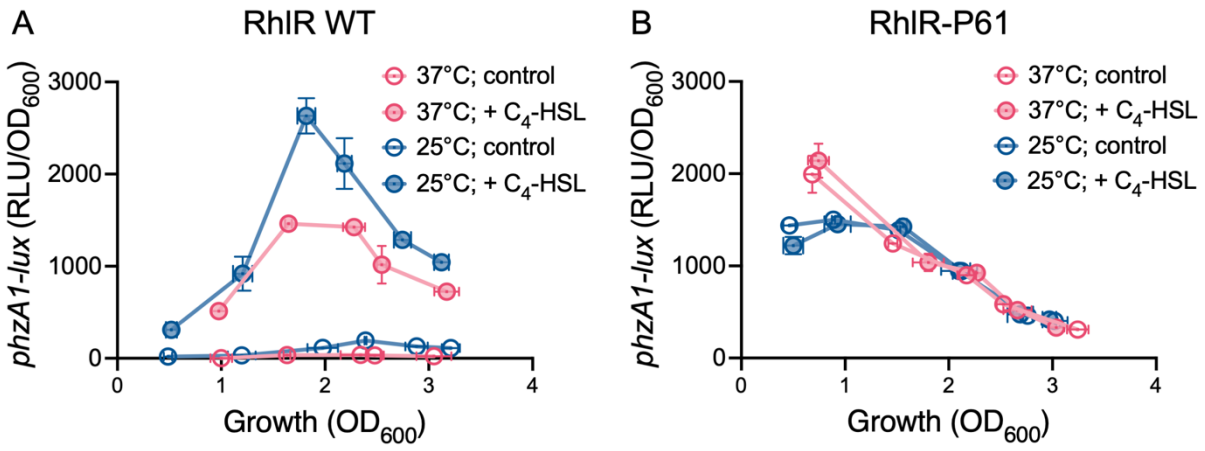

Figure 4.1.9. A stabilized variant of RhlR is not sensitive to temperature variations.

Transcription of the chromosomal reporter *phzA1-lux* in the triple mutant Δ*rhlI* Δ*pqsE* Δ*rhlR* during growth in TSB. Cultures were incubated at 37°C (red) or 25°C (blue). C_4-HSL was supplemented at the final concentration of 1.5 μM (solid symbols and lines). Solvent alone (acetonitrile) was used in the controls (open symbols and dotted lines). **(A)** in the presence of a RhlR-WT encoding plasmid vector. **(B)** in the presence of a RhlR-P61 encoding plasmid vector. The values are means ± standard deviation (error bars) from three replicates.

4.1.5.6 Temperature affects the virulence of the Δ*rhlI* mutant

Considering that *P. aeruginosa* is an opportunistic pathogen and several of its virulence factors are regulated by QS, the implications of the induction of RhlR activity in response to bacterial growth at environmental temperatures are instrumental in understanding its virulence. The functionality of the *rhl* system has been previously associated with bacterial virulence in several infection models (Haller *et al.*, 2018; Mukherjee *et al.*, 2018). While *rhlR* mutants are unable to produce pyocyanin, a Δ*rhlI* mutant is not. Indeed, this mutant regains the ability to produce this survival determinant at a lower temperature (**Fig. 4.1.4**). We hypothesized that a Δ*rhlI* mutant would manifest distinct virulence profiles when exposed to temperatures lower than those resembling mammalian infection conditions. Consequently, we expected that the absence of C_4-HSL could be compensated for by a lower temperature, presumably maintaining the production of RhlR-controlled survival determinants. We expected that the virulence profiles of this mutant would vary between the tested temperatures, while no significant difference in virulence was foreseen for the Δ*rhlR*. To conduct this investigation, we needed an infection model capable of operating at different temperatures. Several infection models have been developed for *P. aeruginosa*, each with their unique advantages (Apidianakis *et al.*, 2009; Miyata *et al.*, 2003; Rahme *et al.*, 1997; Stieritz *et al.*, 1975; Tan *et al.*, 2000). Among these, the utilization of *Galleria*

mellonella larvae, commonly known as the Greater wax moth, provides the necessary flexibility to investigate temperature-dependent variations (Koch *et al.*, 2014). According to the expected response, at 37°C, strains with mutations in Δ*rhlR* and Δ*rhlI* exhibit reduced virulence compared to the PA14 wild-type strain (**Fig. 4.1.10A**). Fluctuations in temperature significantly influence the virulence profile of *P. aeruginosa*. At 25°C, while the virulence of Δ*rhlR* mutant towards *G. mellonella* remains significantly different from that of PA14, the virulence of the Δ*rhlI* mutant does not (**Fig. 4.1.10B**). This thermo-dependent response aligns with the predicted outcome, indicating that the restoration of RhlR-controlled determinants' production in the Δ*rhlI* strain at lower temperatures significantly impacts its virulence. The mortality rate of larvae infected with a Δ*rhlR* mutant reinforces the impact of RhlR-controlled survival determinants in this infection model (**Fig. 4.1.10**).

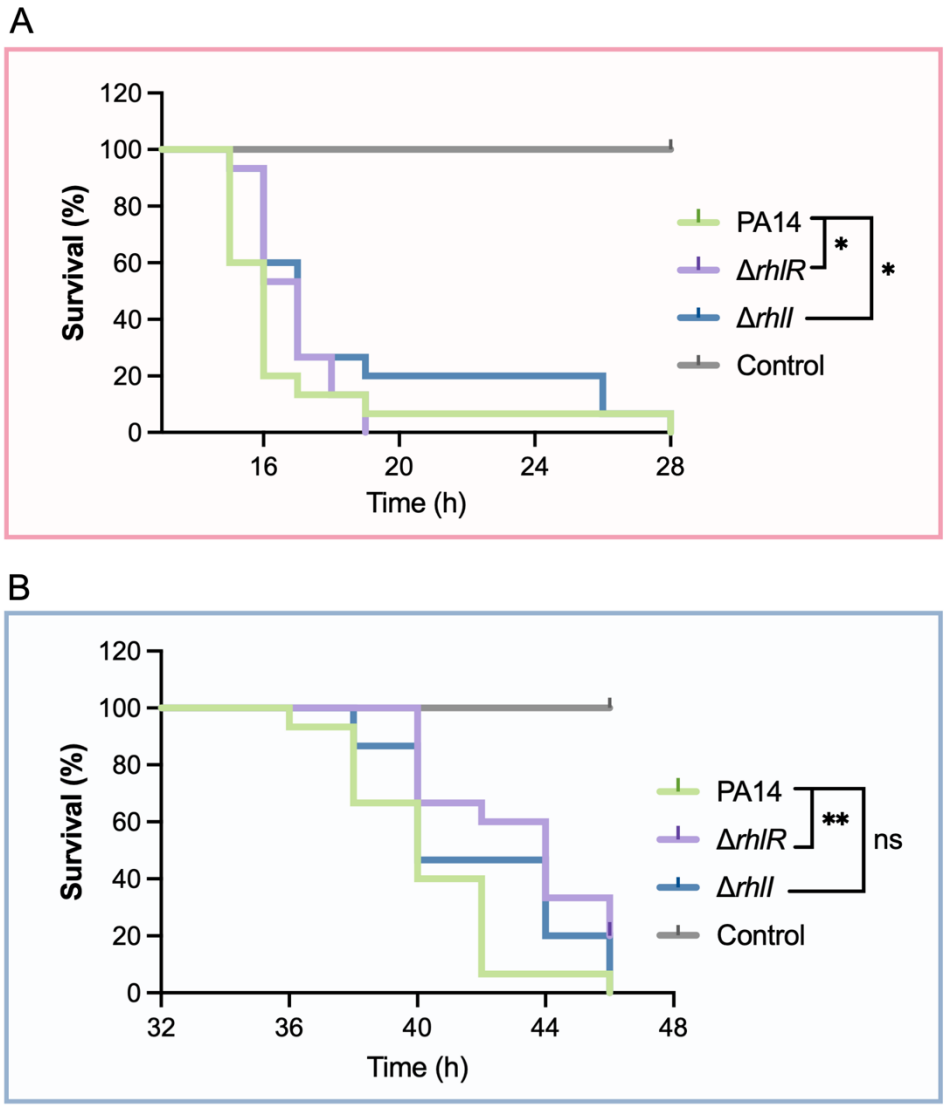

Figure 4.1.10. Temperature impacts the virulence profile of a Δ*rhlI* mutant of *P. aeruginosa* toward *G. mellonella*. **(A)** The great wax larvae were infected with a suspension of $5x10^3$ CFU mL^{-1} (50 bacterial cells per larvae) and incubated at 37°C (top panel, red) or **(B)** at 25°C (bottom panel, blue). Larvae mortality was monitored over time. *N* = 15 larvae per group for each experiment. Statistical significance was determined using Kaplan-Meier analysis and survival curves were compared using Gehan-Breslow-Wilcoxon. ns, nonsignificant, * $P \leq 0.05$, ** $P \leq 0.01$.

4.1.6 Conclusion

P. aeruginosa is an adaptable and versatile saprophyte bacterium, isolated from multiple environments, as a free-living bacterium in aquatic and soil habitats and also in association with several hosts. In these environments, similarly to the requirement encountered in human infection settings, the expression of factors under QS control is important.

A paradox within *P. aeruginosa*'s adaptation strategy is the prevalent deficiency in LasR, a critical QS transcriptional regulator. This deficiency is found in approximately 40% of *P. aeruginosa* isolates from various environments and highlights the need for a functioning of the *rhl* system for QS responsiveness (Groleau *et al.*, 2022; Trottier *et al.*, 2024). RhlR, an inherently unstable protein, depends on the presence of both its cognate ligand C_4-HSL and the chaperone-like protein PqsE for its stability and activity. Within the canonical QS circuitry, LasR is the primary regulator of C_4-HSL production (Groleau *et al.*, 2020). Additionally, LasR indirectly stimulates the production of PqsE through MvfR (Gilbert *et al.*, 2009; Xiao *et al.*, 2006b). Consequently, the absence of LasR has a detrimental effect on both key elements required for RhlR function.

We have found that a lower temperature, resembling those encountered in environmental niches, is sufficient to reduce the requirement of RhlR to C_4-HSL, as it serves as a third stabilizing element of RhlR. This temperature-dependent stabilization exerts a profound influence on the expression of genes under RhlR control, as exemplified by our findings with *phz1*. This thermoregulation could be a strategic adaptation exploited by LasR-deficient cells to maintain QS function, effectively compensating for reduced levels of C_4-HSL and PqsE. Indeed, this compensatory response extends to situations where C_4-HSL is absent, as indicated by the continued production of pyocyanin at 25°C.

Our findings have significant implications, particularly concerning the evolution of *P. aeruginosa* and the *pqsABCDE* operon. Within the *Pseudomonas* genus, *P. aeruginosa* is the most prominent opportunistic pathogen, whereas the other species in the genus are predominantly associated with environmental habitats such as water, soil, or plants (Saati-Santamaría *et al.*, 2022). Notably, the *pqs* operon, and thus the associated protein PqsE, is found solely in *P. aeruginosa* within this genus. This observation raises intriguing questions about the role of the *pqs* operon in the transition of *P. aeruginosa* from environmental niches to warm-blooded hosts. The emergence of PqsE may have enabled the activity of the *rhl* system in mammalian infection settings, potentially contributing to the pathogenicity of *P. aeruginosa* in human infections. Interestingly, the sole known homologue of PqsE, HmqE (Diggle *et al.*, 2006; Folch *et al.*, 2013), is also present in

opportunistic pathogens of the *Burkholderia* genus (Coulon *et al.*, 2019). This observation hints at a potential conversion of evolutionary traits favouring QS mechanisms in response to environmental cues among opportunistic pathogens. Also interesting, *rhlI* mutations were recently reported to accumulate in naturally occurring LasR-negative isolates (Simanek *et al.*, 2023). In these cases, the *rhl* system could remain functional at lower temperatures, facilitating bacterial adaptation and enhancing virulence, particularly in non-warm-blooded hosts. These findings underscore our work's ecological and clinical relevance, emphasizing the remarkable adaptability of *P. aeruginosa*.

4.1.7 Acknowledgments

We thank Wulf Blankenfeldt from the Helmholtz Centre for Infection Research and the Technical University of Braunschweig for generously providing materials that greatly contributed to the progress of this research. We acknowledge the support of PhD scholarships from the Fondation Armand-Frappier, which were awarded to T.O.P.

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4.2 Supplemental data related to the article "Temperature-responsive control of *Pseudomonas aeruginosa* virulence determinants through the stabilization of quorum sensing transcriptional regulator RhlR"

4.2.1 Contextualization

The article provides insights into the activity of the transcriptional reporter *phzA1-lux* under various culture conditions, concluding that a lower temperature, resembling environmental conditions, stimulates RhlR activity. This section presents additional data that enhances the overall understanding of this thermoregulation phenomenon.

4.2.2 Material and Methods

4.2.2.1 Bacterial growth conditions

Bacterial cultures were routinely cultivated in tryptic soy broth (TSB; BD Difco, Canada) at 37°C in a TC-7 roller drum (NB, Canada) at 240 rpm. For gene expression experiments, cultures were grown in King's A medium (King *et al.*, 1954) supplemented with 100 μM $FeCl_3$ or TSB and incubated at 37°C or 25°C in a Multitron Pro incubator (INFORS HT, Switzerland) at 240 rpm. When necessary, 15 μg/ml gentamicin was added to maintain the plasmid during routine manipulations. No antibiotics were used in the gene expression assays. In experiments involving the addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), cultures were either buffered with 100 mM HEPES from a stock solution prepared in ultrapure water or left unbuffered, with only water added to the control samples. Plasmids used are listed in **Table 4.2.1**.

Table 4.2.1. Plasmids used in this supplementary study.

Plasmid	Description	Reference
pP_{rhlA}-*gfp*	Broad-host-range expression plasmid (pBBR1MCS-5) with *rhlA* promoter fused to *gfp*, Gm^R;	(Kostylev *et al.*, 2023)
pCTX-1-P_{hcnA}-*lux*	Promoter of *hcnA* in mini-CTX-*lux*, Tet^r	(Cabeen, 2014)
pUCP20-*pqsE*	*pqsE* gene under control of the *lac* promoter in pUCP20; $Carb^R$	(Yu *et al.*, 2009)
pUCP20-PqsE E187R	*pqsE* gene variant (PqsE E187R) under control of the *lac* promoter in pUCP20; $Carb^R$	This study

4.2.2.2 Construction of plasmids

The sequence encoding the PqsE variant E187R (Borgert *et al.*, 2022) was synthesized and cloned into the pUCP20 vector backbone (West *et al.*, 1994b) by Bio Basic Inc.

4.2.2.3 Gene expression reporter measurements

Gene expression was assessed employing luminescence (*lux*) or fluorescence (*gfp)* reporters. The activity of the reporters was quantified using Cytation 3 multimode microplate reader (BioTek Instruments, USA). For fluorescence measurements, the excitation was set at 485 nm and the emission, at 540 nm. Relative light units (RLU, for *lux* readings) and relative fluorescence units (RFU, for *gfp* readings) were normalized by bacterial growth (OD_{600}). Optical density measurments were performed utilizing the NanoDrop ND100 spectrophotometer (Thermo Fisher Scientific, Canada). When mentioned, C_4-HSL and mBTL were added to the indicated concentrations from a stock prepared in high-performance liquid chromatography (HPLC)-grade acetonitrile (C_4-HSL) and DMSO (mBTL). Solvent only was added as controls.

4.2.3 Results

4.2.3.1 Manipulating the structural dynamics of the active RhlR complex reinforces the notion that temperature affects RhlR's activity

The manuscript presents results that indicate that an environmental-like temperature acts as a stabilizing factor for the RhlR/C_4-HSL/PqsE complex. Additional experiments were conducted to confirm this finding. As mentioned, both RhlR and PqsE are part of a homodimeric structure within the active complex [refer to the structure in (Borgert et al., 2022)]. These homodimers interact with each other, and this interaction represents a critical interaction for stabilizing RhlR. Indeed, Borgert and colleagues have emphasized its importance by demonstrating that introducing a mutation in the dimer interface of PqsE, resulting in a monomeric structure, weakens the protein-protein interaction with RhlR and significantly reduces pyocyanin production (Borgert *et al.*, 2022).

To verify whether the thermoregulation of *phz1* transcription stemmed from the stabilization of RhlR, the monomeric variant of PqsE (E187R) was utilized (Borgert *et al.*, 2022). It is worth noting that aside from acting as a stabilizing factor for RhlR, PqsE also exhibits enzymatic activity (Drees *et al.*, 2015; Zender *et al.*, 2016), a trait retained in the PqsE E187R variant. Consequently, this

variant allows the distinction between the structural role of PqsE and its enzymatic function in modulating RhlR activity. The transcription of *phz1* was evaluated in the double Δ*rhlI* Δ*pqsE* mutant expressing either PqsE WT or the PqsE E187R variant through an expression vector in response to C_4-HSL at both 37°C and 25°C.

In line with the results presented in the manuscript, the expression of *phz1* in this genetic background is virtually abolished and the assessment of the impact of the PqsE's variants on transcription requires the addition of exogenous C_4-HSL. Under an equivalent concentration of C_4-HSL, the *phz1* transcription was higher at 25°C compared to 37°C for both variants of PqsE (**Fig. 4.2.1**). The transcriptional profile of the strain carrying PqsE WT was comparable to that of the Δ*rhlI,* while complementation with the PqsE E187R variant leads to the response profile of the Δ*rhlI* Δ*pqsE* mutant (compare **Fig. 4.2.1A** with **Fig. 4.1.5A** and **Fig. 4.2.1B** with **Fig. 4.1.7A**). Therefore, disrupting the interaction of RhlR and PqsE (i.e., through the use of the PqsE E187R variant) has the same impact on *phz1* transcription as the deletion of the latter, underscoring the previously reported significance of PqsE homodimer formation for RhlR activity.

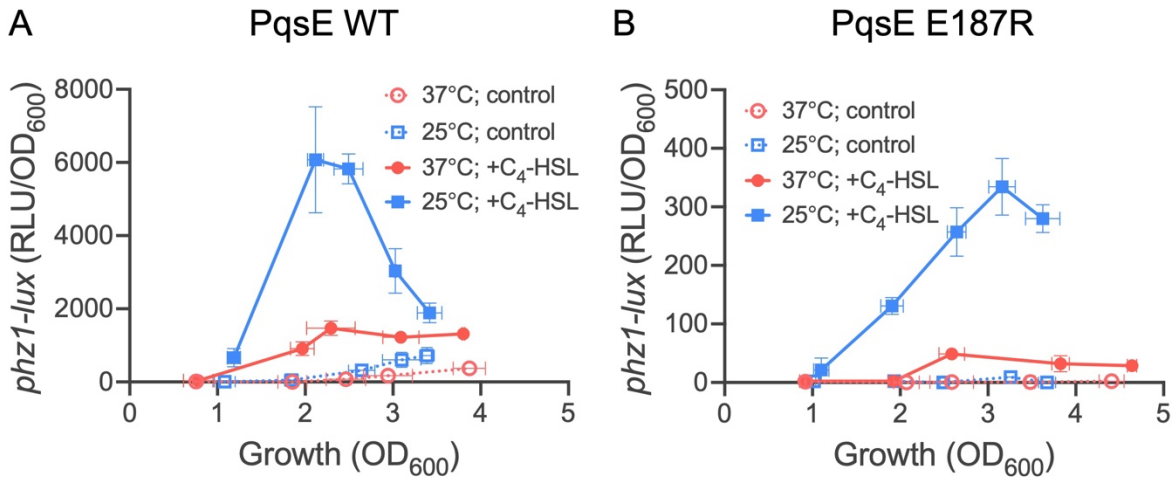

Figure 4.2.1. Transcription of *phz1* is reduced when a PqsE variant unable to interact with RhlR is present. Transcriptional activity of the chromosomal reporter *phzA1-lux* in the Δ*rhlI* Δ*pqsE* genetic background in response to 0.75 µM of C_4HSL (solid symbols and solid lines). Solvent only was used as controls (open symbols and dotted lines). Cultures were incubated at 37°C (red) or 25°C (blue). **(A)** Cultures with a PqsE WT encoding vector. **(B)** Cultures with a PqsE E187R encoding vector. The values are means ± standard deviation (error bars) from three replicates.

The response measured with increasing concentrations of C_4-HSL was analyzed at the peak of *phz1* transcription under the tested temperatures (i.e., OD_{600} of 2.5; **Fig. 4.2.1**). One method for interpreting the data involves calculating the ratio between *phz1* expression at 25°C and that at

37°C. This analytical approach allows a detailed examination of how temperature influences *phz1* transcription under the tested conditions. Variations in these ratios would likely be due to the impact of the PqsE variant on RhlR activity/function. In the Δ*rhlI* Δ*pqsE* mutant complemented with PqsE WT, thermally induced *phz1* transcription remained consistent across all conditions (**Fig. 4.2.2**). The interaction of PqsE WT with RhlR compensates for C_4-HSL absence, yielding comparable induction ratios regardless of C_4-HSL presence and concentration, resulting in comparable induction ratios regardless of C_4-HSL presence or concentration, although C_4-HSL does enhance luminescent values. In contrast, the PqsE E187R variant exhibits a distinct ratio for thermo-induced *phz1* transcription, indicating temperature's limited modulation on the transcription ratio due to hindered interaction between PqsE E187R and RhlR. However, with C_4-HSL present, thermo-induced upregulation of *phz1* transcription is restored regardless of concentration. This suggests that RhlR's interaction with its autoinducer is sufficient for the response to temperature changes. These results confirm that PqsE's involvement in *phz1* thermoregulation is linked to the active RhlR complex structure (i.e., RhlR/C_4-HSL/PqsE), rather than an alternative function in *P. aeruginosa*.

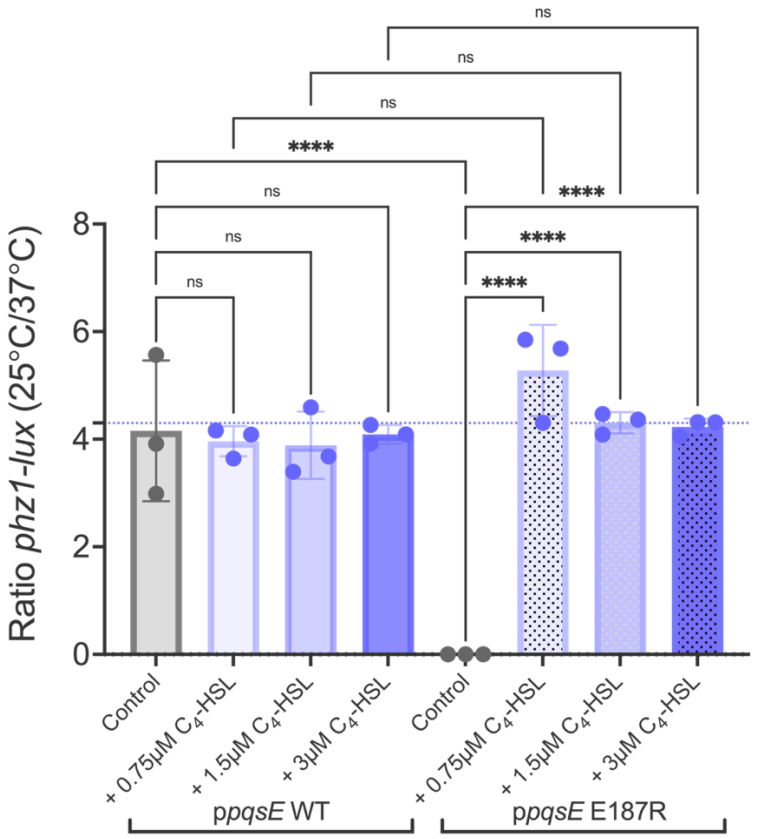

Figure 4.2.2. *phz1* transcription remains unaffected by temperature variations when the formation of an active RhlR complex is compromised.

The transcription of the chromosomal reporter *phzA1-lux* was assessed in the double Δ*rhlI* Δ*pqsE* mutant in the presence of a plasmid vector encoding either PqsE-WT or PqsE E187R, as indicated. Exogenous C_4-HSL was added at specified concentrations, and solvent was used as a control. Cultures were incubated at 37°C or 25°C. The obtained RLU values, corrected per OD_{600} at 25°C, were divided by values obtained at 37°C, resulting in the presented ratio. The comparison was performed between cells at approximately OD_{600} of 2.5 in both conditions, and the dotted line indicates the average temperature-induced ratio. The values are means ± standard deviation (error bars) from three replicates. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Šídák's multiple comparisons posttest. ns, nonsignificant; **** $P \leq 0.0001$.

The instability of RhlR has long posed challenges for biochemical studies of this regulator. While crucial for RhlR function, the cognate autoinducer C_4-HSL fails to sufficiently stabilize RhlR for protein purification in heterologous systems (O'Loughlin *et al.*, 2013). However, the utilization of the synthetic molecule *meta*-bromo-thiolactone (mBTL, 4-(3-bromophenoxy)-*N*-(2-oxotetrahydrothiophen-3-yl)butanamide) has proven effective in enhancing the stability of RhlR, thus overcoming this limitation (Borgert *et al.*, 2022; Feathers *et al.*, 2022; McCready *et al.*, 2019). Moreover, similar to C_4-HSL, mBTL can induce the expression of genes under RhlR control in

both autologous and heterologous systems (Borgert *et al.*, 2022; McCready *et al.*, 2019; O'Loughlin *et al.*, 2013). Inspired by these findings, the effects of artificially induced RhlR stability by mBTL were explore aiming to reinforce the idea that temperature influences RhlR-controlled processes through modulation of RhlR stability.

To achieve this objective, the activity of the *phzA1-lux* in the double Δ*rhlI* Δ*pqsE* mutant when exposed to the artificial ligand mBTL at both 37°C and 25°C was assessed. In line with prior research (Borgert *et al.*, 2022), a concentration of 50 μM of mBTL to stabilize the RhlR structure was maintained. The impact of this concentration of mBTL on *phz1* expression was compared with that of an equivalent concentration of C_4-HSL. Upon stabilization of RhlR with mBTL, *phz1* transcription is induced at 37°C compared to 25°C (**Fig. 4.2.3**), in contrast to the results depicted in **Figures 4.1.5** and **4.1.7**, in response to the exogenous presence of an RhlR stabilizing element. Interestingly, the presence of 50 μM of C_4-HSL had a comparable impact on *phz1* expression when compared to mBTL (**Fig. 4.2.4**), indicating that this non-physiological concentration of autoinducer overrides the positive influence of temperature.

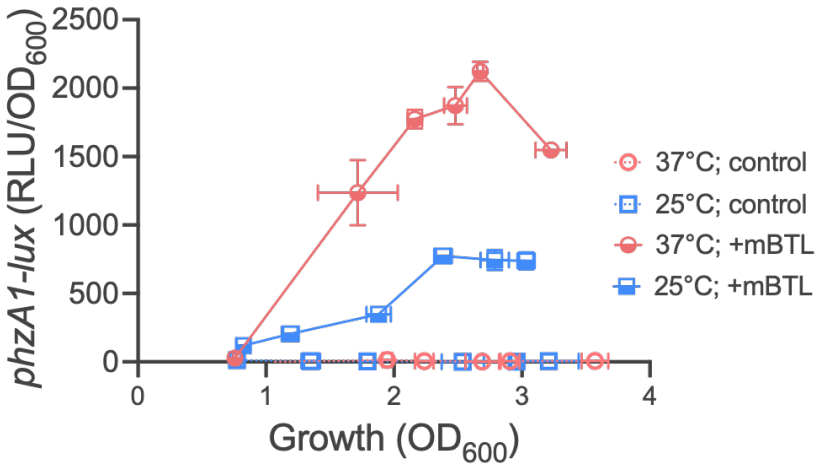

Figure 4.2.3. Expression of *phz1* driven by the complex RhlR:mBTL is not induced at an environmental-like temperature.

Transcription of the chromosomal reporter *phzA1-lux* in the genetic background Δ*rhlI* Δ*pqsE* in response to the presence of 50 μM of mBTL (solid symbols and lines). The solvent only was used as a control (open symbols and dotted lines). Cultures were incubated at 37°C (red) or 25°C (blue). The values are means ± standard deviation (error bars) from three replicates.

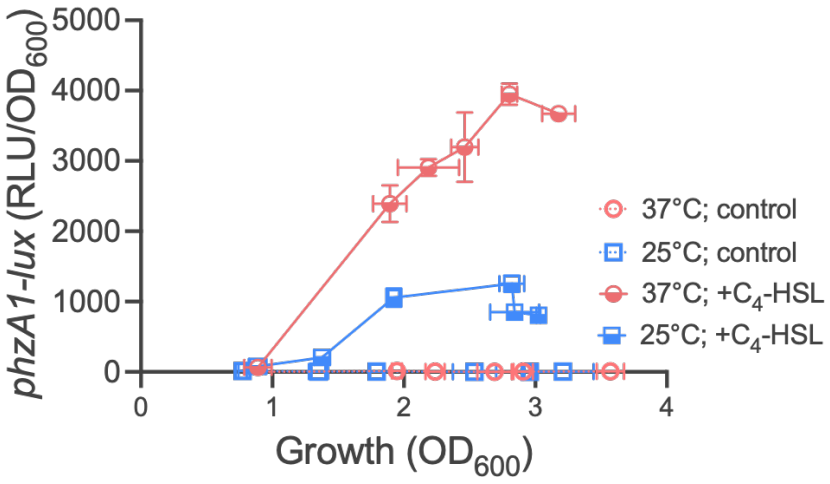

Figure 4.2.4. Loss of the impact of an environmental-like temperature in RhlR stabilization at high C_4-HSL concentrations.

Transcription of the chromosomal reporter *phzA1-lux* in the genetic background Δ*rhlI* Δ*pqsE* in response to the presence of 50 μM of C_4-HSL (solid symbols and lines). The solvent only was used as a control (open symbols and dotted lines). Cultures were incubated at 37°C (red) or 25°C (blue). The values are means ± standard deviation (error bars) from three replicates.

Notably, at the same concentration, the addition of C_4-HSL elicits a more pronounced effect on *phz1* transcription than mBTL, despite the known impact of the latter on RhlR stability. Both stabilizing elements, C_4-HSL and mBTL, occupy a similar pocket within the structure of RhlR, and such discrepancy in activity induction was not predicted based on this parameter (Borgert *et al.*, 2022). This finding suggests that while RhlR stability is a contributing factor to its activity, other dynamic processes are also involved in this phenomenon. Factors such as the membrane permeability of these compounds in *P. aeruginosa* may additionally contribute to the observed response. Regardless of the mechanism(s) underlying the differential response to C_4-HSL and mBTL, these results indicate that artificially inducing the *rhl* system, either by mBTL or an excess of C_4-HSL, disrupts the typical thermoregulation of *phz1* transcription, which is usually induced at lower temperatures. Therefore, artificially inducing the *rhl* system can bypass the stabilizing effect of lower temperatures on RhlR activity.

4.2.3.2 Changes in pH are not the main driver for variation in *phz1* expression in response to different growth temperatures

As previously mentioned, all AHLs include a homoserine lactone moiety, as depicted in **Fig. 1.1.3**. This structure remains stable under neutral and slightly acidic pH conditions, but the lactone ring undergoes hydrolysis in basic environments (Horswill *et al.*, 2007). An AHL structure with an open ring lacks biological activity. Notably, the metabolism of cells grown at 37°C and 25°C differs significantly, resulting in variations in bacterial waste released into the growth medium. One plausible consequence of this metabolic difference is a change in pH (Ratzke *et al.*, 2018; Sánchez-Clemente *et al.*, 2018), which is relevant since TSB is not a buffered growth medium. In fact, a report on *P. aeruginosa* has described the link between growth temperature and the rate of C_4-HSL lactonization, which increases with temperature enhancements in a growth-independent manner (Yates *et al.*, 2002). To investigate the potential role of pH in *phz1* expression under our conditions, experiments were conducted using HEPES-buffered TSB. The expression profile of *phz1* remained similar between the buffered and unbuffered conditions (**Fig. 4.2.5** vs **Fig. 4.1.5A**), suggesting that any degradation of C_4-HSL due to alkaline conditions is not the primary factor driving the thermoresponse described in this manuscript. However, the fold change of expression between 25°C and 37°C is slightly less pronounced in experiments carried out in HEPES-buffered TSB, indicating a minor contribution of pH variations to the overall thermo-induced *phz1* expression.

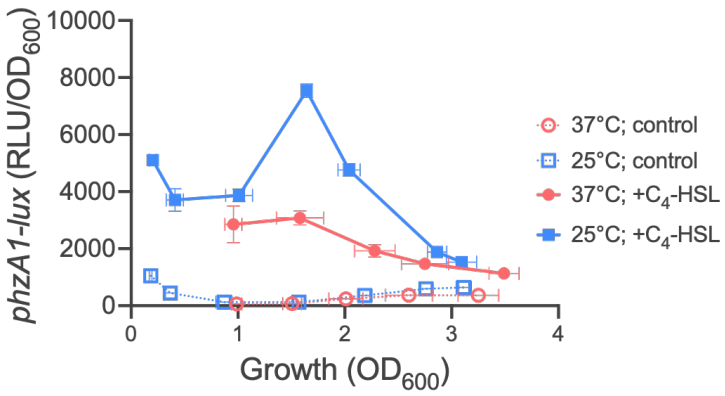

Figure 4.2.5. The upregulation of *phz1* under an environmental-like temperature condition is consistently observed in a HEPES-buffered TSB growth medium.

Transcription of the chromosomal reporter *phzA1-lux* in Δ*rhlI* mutant during growth in HEPES-buffered TSB. Cultures were incubated at 37°C (red) or 25°C (blue). C_4-HSL was supplemented at the final concentration of 3 μM (solid symbols and lines). Solvent alone (acetonitrile) was used in the controls (open symbols and dotted lines). The values are means ± standard deviation (error bars) from three replicates.

4.2.3.3 The thermo-induced expression of *phz1* extends beyond growth on TSB

The bacterial responses to an environmentally relevant temperature presented in the article were conducted using TSB as a growth medium. Specific QS responses have been reported to depend on nutritional conditions (Dubern *et al.*, 2023; García-Reyes *et al.*, 2021; Mellbye *et al.*, 2014; Scribner *et al.*, 2022; Shrout *et al.*, 2006; Soto-Aceves *et al.*, 2022). To ascertain whether the described temperature-related response is limited to TSB growth conditions, *phz1* transcription was assessed in cells cultured in King's A medium.

The *phz1* expression profile noticeably differs from cells grown in TSB (**Fig. 4.2.6A**; compare with **Fig. 4.1.1**). Specifically, there is no evident expression peak in cells incubated at 37°C. However, at 25°C in PA14, *phz1* expression is induced sevenfold, and there is a discernible peak of expression at a bacterial quorum comparable to that of TSB (OD_{600} of 1.6) (**Fig. 4.2.6A**). Similar to the response observed in TSB, the absence of *rhlR* (Δ*rhlR*) abolishes *phz1* transcription (**Fig. 4.2.6A**).

A comparable response to the one reported is also evident in mutants affecting RhlR stability: Δ*rhlI*, Δ*pqsE*, and Δ*rhlI* Δ*pqsE*. When grown at 25°C, these mutants show an induction of *phz1* expression compared to growth at 37°C (**Fig. 4.2.6B**). However, this induction pattern is observed in cells at a higher cellular density, occurring later in the growth phase for both Δ*rhlI* and Δ*pqsE* strains (**Fig. 4.2.6B**). This profile contrasts with growth in TSB, where expression at 37°C never exceeds that at 25°C (**Fig. 4.1.3B**). The relatively elevated expression of *phz1* in the absence of either C_4-HSL or PqsE suggests that RhlR is more stable or active under the tested basal conditions (i.e., growth at 37°C) in King's A medium. This observation justifies the delay in observing thermoregulation in this growth medium. Nevertheless, these findings align perfectly with those presented in the article, confirming that the reported thermoregulation is not limited to cells cultivated in TSB.

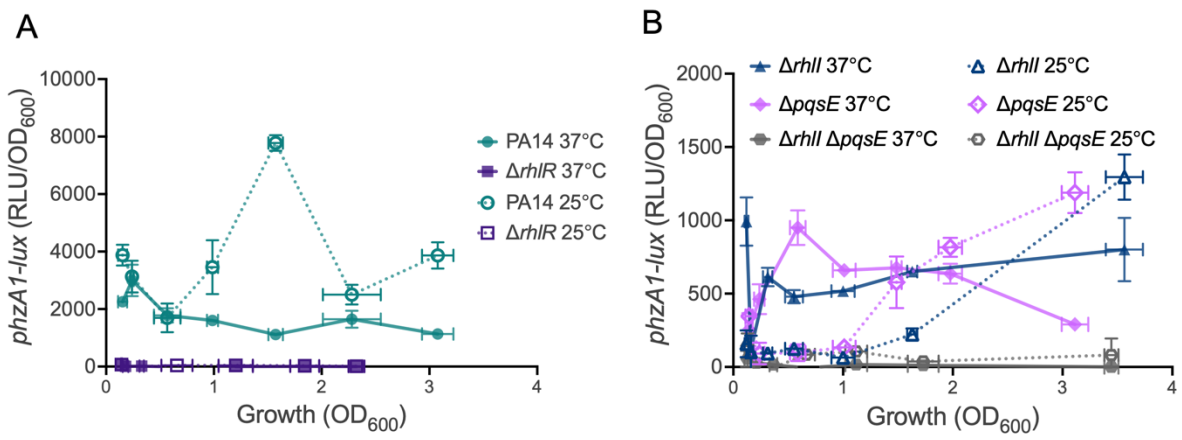

Figure 4.2.6. An environmental-like temperature also induces *phz1* expression in King's A medium cultured cells

The transcription of the chromosomal reporter *phzA1-lux* was assessed in **(A)** PA14 and its isogenic *rhlR* mutant (Δ*rhlR*) and **(B)** mutants Δ*rhlI*, Δ*pqsE*, and Δ*rhlI* Δ*pqsE*. The cultures were incubated at 37°C (solid symbols and solid lines) and 25°C (open symbols and dotted lines). The values are means ± standard deviation (error bars) from three replicates.

4.2.3.4 Expression of genes under RhlR presents variable profiles in response to an environmental-like temperature: is it due to the presence of several RhlR subregulons?

As previously mentioned, the data presented in the article exclusively relies on the *phzA1-lux* reporter. This choice was motivated by two factors: firstly, the model illustrating how PqsE enhances RhlR stability was initially established using *phzA1* expression (Borgert *et al.*, 2022); secondly, RhlR serves as this gene's primary direct transcriptional regulator. However, RhlR also plays a central role in activating the transcription of the *rhlA* gene, which is related to rhamnolipid production (Medina *et al.*, 2003; Ochsner *et al.*, 1994; Pearson *et al.*, 1997). Consequently, measurements using the transcriptional reporter *rhlA-gfp* were conducted to investigate whether the proposed model of RhlR activity is valid for its impact on *rhlA* transcription.

At 37°C, there is a gradual increase in *rhlA* expression during bacterial growth, peaking at an OD_{600} of 3 in the PA14 background (**Fig. 4.2.7**). Conversely, at 25°C, the expression of this gene is barely detectable. The relatively high expression observed at the initial time point at 25°C is likely an artifact, possibly due to the residual presence of C_4-HSL and an extremely low OD_{600}, leading to an overestimation of relative fluorescence produced per OD_{600}. Therefore, under the conditions employed to describe the thermoregulation induced by RhlR, the expression of *rhlA*, a gene under RhlR control, remains undetectable (**Fig. 4.2.7**). Indeed, *rhlA* transcription is

intrinsically linked to the employed culture conditions (Déziel *et al.*, 2003; Guerra-Santos *et al.*, 1986) and could partially account for the lack of expression in cells grown at 25°C. Importantly, the same pattern is observed in cells cultivated in King's A medium (data not shown). The absence of expression under the tested conditions limits the conclusions that can be drawn from the presented data. However, it suggests that the thermoregulation model we propose for *phz1* does not adequately explain *rhlA* transcription.

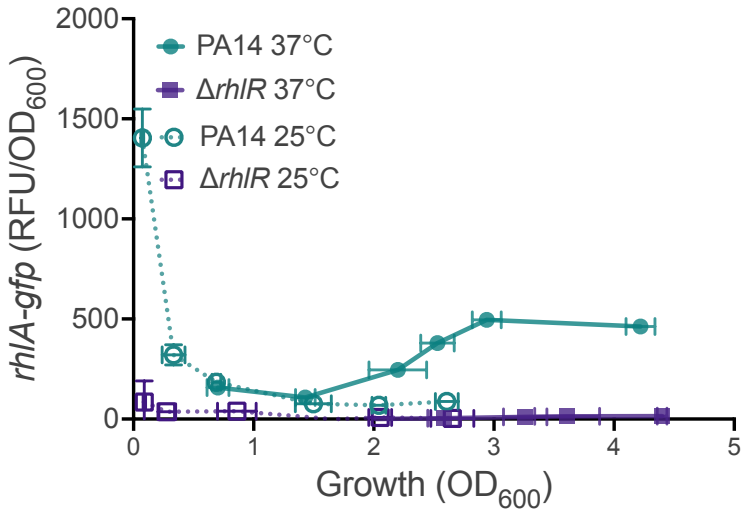

Figure 4.2.7. The gene *rhlA*, which is under the control of RhlR, is not induced at an environmental-like temperature.

The transcription of the vector-encoded reporter *rhlA-gfp* was measured during growth in TSB using both the PA14 strain and its isogenic Δ*rhlR* mutant. The cultures were incubated at 37°C (solid symbols and solid lines) and 25°C (open symbols and dotted lines). The values are means ± standard deviation (error bars) from three replicates.

The findings indicate that, under specific conditions, the expression of genes regulated by RhlR may diverge, as observed for the transcription of *phz1* and *rhlA*. This divergence has been noted previously (Letizia *et al.*, 2022; Mukherjee *et al.*, 2018), so it was anticipated that *rhlA* transcription would not align with the proposed model. As mentioned in this document, RhlR activity relies on C_4-HSL and PqsE. Notably, the extent of PqsE's impact on the regulation of *phz1* and *rhlA* varies by strain (Keegan *et al.*, 2023; Letizia *et al.*, 2022; Mukherjee *et al.*, 2018). In the PA14 strain used throughout the experiments in this document, *rhlA* transcription does not appear to be dependent on RhlR, unlike *phz1*. Since the model we propose for the thermoregulation of RhlR activity is based on the responses of PA14, where RhlR depends on PqsE for *phz1* transcription, not *rhlA*, it underscores the role of PqsE in this response. Importantly, it also indicates a limitation

of the model, which would only describe the thermoregulation of RhlR targets that, like *phz1*, depend on PqsE.

To further explore thermoregulation in a gene regulated by RhlR, we examined transcription from the *hcnA* promoter using a chromosomal *hcnA-lux* reporter. Similar to the *phz1* profile, growth at 25°C induces *hcnA* expression in wild-type PA14 (**Fig. 4.2.8**). However, in the absence of RhlR, *hcnA* transcription is also induced at 25°C. Importantly, under both growth temperatures, the absence of RhlR results in reduced *hcnA* transcription compared to its wild-type counterpart. It suggests that while RhlR plays a role in *hcnA* regulation, other regulatory mechanisms are also at play. Thus, thermoregulation of *hcnA* transcription is partially mediated by RhlR, and, like *phz1* transcription, it is induced at a lower temperature. As previously noted in this document, *hcnA* expression is not solely controlled by RhlR, aligning this result with what was expected.

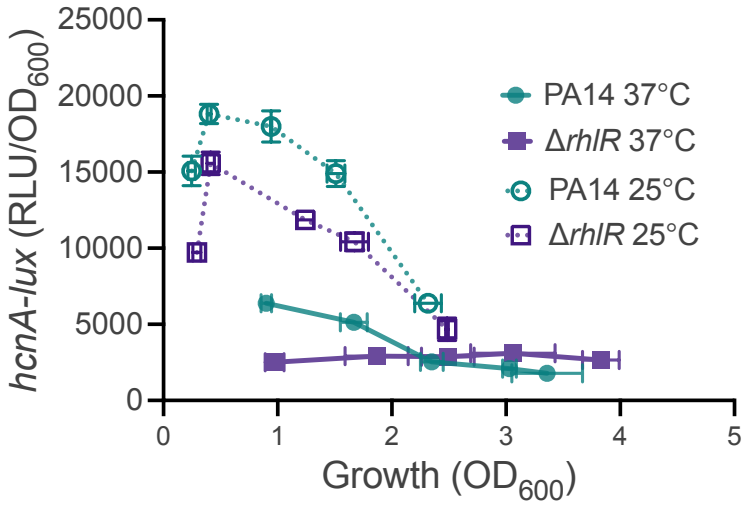

Figure 4.2.8. *hcnA* expression is induced at an environmentally relevant temperature, but this response is not solely attributed to RhlR.

The transcription of the chromosomal reporter *hcnA-lux* was measured during growth in TSB using both the PA14 strain and its isogenic Δ*rhlR* mutant. The cultures were incubated at 37°C (solid symbols and solid lines) and 25°C (open symbols and dotted lines). The values are means ± standard deviation (error bars) from three replicates.

4.3 Article: "Unravelling the plasticity of quorum sensing in *Pseudomonas aeruginosa*: Insights from a naturally evolved LasR variant"

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Laboratory experiments done by: TOP

Data analysis and interpretation: TOP, MCG, and ED

Manuscript writing: TOP

4.3.1 Abstract

One remarkable feature of *Pseudomonas aeruginosa* is its ability to adapt and survive in various conditions. The emergence of LasR deficiency, a critical component of its quorum sensing (QS), has been observed across strains from various isolation sites, indicating its widespread occurrence as an adaptation feature. For instance, LasR-defective isolates readily emerge in experimental evolution within the laboratory. Notably, a deficiency in the *las* system does not necessarily result in the loss of responses governed by the QS system. The *rhl* system, mediated by the regulator RhlR, can still regulate these responses. The autoinducer C_4-HSL is instrumental for RhlR functionality. Here, we explored the requirement of C_4-HSL for RhlR functionality to investigate plasticity within the QS regulatory network. We conducted an experimental evolution in a C_4-HSL-negative background to investigate whether such conditions decrease the emergence of naturally occurring LasR-defective isolates. One isolate that emerged from this evolution maintained some behaviours regulated by QS, even in the impairment of both LasR and RhlR functions. We confirmed that the observed response was due to the LasR A158P variant. To eliminate potential interference from other mutations that may have arisen during evolution, we transferred the LasR A158P variant into a wild-type PA14 background. This variant exhibited functional LasR activity only at lower temperatures, specifically at 25°C or room temperature, but not at 37°C. To understand the impact of LasR A158P, we monitored the expression of several genes regulated by QS at these temperatures. However, we observed significant effects only on the expression of *lasI*, *rhlI*, and *phz1*. Notably, since this variant was initially identified in a C_4-HSL background, it became evident that the deregulation of *rhlI*, which affects RhlR function and subsequently influences *phz1* expression, is not the sole mechanism by which this variant impacts RhlR-dependent QS responses. This highlights the intricate and multifaceted regulatory mechanisms that mediate QS functionality in *P. aeruginosa*.

4.3.2 Introduction

Quorum sensing (QS) is a cell-to-cell communication system that allows bacteria to favour the adoption of group behaviours over individual, asocial ones. The detection of fluctuations in cell number is the fundamental basis of this response. Bacteria produce small signalling molecules called autoinducers that accumulate in the function of cell density to the concentration in which they bind to and activate cognate receptors. Gene expression of the bacterial population is synchronized by this process (Diggle *et al.*, 2007; Waters *et al.*, 2005). In Gram-negative bacteria, acyl-homoserine lactones (AHLs), produced by LuxI synthases, are the class of autoinducers

most often used in QS and regulate the activity of LuxR transcriptional regulators (Fuqua *et al.*, 1994b; Papenfort *et al.*, 2016).

The versatile bacterium *Pseudomonas aeruginosa* employs QS to thrive in diverse ecological niches. Environmental isolates of this bacterium can be found in niches closely associated with human activities, including soil and water reservoirs (Crone *et al.*, 2020; Green *et al.*, 1974; Mena *et al.*, 2009). With a broad host range, *P. aeruginosa* can also infect a wide array of organisms, from plants, insects, and worms to warm-blooded animals, such as humans (Apidianakis *et al.*, 2009; Reynolds *et al.*, 2021; Starkey *et al.*, 2009; Stieritz *et al.*, 1975; Stotland *et al.*, 2000; Tan *et al.*, 2000). Notably, *P. aeruginosa* is one of the primary agents responsible for nosocomial infections, posing a significant threat to individuals with cystic fibrosis (CF), and thus, ranks among the most clinically important opportunistic pathogen (Reynolds *et al.*, 2021).

Within *P. aeruginosa*, the QS circuitry elegantly integrates three systems, each activated by detecting a different signal molecule. The *las* and *rhl* systems represent AHL-based QS systems, wherein the LuxR-type transcriptional regulators, LasR and RhlR, respond to the products of LuxI-type synthases, LasI and RhlI. These signalling molecules are *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), respectively (Passador *et al.*, 1993). Upon LasR activation, numerous genes, including those encoding virulence determinants and other QS elements, are transcribed. The *rhl* system, also influenced by LasR, further drives the expression of virulence determinants upon activation. However, full activation of the *rhl* circuitry necessitates the involvement of a third element: PqsE (Borgert *et al.*, 2022; Farrow *et al.*, 2008; Groleau *et al.*, 2020; Letizia *et al.*, 2022). The remaining QS system, the *pqs* system, produces this protein. MvfR (also known as PqsR) is this system's transcription regulator and induces the transcription of the *pqsABCDE* operon (Gallagher *et al.*, 2002). The enzymes PqsABCD are involved in the synthesis of 4-hydroxy-2-alkylquinolines (HAQs), with the primary product of their activity being 4-hydroxy-2-heptylquinoline (HHQ) (Déziel *et al.*, 2004). HHQ undergoes conversion to *Pseudomonas* quinolone signal (PQS; 3,4-dihydroxy-2-alkylquinoline) catalyzed by the enzyme PqsH (Déziel *et al.*, 2004; Schertzer *et al.*, 2010). MvfR responds to dual ligands, HHQ and PQS, creating an autoregulatory loop that amplifies HAQ production and, consequently, the synthesis of PqsE – the final protein encoded in the *pqsABCDE* operon (Déziel *et al.*, 2005; Rampioni *et al.*, 2016; Xiao *et al.*, 2006a). PqsE, in collaboration with the cognate signal C_4-HSL, enhances the stability and the activity of RhlR, thereby mediating the complete activation of the *rhl* system targets (Borgert *et al.*, 2022; Feathers *et al.*, 2022).

Isolates of *P. aeruginosa* from both environmental and clinical settings often are LasR deficient, and the high prevalence of this phenotype strongly suggests it is a widespread adaptation of natural populations of *P. aeruginosa* (Groleau *et al.*, 2022; Trottier *et al.*, 2024). One of the most frequently reported genetic alterations in *lasR* involves single-nucleotide substitutions, resulting in functional or non-functional protein (Feltner *et al.*, 2016; Groleau *et al.*, 2022). While this adaptation might seem to contradict the significance of QS, numerous reports have highlighted the plasticity within the QS circuitry regulation, demonstrating sustained QS functionality even in the absence of LasR (Asfahl *et al.*, 2022; Chen *et al.*, 2019a; Cruz *et al.*, 2020; Feltner *et al.*, 2016; Groleau *et al.*, 2022; Kostylev *et al.*, 2019). Thus, it is likely that several convergent mechanisms drive the reorganization of the QS system, ultimately leading to the activation of QS through the *rhl* system.

In this investigation, we explored the potential plasticity of QS in the laboratory strain *P. aeruginosa* PA14, utilizing an experimental evolution approach known to generate LasR-deficient isolates. In these strains, RhlR becomes the primary functional LuxR protein, and its activity becomes central to the QS system. Given the critical importance of RhlR functionality in this context, we initiated the study with a C_4-HSL-negative background as the parental strain. This approach allowed us to probe whether bacteria face pressure to conserve a functional LuxR protein. Interestingly, in the evolved population, comprising a mixture of LasR-functional and -defective isolates, the transcription of RhlR-controlled genes, initially at a basal level in the parental strain, is induced. Our findings reveal that sustained QS responses persist in the C_4-HSL-negative background harbouring the *lasR* allele, characterized by a single-nucleotide substitution at *lasR* base pair 472. Moreover, we delve into the significance of this allele in QS regulation and demonstrate that temperature plays a pivotal role in determining the expression pattern of the rhl system under these conditions.

4.3.3 Material and Methods

4.3.3.1 Bacterial strains and growth conditions

Bacterial strains and plasmids employed in this study are listed in **Tables 4.3.1** and **4.3.2**, respectively. Routine bacterial cultures were grown in tryptic soy broth (TSB; BD Difco, Canada) at 37°C within a TC-7 roller drum (NB, Canada) set to 240 rpm. Alternatively, cultures were cultivated on lysogeny broth (LB; BD Difco, Canada) agar plates. To observe the expression of QS-controlled genes, cultures were grown in TSB at either 37°C or 25°C, within a Multitron Pro

incubator (INFORS HT, Switzerland) operating at 240 rpm. Antibiotics were supplemented as needed, with the following concentrations: for *P. aeruginosa* PA14, 300 µg/ml carbenicillin, 30 µg/ml gentamicin, and tetracycline at 125 µg/ml (solid) or 75 µg/ml (liquid). For *Escherichia coli*, the antibiotic concentrations were 100 µg/ml carbenicillin, 15 µg/ml gentamicin, and 15 µg/ml tetracycline. Irgasan (20 µg/ml) served as a counter-selection agent against *E. coli*.

Table 4.3.1. Strains used in this study

Strain	**Lab ID #**	**Relevant genotype or description**	**Reference**
P. aeruginosa			
PA14	ED14	Clinical isolate from a human burn patient UCBPP-PA14	(Rahme *et al.*, 1995)
PA14 Δ*rhlI*	ED4407	PA14 derivate; unmarked in-frame *rhlI* deletion	This study
PA14 *rhlI*-	ED297	PA14 derivate; marked mutant (MrT7)	(Liberati *et al.*, 2006)
Evolved Δ*rhlI* *lasR*^{A158P}	ED4569	Naturally evolved PA14 Δ*rhlI* mutant carrying a missense *lasR* mutation (LasR A158P)	This study
Evolved Δ*rhlI* *lasR*^{WT}	ED4701	Naturally evolved PA14 Δ*rhlI* mutant complemented with a wild-type *lasR* allele (LasR WT)	This study
PA14 Δ*rhlI* Δ*lasR*	ED4541	PA14 derivate; unmarked in-frame double *rhlI* and *lasR* deletion	(de Oliveira Pereira *et al.*, 2023)
PA14 Δ*lasR*	ED4409	PA14 derivate; unmarked in-frame *lasR* deletion	(de Oliveira Pereira *et al.*, 2023)

PA14 *lasR*^{A158P}	ED4702	PA14 Δ*lasR* background in which the mutant allele was replaced *in situ* by an allele coding the LasR A158P variant	This study
PA14 Δ*rhlI* *lasR*^{A158P}	ED4703	PA14 Δ*rhlI* Δ*lasR* background in which the mutant allele was replaced *in situ* by an allele coding the LasR A158P variant	This study
PA14 Δ*rhlR* *lasR*^{A158P}	ED4704	PA14 Δ*rhlR* Δ*lasR* background in which the mutant allele was replaced *in situ* by an allele coding the LasR A158P variant	This study
PA14 Δ*pqsE* *lasR*^{A158P}	ED4705	PA14 Δ*pqsE* Δ*lasR* background in which the mutant allele was replaced *in situ* by an allele coding the LasR A158P variant	This study
E. coli			
SM10(λ*pir*)	ED222	*thi thr leu tonA lacY supE recA*::*RP4-2*-Tc::*Mu* Km λ*pir*	Lab collection

Table 4.3.2. Plasmids used in this study

Plasmid	Description	Reference or source
pTOP03	Gene replacement vector for the in-frame deletion of *rhlI* by allelic recombination; $Carb^R$	(de Oliveira Pereira *et al.*, 2023)
pTOP09	Gene replacement vector for in situ complementation of variant LasR A158P; $Carb^R$	This study
pTOP10	Gene replacement vector for in situ complementation of WT LasR; $Carb^R$	This study
pCDS101	Promoter of *phz1* in mini-CTX-*lux*; Tet^R	(Sibley *et al.*, 2008)
pCTX-1-P_{phzA1}-lux	Promoter of *phz1* in mini-CTX-*lux*; Tet^R	(Cabeen, 2014)
pTOP05	Promoter of *lasI* in mini-CTX-*lux*; Tet^R	(de Oliveira Pereira *et al.*, 2023)
pCTX-1-P_{rsaL}-lux	Promoter of *rsaL* in mini-CTX-*lux*; Tet^R	(Cabeen, 2014)
pCTX-1-P_{lasB}-lux	Promoter of *lasB* in mini-CTX-*lux*; Tet^R	(Cabeen, 2014)
mini-CTX *pqsA*::lux	Promoter of *pqsA* in mini-CTX-*lux*; Tet^R	(Diggle *et al.*, 2007)
pP_{rhlA}-*gfp*	Broad-host-range expression plasmid (pBBR1MCS-5) with *rhlA* promoter fused to *gfp;* Gm^R	(Kostylev *et al.*, 2023)
pME3846 (*rhlI-lacZ*)	*rhlI-lacZ* translational reporter; Tet^R	(Pessi *et al.*, 2001)
pPCS1002 (*rhlR-lacZ*)	*rhlR-lacZ* transcriptional reporter; $Carb^R$	(Pesci *et al.*, 1997)

4.3.3.2 Experimental evolution

The parental strains employed for the evolution included PA14, PA14 Δ*rhlI*, PA14::CTX *phzA1-lux*, and PA14 *rhlI*::CTX *phzA1-lux*. Fresh TSB medium (5 mL) was inoculated with glycerol bacterial stock stored at -80°C and incubated overnight at 37°C on a roller drum. Subsequently, the overnight cultures were adjusted to an optical density of 0.05 (OD_{600} of 0.05) and further cultivated at 37°C until they reached an OD_{600} of 1. At this stage, the cultures were re-diluted to an OD_{600} of 0.05 and distributed into three tubes, each containing 5 mL of fresh TSB, marking the initiation of the evolution experiment (i.e., time 0). Thus, three independent evolution trajectories were carried out for each strain during the experiment. Passaging occurred every 24 hours, involving the transfer of 50 µL of culture into 5 mL of fresh media at a 1:100 dilution. The experimental evolution was based on five passages, and samples from each passage were preserved in glycerol stock at -80°C. The percentage of LasR-defective isolates within the evolved population was determined based on the phenotype of one hundred randomly selected colonies grown on 1.5% Tryptic Soy agar (TSA, AlphaBiosciences) supplemented with 1.5% skim milk (Robitaille *et al.*, 2020). These plates were incubated overnight at 37°C and then left at room temperature to allow for phenotype development.

4.3.3.3 Whole genome sequencing

Genomic DNA from both the parental PA14 Δ*rhlI* strain and the evolved Δ*rhlI* (LasR A158P) strain was extracted utilizing the EasyPure genomic DNA kit (TransGen Biotech) as per the manufacturer's protocol. Whole-genome sequencing of these isolates was conducted using the Illumina NextSeq 2000 platform at the Microbial Genome Sequencing Center (MiGS). The sequencing generated 2x151bp paired-end reads, which were subsequently subjected to trimming. To align and compare the genomes of the parental and evolved strains, the variant caller breseq (version 0.35.4) (Deatherage *et al.*, 2014) was employed, and this service was provided by MiGS.

4.3.3.4 Construction of in-frame deletion mutants

Gene knockout deletions were constructed based on allelic recombination employing the use of a suicide vector (Hmelo *et al.*, 2015). pTOP03 was introduced into *P. aeruginosa* by conjugation with *E. coli* SM10 donor strain. Carbenicillin and Irgasan were used to select recipient merodiploid

cells and counter-select *E. coli*, respectively. Double-crossover mutants were selected by sucrose counterselection and confirmed by PCR.

4.3.3.5 *In situ* complementation of LasR variants

Complementation was performed through allelic replacement, following a methodology similar to constructing in-frame deletions. In this process, the upstream, coding, and downstream sequences of *lasR* were amplified via PCR, utilizing the genomic DNA of the evolved Δ*rhlI* (LasR A158P) isolate as the template. The oligonucleotides used are listed in **Table 4.3.3**. Subsequently, this sequence was cloned into the pEX18Ap backbone using the seamless ligation-independent cloning strategy, yielding pTOP09 and pTOP10. The sequence of these constructs was verified through Sanger sequencing, conducted at the Institut de Recherches Cliniques de Montréal (Montréal, QC, Canada). The vectors were then introduced into the *P. aeruginosa* Δ*lasR* mutant through conjugation with the donor *E. coli* SM10 strain. Carbenicillin and Irgasan were used to select recipient merodiploid cells and counter-select *E. coli*, respectively. Double-crossover mutants were selected by sucrose counterselection and confirmed by PCR. Finally, the replacement of the Δ*lasR* allele with the LasR A158P variant was confirmed by PCR.

Table 4.3.3. Oligonucleotides used in this study.

Name	Sequence (5'-3')	Description
TO_lasR_up_fwd	taaaacgacggccagtgccaAACTGGAAAAGTGGCTATG	Design for in situ allelic replacement of *lasR* by cloning a PCR-amplified fragment into a pre-digested pEX18Ap vector (using HindIII and BamHI). Both the wild-type and LasR A158P coding alleles were amplified using these oligonucleotides.
TO_lasR_down_rev	attcgagctcggtacccgggAACTTGTGCATCTCGCCC	

4.3.3.6 Construction of reporter strains

Luminescence-based reporters (mini-CTX-*lux* backgrounds) were integrated into the chromosomal *attB* site of PA14 and isogenic mutants through conjugation on LB plates. Selection of *P. aeruginosa* transformants was achieved using tetracycline, while Irgasan served as the counter-selection agent for donor E. coli SM10. Additionally, non-integrative plasmids carrying reporter fusions were introduced into electrocompetent *P. aeruginosa* cells and subsequently selected using the appropriate antibiotics (Choi *et al.*, 2006).

4.3.3.7 Gene expression reporter measurements

Gene expression, either transcriptional or translational, was evaluated through luminescence/ fluorescence reporter measurements, or β-galactosidase activity assays. Strains containing the reporter fusions were first cultured overnight in TSB with the appropriate antibiotics and subsequently diluted to an OD_{600} of 0.1 in fresh TSB. For the *lux* and *gfp* reporter assays, activity was quantified using the Cytation3 multimode microplate reader (BioTek Instruments, USA). Excitation and emission parameters were set at 485 nm and 540 nm, respectively, for fluorescence measurements. Relative light units (RLU, for *lux* readings) and relative fluorescence units (RFU, for *gfp* readings) were normalized based on growth (OD_{600}). Optical density measurements were performed utilizing the NanoDrop ND100 spectrophotometer (Thermo Fisher Scientific, Canada). The activity of *lacZ* reporters was determined through β-galactosidase activity assays, following the standard protocol (Miller, 1972).

4.3.3.8 Pyocyanin quantification

Different methods were employed to quantify pyocyanin based on the bacterial growth conditions. When bacteria were cultured under surface-associated conditions, pyocyanin was chloroform extracted and acidification before measuring its absorbance at 520 nm, following a previously described protocol (de Oliveira Pereira *et al.*, 2023). The pigment extraction in this growth condition was conducted after 72 hours of incubation at room temperature.

For bacteria cultivated in King's A broth, pyocyanin levels were measured after 24 hours of growth. At this point, a portion of the culture was subjected to centrifugation at 10,000 × *g* for 15 minutes. The resulting supernatant was then transferred to a 96-well plate, and the OD_{695} was determined using a Cytation microplate reader (BioTek, Winooski, VT, USA). Alternatively, pyocyanin

quantification was carried out using high-performance liquid chromatography-tandem mass spectrometry (LC/MS/MS).

4.3.3.9 Quantification of AHLs signalling molecules

The quantification of AHL concentrations in cultures was carried out for bacteria cultivated in King's A broth using LC/MS/MS, following the previously established procedure (Lépine *et al.*, 2018). In summary, at specified time points, cultures were combined with acetonitrile containing the internal standard tetradeuterated 4-hydroxy-2-heptylquinoline (HHQ-d4) in a ratio of 4:1 (culture to solvent). This resulted in a final concentration of HHQ-d4 at 3 ppm. The mixture was vigorously vortexed and subsequently centrifuged at maximum speed for 5 minutes to pellet bacterial cells. The resulting supernatants were then transferred into vials for LC/MS/MS analysis.

4.3.4 Results

4.3.4.1 LasR defect positively modulates RhlR-regulated behaviours in *P. aeruginosa*, even in the absence of the cognate synthase RhlI

The emergency of LasR-defective isolates in the prototypical *P. aeruginosa* strain UCBPP-PA14, referred to as PA14, has recently been described using an experimental evolution model (Mould *et al.*, 2022). Interestingly, when employing a similar model, we observed a parallel trend in the PA14 wild-type strain (**Fig. 4.3.1A**). Given the widespread presence of LasR-defective strains within the *P. aeruginosa* ecology, the need for flexibility within the QS circuitry becomes evident, as we discussed recently (Trottier *et al.*, 2024). Without LasR activity, RhlR becomes the primary regulator of the QS circuitry. This reorganization phenomenon has been documented in wild-type laboratory strains under specific nutrient conditions, rendering LasR activity obsolete, and is also evident in some naturally occurring LasR-deficient isolates (Asfahl *et al.*, 2022; Chen *et al.*, 2019a; Cruz *et al.*, 2020; Feltner *et al.*, 2016; Kostylev *et al.*, 2019; Soto-Aceves *et al.*, 2021). The activity of RhlR relies on its cognate signal ligand C_4-HSL, produced by the synthase RhlI, and is further activated by the chaperone-like protein PqsE (Borgert *et al.*, 2022; Feathers *et al.*, 2022; Groleau *et al.*, 2020; Letizia *et al.*, 2022).

Considering the importance of QS functionality, we aimed to explore its potential flexibility under controlled laboratory conditions. Initially, we hypothesized the emergence of LasR loss-of-function isolates would be limited but not abolished in a QS-defective background. To test this hypothesis,

we employed a Δ*rhlI* mutant as the parental strain in an experimental evolution assay. Given the central role of RhlR in reorganizing the QS circuitry in the absence of LasR function, we opted to use a C_4-HSL negative background. Similar to our observations with the PA14 WT strain, LasR-defective isolates also emerged within the Δ*rhlI* mutant (**Fig. 4.3.1A**). However, contrary to our initial hypothesis, they emerge even more rapidly than in the wild-type background.

This unexpected result prompted us to investigate the adaptability of the QS system further. Notably, throughout the experimental evolution assay, an apparent defect of pyocyanin production was observed in the evolved populations of the wild-type strain (data not shown). This observation led us to measure the activity of a chromosomal *phzA1-lux* reporter in a *rhlI*-negative background before and after the experimental evolution. The *phz1* operon, in conjunction with the autologous *phz2* operon, plays a central role in synthesizing phenazines such as phenazine-1-carboxylic acid (PCA), which serves as a precursor for the redox-active blue pigment pyocyanin, giving *P. aeruginosa* cultures their characteristic coloration (Mavrodi *et al.*, 2001). Transcription of the operon *phz1* relies on the RhlR activity (Groleau *et al.*, 2020; Whiteley *et al.*, 2001). As expected, prior to the evolution experiment, expression of *phz1* was not detected in the *rhlI*-negative background (**Fig. 4.3.1B**). However, after five rounds of passages, transcription *phz1* in the evolved *rhlI*-negative population increased by twentyfold (**Fig. 4.3.1B**), highlighting the dynamic nature of QS function.

Next, we asked whether the emergence of LasR-defective isolates was merely correlated with increased *phz1* expression or if a cause-and-effect relationship existed between these results. We isolated individual clones from several passages of the Δ*rhlI* mutant experimental evolution and cultured them on skim milk agar to assess LasR-regulated phenotypes. Considering the impact of the experimental evolution on *phz1* transcription, we focused on the potential restoration of pyocyanin production by clones exhibiting reduced proteolysis activity (a characteristic indicative of LasR loss-of-function).

Most of the isolated clones exhibited the anticipated phenotype of a double *rhlI lasR* mutant, characterized by reduced proteolysis activity and the absence of detectable pyocyanin production. However, one of the isolates displayed an intriguing phenotype: it simultaneously exhibited pyocyanin production and reduced proteolysis (**Fig. 4.3.1C**). This phenotype closely mirrored the outcomes of our previous experimental evolution assays, suggesting a direct association between LasR loss-of-function and induced *phz1* expression. Indeed, the activation of *phz1* transcription in the *rhlI* background (**Fig. 4.3.1B**) indicates that such a clone emerged multiple times throughout

passages, and its isolation proves a concept. Therefore, we decided to delve deeper into its characterization.

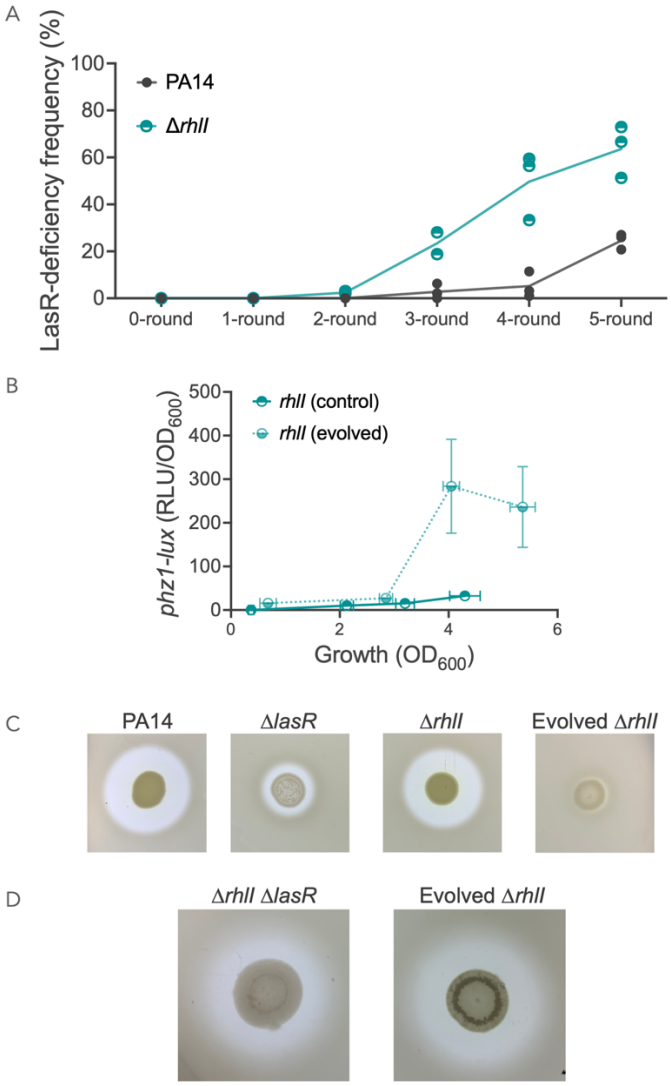

Figure 4.3.1. The emergency of LasR-defective isolates during experimental evolution is related to the induction of RhlR-controlled factors in a C_4-HSL negative background.

(A) Percentage of LasR-defective isolates observed during the course of evolution in TSB, with passages occurring every 24 hours. The data is presented for both the WT PA14 strain (black) and its isogenic Δ*rhlI* mutant (green). The experimental evolution encompassed five passages at 37°C. **(B)** The transcriptional activity of the population is depicted for the parental *rhlI* strain, which carries the chromosomal reporter *phzA1-lux* (*rhlI*::CTX *phzA1-lux*), prior (solid line) and after five rounds of experimental evolution (dashed line). **(C)** Casein degradation by colonies grown on skim milk agar after overnight incubation at 37°C. The strains are presented in the following order: PA14, Δ*lasR*, Δ*rhlI*, and the pyocyanin producer Δ*rhlI* evolved strain. **(D)** Pyocyanin production within colonies after 72 hours of incubation at room temperature.

4.3.4.2 The evolved Δ*rhlI* clone possesses a missense *lasR* mutation

To identify genetic modifications that occurred during our experimental evolution, we extracted and sequenced the genomic DNA of the pyocyanin-producing evolved Δ*rhlI* clone. As expected, given the reduction in the proteolytic zone on skim milk agar, a point mutation in the coding sequence of *lasR* was found. This mutation involves a G-to-C nucleotide change at position 472, resulting in an alanine-to-proline substitution at position 158 of the protein (A158P) (**Fig. 4.3.2A**). Additionally, the evolved clone carries a mutation in the *ftsY* coding sequence (*PA14_04900*), a gene associated with the bacterial signal-recognition particle receptor in the general secretory pathway (Cao *et al.*, 2003). We also identified two deletions in the intergenic region between *rpsF* (*PA14_65180*) and *PA14_65190*.

To confirm whether the missense *lasR* mutation was responsible for the QS-modulated phenotype of the evolved strain, we replaced the LasR A158P coding allele with WT LasR and then assessed pyocyanin production in colonies. As a control, we used the double mutant Δ*rhlI* Δ*lasR*. In this control, pyocyanin is absent and only present in minor concentrations in colonies of PA14 and the parental Δ*rhlI* strains. In contrast, the evolved Δ*rhlI* *lasR*^{A158P} strongly induces the production of this phenazine, and the replacement of WT LasR abolishes this. This result suggests that the other mutations did not significantly modulate QS responsiveness (**Fig. 4.3.2B**). Notably, the development of pyocyanin production within the colony required prolonged incubation at room temperature.

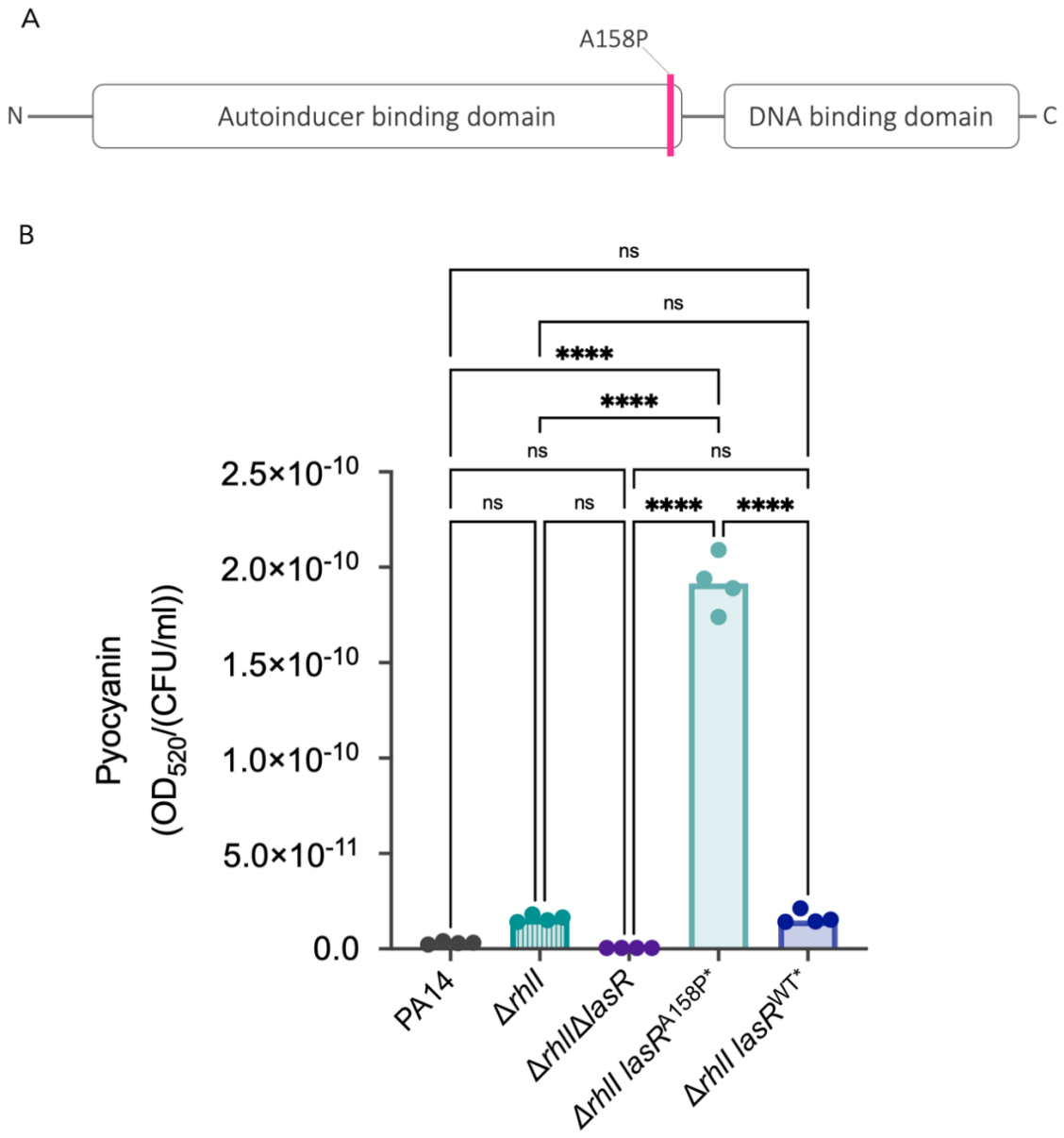

Figure 4.3.2. The naturally evolved LasR A158P variant is sufficient to induce pyocyanin production in a *rhlI*-negative background.

(A) The location of the single nucleotide change from G to C at position 472 is mapped onto the LasR amino acid sequence resulting from experimental evolution, leading to the substitution of an alanine with a proline at position 158 (A158P). The autoinducer binding domain (position 16-161) and the DNA binding domain (position 174 to 231) are highlighted for reference. **(B)** Pyocyanin produced within colonies grown on King's A agar was extracted using chloroform and measured after 72h incubation at room temperature. The values were corrected per CFU/mL of PA14, Δ*rhlI*, a double Δ*rhlI* Δ*lasR*, Δ*rhlI lasR*^{A158P}, and Δ*rhlI lasR*^{WT}. Strains marked with an asterisk (*) indicate evolved background. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test were used to quantify statistical significance. ns, nonsignificant; ****$P \leq 0.0001$.

4.3.4.3 LasR A158P modulation of QS-dependent factors is influenced by temperature

Based on current knowledge, the simultaneous impairment of the *las* and *rhl* systems results in deficient expression of QS-dependent factors, such as exoproteases and pyocyanin. Therefore, the presence of a strain with reduced LasR activity, as evidenced by reduced casein proteolysis, that still retains the ability to produce pyocyanin in a C_4-HSL-negative background (thus, with impaired RhlR activity) is intriguing. Because the *las* and *rhl* systems are interconnected, interpreting QS-modulating inputs from each system individually in the Δ*rhlI lasR*^{A158P} strain is challenging.

To dissect the role of LasR A158P, we decoupled the *las* and *rhl* circuitries by substituting the wild-type LasR protein with LasR A158P in PA14 WT (designated as PA14 *lasR*^{A158P}). To confirm whether this LasR variant encodes a functional protein, we measured the activity of a chromosomal *lasI-lux* reporter, encoding the cognate synthase of the *las* system, for which LasR is the essential activator. Transcription of *lasI* is severely affected by LasR A158P, resembling the PA14 Δ*lasR* mutant (**Fig. 4.3.3A**). As a complementary approach, we measured concentrations of 3-oxo-C_{12}-HSL in cultures of PA14 *lasR*^{A158P} using liquid chromatography-tandem mass spectrometry (LC/MS/MS). The concentration of this molecule was drastically reduced compared to the WT strain; however, unlike the Δ*lasR* mutant, 3-oxo-C_{12}-HSL production had a pattern of production resembling the WT, which might indicate that LasR A158P displays residual activity under these conditions (**Fig. 4.3.3B**).

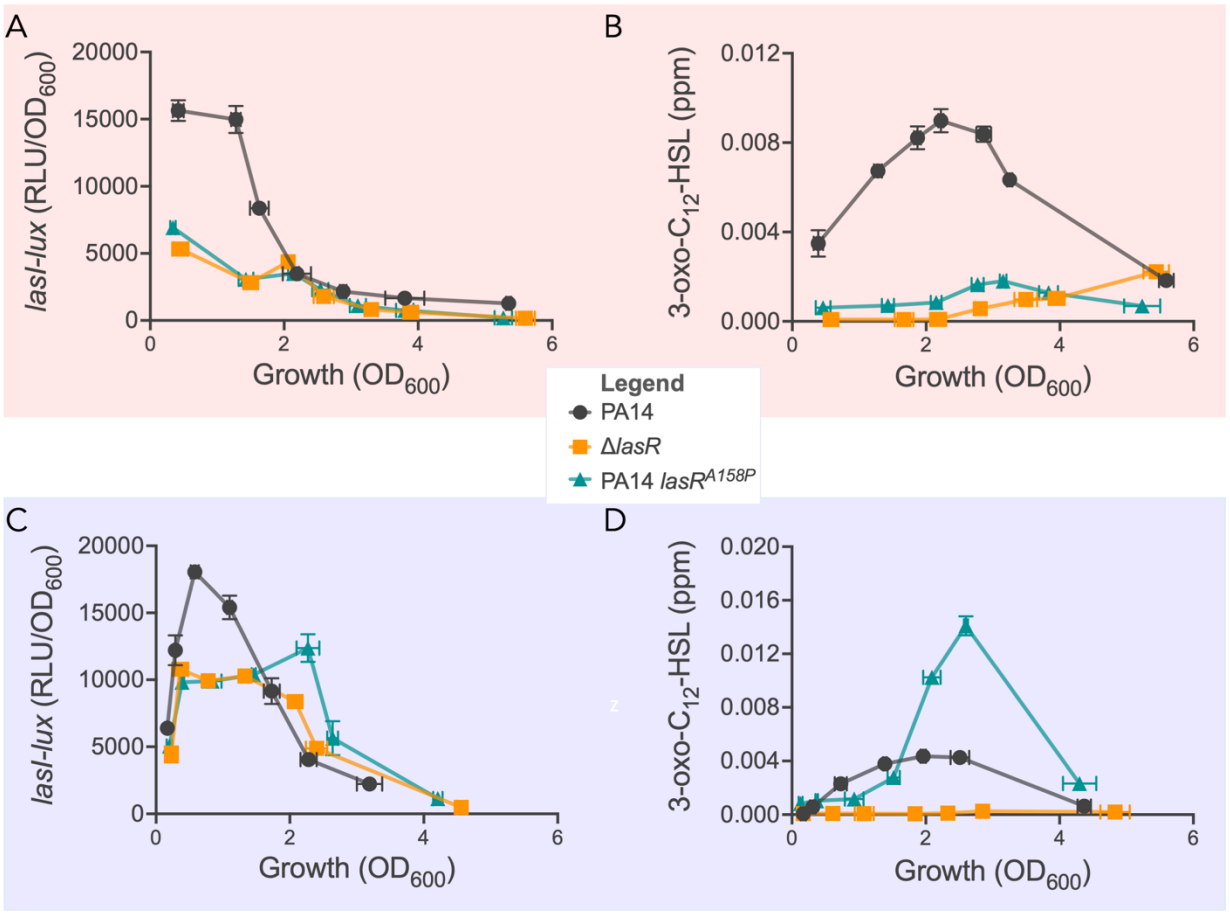

Figure 4.3.3. The activity of the LasR A158P variant is repressed under standard culture conditions.
LasR activity was evaluated by monitoring the expression of the chromosomally integrated *lasI-lux* reporter in strains PA14, its isogenic lasR mutant (Δ*lasR*), and PA14 carrying the mutated *lasR*^{A158P} allele (PA14 *lasR*^{A158P}) during growth in King's A **(A)** at 37°C (red panel) and **(C)** at 25°C (blue panel). Concentrations of 3-oxo-C12-HSL were measured at various time points using liquid-chromatography-mass spectrometry during growth in King's A media **(B)** at 37°C (red panel) and **(D)** at 25°C (blue panel). The values are presented as means ± standard deviations (depicted by error bars) from three replicates.

Longer incubation at room temperature was necessary to induce pyocyanin production in the evolved Δ*rhlI lasR*^{A158P} background, as indicated in **Figures 4.3.1D** and **4.3.2B**. Given our recent investigation into the relationship between environmental temperatures and RhlR activity (refer to **subsection 4.1**), we posited that the room temperature, significantly cooler than the standard 37°C, might be the triggering factor for the induction of QS factors, such as pyocyanin production. To explore this hypothesis, we quantified pyocyanin production at 25°C and 37°C using the engineered Δ*rhlI lasR*^{A158P} strain. Reinforcing the results presented earlier, the Δ*rhlI lasR*^{A158P}

produces pyocyanin when cultivated at 25°C, but the production of this redox-active molecule is not detected when grown at 37°C (**Fig. 4.3.4**). Notably, this strain demonstrates the same phenotype as the evolved isolate Δ*rhlI lasR*^{A158P}. We inferred that this result indicated a thermoregulated functionality for LasR A158P.

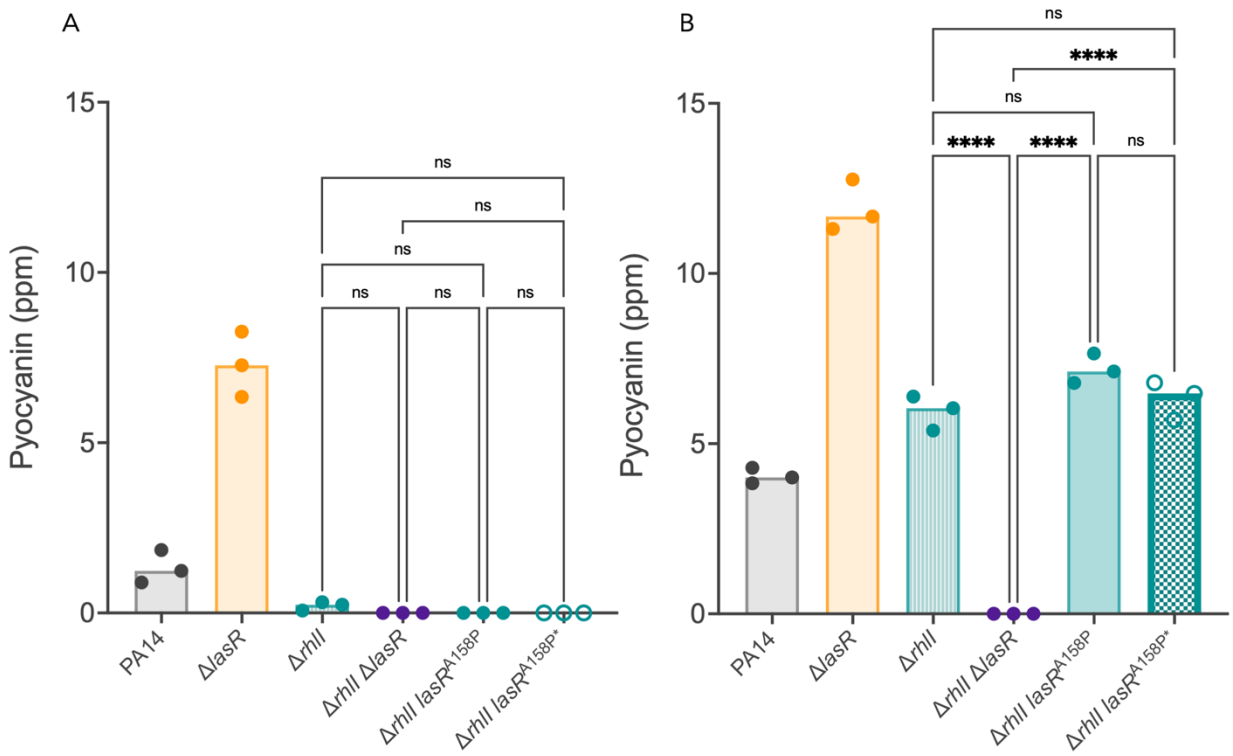

Figure 4.3.4. The LasR A158P variant produces pyocyanin in a temperature-dependent manner.
Pyocyanin levels were quantified following a 24h incubation at either **(A)** 37°C or **(B)** 25°C in King's A broth using liquid chromatography/mass spectrometry. PA14, and the mutants Δ*lasR*, Δ*rhlI*, Δ*rhlI* Δ*lasR*, Δ*rhlI lasR*^{A158P}, and Δ*rhlI lasR*^{A158P*} were employed. The latter, marked with an asterisk (*), indicates the evolved background. The reported values represent the means ± standard deviation (error bars) obtained from three replicates. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test were used to quantify statistical significance. ns, non-significant; ****$P \leq 0.0001$.

We confirmed the thermoregulated functionality of LasR A158P by conducting a new round of measurements using the *lasI-lux* reporter and 3-oxo-C_{12}-HSL at 25°C. At this temperature, the transcription of *lasI-lux* showed similarities between the Δ*lasR* mutant and PA14 *lasR*^{A158P} until the late stationary phase (OD_{600} of 2); at this point, *lasI* was activated by the LasR A158P variant. In concordance with this induction, the concentration of 3-oxo-C_{12}-HSL in the PA14 *lasR*^{A158P} culture significantly increased, even surpassing the production levels of this signal molecule

observed in the WT strain (**Figs. 4.3.3C and D**). Taken together, these data suggest that the LasR A158P function is bimodal: under standard culture conditions (37°C), this LasR variant displays only residual activity, whereas at 25°C, it becomes fully active.

4.3.4.4 Expression of RhlR-controlled genes, not LasR-dependent, is regulated by LasR A158P

At 25°C, the profile of pyocyanin production differs significantly between Δ*rhlI*, which encodes wild-type LasR, and Δ*rhlI lasR*^{A158P}, encoding the LasR A158P variant (**Fig. 4.3.2**). The latter exhibits robust overproduction of the redox-active pigment compared to Δ*rhlI*. This stark contrast indicates that the activity of LasR alone cannot account for the expression of pyocyanin (and potentially other QS-controlled genes) and suggests that the LasR A158P variant reorganizes the expression of target genes. To validate this hypothesis, we investigated the expression of several well-known quorum-regulated genes at two different temperatures: 37°C (representing the residual activity state) and 25°C (representing the functional state).

As LasR is central to the *las* system, we initiated our investigation by assessing LasR activity through two of its primary targets: *rsaL* and *lasB*. These genes encode the transcriptional repressor RsaL, responsible for the *las* system homeostasis, and the elastase LasB, respectively (Gambello *et al.*, 1991; Rampioni *et al.*, 2007; Schuster *et al.*, 2004b). Using chromosomally integrated reporter assays, we observed that expression from both *rsaL* and *lasB* promoters in PA14 *lasR*^{A158P} is similar to the *lasR* negative background at both temperatures, demonstrating that whatever the distinct nature of LasR A158P regulatory activity, it does not contribute to the transcription of these traditional LasR-regulated genes (**Figs. Fig. 4.3.5**).

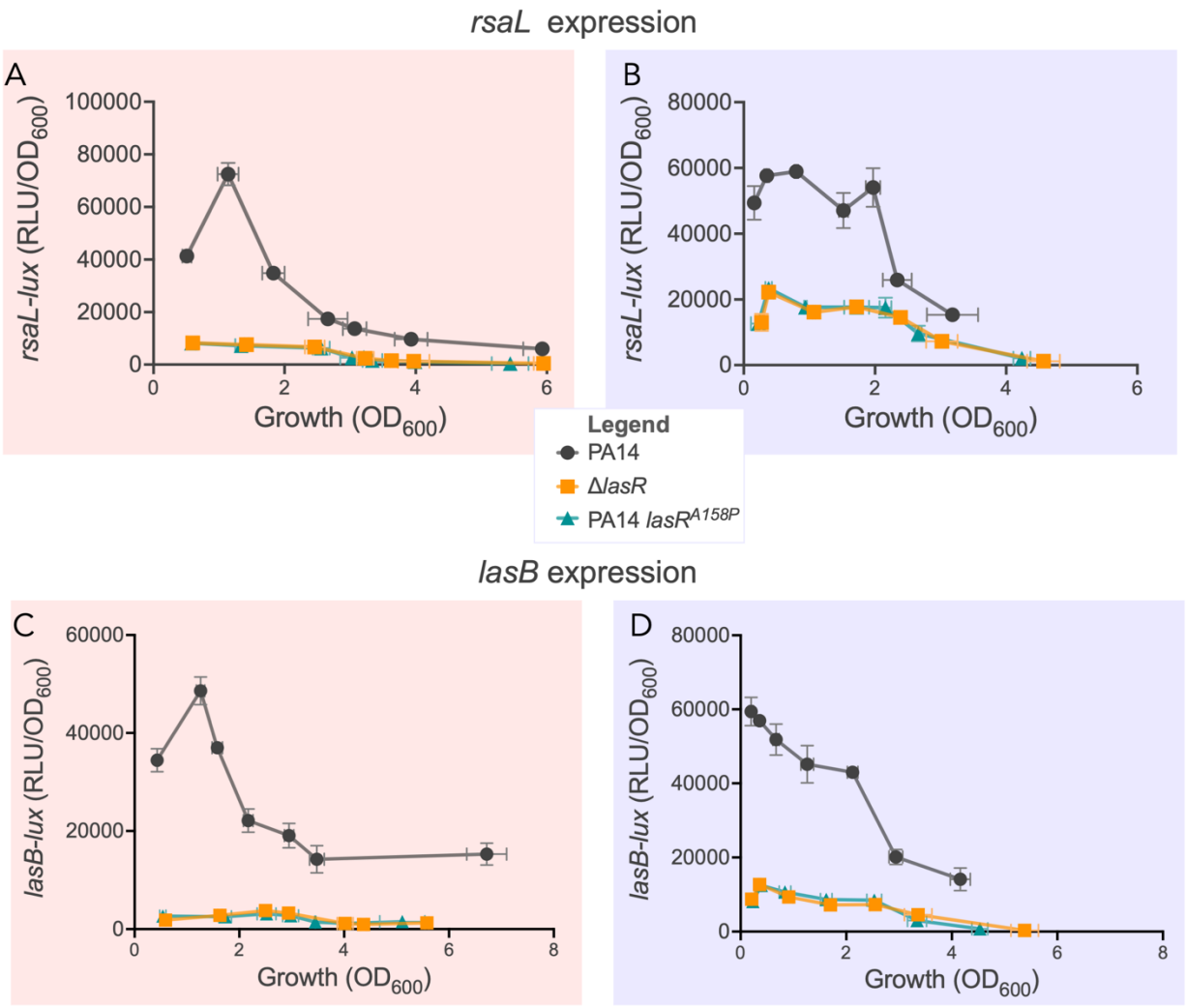

Figure 4.3.5. Expression of LasR-controlled genes is not differently induced by LasR A158P activity.
The expression of the chromosomally integrated **(A-B)** *rsaL-lux* reporter and **(C-D)** *lasB-lux* reporter was monitored during growth in King's A broth at 37°C (red panels) or at 25°C (blue panels). The WT PA14 strain, its isogenic *lasR* mutant (Δ*lasR*), and the PA14 carrying the mutated *lasR*^{A158P} allele strain (PA14 *lasR*^{A158P}) were employed. The values are means ± standard deviations (error bars) from three replicates.

Subsequently, we explored the role of the *rhl* system in the QS activation mediated by LasR A158P. We investigated the expression from the *rhlA* and *phzA1* promoters, regulated by RhlR and involved in producing rhamnolipids and phenazines (including pyocyanin), respectively. The expression profile from the *rhlA* promoter remained similar when PA14 *lasR*^{A158P} was compared with a *lasR* deletion mutant at both tested temperatures (**Fig. 4.3.6A and B**). Similar to the expression of *rhlA*, transcription from the *phzA1* promoter in PA14 *lasR*^{A158P} mirrors a *lasR*-

negative background at 37°C. However, at 25°C, the *phzA1* reporter is induced in PA14 *lasR*^{A158P} compared to the *lasR* mutant, leading to an expression profile similar to the one observed in the WT strain (**Fig. 4.3.6C and D**). This finding indicates that the A158P mutation changes the activity target profile of LasR at 25°C and can now activate *phz1* expression, primarily mediated by RhlR. This could mean that the effect of LasR on the *rhl* system is modulated by the A158P mutation.

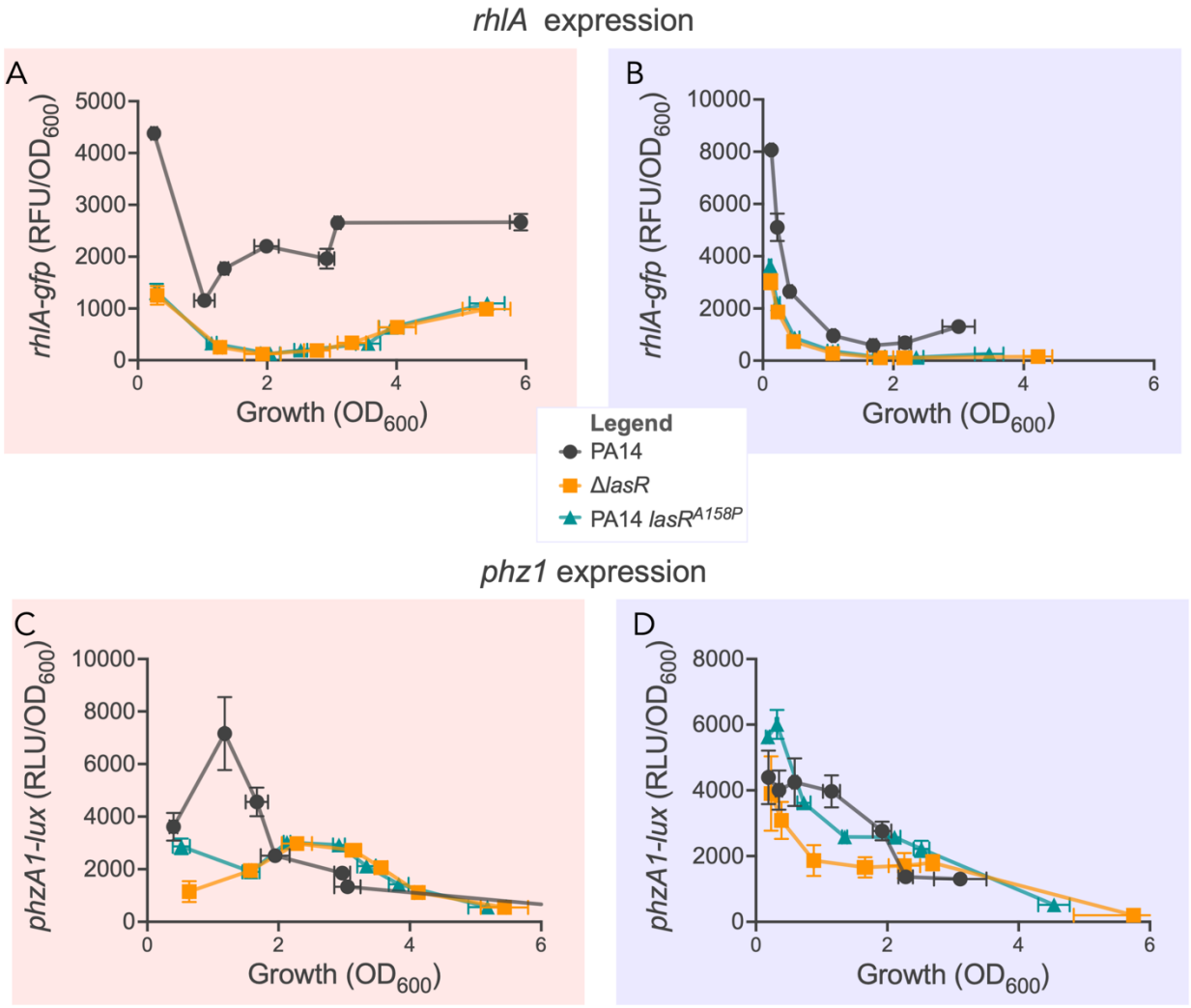

Figure 4.3.6. LasR A158P activity modulates the expression of *phz1*, a RhlR-controlled gene.
(A-B) Transcription of *rhlA* or **(C-D)** *phzA1* in strains PA14, isogenic *lasR* mutant (Δ*lasR*), and PA14 carrying the mutated *lasR*^{A158P} allele (PA14 *lasR*^{A158P}) grown in King's A broth at 37°C (red panel) or at 25°C (blue panel). Error bars represent the standard deviations of three replicates.

To better comprehend the unique effect of the A158P variant on the *rhl* system, we introduced a *rhlR* deletion into the PA14 *lasR*^{A158P} background (i.e., Δ*rhlR lasR*^{A158P}) to assess pyocyanin production at both 37°C and 25°C. As expected, the absence of RhlR led to a complete absence of pyocyanin production, regardless of the growth conditions, aligning with the essential role of this regulator in pyocyanin production (**Fig. 4.3.7**). To further investigate this regulation, we engineered a *pqsE* deletion (i.e., Δ*pqsE lasR*^{A158P}), which, similar to Δ*rhlI lasR*^{A158P}, is expected to exhibit compromised RhlR activity. We measured pyocyanin production in these *lasR*^{A158P} strains with impaired RhlR function at 37°C and 25°C. Interestingly, Δ*pqsE lasR*^{A158P} exhibited behaviour similar to that of Δ*rhlI lasR*^{A158P}, where an environmental-like temperature induced pyocyanin production in this strain (**Fig. 4.3.7**). However, the production of this molecule was significantly reduced compared to the *lasR*^{A158P} background, indicating that the absence of RhlR-stabilizing factors negatively affected this response, thus confirming the pivotal role of RhlR in its mediation.

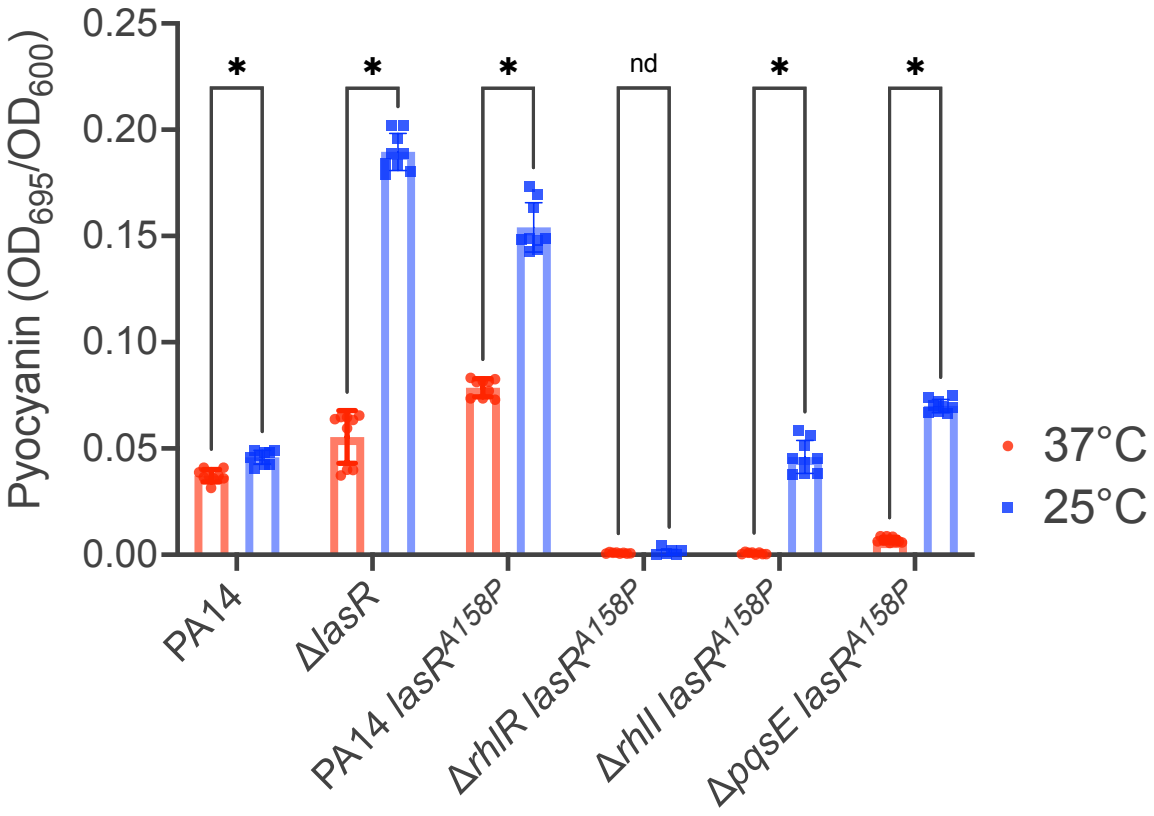

Figure 4.3.7. The impact of the LasR A158P variant on pyocyanin production is mediated by RhlR.
Pyocyanin levels were quantified following a 24h incubation at either 37°C (red bars) or 25°C (blue bars) in King's A broth. The reported values represent the means ± standard deviation (error bars) obtained from three replicates. Multiple unpaired t-tests and the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli were utilized to quantify statistical significance. nd, not defined; *$P \leq 0.0001$.

4.3.4.5 LasR A158P modulates C_4-HSL concentrations

On prototypical *P. aeruginosa* strains, LasR activity positively regulates the *rhl* system by directly inducing the transcription of *rhlI*, *rhlR*, and indirectly *pqsE* (de Kievit *et al.*, 2002; Groleau *et al.*, 2020; Pesci *et al.*, 1997; Wade *et al.*, 2005). Thus, we aimed to investigate whether the influence of the LasR A158P variant on these regulatory elements followed the same pattern. Expression of *rhlR* remains unaltered in response to LasR A158P, as temperature did not induce differential regulation between PA14 *lasR*^{A158P} and the *lasR* mutant (**Fig. 4.3.8A and B**). In contrast, the expression of *rhlI* is higher in the LasR A158P variant during the late stationary growth phase than in the *lasR* deletion mutant (**Fig. 4.3.8C and D**). This finding aligns with the higher C_4-HSL concentrations in PA14 *lasR*^{A158P} cultures compared with the *lasR* mutant, although not reaching WT levels (**Fig. 4.3.9**). At 37°C, C_4-HSL concentrations are similar between the PA14 *lasR*^{A158P} and the *lasR*-negative background, reinforcing the necessity of LasR activity for inducing C_4-HSL production (Groleau *et al.*, 2020) (**Fig. 4.3.9**).

We indirectly assessed the transcription of *pqsE* by LasR A158P through the activity of a *pqsA* reporter, given its presence in an operon (Déziel *et al.*, 2004; Gallagher *et al.*, 2002). Notably, LasR is required fo the full activity of this operon as it activates MvfR (Gilbert *et al.*, 2009; Xiao *et al.*, 2006b). Transcription from the *pqsA* promoter remains comparable between strains PA14 *lasR*^{A158P} and the *lasR* mutant at both tested temperatures, suggesting equivalent PqsE levels due to similar transcriptional regulation (**Fig. 4.3.8E and F**).

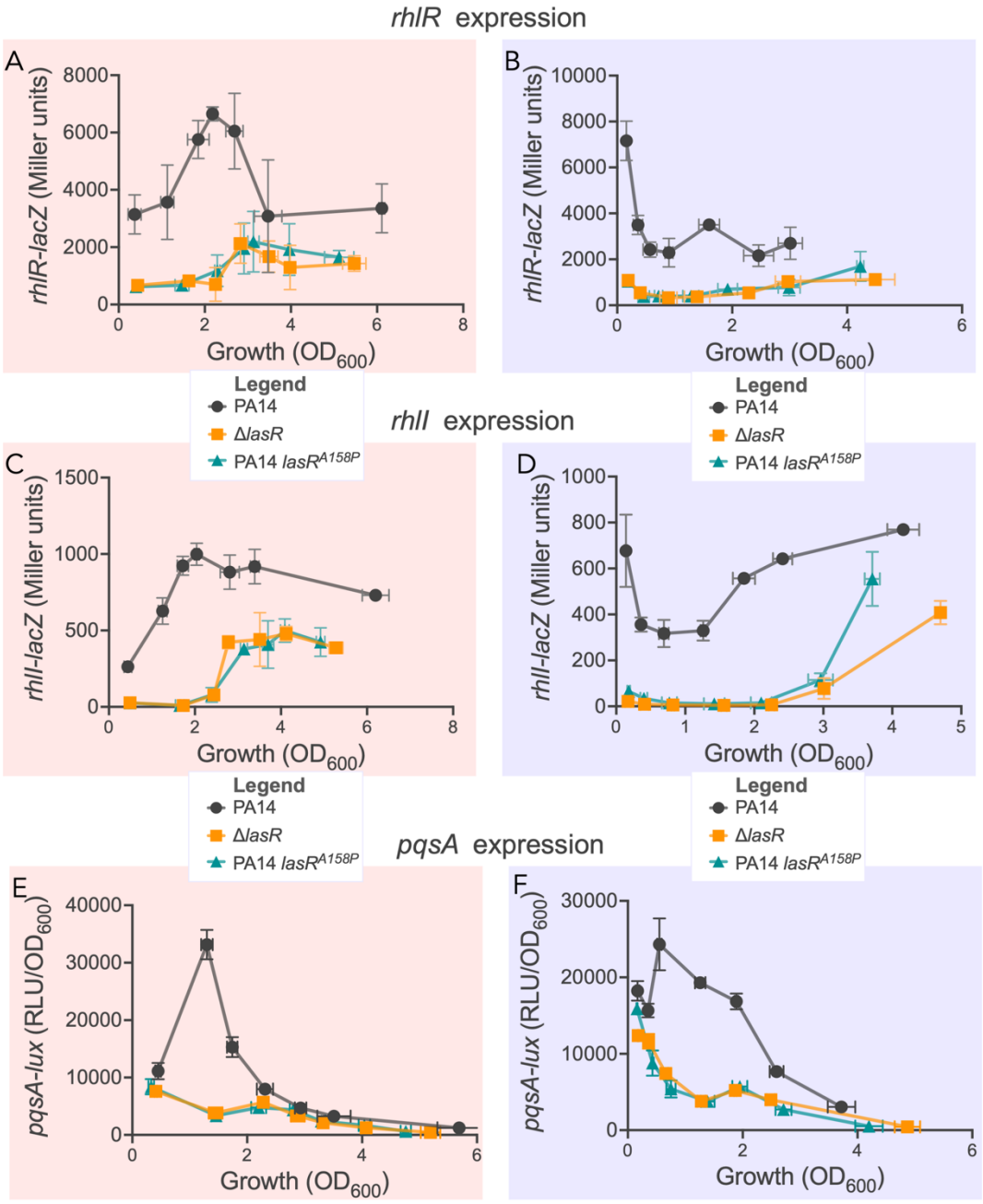

Figure 4.3.8. The LasR A158P variant exclusively induces the expression of *rhlI*, with no discernible effect on the expression of either *rhlR* or *pqsABCDE*.

(A-B) Transcription of *rhlR*, **(C-D)** *rhlI* and **(E-F)** *pqsA* in strains PA14, isogenic *lasR* mutant (Δ*lasR*), and PA14 carrying the mutated *lasR*^{A158P} allele (PA14 *lasR*^{A158P}) grown in King's A broth at 37°C (red panel) or at 25°C (blue panel). The promoter of the *pqsA*-E operon is located upstream of *pqsA,* and the expression of the latter was used to assess the expression of the operon. Error bars represent the standard deviations of three replicates.

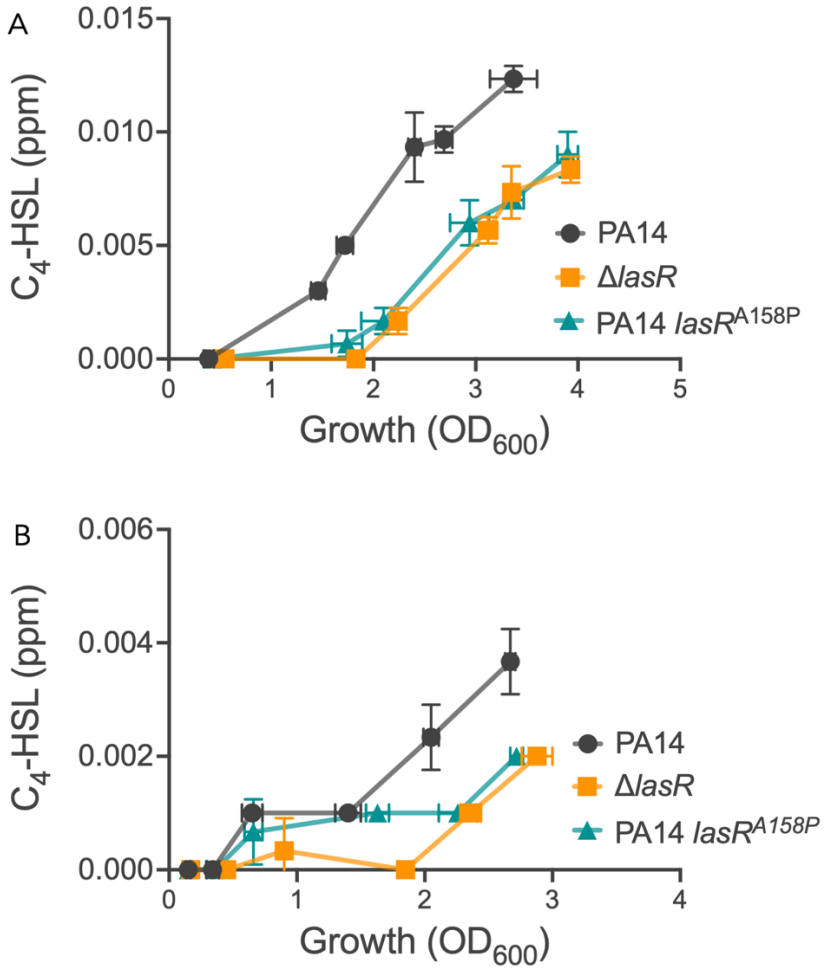

Figure 4.3.9. The LasR A158P variant stimulates C_4-HSL production at a temperature resembling environmental conditions.

Production of C_4-HSL was monitored during growth in King's A broth at both **(A)** 37°C and **(B)** 25°C using liquid chromatography/mass spectrometry in the background strains PA14, Δ*lasR*, and PA14 *lasR*^{A158P}. The reported values represent the means ± standard deviation (error bars) obtained from three replicates.

4.3.5 Discussion

The significance of LasR-defective *P. aeruginosa* isolates in the ecology of this bacterium is a topic of central importance, and we have recently discussed this topic (Trottier *et al.*, 2024). Initially believed to primarily emerge in response to chronic infections in hosts, such as individuals with CF, this perception shifted as a substantial proportion of *P. aeruginosa* isolates were identified as *lasR* mutants and LasR-defective (Groleau *et al.*, 2022; O'Connor *et al.*, 2022; Trottier *et al.*, 2024). This suggests that LasR deficiency represents a widespread adaptation strategy for this bacterium. Importantly, LasR deficiency does not equate to a loss of QS function, as RhlR activity can sustain these responses (Chen *et al.*, 2019a; Cruz *et al.*, 2020; Feltner *et al.*, 2016; Groleau *et al.*, 2022; Kostylev *et al.*, 2019; Morales *et al.*, 2017). Recent research has further highlighted that LasR-defective strains frequently emerge in controlled experimental evolution assays using rich media in the *P. aeruginosa* PA14 background (Mould *et al.*, 2022) or under swarming motility conditions (Robitaille *et al.*, 2020). Here, we harnessed the potential to induce environmentally driven adaptations under laboratory conditions to study the mechanisms underlying plasticity within the QS regulatory network.

Considering the critical role of RhlR functionality in the QS system of LasR-defective isolates, we aimed to investigate the potential for QS plasticity using a C_4-HSL-negative background (i.e., *rhlI* mutant) as the source strain for experimental evolution. We conducted a parallel evolution experiment with the wild-type PA14 as the parental strain, enabling a comparison with a previously published study (Mould *et al.*, 2022). LasR-defective isolates emerged in the C_4-HSL-negative background and, as reported, in the PA14 background (**Fig. 4.3.1**). In our study, approximately 25% of the evolved PA14 population consists of these mutants (after five passages) in contrast to around 80% of the population being LasR-defective (after six passages) (Mould *et al.*, 2022). However, direct comparisons of the evolutionary trajectory leading to the accumulation of these isolates are challenging due to some significant differences in our experimental protocols. Mould and colleagues have reported the involvement of the CbrAB/Crc system in the emergence of LasR-defective isolates (Mould *et al.*, 2022). The CbrAB/Crc regulates substrate prioritization by influencing the catabolite repression control pathway (Rojo, 2010; Sonnleitner *et al.*, 2009). This system drives LasR-defective isolate emergence in response to the nutrient conditions to which *P. aeruginosa* is exposed. Several differences in our experimental design significantly impact nutrient conditions compared to the previous study. Firstly, we employed TSB as our growth medium, conducting passages every 24 hours with a 1:100 dilution, while they used LB with passages every 48 hours and a 1:200 dilution. TSB is a

richer growth medium than LB, containing, for instance, D-glucose. Furthermore, more frequent passages help prevent nutrient depletion resulting from bacterial growth and the over-accumulation of detrimental metabolic wastes. These factors collectively alter the nutrient status experienced by the cells, leading to variations in the proportion of LasR-defective isolates.

While the emergence of LasR-negative strains in the C_4-HSL-negative background may not, on its own, signify inherent plasticity within the QS-regulatory network, we established a straightforward correlation between these factors employing strains carrying a transcriptional reporter of a gene regulated by RhlR. We selected the *phz1* transcriptional reporter, one of RhlR's primary targets, which facilitated assessing RhlR activity in evolved populations.

Our observations revealed distinct patterns in *phz1* expression within the evolved populations. In the PA14 background, the evolved population exhibited reduced *phz1* expression compared to the parental strain before the experimental evolution (**Fig. 4.3.10**). In stark contrast, the C_4-HSL-negative evolved population displayed a significant increase in *phz1* expression (**Fig. 4.3.1B**). Notably, we confirmed the prevalence of LasR-negative isolates within these evolved populations, aligning with the proportions presented in **Fig. 4.3.1A**.

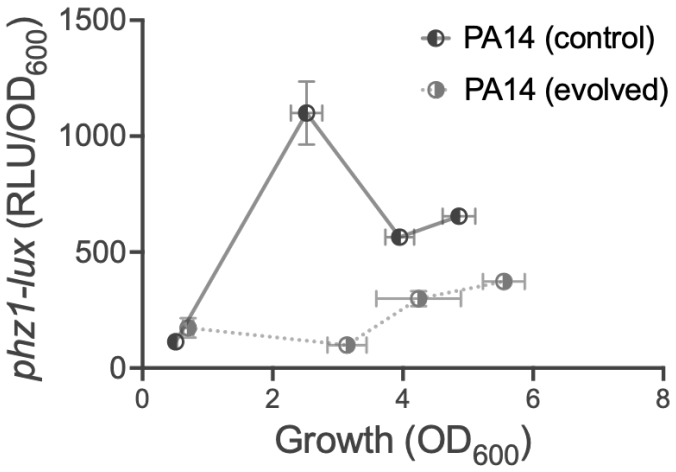

Figure 4.3.10. The expression of *phz1* is significantly reduced in evolved populations of *P. aeruginosa* PA14. The transcriptional expression of *phz1* was monitored during growth in TSB of PA14 carrying a chromosomal reporter *phzA1-lux* (PA14::CTX *phzA1-lux*). This activity was measured in populations prior (solid line) and after five rounds of experimental evolution (dashed line). The reported values represent the means ± standard deviation (error bars) obtained from three replicates.

The correlation between the emergence of LasR-defective strains and *phz1* expression is readily explained in the PA14 background. LasR directly activates the transcription of *rhlR* and *rhlI* while indirectly inducing *pqsE* expression, all of which are required for the transcription of *phz1*, as this gene is under RhlR regulation. Previous research has already documented the negative impact of a *lasR* mutant on *phz1* expression (Groleau *et al.*, 2020). However, the situation becomes more complex in the Δ*rhlI* background.

The concept we have presented, which can be summarized as the potential restoration of group behaviour signalling through combined mutations within the *las* and *rhl* systems, particularly in the RhlI-encoding gene, was recently addressed. As previously mentioned, the prevalence of LasR-defective isolates is well documented, primarily in clinical settings. Simanek and colleagues analyzed *P. aeruginosa* clinical isolates, revealing that mutations in *lasR* often coincide with concurrent mutations in *rhlI* (Simanek *et al.*, 2023). The co-occurring mutations in these genes were proposed as an adaptive response to balance RhlI activity, as their conditions showed that a *las* mutant tends to overproduce C_4-HSL, and a selected subset of these *rhlI* mutations resulted in a reduction in RhlI activity (Simanek *et al.*, 2023). However, as shown in **Figure 4.3.9**, a *lasR* mutant does not exhibit C_4-HSL overproduction under the conditions tested in our study. Instead, a significant deficiency of this autoinducer is observed throughout growth, aligning with the role of LasR as the primary driver of *rhlI* expression (Groleau *et al.*, 2020). Regardless of the underlying mechanisms, it underscores the notion that the interplay between the *las* and *rhl* systems can influence the organization of the QS system.

In a similar rationale as to Simanek and colleagues, we isolated a naturally occurring LasR-deficient variant in a laboratory-engineered C_4-HSL-negative background within an experimental evolution setting. This mutated *lasR* allele encoded the LasR A158P variant, where the alanine at position 158 of the polypeptide is substituted by proline. Intriguingly, the LasR A158P variant effectively restores pyocyanin production, an exoproduct regulated by RhlR, in the C_4-HSL-negative background. However, this response exhibits thermoregulation, with pyocyanin production observed exclusively when the evolved strain is cultivated at environmental-like temperatures. This thermo-induced response is intricately linked to the activity of the LasR A158P variant, which, akin to pyocyanin production, is only detected at environmental-like temperatures. This specific thermo-induced behaviour is likely attributed to the structural features of the LasR A158P variant protein. The amino acid substitution at residue 158 is situated within the autoinducer binding domain, where residue 158 is involved in LasR dimerization (Bottomley *et al.*, 2007). Dimerization of LasR is essential for its function as a transcriptional regulator

(Bottomley *et al.*, 2007). We hypothesize that lower temperatures favour low-affinity interactions, facilitating dimerization, while such interactions are hindered at higher temperatures, such as those mimicking human infection conditions (37°C). Within this framework, we presume the proline at position 158 reduces the affinity of LasR for its monomer, allowing this interaction to occur primarily at lower temperatures.

The intricate nature of QS poses significant challenges when attempting to isolate and characterize individual QS systems. Several sophisticated strategies have been employed to disentangle individual QS responses (Letizia *et al.*, 2022; Rampioni *et al.*, 2016; Rampioni *et al.*, 2010). In our study, we opted to introduce the LasR A158P coding allele into the PA14 background to avoid the complications associated with a RhlI-negative mutant. To gain insights into how this LasR variant impacts the QS system, we closely monitored the expression of several well-known genes regulated by QS under temperature conditions that induced the activity of this variant. Interestingly, among the genes controlled by both LasR and RhlR, the LasR A158P variant exhibited specific deregulation of the *phz1* gene when compared to a *lasR* mutant, along with the genes coding the synthases LasI and RhlI (**Fig. 4.3.6**). This observation was quite unexpected, as we initially anticipated that genes regulated directly by LasR itself would exhibit differences under our tested conditions. Indeed, our experiments revealed that the activity of LasR A158P led to the production of C_4-HSL, which could explain the increased expression of *phz1* through RhlR under these specific conditions. Surprisingly, these were the only discernible effects of this variant on QS regulation.

These findings underscore the limited impact of LasR A158P on the QS regulatory network, as it mostly mirrors the expression pattern of a *lasR* mutant. Notably, while the influence of this variant is not confined solely to the C_4-HSL-negative background, it accentuates the effects of the variant within this context. Under these conditions, the presence of the LasR A158P variant induces pyocyanin production in bacteria cultivated at room temperature, as depicted in **Figure 4.3.2**. This observation implies that regulating this redox-active molecule may stimulate RhlR activity independently of C_4-HSL, although it remains reliant on RhlR (**Figure 4.3.7**). Furthermore, introducing the A158P mutation into a *pqsE* mutant yields a similar response to that observed for Δ*rhlI lasR*^{A158P}, suggesting that this regulation is not dependent on either of these factors.

Importantly, we have demonstrated that an environmental-like condition, characterized by a temperature of 25°C, enhances RhlR activity by stabilizing this inherently unstable transcriptional regulator (refer to **subsection 4.1**). This thermo-induced RhlR activity, similar to the one

presented here, operates independently of the requirement for C_4-HSL and PqsE. Notably, growth at 25°C restores pyocyanin production of a *rhlI* mutant, prompting us to consider whether this response might elucidate the results presented in this study. However, it is important to mention that the responses previously reported are observed in the presence of a functional LasR, as confirmed by the absence of pyocyanin production at both growth temperatures in the double Δ*rhlI* Δ*lasR* mutant. Assuming that LasR A158P is fully functional at 25°C (despite evidence suggesting otherwise in **Fig. 4.3.5**), the mechanism under study here would align with our previously published findings. However, the production of pyocyanin by the Δ*pqsE lasR*^{A158P} strain indicates strain introduces complexity into this response (**Fig. 4.3.7**). In most conditions tested, a *pqsE* mutant cannot produce pyocyanin, except in the case of a double Δ*pqsE* Δ*lasR* mutant (Dekimpe, 2010). In this context, pyocyanin production by the Δ*pqsE lasR*^{A158P} strain suggests that LasR A158P is not functional. The most plausible explanation that reconciles these results is that LasR A158P induces RhlR activity independently of its autoinducer C_4-HSL and PqsE. This mechanism remains elusive and warrants further investigation.

The sole determinant of this elusive regulation, evident in our data, is strong responsiveness to surface conditions. Surface sensing has been established to modulate various bacterial responses, including those within the QS regulatory network (Chuang *et al.*, 2019; de Oliveira Pereira *et al.*, 2023). Specifically, in surface-grown cells, both the evolved Δ*rhlI lasR*^{A158P} isolate, and the laboratory-engineered Δ*rhlI lasR*^{A158P} strain exhibit a substantial upregulation in pyocyanin production compared to the Δ*rhlI* parental strain (**Fig. 4.3.2**). In contrast, these strains display comparable pyocyanin production levels in planktonic cells (**Figs. 4.3.4** and **4.3.7**). This potential variance in regulation should be explored to offer a comprehensive understanding of the impact of the naturally occurring LasR A158P variant on QS regulation.

4.3.6 Acknowledgments

We thank Nicolas Doucet for valuable discussions that significantly advanced our research. Additionally, we extend our acknowledgment to the Fondation Armand-Frappier for PhD scholarships awarded to T.O.P.

4.4 Supplemental data related to the article "Unravelling the plasticity of quorum sensing in *Pseudomonas aeruginosa*: Insights from a naturally evolved LasR variant"

4.4.1 Contextualization

The manuscript offers insights into the influence of the naturally occurring LasR A158P variant on the QS circuitry of *P. aeruginosa*. In this section, we present these additional results, which serve to expand our understanding of the emergence of LasR-defective isolates under controlled culture conditions.

4.4.2 Material and Methods

Table 4.4.1. Strains used in this supplementary study.

Strain	ED #	Description	Reference
PA14 Δ*rhlI* Δ*pqsE*	ED4408	PA14 derivate; unmarked in-frame double *rhlI* and *pqsE* deletion	**Subsection 4.1**
rhlR⁻	ED503	Marked (::Gm) *rhlR* mutation	(Hazan *et al.*, 2010)

4.4.2.1 Surface-associated experimental evolution

The evolved Δ*rhlI lasR*^{A158P} isolate was used as the parental strain. To initiate the experiment, 5 mL of fresh TSB medium was inoculated with a glycerol bacterial stock stored at -80°C and incubated overnight at 37°C on a roller drum. Subsequently, 5 µL of the overnight culture was used to inoculate agar plugs. These agar plugs were prepared by pouring King's A agar supplemented with 100 µM $FeCl_3$ into each well of a 96-well plate (200 µl per well) and were allowed to solidify in a biosafety cabinet. Once inoculated, the plates were sealed with parafilm to prevent excessive water loss. The sealed plates were then incubated at room temperature for three days (72 hours). After this incubation period, agar plugs were carefully removed, and the associated bacteria were recovered by vigorous vortexing in 1 mL of PBS. Each replicate comprised the bacterial pool from two agar plugs, and this process was repeated for three independent evolution trajectories. To maintain the evolution experiment, 5 µL of the suspension

was used to re-inoculate fresh King's A agar plugs, thus restarting the process. Passages were conducted every 72 hours. This experimental evolution consisted of five passages, and samples from each passage were preserved as glycerol stocks at -80°C.

4.4.3 Results

4.4.3.1 The emergence of LasR-deficient isolates is a widespread feature, and not restricted to a certain genetic background

As described in the **subsection 4.3**, we monitored the emergence of LasR-deficient isolates, not only in the wild-type PA14 strain but also in *rhlI* background (**Fig. 4.3.1A**). In these backgrounds, while a significant fraction of the QS system is compromised in LasR-defective strains, some isolates might still retain QS function due to the activity of RhlR. Therefore, we can hypothesize that the emergence of LasR-defective isolates could be limited to genetic backgrounds where a minimal level of QS function can still be maintained, even if some reorganization of the QS system is necessary. This leads to some questions: Do bacteria retain LasR-deficient isolates within their population only when a viable alternative pathway to activate QS exists? Or is the emergence of LasR-defective isolates not inherently tied to QS but rather serves as an alternative adaptive feature for *P. aeruginosa* populations? To explore this, we also tracked the evolutionary trajectory of strains in which RhlR is absent or non-functional, specifically the *rhlR* mutant and the double *rhlI pqsE* mutants. Surprisingly, LasR-defective isolates emerged in both of these strains despite the likely inactivity of the QS system (**Fig. 4.4.1**). Moreover, after five rounds of passages, a significant fraction of the evolved population consisted of LasR-defective isolates, accounting for approximately 60% when considering the average of the three trajectories. However, notably, these passages were not sufficient to stabilize the proportion of LasR-defective isolates in the population, a phenomenon previously reported (Dandekar *et al.*, 2012; Mould *et al.*, 2022; Robitaille *et al.*, 2020; Sandoz *et al.*, 2007), suggesting an equilibrium between LasR-functional and -defective isolates is likely to be achieved in populations comprising more than 60% of the latter isolates. This strongly suggests that LasR-defective isolates emerge as an adaptive feature beyond the activation of the QS regulatory system in *P. aeruginosa*.

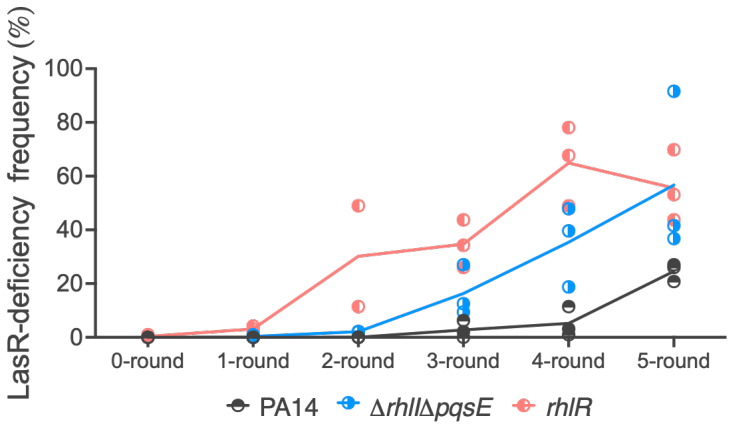

Figure 4.4.1. LasR-defective strains can emerge across various genetic backgrounds, regardless of *rhl* system activation.

Percentage of LasR-defective isolates observed during the course of evolution in TSB, with passages occurring every 24 hours. The data is presented for both the WT PA14 strain (gray), *rhlR* (orange), and the double Δ*rhlI* Δ*pqsE* mutant (blue). The experimental evolution encompassed five passages at 37°C.

4.4.3.2 Pyocyanin production is not the by-product of a secondary mutation in the evolved Δ*rhlI lasR*^{A158P} isolate

Pyocyanin production by the evolved Δ*rhlI lasR*^{A158P} isolate was initially detected on TSA milk agar plates, commonly used for phenotypic screening of LasR-defective isolates. Notably, the visual identification of pyocyanin in this evolved isolate necessitated several days of incubation at room temperature. This delay in the appearance of the pigment piqued our curiosity, especially as we later observed that pyocyanin production was significantly enhanced in surface-grown cells, in contrast to planktonic cells, which exhibited earlier but less pronounced pyocyanin production. This prompted us to consider whether secondary mutations might have arisen during this delay, potentially influencing pyocyanin production independently of LasR A158P.

To investigate this possibility, we conducted an experimental evolution experiment using the evolved Δ*rhlI lasR*^{A158P} strain as the parental isolate, with passages occurring every three days (72 hours). This timing was chosen because it allows for pyocyanin production. After five passages, we compared the pyocyanin production profile of the evolved populations to that of the parental isolate. As shown in **Fig. 4.4.2**, the production profile remained consistent between these conditions, confirming that pyocyanin production in the evolved isolate is not a result of secondary mutations. Instead, the delay could be caused by a non-canonical pyocyanin-inducing mechanism.

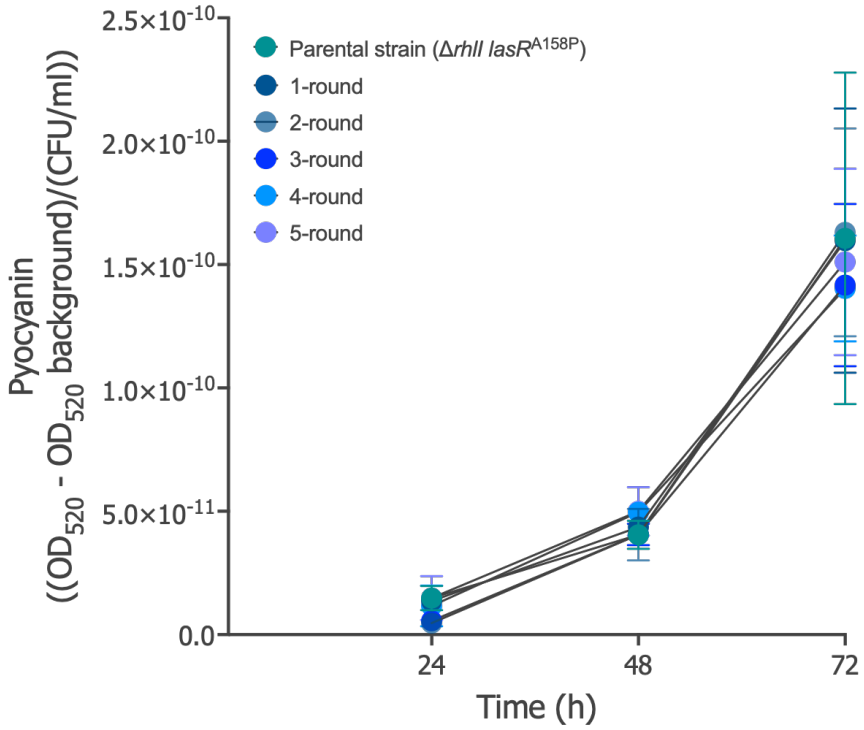

Figure 4.4.2. Pyocyanin production by the evolved isolate is not a result of secondary mutations.

The evolved Δ*rhll lasR*^{A158P} isolate was used to initiate an experimental evolution assay in surface-grown conditions. King's A agar was used as the growth medium. Five rounds of evolution were performed, and pyocyanin production by the evolved populations, thus corresponding to each passage, was monitored for 72h. Pyocyanin was chloroform extracted, and the values were corrected by CFU/mL. The reported values represent the means ± standard deviation (error bars) obtained from three replicates.

4.5 Overall discussion of temperature as a QS modulator

The manuscripts in this chapter have distinct starting points, yet they converge on the importance of growth temperature in regulating QS-controlled behaviours. The first article, presented in **subsection 4.1**, began with the hypothesis that temperature could influence the activity of the transcriptional regulator RhlR. In the manuscript, through a genetic approach, this hypothesis was confirmed; however, unlike anticipated, RhlR function/activity was induced by a lower temperature, akin to the ones encountered by environmental isolates. The subsequent subsection, **subsection 4.2**, provided additional insights related to this manuscript.

One common misconception about QS systems is the assumption that their activity depends solely on the concentration of the cognate autoinducer. In reality, these signal molecules may exist in an inactive form, rendering them incapable of inducing QS responses (Czajkowski *et al.*, 2009). For instance, the accumulation of products released into the extracellular environment during bacterial growth alters its chemical properties. *P. aeruginosa* cultivated in the rich LB medium releases ammonia into the medium due to the degradation of peptides used as carbon and energy sources, increasing the pH (Yates *et al.*, 2002). An alkaline environment promotes the hydrolysis of the lactone ring in all AHLs, as homoserine lactone is a common feature of these molecules (Horswill *et al.*, 2007). Importantly, these open forms are biologically inactive. It is worth noting that while all AHLs are susceptible to lactone ring hydrolysis, molecules with shorter acyl chains are more vulnerable. A list of AHLs with short acyl chains includes the autoinducer C_4-HSL (Yates *et al.*, 2002), which was studied.

To ensure a controlled response, bacterial backgrounds unable to produce the ligand C_4-HSL were employed, enabling the exogenous addition of known concentrations of this molecule before incubation at either 37°C or 25°C. The chemical nature of C_4-HSL results in hydrolysis rates that increase with temperature, thus exhibiting a direct and positive correlation (Yates *et al.*, 2002). In the tested experimental settings, this could lead to lower bioavailable concentrations of C_4-HSL in cells incubated at 37°C. Accordingly, the transcriptional activity induced by C_4-HSL at 37°C was less pronounced than at 25°C. It raised the possibility that the effects described in **subsection 4.1** might be an indirect product of differential hydrolysis rates under the tested temperatures.

To investigate this possibility, *phz1* transcription was measured using a HEPES-buffered TSB medium, ensuring pH homeostasis. HEPES has been previously used to buffer TSB and was effective in avoiding AHL hydrolysis arising from alkaline stress in *Burkholderia thailandensis* (Guillouzer *et al.*, 2020). Like the results obtained using unbuffered TSB, *phz1* transcription is higher in cells cultivated at 25°C than at 37°C in response to equivalent concentrations of C_4-

HSL. This confirmed that the thermoregulation of *phz1* transcription is not an artifact caused by pH variations during growth. However, the induction rate (i.e., the comparison of induction at 25°C to that at 37°C) appeared to be reduced under buffered conditions, indicating that differential hydrolysis might have a small impact on this response under the tested conditions.

The thermoregulation of RhlR function/activity was investigated in bacteria cultured in the rich medium TSB. Notably, this response is not limited to bacteria grown in TSB but is also observed in King's A broth. The proposed impact of temperature variations on RhlR is based on physical factors rather than chemical ones. Therefore, the composition of the growth medium was not expected to influence this response significantly. Accordingly, the growth medium would only be anticipated to interfere if there were impacts on the production or stability of the autoinducer C_4-HSL, the chaperone PqsE, or RhlR. As long as these elements remain present and stable, a lowered temperature should prompt the expression of *phz1* and pyocyanin production in *P. aeruginosa*. This consistency across more than one culture media reinforces the model and supports the notion that temperature plays a significant role in this response.

The proposed model was also tested on other genes under RhlR control, namely *rhlA* and *hcnA*. These genes were included in the study to provide a comprehensive assessment because they, along with *phz1*, represent a group of RhlR-regulated genes with varying dependencies on PqsE (Feathers *et al.*, 2022; Letizia *et al.*, 2022; Mukherjee *et al.*, 2018). In the prototypical strain PA14, this dependency increases from *rhlA* to *hcnA* and *phz1* (Feathers et al., 2022). However, as mentioned extensively in this chapter, the current structural model of the RhlR/C4-HSL/PqsE complex does not account for these differences because the mechanisms behind them remain elusive (Borgert *et al.*, 2022). Therefore, the coexistence of multiple functional versions of RhlR within the cell, some associated with PqsE and others not, remains a possibility that is supported by accumulating experimental data (Feathers *et al.*, 2022; Letizia *et al.*, 2022; Mukherjee *et al.*, 2018). However, a clearer understanding of the factors that may govern these variations would be required. Accordingly, while the proposed thermoregulation model effectively predicts the expression of *phz1*, which is attributed to RhlR function/activity, it does not explain the behaviour of other genes regulated by RhlR under the same environmental conditions. This limitation is likely because the model primarily covers a subset of genes regulated by RhlR, specifically those that, like *phz1*, rely on PqsE for their activity. It partially accounts for the temperature regulation of genes with lesser dependence on PqsE, such as *hcnA*, but does not encompass PqsE-independent genes like *rhlA* in the prototypical strain PA14.

The second part of this chapter delves into the behaviour of the LasR A158P variant and its regulation by temperature (**subsections 4.3 and 4.4**) This naturally occurring LasR variant was isolated through experimental evolution using a C_4-HSL-negative background, as it still produces pyocyanin in this context. However, the accumulation of this pigment occurs gradually during surface growth and is not evident after extended incubation periods (72 hours). During this time, bacteria continue to grow and divide, creating conditions that could potentially favour the emergence of isolates carrying secondary mutations.

To ensure that pyocyanin production was not a result of the emergence of mutated isolates, an evolution assay in surface-grown settings was conducted. The initially evolved and pyocyanin-producing Δ*rhlI lasR*^{A158P} isolate served as the parental strain in this experiment. The results showed that the evolved populations did not favour pyocyanin production compared to the parental strain's profile. This indicates that this behaviour resulted from genetic alterations during the initial evolution and was uniquely attributed to LasR A158P.

As a supplemental result, the emergence of LasR-defective strains was demonstrated not to be directly linked to the QS function status of bacteria. The reported LasR-defective strains isolated to date retaining the ability to function as a group of cells were associated with RhlR activity (RAIL strains (Groleau *et al.*, 2022)), thus relying on a functional *rhl* system. In **subsection 4.3**, a strain carrying the LasR A158P variant presented a flexible *rhl* system, producing RhlR-controlled molecules such as pyocyanin independently of C_4-HSL. This adaptability, however, is lost in the absence of RhlR or, presumably, when both its stabilizing factors, C_4-HSL and PqsE, are absent.

While one might anticipate that bacteria would retain a functional LuxR regulator, resulting in a lower emergence rate of LasR-negative mutants in strains with an impaired *rhl* system compared to wild-type strains, the frequency of these mutants is surprisingly higher when *rhl* defective strains are used as the parental background for experimental evolution. This discrepancy raises questions about the importance of maintaining QS responses at the individual cell level, which contradicts the current understanding of the QS system. Instead, the widespread emergence of LasR-defective isolates, which can confer advantages to the bacterial population, suggests a common feature for which the driving factors are yet to be identified. In fact, we recently published an opinion article on this subject based on our accumulated knowledge about the isolation and emergence of LasR-defective isolates (Trottier *et al.*, 2024). Our conclusion suggests that these mutants arise as part of a mixed population comprising LasR-functional and -defective isolates, benefiting the population as a whole in ways that extend beyond their ability to perform QS functions themselves.

Considering this apparent "fragility" of the QS system, *P. aeruginosa* may have developed a complex regulatory network that allows for QS functionality even without a functional LasR. Modulators of RhlR, such as the chaperone-like protein PqsE or external factors like temperature, may have evolved to promote *rhl* system activity/function under various conditions. The combination of these factors might even permit RhlR to function in the absence of its cognate ligand C_4-HSL in contrast with traditional AHL-mediated QS. Notably, while mutations in *lasR* are frequently observed in *P. aeruginosa* isolates, the genes encoding RhlR and PqsE are rarely affected (O'Connor *et al.*, 2022). Therefore, despite the experimental possibility of LasR-defective isolates in *rhl*-inactive backgrounds, such strains are relatively uncommon in natural environments.

5 GENERAL OVERVIEW

5.1 Conclusion

This thesis aimed to enhance our understanding of the environment on the functioning of the QS regulatory network in *P. aeruginosa*. In particular, the effect of surface growth and the influence of growth temperature on modulating the activity of this communication system were investigated. This goal was established due to the significance of these environmental factors in natural *P. aeruginosa* populations, both in non-clinical and clinical environments. More precisely, the primary focus of this thesis centred on elucidating the molecular mechanisms responsible for driving these environment-responsive regulations.

Another significant factor influencing the point-of-view taken in this thesis is the prevalence of LasR-defective cells among *P. aeruginosa* isolates. This concept served as the foundation for our first publication, as presented in **Chapter 3**. When we observed that, unlike planktonic cells, LasR was dispensable to produce its cognate autoinducer 3-oxo-C_{12}-HSL in surface-grown cells, we became intrigued by the implications of this phenomenon in natural populations consisting of LasR-defective cells. The demonstrated impact of 3-oxo-C_{12}-HSL on the bacterial population can provide partial insight into the prevalence of LasR-defective isolates. The differential regulation between planktonic and surface-grown cells within the core of the QS regulatory network underscores the limited and condition-specific nature of our overall understanding of the QS system. A surface-conditioned response has been attributed to wild-type *P. aeruginosa* PA14 and its sensitivity of 3-oxo-C_{12}-HSL upon surface association (Chuang *et al.*, 2019), which aligns with the implications of our publication. Along with ours, this is the sole report delving into how surface-sensing modulates QS behaviours, and both focus on LasR and its autoinducer.

While these publications share a common subject, they diverge significantly regarding the underlying mechanisms driving the observed responses. In the prototypical PA14 strain, the presence of the regulator RhlR is indispensable for inducing the production of 3-oxo-C_{12}-HSL in LasR-defective cells (refer to **Chapter 3**). This regulatory relationship, though not surprising given the previous publication that underscored RhlR's role as a surrogate regulator of genes controlled by LasR when the latter is inactive (Dekimpe *et al.*, 2009), presents some unexpected nuances. We confirmed the dependency of RhlR in the production of 3-oxo-C_{12}-HSL. However, what surprised us was that this dependency is not reflected in the transcriptional profile of *lasI*, the gene responsible for encoding the 3-oxo-C_{12}-HSL synthase. Instead, our observations point to the involvement of RhlR in the translational regulation of this gene, a finding that defies our initial

expectations. As previously introduced (refer to **section 1**), RhlR is a transcriptional regulator, and we initially hypothesized that it directly activates the expression of *lasI* by binding to the *las* box, a motif recognized by both LasR and RhlR in conjunction with the RNA polymerase (Soberón-Chávez *et al.*, 2005; Whiteley *et al.*, 2001; Whiteley *et al.*, 1999). Our findings, however, strongly suggest an indirect dependency, leading us to propose the existence of a potential RhlR-regulated translational regulator that exerts its influence on *lasI*. Notably, to the best of our knowledge, there is no documented report of such a regulator, either a protein or a regulatory RNA. Therefore, an exploratory screening becomes imperative to uncover the elusive link between *lasI* expression and RhlR. Moreover, identifying such a regulator is essential in clarifying its role in both levels of *lasI* expression (transcriptional and translational and whether they are coupled) and understanding why it is exclusively active in surface-grown cells and the absence of LasR.

The putative RhlR-controlled regulator of *lasI* could provide valuable insights into comprehending the QS regulatory networks across different *P. aeruginosa* isolates, extending our knowledge beyond the prototypical PAO1 and PA14 strains. Notably, RhlR is not universally required to produce 3-oxo-C_{12}-HSL in LasR-defective isolates (**Fig. 3.2.1**). In these strains, both the transcription and translation of *lasI* persist even in the absence of LasR and RhlR functionality. The remaining question is whether the putative regulator, previously identified in PA14, would play a similar role in these "atypical" LasR-defective isolates but be subject to different regulations. Otherwise, it would suggest the convergence of multiple regulatory networks controlling the induction of 3-oxo-C_{12}-HSL production during surface growth in various *P. aeruginosa* isolates, highlighting the ecological significance of this response. For instance, additional LuxR regulators might be encoded in their genomes, enabling direct activation of *lasI* transcription. Importantly, LasR, RsaL, and PhoB are the sole regulators shown to bind to the *lasI* promoter region to date (Meng *et al.*, 2020; Rampioni *et al.*, 2006; Schuster *et al.*, 2004b). Still, additional regulators, not present in prototypical strains, could also be involved in this “atypical” response.

The alteration of 3-oxo-C_{12}-HSL regulation in LasR-defective cells is just one example of the changes observed in surface-grown cells compared to their planktonic counterparts within the QS regulatory network. The investigation extended to the production profiles of other signalling molecules in the wild-type PA14 background, revealing that surface conditions also influence the production of C_4-HSL and the overall activity of the *pqs* system. These findings have led to the proposal of an alternative model for the organization of the QS system in surface-grown cells, as illustrated in **Figure 3.2.6**. While some of these regulatory patterns resemble canonical ones,

others appear to deviate from the norm, adding complexity to the overall regulation of the QS system. These specific surface-modulated regulations require further in-depth study, like the one presented in **subsection 3.1**, to confirm their validity.

In the second half of the investigation, the focus remained on exploring the significance of LasR-defective isolates in *P. aeruginosa*. Without LasR, RhlR should become the crucial component sustaining QS-responsiveness in cells. The initial inquiry revolved around the unique activation requirements of RhlR, an inherently unstable LuxR-type protein, and how it might function as a regulatory element. Upon further investigation, it was uncovered that growth temperatures have a significant impact on the stability of the active RhlR/C_4-HSL/PqsE complex. Contrary to prevailing notions in the literature, the findings indicate that RhlR activity is favoured at lower temperatures, akin to environmental conditions, rather than the higher temperatures typically associated with human infection settings. This discovery carries crucial implications as it was demonstrated that the stabilizing effect of temperature can compensate for the absence of C_4-HSL or PqsE in the expression of genes under RhlR control.

Interestingly, these self-produced stabilizing elements, namely C_4-HSL and PqsE, are under the control of LasR. Therefore, their availability is limited in LasR-defective cells. The frequency in the isolation of such isolates in populations of *P. aeruginosa* prompts a reflection: Did the thermoregulation of RhlR evolve to ensure its activity, and more broadly, the functionality of the QS system itself, when LasR function is lost? If so, since this thermoregulation is not linked to LasR itself, it should affect the population as a whole, including both LasR-functional and -defective cells. However, the impact of this regulation would likely be more pronounced in cells with limited production of RhlR-stabilizing elements. If this notion holds true, it implies that QS responsiveness may be of greater importance to free-living bacteria and infections in non-warm-blooded hosts than in infection settings involving warm-blooded mammals, such as humans. Furthermore, within this framework, it could be suggested that the requirement for PqsE stabilizing function evolved as an adaptation to environments that do not inherently support the activity of RhlR, such as in mammalian infections. Over time, the dependency on PqsE may have evolved to be stronger, potentially accompanied by modifications in RhlR itself to facilitate this interaction.

The investigation into the impact of growth temperature on the functionality of the *rhl* system encompassed experiments conducted on the wild-type PA14, and its implications were extended to LasR-defective isolates. The aim was to establish a direct connection between these two communication networks, namely the *las* and *rhl* systems, as we delved into the adaptability of the QS circuitry. To achieve this, an experimental evolution assay , known for the emergence of

LasR-defective mutants, was initiated using a strain incapable of producing C_4-HSL. The LasR A158P variant emerged from this assay, exhibiting a robust induction of pyocyanin production in a C_4-HSL-negative background, suggesting potential modulation of RhlR activity through an elusive mechanism. Interestingly, the optimal pyocyanin induction was observed at room temperature (around 23°C to 25°C) and in surface-grown cells. The regulatory elements explored thus far likely play a role in this response, but further research is needed to elucidate the precise interaction between the LasR A158P variant and these factors. Regardless of the mechanism, this naturally occurring LasR variant underscores the significance of comprehending how the QS regulatory network responds to environmental cues, as these cues can converge to modulate QS-regulated responses within the naturally evolved QS circuitry of *P. aeruginosa.*

Moreover, perhaps the most relevant aspect of this investigation is its focus on only one evolved isolate (thus, one *lasR* allele), while several others emerge during controlled conditions in the laboratory and natural environments. In both instances, they become members of mixed populations, and their genetic diversity might favour exchanges among the cells, benefiting the population as a whole. Therefore, understanding the contributions of individual isolates to the population might help comprehend more complex population-wise behaviours.

In summary, the findings presented in this thesis highlight the need for further research to comprehensively understand the QS network under conditions more closely resembling those found in natural environments. One important avenue for exploration is to re-evaluate the QS transcriptome and phenome that has predominantly been established under broth conditions and apply it to cells grown in association with surfaces. Additionally, it is essential to investigate how bacterial responses are influenced when exposed to temperatures significantly different from those of the human body. The observed effects of these environmental cues on QS regulation suggest that other factors also play a relevant role in this communication system. The individual impact of each environmental factor must be investigated before considering their combined effects, ultimately providing a holistic view of the functionality of the *P. aeruginosa* QS regulatory network across various conditions commonly encountered by this bacterium.

5.2 Challenges inherent to this study and future prospects

This section will be partitioned into three subsections, aligning with one of the research manuscripts presented in this thesis. Within each subsection, the distinct challenges encountered will be addressed, accompanied by a brief discussion and potential future directions to advance these topics.

5.2.1 QS organization in surface-grown cells

As extensively discussed in this document, there are noticeable differences in bacterial behaviours when comparing planktonic and surface-grown cells. Investigating the mechanisms underlying the bacterial response to encounters with surfaces is a relatively recent area of study, and much remains to be understood about the governing processes. The mechanisms triggered by surface sensing were introduced in **subsection 1.3.1**, describing how physical signals are translated into biochemical responses. However, fundamental questions about this process still linger. For instance, can bacteria distinguish between a physical encounter with a cluster of bacterial cells and an encounter with an abiotic substrate? What signals do bacteria emit when encountering either a biotic or abiotic surface? While these questions remain open, their answers directly affect the surface-associated growth model we have employed.

As a reminder, agar plugs were employed to cultivate bacteria. In this model, the surface area available for direct bacterial contact is limited and quickly becomes covered by a layer of cells. This compact configuration promotes direct cell-to-cell contact, in contrast to broth growth, which allows for a more dispersed distribution of cells. Moreover, other variables inherent to surface living, such as the creation of gradients in oxygen, nutrients, and waste products, come into play (Jo *et al.*, 2022). All these factors are encompassed within the "surface-associated" model. The impact of this mode of growth on the QS regulatory network described in this document likely results not solely from how bacteria sense surfaces *per se* but rather from the interplay of how these signals are perceived and integrated by *P. aeruginosa*.

The protocol employed to measure the production of QS signal molecules does not account for the cellular heterogeneity between surface-grown cells. Consequently, the results represent the average production of these molecules across the cell population. Indeed, localized differences in the production of these signals may occur due to a regulatory mechanism triggered by an unknown stimulus, which may not be uniform across the entire surface-associated population. Furthermore, there is always a diversification/radiation of populations, with various clones that the heterogeneous nature of the culture system environment may favour. To explore the heterogeneity, the production of 3-oxo-C_{12}-HSL in surface-grown LasR-negative cells was investigated. To do this, attempts were made to visualize the expression of *lasI* by employing a transcriptional *lasI* reporter fused with the *lux* operon in colony biofilms of a *lasR* mutant. This approach was inspired by the *in-situ* gene expression under these growth conditions performed previously by Wang and colleagues (Wang *et al.*, 2021). The rationale was that variations in the intensity of light emitted by different colony sections might be observed if such regulation

occurred. As a control, a colony biofilm of PA14 was used, but unfortunately, no significant differences in light intensity were observed. This lack of distinction was evident even when compared to the control, which produces considerably less 3-oxo-C_{12}-HSL in surface-associated conditions. Further exploration of this avenue of investigation could be undertaken to identify the specific signal triggering the described response, but it requires optimization of the light emission protocol.

Studying the QS of surface-grown cells presents another challenging aspect, requiring an open-minded approach due to the potential differences from well-established pathways observed in broth cultures. Variability in QS regulation is not uncommon and is exemplified by the slightly distinct QS circuitries in various *P. aeruginosa* isolates. These variations expand the boundaries of the communication system beyond what is known for the prototypical strains PAO1 and PA14. The surprising aspect of the findings goes beyond the mere existence of differences within this system, as this was an initial assumption of the project. The genuine surprise lies in the extent of these differences, as outlined in **Chapter 3**. In this chapter, a physiological condition, specifically surface association, that significantly upregulates the production of 3-oxo-C_{12}-HSL in LasR-defective cells is discussed.

LasR-negative isolates produce trace amounts of 3-oxo-C_{12}-HSL in broth cultures. The mechanism governing the production of this signalling molecule remains consistent across these growth conditions, relying on RhlR. Thus, this regulation is induced upon surface association, but its mechanisms are operational to a lesser extent in broth cells. The hypothesis suggests that the activation of this pathway in surface-grown cells indicates that the presence of 3-oxo-C_{12}-HSL is more vital to bacterial physiology than in planktonic cells despite the absence of its cognate transcriptional regulator, LasR. As previously introduced and discussed, RhlR does not directly activate *lasI* transcription in planktonic and surface-grown cells. The relationship between RhlR and the production of 3-oxo-C_{12}-HSL is central to understanding core QS regulations and warrants further exploration in future research. Such investigations should ideally employ a model with surface-grown cells, as it better reflects the physiological conditions favouring the RhlR-controlled production of 3-oxo-C_{12}-HSL.

The concept of promiscuity within LuxR regulators was initially introduced in **subsection 1.2.3.7** and further discussed in detail in **subsection 1.3.1**. This concept becomes particularly relevant when considering the production of 3-oxo-C_{12}-HSL in the absence of LasR, as the orphan LuxR-type protein QscR also responds to this long-chain AHL. The ability of QscR to use 3-oxo-C_{12}-

HSL raises the question of whether the induction of 3-oxo-C_{12}-HSL might enhance the activity of QscR, potentially leading to the autologous production of pyocyanin, as described in **subsection 3.1.5**. Previous discussions (refer to **subsection 3.1.6**) outlined why it is believed QscR is not the primary factor responsible for the physiological outcomes described. Nevertheless, QscR may play a role in this behaviour, responding to the physiological induction of 3-oxo-C_{12}-HSL in surface-grown cells. Further experimental investigations are required to fully elucidate this potential connection between 3-oxo-C_{12}-HSL, QscR, and pyocyanin production in LasR-defective cells. While acknowledging the possibility of a QscR-mediated response to the physiological induction of 3-oxo-C_{12}-HSL in surface-grown cells, a clear and direct link between the known effects of QscR, acting as an anti-activator of the QS system, and pyocyanin production has yet to be established. Moreover, results suggest that 3-oxo-C_{12}-HSL directly affects RhlR, impacting pyocyanin production. However, the underlying mechanisms of this regulatory interaction remain elusive.

Unlike QscR, RhlR is an exceptionally selective LuxR-type protein not activated by most laboratory-tested AHLs, including 3-oxo-C_{12}-HSL (Wellington *et al.*, 2019). This selectivity aligns with our findings, as the induction of pyocyanin production, serving as a proxy for RhlR activation, is observed only in the concurrent presence of C_4-HSL. This necessity suggests that 3-oxo-C_{12}-HSL exclusively impacts the bound RhlR conformation, a phenomenon not previously reported in the literature. Investigating this structural alteration would entail the co-crystallization of RhlR with its cognate autoinducer, C_4-HSL, and eventually 3-oxo-C_{12}-HSL. However, as mentioned extensively, RhlR is inherently unstable, and binding with C_4-HSL alone fails to provide the stabilization required for purification.

Hence, confirming the putative interaction of 3-oxo-C_{12}-HSL with bound RhlR/C_4-HSL poses significant challenges but should not be dismissed. Meanwhile, an indirect approach can reinforce this possibility. The impact of 3-oxo-C_{12}-HSL in LasR-deficient cells under conditions where the production of this signalling molecule is naturally or artificially induced could be investigated, corresponding respectively to surface-grown and planktonic cells in LasR-defective cells. Conducting high-throughput RNA sequencing and comparing it to a condition where 3-oxo-C_{12}-HSL is absent could help identify differentially regulated genes. Such data may provide crucial insights into which regulator(s) are involved in the 3-oxo-C_{12}-HSL-induced response. Moreover, multiple genes under RhlR control would likely be identified. If so, it would strongly suggest that 3-oxo-C_{12}-HSL plays a role in modulating RhlR activity, motivating further investigation into the structure of this regulator in the presence of 3-oxo-C_{12}-HSL.

This strategy could also contribute to identifying the link between *lasI* expression and RhlR, which, as previously mentioned in this subsection, is likely connected, given that QS systems often form positive feedback loops. Therefore, this avenue holds promise for expanding the understanding of surface-grown QS regulation in LasR-negative cells and is currently the subject of ongoing laboratory investigation.

5.2.2 Growth temperature and RhlR activity

The intrinsic instability of RhlR requires the presence of both the chaperone PqsE and its cognate autoinducer C_4-HSL for its full activation under laboratory conditions. These conditions typically mimic the temperature of the human body to cultivate *P. aeruginosa*. Combining these elements stabilizes RhlR, resulting in an active RhlR/C_4-HSL/PqsE complex. Our research has shown that a lower temperature, similar to that encountered in natural environments, acts as a third stabilizing factor, enhancing the activity of the active RhlR complex.

Genetic tools were utilized to disrupt the RhlR/C_4-HSL/PqsE complex to reach these conclusions. Various approaches targeting the autoinducer, PqsE, and RhlR were employed, leading to the same outcomes. However, this study primarily focuses on the activity aspect of RhlR, without including any biochemical data to support the conclusions. Ideally, a follow-up study should concentrate on the structural facet of this regulation to validate the findings. Nevertheless, this prospective project presents significant challenges. The structural description of RhlR has only recently emerged, delayed by the technical difficulties faced by previous researchers.

Additionally, purifying RhlR is simplified by using the artificial ligand mBTL, while purifying the natural and biologically active conformation remains challenging. This prospective project would require an examination of the native RhlR complex, and its feasibility is currently uncertain. This concept should be revisited when advancements in RhlR purification techniques become available.

At this point, a couple of methodological questions should be considered. Is temperature solely a stabilizer of the active RhlR biological systems, or does it indicate a pre-established physical factor? The answer to this question is important, as it directly impacts protein purification protocols that should be employed. For instance, could temperature variations during the execution of the protocols influence the conformation of this active complex?

The model, based on the influence of temperature on RhlR activity, primarily focuses on the expression of *phz1*, a gene controlled by RhlR, and pyocyanin production. While this model

explains the connection between temperature, RhlR and pyocyanin production, it covers only a subset of genes regulated by this transcription factor. Indeed, observations have indicated that this proposed model does not apply to other genes regulated by RhlR, such as *rhlA*, and only partially aligns with *hcnA* expression. It is well documented that the dependency of these genes on PqsE differs, but the exact mechanisms remain unclear. Could different structural conformations of RhlR coexist within the cells, allowing the expression of these genes? If so, how is the equilibrium between these structural forms achieved? Examining the timing of expression for these factors might provide some insights, but both factors are also regulated by LasR, adding complexity to the picture. There is still much to discover about these regulatory networks, and the model, though valuable, remains a work in progress. The understanding of these intricate regulatory processes will evolve as relevant data continues to be gathered, and the model should adapt accordingly.

5.2.3 The LasR A158P variant: Conversion of surface and temperature cues?

Plasticity within the QS regulatory circuitry has been observed in various settings of *P. aeruginosa*, both in non-clinical and clinical environments. An experimental evolution model was employed to investigate this phenomenon. Under laboratory conditions, LasR-defective isolates emerge during repeated passages of a parental strain. Given that the absence of functional LasR compromises QS responses in PA14, curiosity arose regarding the potential effects when a strain with already compromised QS was used as the parental strain for this evolution assay.

In a C_4-HSL-negative background, the emergence of LasR-defective strains was indeed observed. The frequency of these isolates in such conditions suggests that the factors driving LasR loss-of-function persist even without an active *rhl* system. This indicates specificity to LasR and implies that these factors are likely not solely QS-regulated. While RhlR can compensate for some LasR-controlled functions (Dekimpe *et al.*, 2009), certain LasR-specific functions cannot be fully replaced by RhlR, suggesting that these drivers may fall into that category. Furthermore, an intriguing correlation was noticed, suggesting that these mutants might induce RhlR activity at a population level without C_4-HSL. This correlation caught attention and prompted the establishment of a direct connection between these factors by isolating a strain containing an allele encoding the LasR A158P variant in the C_4-HSL-negative background. Strikingly, this strain exhibited a phenotype associated with LasR deficiency while simultaneously displaying increased pyocyanin production, an exoproduct regulated by RhlR.

The production of pyocyanin by the evolved isolate exhibited conditional behaviour, and it took some time to identify the factor responsible for this differential regulation. Initially, the assumption was that this response was triggered by surface growth, as neither precultures nor cultures of this strain produced the pigment. However, further investigation revealed that even surface-grown cells incubated continuously at 37°C failed to produce pyocyanin, highlighting the role of temperature in this response. Indeed, the LasR A158P variant is functional at 25°C, as evidenced by *lasI* expression and the production of 3-oxo-C_{12}-HSL. However, this activity is lost at 37°C. Importantly, even in its active state, the LasR A158P variant did not exhibit differential regulation of known genes under LasR control at either temperature when compared to a *lasR* mutant. Consequently, there were partially contradictory results, making it challenging to assess LasR functionality conclusively.

One potential approach to overcome this limitation is to employ a phenotypic profiling method based on the quantification of QS-dependent extracellular molecules developed in the laboratory to assess the activity status of the LasR A158P variant (Groleau *et al.*, 2022). This method allows for categorizing strains unbiasedly based on their phenotypic profiles. This method enables the differentiation between LasR-defective and -functional isolates. Performing this phenotypic assessment at both growth temperatures used in this study (37°C and 25°C) could confirm whether the isolate carrying the LasR A158P variant clusters with the wild-type PA14 strain at only one temperature or consistently exhibits the profile of a *lasR* mutant. Notably, this methodology relies on comparing multiple strains to achieve significant results, which would require a substantial amount of time. Due to the choices made in this project, this analysis was not conducted. However, the approach remains valid and represents the most suitable strategy for determining the functional status of the LasR A158P variant in response to growth temperatures.

Understanding the impact of the LasR A158P variant on the QS system is a complex endeavour that requires thorough investigation. The allele encoding this protein variant was introduced into the PA14 background to isolate its effects on the *las* system and eliminate any secondary mutations acquired during experimental evolution. Throughout the study, the PA14 *lasR*^{A158P} strain is constantly compared with the PA14 wild-type strain and its isogenic *lasR* mutant.

As previously mentioned, pyocyanin production was one notable outcome of the presence of LasR A158P in a C_4-HSL-negative background. However, we should note that a *lasR* mutant of PA14 also produces significant amounts of this pigment when cultivated in King's A broth, the culture medium employed in our study. This complicates the assessment of whether LasR A158P

has a substantial impact on the QS regulatory system. Consequently, only minor indications of a role for this variant in the system were identified.

An intriguing possibility worth exploring is whether the impact of LasR A158P is specific to strains incapable of producing C_4-HSL. This variant appears to initiate an alternative regulatory pathway leading to RhlR activation, as RhlR is required for this response. To delve deeper into this phenomenon, a series of transcriptional reporters previously employed in the Δ*rhlI lasR*^{A158P} background should be utilized, along with the corresponding controls. Furthermore, the study could be extended to Δ*pqsE lasR*^{A158P} and compare its responses to Δ*rhlI lasR*^{A158P}. This comparative analysis could indicate whether LasR A158P predominantly influences the QS regulatory network when the *rhl* system is compromised.

To account for the temperature-dependent response, conducting these investigations at 37°C and 25°C in planktonic cells is essential. Suppose the analysis reveals no significant differences at the transcriptional level in strains with compromised *rhl* systems. In that case, it may be prudent to consider the possibility of post-transcriptional regulations, such as those mediated by small RNAs (as presented in **subsection 1.2.3.6**). Additionally, in parallel, it could be explored whether the presence of LasR A158P alters how RhlR responds to C_4-HSL, providing further insights into the complexity of this variant's effects on the QS system.

Notably, the evolved strain's maximum induction of pyocyanin production was observed under surface-grown conditions, suggesting that this growth mode might lead to different regulatory patterns than broth conditions. It would be valuable to replicate the most intriguing results obtained in planktonic cells in a surface-grown model, accounting for potential differential regulation between these growth conditions while considering different growth temperatures. Unfortunately, direct investigation of these potential regulations in a surface-associated study model is challenging due to the limited information about the regulations under these growth conditions.

In summary, unravelling the impact of the LasR A158P variant requires a step-by-step approach. The process was initiated by examining the effects of LasR A158P in PA14 under various growth temperatures. However, a comprehensive understanding of this variant necessitates further investigation in several genetic backgrounds, as a clear indication of the mechanism through which this variant affects the QS system has yet to be identified. Only when a comprehensive picture of the potential regulatory changes influenced by this variant is established based on broth cultures can specific pathways be validated in surface-grown cells to confirm whether surface association serves as a regulatory cue to influence gene regulation.

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7 ANNEXES

7.1 **ANNEX I -** Scientific communication

7.1.1 Oral presentations

i. **Thays de Oliveira Pereira**, Marie-Christine Groleau, Eric Déziel

Congrès de Bactériologie intégrative : Symbiose & Pathogénèse (BiSP) 2023, Montréal, Québec, Canada (**September 2023**)

Presentation in French titled: « La température influence l'expression des facteurs de survie via les systèmes de *quorum sensing* chez le pathogène opportuniste *Pseudomonas aeruginosa* »

Translated title to English : "The temperature influences the expression of survival factors through quorum sensing systems in the opportunistic pathogen *Pseudomonas aeruginosa*"

ii. **Thays de Oliveira Pereira**, Marie-Christine Groleau, Eric Déziel

Congrès Armand-Frappier (CAF) 2021. This conference was held online (**November 2021**)

Presentation in French titled: « L'évolution expérimentale de *Pseudomonas aeruginosa* sélectionne des mutants dont la régulation du *quorum sensing* est altérée »

Translated title to English : "Experimental evolution of *Pseudomonas aeruginosa* selects for mutants with altered quorum sensing regulation"

iii. **Thays de Oliveira Pereira**, Marie-Christine Groleau, Eric Déziel

Congrès de Bactériologie intégrative : Symbiose & Pathogénèse (BiSP) 2021. This conference was held online (**May 2021**)

Presentation in French titled: « L'évolution expérimentale de *Pseudomonas aeruginosa* sélectionne des mutants dont la régulation du *quorum sensing* est altérée »

Translated title to English : " Experimental evolution of *Pseudomonas aeruginosa* readily selects for mutants with impaired quorum sensing regulation"

7.1.2 Poster presentations

i. **Thays de Oliveira Pereira**, Marie-Christine Groleau, Eric Déziel

Congrès Armand-Frappier (CAF) 2023. Saint-Sauveur, Québec, Canada (**October 2023**)

Presentation in French titled: « La température influence l'expression des facteurs de survie via les systèmes de *quorum sensing* chez le pathogène opportuniste *Pseudomonas aeruginosa* »

Translated title to English: "The temperature influences the expression of survival factors through quorum sensing systems in the opportunistic pathogen *Pseudomonas aeruginosa*"

ii. **Thays de Oliveira Pereira**, Marie-Christine Groleau, Eric Déziel

Conference *Pseudomonas* 2022. Atlanta, Georgia, United States (**April 2022**)

Experimental evolution of *Pseudomonas aeruginosa* readily selects for mutants with impaired quorum sensing regulation

iii. **Thays de Oliveira Pereira**, Marie-Christine Groleau, Eric Déziel

Conference of the Canadian Society of Microbiologists (CSM) 2021. This conference was held online (**June 2021**)

Experimental evolution of *Pseudomonas aeruginosa* readily selects for mutants with impaired quorum sensing regulation

iv. **Thays de Oliveira Pereira**, Marie-Christine Groleau, Eric Déziel

Congrès Armand-Frappier (CAF) 2019. Saint-Sauveur, Québec, Canada (**October 2023**)

Presentation in French titled: « Nouvelle interaction entre les systèmes de communication intercellulaire chez *Pseudomonas aeruginosa* »

Translated title to English: " New interaction between intercellular communication systems in *Pseudomonas aeruginosa* "

7.2 **ANNEX II** - Articles in collaboration

Trottier MC, **de Oliveira Pereira T**, Groleau M-C, Hoffman LR, Dandekar AA, Déziel E (2024) The end of the reign of a “master regulator”? A defect in function of the LasR quorum sensing regulator is a common feature of *Pseudomonas aeruginosa* isolates. *mBio* 0(0):e02376-02323.

Singh VK, Almpani M, Maura D, Kitao T, Ferrari L, Fontana S, Bergamini G, Calcaterra E, Pignaffo C, Negri M, **de Oliveira Pereira T**, Skinner F, Gkikas M, Andreotti D, Felici A, Déziel E, Lépine F, Rahme LG (2022) Tackling recalcitrant *Pseudomonas aeruginosa* infections in critical illness via anti-virulence monotherapy*. Nature Communications* 13(1):5103.

Groleau M-C, **de Oliveira Pereira T**, Dekimpe V, Déziel E (2020) PqsE is essential for RhlR-dependent quorum sensing regulation in *Pseudomonas aeruginosa*. *mSystems* 5(3):e00194-00120.