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Pseudomonas aeruginosa isolates defective in function of the LasR quorum sensing regulator are frequent in diverse environmental niches

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Summary

The saprophyte *Pseudomonas aeruginosa* is a versatile opportunistic pathogen causing infections in immunocompromised individuals. To facilitate its adaptation to a large variety of niches, this bacterium exploits population density-dependent gene regulation systems called quorum sensing (QS). In *P. aeruginosa*, three distinct but interrelated QS systems (*las*, *rhl* and *pqs*) regulate the production of many survival and virulence functions. In prototypical strains, the *las* system, through its transcriptional regulator LasR, is important for the full activation of the *rhl* and *pqs* systems. Still, LasR-deficient isolates have been reported, mostly sampled from the lungs of people with cystic fibrosis, where they are considered selected by the chronic infection environment. In this study, we show that a defect in LasR activity appears to be an actually widespread mechanism of adaptation in this bacterium. Indeed, we found abundant LasR-defective isolates sampled from hydrocarbon-contaminated soils, hospital sink drains and meat/fish market environments, using an approach based on phenotypic profiling, supported by gene sequencing. Interestingly, several LasR-defective isolates maintain an active *rhl* system or are deficient in *pqs* system signalling. The high prevalence of a LasR-defective phenotype among environmental *P. aeruginosa* isolates questions the role of QS in niche adaptation.

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Introduction

Many Gram-negative bacteria found in natural habitats, such as members of the *Pseudomonas* genus, are also capable of causing opportunistic infections in plants and animals, including humans. Clinical and environmental strains of *Pseudomonas aeruginosa* possess a high degree of genomic conservation but still show significant phenotypic diversity (Grosso-Becerra *et al*., 2014). This opportunistic pathogen is an adaptable organism mostly distributed in environments closely related to human activities and is a major cause of nosocomial infections (Crone *et al*., 2020). To promote survival in various environmental niches, *P. aeruginosa* coordinates group behaviours to act as a community through density-dependent intercellular communication systems named 'quorum sensing' (QS). QS is important for the coordination of competitive and cooperative interactions between organisms within a species or between species (Abisado *et al*., 2018). In *P. aeruginosa*, the QS circuitry is built on three hierarchically arranged systems, each mediated by the production and detection of distinct signalling molecules. The *las* system is generally considered to initiate the regulatory cascade (Gilbert *et al*., 2009). LasI is a LuxI-type synthase responsible for the production of *N*-3-oxododecanoyl-homoserine lactone (3-oxo-C_{12}-HSL), the signalling molecule that binds and activates the LuxR-type transcriptional regulator LasR. LasR then induces the transcription of many target genes, including those encoding various exoproteases (e.g. LasA and LasB) as well as the *lasI* gene, thus causing a positive feedback loop (Gambello and Iglewski, 1991; Toder *et al*., 1991; Whiteley *et al*., 1999). LasR bound to its autoinducer 3-oxo-C_{12}-HSL also activates the downstream QS system *rhl*, by inducing the transcription of both *rhlI* and *rhlR* (Latifi *et al*., 1996; Pesci *et al*., 1997; Whiteley *et al*., 1999; Wagner *et al*., 2003; Gilbert *et al*., 2009). The RhlI synthase catalyses the production of *N*-butanoyl-homoserine lactone (C_4-HSL) that can bind to the LuxR-type regulator RhlR, which controls the transcription of other genes, several encoding for exoproducts such as phenazines (*phz1* and *phz2* operons), rhamnolipids (*rhlAB*) and hydrogen cyanide (*hcnABC*) (Ochsner *et al*., 1994; Brint and Ohman, 1995;

Albus *et al*., 1997; Pesci *et al*., 1997; Pessi and Haas, 2000).

The third QS system is based on the production of 4-hydroxy-2-alkylquinolines (HAQs), which are synthesized through the enzymes encoded by the *pqsABCDE* operon. The main product of the PqsABCD enzymes, 4-hydroxy-2-heptylquinoline (HHQ) is converted in the *Pseudomonas* quinolone signal (PQS) by the PqsH enzyme (Déziel *et al*., 2004). Both HHQ and PQS act as autoinducing ligands of the LysR-type transcriptional regulator MvfR (PqsR), which in turn activates the transcription of the *pqs* operon. The PqsABCD enzymes coupled with the activity of the PqsL monooxygenase also synthetize 4-hydroxy-2-heptylquinoline *N*-oxide (HQNO), a secondary metabolite inhibiting Gram-positive bacteria respiration and inducing self-poisoning (Lightbown and Jackson, 1956; Machan *et al*., 1992; Hazan *et al*., 2016). Considering that LasR also controls the transcription of both the *pqsH* and *pqsL* genes (Whiteley *et al*., 1999), the *pqs* system is in fact intertwined in the QS circuitry, with LasR positively regulating the expression of *pqsABCDE* and RhlR acting as a repressor. Furthermore, the PqsE protein is not required for HAQ synthesis but is important for full activation of RhlR-dependent QS (Mukherjee *et al*., 2018; Groleau *et al*., 2020). Importantly, the above-mentioned hierarchy of the QS circuitry is flexible, since RhlR can take over the role of LasR in the stationary phase (Dekimpe and Déziel, 2009), and several clinically isolated LasR-defective mutants are still able to produce RhlR-dependent factors (Feltner *et al*., 2016; Chen *et al*., 2019).

LasR-defective mutants are frequently identified among clinical samples, especially from chronic infections such as cystic fibrosis (CF) (Cabrol *et al*., 2003; D'Argenio *et al*., 2007; Hoffman *et al*., 2009; Bjarnsholt *et al*., 2010; Feltner *et al*., 2016). The presence of these mutants is associated with an adaptation to unique conditions. For example, they possess growth advantages on specific amino acids largely found in CF secretions or have an increased resistance to some antimicrobials, suggesting a selective pressure from this kind of environment (D'Argenio *et al*., 2007; Hoffman *et al*., 2009). On the other hand, LasR-defective mutants were also exceptionally isolated from corneal ulcers, considered an acute infection (Hammond *et al*., 2016). Surprisingly, the conservation of QS activity has been rarely investigated among isolates from environmental sources (Cabrol *et al*., 2003; Vincent *et al*., 2017). LasR-deficient isolates of *P. aeruginosa* have been thoroughly characterized in several reports, but essentially in studies investigating their prevalence in the lungs of patients with CF (D'Argenio *et al*., 2007; Feltner *et al*., 2016). Focus is often on the findings of variations in the sequence of the *lasR* gene. However, sequences are compared to those of reference strains and variations may not always indicate a defective LasR activity. In addition, variations in regulatory elements affecting the functionality of LasR are set aside in these studies, even more so when focusing on the sole sequencing of the *lasR* gene rather than whole-genome sequencing. Elements such as anti-activator proteins such as QslA, QteE and QscR are able to suppress QS-activation through the blocking of LasR activity (Siehnel *et al*., 2010; Lintz *et al*., 2011; Fan *et al*., 2013; Asfahl and Schuster, 2018). A phage-encoded anti-activator named Aqs1 that inhibits LasR activity was also described (Shah *et al*., 2021). Phenotypical traits of LasR-defective strains are well known. A non-functional LasR typically leads to a characteristic surface iridescent sheen and autolysis of colonies on agar plates, a result of imbalance in HAQ production (D'Argenio *et al*., 2007). The production of the blue pigment pyocyanin is also negatively altered in exponential phase cultures grown in rich media. On the other hand, pyocyanin accumulates at high levels in cultures of LasR-deficient strains during the late stationary phase (Dekimpe and Déziel, 2009; Cabeen, 2014; Wang *et al*., 2018). Since LasR controls the production of various proteases, a LasR deficiency also leads to an inability to degrade casein (Gambello and Iglewski, 1991; Toder *et al*., 1991).

In this study, we tested the hypothesis that a defect in LasR activity is not restricted to clinical strains originating from human infections. To estimate the prevalence of a LasR defect in a collection of strains isolated from various environments other than clinical, we chose an approach based on phenotypic profiling rather than strictly on gene sequencing. The phenotypic profile of 176 environmental isolates originating from hospital sink drains, hydrocarbon-contaminated soils and meat/fish market environment and products were characterized and integrated into a classification scheme providing a non-ambiguous assessment of their LasR activity status. Unexpectedly, we found that LasR-defective isolates are ubiquitous in these environments. Our methodology also uncovered a diversity in QS profiles with an abundance of LasR-defective isolates maintaining an active *rhl* system as well as isolates with a functional LasR but producing no HAQ molecules. This study thus reveals a high prevalence of the LasR-defective phenotype among *P. aeruginosa* natural isolates. Their incidence in CF infections is associated with their selection by the chronic infection environment. Rather, loss of LasR activity appears a widespread mechanism of adaptation in this bacterium.

Results

Many environmental P. aeruginosa *isolates are deficient in LasR activity*

To investigate the prevalence of a LasR deficiency among non-clinical *P. aeruginosa* isolates, and since

sequencing is not sufficient to identify a defect in LasR activity, we performed a phenotypic characterization of 176 isolates sampled from diverse environments (Table S1), based on known traits of LasR-defective strains. We first looked at unique phenotypes (iridescence and autolysis) of colony on agar, at pyocyanin production patterns in both liquid Tryptic Soy Broth (TSB) and King's A medium, and at the ability to grow on casein as a sole carbon source, indicative of exoprotease production. PA14 strain and its isogenic *lasR*-null mutant were included as references. Results are detailed in Table S2. Based on these often-used criteria, we were unable to clearly determine which isolates had a LasR deficiency. We observed variations in the ability to grow on casein; inconsistencies in the correlation between factors, for instance, some isolates were iridescent and autolytic, overproduced pyocyanin in King's A but could still grow on casein agar. It appears that these traits, although typically used as markers of LasR functionality, are not reliable enough for efficient determination of LasR deficiency in phenotypically-diverse environmental isolates, since there is no specific threshold delineating this characteristic.

We thus looked for an alternative approach. LasR controls the production of extracellular metabolites such as acyl-homoserine lactones (AHLs), HAQs and pyocyanin, whose concentration profiles over time could be adapted to a classification scheme to establish a LasR phenotype. That strategy was explored by measuring 3-oxo-C_{12}-HSL, C_4-HSL as well as HHQ, HQNO, PQS and pyocyanin concentrations at various time points during growth of a *lasR* mutant of PA14, to define reference production profiles. Biosynthesis of these metabolites is directly or indirectly dependent on the regulatory activity of LasR. In the *lasR* mutant background, the production of C_4-HSL was not observed until early stationary phase due to the delayed activation of *rhlI* by RhlR whereas there is an absence of 3-oxo-C_{12}-HSL (Fig. 1A and B) (Groleau *et al*., 2020). In addition, HQNO and PQS are expectedly only produced in minute levels in a LasR-deficient mutant (Fig. 1E and F). Accordingly, the mutant accumulates HHQ, the precursor of PQS (Fig. 1D) since in wild-type PA14, the levels of HHQ stabilize when concentrations of HQNO and PQS start augmenting (Fig. 1D–F) (Déziel *et al*., 2004). As expected, pyocyanin production is heightened in the *lasR*::Gm mutant when compared to PA14 (Fig. 1C). Taken together, the distinct production profiles of all these metabolites suggest that they could be used as markers of LasR deficiency.

Convergence of metabolic profiles with the LasR-defective phenotype was further examined with a subset of 30 isolates from various environments. Based on our initial qualitative phenotypic profiling (Table S2), we purposely chose a majority of isolates (in bold in Table S2) with profiles possibly corresponding to a LasR-deficiency as well as some with patterns in accordance with a functional LasR. Timing of sampling was chosen based on the profiles observed in the LasR mutant versus wild-type (WT) PA14 (Fig. 1). Samples for the analysis of AHLs were collected after 3 and 6 h, which correspond to OD_{600} of 1 and 3, respectively, to generate variables C4-HSL_1 and _2 and 3-oxo-C12-HSL_1 and _2. HHQ, HQNO and PQS were sampled at 6 and 24 h to generate variables HHQ_1 and _2, HQNO_1 and _2, PQS_1 and _2 respectively. Pyocyanin was measured at 24 h (PYOKA_2). A principal component analysis (PCA) was performed to examine variables contributing the most in distinguishing examined strains and identify redundancy consistency between certain traits (Fig. 2A). PCA is an unconstrained ordination analysis that places isolates in a reduced space defined by the two most important gradients (i.e. dimensions) discriminating them. The relative contribution of each variable in positioning isolates along both axes is depicted by the direction and the length of their vectors: (i) the longer is the vector, the higher is the contribution of the variable; (ii) contribution of the variable is inversely proportional to the angle between the vector and the axis. This is an 'unbiased' approach because no *a priori* relying on reference to prior knowledge is specified before executing the analysis. The principal components explained 75% of the variation of QS molecular profile. Strains PA14 (LasR-functional) and its *lasR*::Gm mutant were included in the analysis to examine their position among the other isolates. The associated cluster dendrogram (Fig. 2B) shows the three groups presented on the PCA. We found that the variable HHQ.C7_2, which corresponds to concentrations of HHQ at late stationary phase (24 h time point), can explain the distribution of strains as well as the AHL concentration data since they show inverse directions on the same axis (Fig. 2A). Vectors for HHQ (second-time point) and 3-oxo-C_{12}-HSL are in the exact opposite direction with the same length along the first axis, meaning both variables are redundant in explaining the most important gradient discriminating the 30 isolates. These results led to the elaboration of a parsimonious classification scheme integrating levels of HHQ, HQNO, PQS and pyocyanin in culture samples to discriminate between strains with a deficient or functional LasR regulator. It is noteworthy that there are methodological benefits in avoiding quantifying AHLs from cultures on a large collection of isolates, as these molecules as present in lower concentrations requiring extraction procedures with solvent and can be unstable. Moreover, classification of our selected isolates performed using only both AHLs (C4-HSL_1 and _2 and 3-oxo-C12-HSL_1 and _2) led to convergent dendrogram topologies confirming that our choice of variables is suitable (Fig. S1).

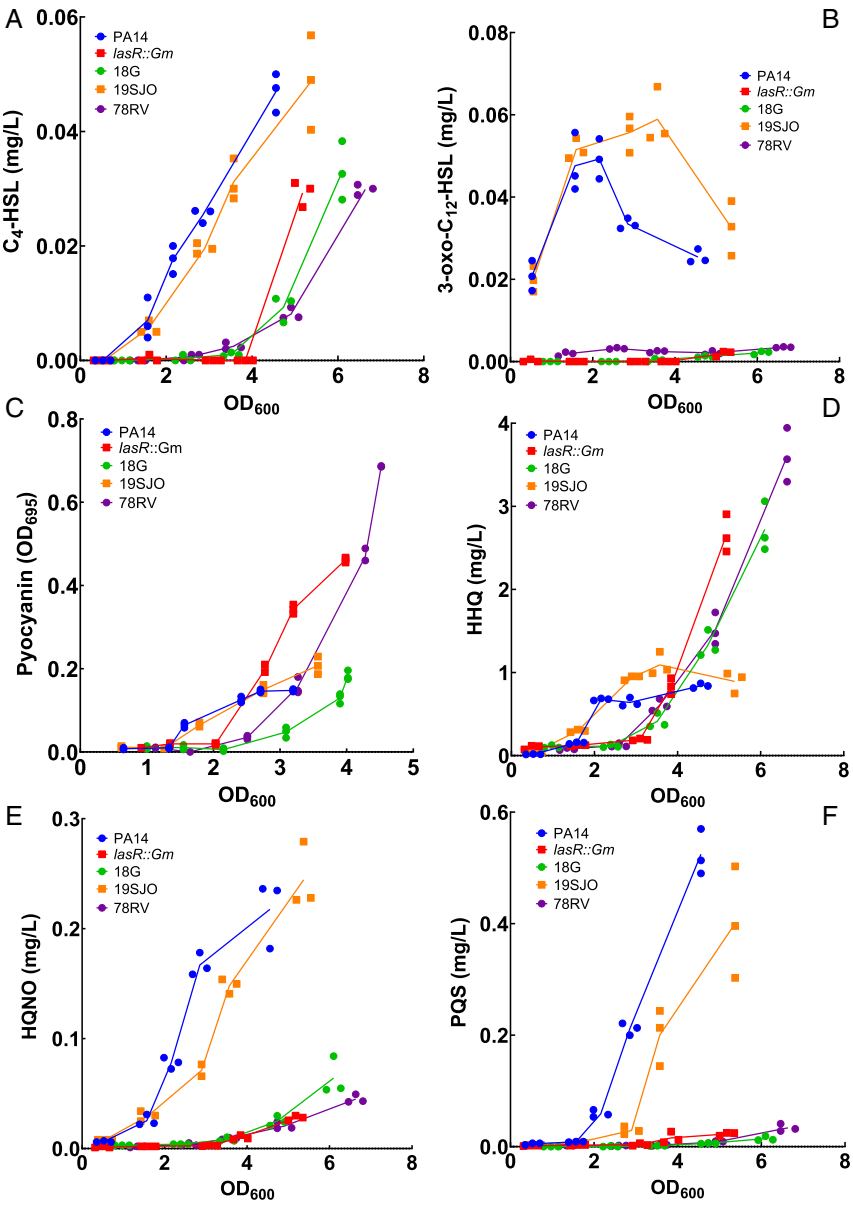

Fig. 1. Production profiles of various extracellular metabolites in strain PA14, its isogenic *lasR*- mutant and selected representative strains. A. C_4-HSL. B. 3-oxo-C_{12}-HSL. C. Pyocyanin (PYO). D. HHQ. E. HQNO. F. PQS. All metabolites were measured by LC/MS, except PYO using A_{695}, in culture supernatant sampled at various time points during growth. Cultures were prepared in triplicates in TSB and incubated at 37°C.

We then performed a new pairwise comparison of the strains based on our parsimonious model and again three different phenotypic profiles were identified among our subset of 30 isolates (Fig. 3). The functional LasR group, behaving typically like WT PA14, encompasses isolates which produce both PQS and HQNO at the end of the exponential phase (PQS.C7_1 and HQNO.C7_1). The LasR-defective group is represented by isolates displaying high concentrations of HHQ at both time points coupled with delayed production of PQS and HQNO and higher late production of pyocyanin in King's A, as illustrated in Fig. 1. The production profiles of the selected variables for an isolate classified in the 'LasR-functional' group (strain 19SJO) indeed matches those of WT PA14 (Fig. 1). Moreover, strains 18G and 78RV, both classified in the 'LasR-defective' group, display production profiles very similar to *lasR*-null mutant of PA14. Our analysis integrating HAQs and AHLs signals revealed a new, unexpected third group of QS variants, which produced very low levels of HHQ, PQS and HQNO but were still able to produce pyocyanin in King's A medium (PA-CL504, 32SB, DCB22 and PA-CL507). Three of these isolates (PA-CL504, PA-CL507 and DCB22) produce AHLs like a WT, meaning their LasR is functional and instead pointing to a defect in the regulation or biosynthesis of HAQs. The other isolate (32SB) produces no 3-oxo-C_{12}-HSL or C_4-HSL at both early and late exponential phases, suggesting it is LasR-defective

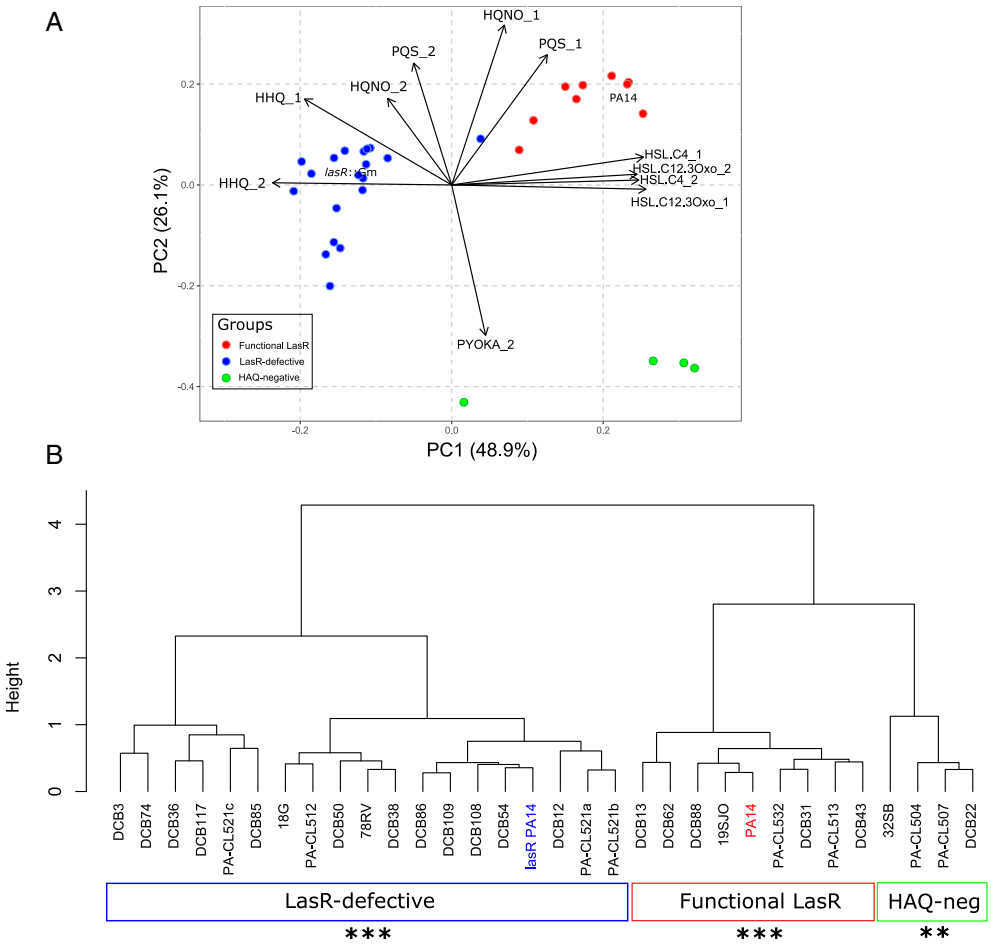

Fig. 2. Principal component analysis (PCA) and clustering analysis elaborated with levels of extracellular metabolites analysed from cultures of a subset of *Pseudomonas aeruginosa* environmental isolates sampled at two time-points. Concentrations of C_4-HSL and 3-oxo-C_{12}-HSL were measured at 3 h (C4-HSL_1 and 3-oxo-C12-HSL_1) and 6 h (C4-HSL_2 and 3-oxo-C12-HSL_2) respectively. Concentrations of HHQ, HQNO and PQS sampled at 6 h (HHQ_1, HQNO_1 and PQS_1) and 24 h (HHQ_2, HQNO_2 and PQS_2) respectively. Pyocyanin (PYOKA_2) was sampled at 24 h. Strains PA14 (LasR-functional) and its *lasR*::Gm mutant were included in the analysis as references. A. Each point on the PCA represents an isolate. Groups represent clusters based on the dendrogram presented in (B) elaborated with the same data. Source data are presented in Table S3. Robustness (R) represents the proportion of clustering runs in which a pair of isolates appeared together in some cluster, given that they were clustered together in at least one run, averaged over all such pairs. R: ****> 90%, ***80%–89% and **70%–79%.

(Table S3). This explains the ambiguous data of Table S2, where this strain has features of a *lasR* mutant but lacks iridescence and autolysis, features, which depend on HAQ production.

Since our parsimonious classification model, including HAQs and pyocyanin production in King's A medium allowed us to cluster with good confidence LasR-deficient isolates (Fig. 3), we next measured the selected variables for the remaining 146 isolates of our investigated collection. As expected, the strains clustered in three distinct phenotypic groups (Fig. S2). One cluster encompasses isolates with a probable defect in LasR function, including PA14 *lasR*::Gm. The second cluster includes isolates with a functional LasR profile. Based on this, at least 40% of our 176 environmental strains display a defective LasR regulatory activity. The third cluster, encompassing 21% of the strains in this collection, contains isolates, which produce negligible levels of HAQs. These isolates could also have a defect in their LasR function, thus further increasing the proportion of LasR-defective strains, and/or only be deficient in their ability to synthesize HAQs.

Whole-genome sequences are available for 45 of the environmental strains investigated here (Table S4) (Freschi *et al*., 2015). We looked for the presence of mutations in the genomes of these strains compared to PA14 to find a genetic explanation for the observed phenotypes. When sequenced isolates had LasR-defective profiles, we indeed found variations in the *lasR* gene

Fig. 3. Clustering of LasR-defective isolates. A cluster dendrogram with a heat map was performed on a subset of isolates using chosen variables HHQ_1 and 2, HQNO_1 and 2, PQS_1 and 2 and pyocyanin (PYOKA_1). Concentrations of HHQ_1 and 2, HQNO_1 and 2, PQS_1 and 2 sampled at 6 and 24 h. Pyocyanin (PYOKA_2) was sampled at 24 h. Strains PA14 (LasR-functional) and its *lasR*::Gm mutant were included in the analysis. Values are listed in Table S3. Robustness (R) represents the proportion of clustering runs in which a pair of isolates appeared together in some cluster, given that they were clustered together in at least one run, averaged over all such pairs. R: ****> 90% and ***80%–89%.

when comparing to the *lasR* gene of WT PA14, with a few exceptions (Table 1). Validating our initial assumption that sequencing alone cannot always predict differences in LasR phenotype, strain 19SJV has a typical LasR-defective profile but no mutation in its *lasR* gene was found. Inversely, a variation in the *lasR* gene of 34JS was found but this strain behaves as if its LasR is functional. Similarly, looking at the upstream promoter region did not reveal any sequence variation pattern unique to LasR-deficient strains. All together, this phenotypic profiling allowed us to highlight for the first time a strong presence of *P. aeruginosa* isolates with defective LasR-dependent signalling in environments not related to human infections.

A subgroup of isolates is unable to produce HAQs

As presented above, the third cluster we observed in Fig. 3 contains a total of 38 isolates, which produce null or extremely low levels of HAQs. To assess the possibility that some of these isolates could also be LasR-defective, we characterized them further. We measured AHL concentrations on this smaller set of isolates and found that 13 of these strains indeed produce both 3-oxo-C_{12}-HSL and C_4-HSL, which indicates a functional LasR (Table S3). When these isolates are included in the final evaluation, we conclude that 48% of our 176 isolates are LasR-defective (Table S3).

Surprisingly, some HAQ-negative LasR-positive strains still produce pyocyanin in King's A (Tables S4 and S5). When looking at the available sequences for some of these strains, we found no mutations in *mvfR* or in the *pqsABCD* genes, which could explain the absence of HAQs. The mechanism explaining the absence of HAQs in these strains is yet to be resolved.

A subgroup of LasR-deficient isolates is still capable of RhlR-dependent QS

A few recent studies reported that a subset of LasR-deficient CF strains conserve a functional RhlR-regulated QS, without the typical requirement for LasR activation, which we call here RAIL strains (for RhlR Active Independently of LasR) (Feltner *et al*., 2016; Chen *et al*., 2019; Cruz *et al*., 2020). We thus asked whether this type of strain in which AHL-dependent QS does not rely on a functional LasR could be also present among our environmental LasR-defective isolates. We looked at

Table 1. SNPs found in the *lasR* gene of sequenced isolates.

Isolates	Description	Classification based on clustering analyses
19SV	Promoter-GCTACGTTCTT ≥ G	Functional LasR[a]
34JS	missense_variant c.251T > C p.Leu84Pro	Functional LasR
57SJ		Functional LasR
PA-CL501		Functional LasR
PA-CL502		Functional LasR
PA-CL504		Functional LasR
PA-CL505		Functional LasR
PA-CL506b		Functional LasR
PA-CL507		Functional LasR
PA-CL508		Functional LasR
PA-CL513		Functional LasR
PA-CL514		Functional LasR
PA-CL515		Functional LasR
PA-CL516		Functional LasR
PA-CL517		Functional LasR
PA-CL518		Functional LasR
PA-CL519		Functional LasR
PA-CL520		Functional LasR
PA-CL527		Functional LasR
PA-CL528		Functional LasR
PA-CL529		Functional LasR
PA-CL532		Functional LasR
PA-CL534a		Functional LasR
PA-CL534b		Functional LasR
PA-CL534x		Functional LasR
PA-CL535		Functional LasR
PA-CL542a		Functional LasR
PA-CL542b		Functional LasR
PA-CL545a		Functional LasR
PA-CL545b		Functional LasR
PA-CL547b		Functional LasR
PA-CL549		Functional LasR
PUPa3		Functional LasR
19SJO		Functional LasR
18G	missense_variant c.654G > C p.Lys218Asn	LasR-defective
19R	missense_variant c.631C > T p.His211Tyr	LasR-defective
19SJV		LasR-defective
32SB	missense_variant c.654G > C p.Lys218Asn	LasR-defective
32SP	missense_variant c.654G > C p.Lys218Asn	LasR-defective
57RV	missense_variant c.611C > T p.Ser204Leu	LasR-defective
78RV	missense_variant c.586G > A p.Glu196Lys	LasR-defective
PA-CL509	missense_variant c.668C > T p.Ser223Phe	LasR-defective
PA-CL511	missense_variant c.668C > T p.Ser223Phe	LasR-defective
PA-CL512	missense_variant c.668C > T p.Ser223Phe	LasR-defective
PA-CL521a	frameshift_variant c.100_112delAAGATCCTCTTCG p.Lys34fs	LasR-defective
PA-CL521b	frameshift_variant c.100_112delAAGATCCTCTTCG p.Lys34fs	LasR-defective
PA-CL522b	missense_variant c.620A > T p.Asn207Ile	LasR-defective
PA-CL523b	missense_variant c.620A > T p.Asn207Ile	LasR-defective
PA-CL524	missense_variant c.620A > T p.Asn207Ile	LasR-defective
PG201	stop_gained c.119 T > A p.Leu40*	LasR-defective

[a]Functional LasR classification includes HAQ-neg isolates, which produced AHLs.
*stop codon.

RhlR-dependent variables among the subgroup of isolates belonging to the LasR-defective group (Fig. 3). Since our clustering key based on HHQ, PQS, HQNO and PYO was not developed to reveal these special LasR-defective strains, we needed to include additional quantitative phenotypes that would be features of RAIL strains. First, since RhlR is a direct activator of the *rhlAB* operon responsible for the production of rhamnolipids in *P. aeruginosa*, we chose to measure the activity of a *rhlA-gfp* reporter in these isolates, since it was used to discriminate RAIL isolates before (Feltner *et al*., 2016). RhlR also positively regulates the *phzA1* operon, which is responsible for the production of phenazines such as pyocyanin. As a second variable, we also measured

pyocyanin levels at the early stationary phase based on the hypothesis that isolates by-passing the traditional LasR-dependent route for the activation of the *rhl* system, and thus phenazine production, would be able to produce pyocyanin earlier than typical LasR-deficient strains. We used the previously well-characterized RAIL strain E90 as a reference (Cruz *et al*., 2020), and performed a clustering analysis. We found that more than half of the LasR-deficient isolates (those studied in Figs 2 and 3) probably retain LasR-independent RhlR activity (Fig. 4 and Table S3). As previously reported, isolates in this cluster indeed showed higher activation at 30 h growth in King's A medium of the *rhlA-gfp* reporter compared to their LasR-defective counterparts (Table S3) (Feltner *et al*., 2016; Cruz *et al*., 2020). The PCA analysis confirmed that this variable was the most determinant for the classification of the strains but that the earlier time point for pyocyanin was not impactful (Fig. 4).

Discussion

Our knowledge on the prevalence of LasR-defective *P. aeruginosa* isolates is essentially based on clinical samples, mostly from CF or other chronic disease patients (D'Argenio *et al*., 2007; Hoffman *et al*., 2009; Ciofu *et al*., 2010; Feltner *et al*., 2016; Hammond *et al*., 2016). It was suggested that adaptation to the CF lungs selects for mutations disturbing LasR activity, resulting in loss of acute virulence factors during chronic infections (D'Argenio *et al*., 2007; Hoffman *et al*., 2010). Mutants in *lasR* have been associated with CF lung disease progression (Hoffman *et al*., 2009; LaFayette *et al*., 2015; Winstanley *et al*., 2016). Still, one study found that LasR-defective isolates were also quite common in severe corneal ulcers, an acute infection and they were inferred to have an environmental origin (Hammond *et al*., 2016). In addition, subpopulations of LasR-defective mutants have been selected *in vitro* during controlled evolution experiments under conditions that require QS, such as swarming motility, growth as a biofilm and in minimal media with casein as the sole carbon source (Sandoz *et al*., 2007; Azimi *et al*., 2020; Robitaille *et al*., 2020) or in a murine chronic wound model (Vanderwoude *et al*., 2020). Thus, the adaptive pressure for loss of LasR function remains to be clearly defined.

In the present study, we used phenotypic profiling based on the quantification of QS-dependent extracellular molecules to evaluate the prevalence of LasR-defective *P. aeruginosa* strains in environments other than clinical. Isolates were recovered from various environments in Canada and Ivory Coast such as oil-contaminated soils, plumbing systems from hospitals as

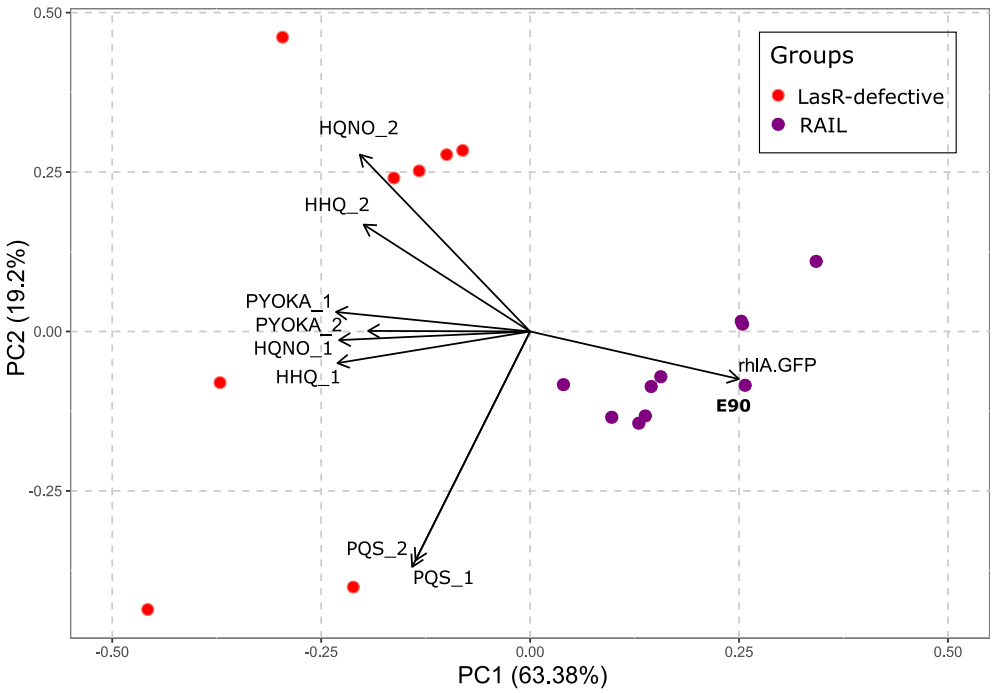

Fig. 4. Principal component analysis elaborated with *Pseudomonas aeruginosa* extracellular metabolites analysed from cultures of LasR-defective isolates shown in Fig. 3. Concentrations of HHQ_1 and 2, HQNO_1 and 2, PQS_1 and 2 sampled at 6 and 24 h respectively. Pyocyanin (PYOKA_1 and PYOKA_2) was sampled at 6 and 24 h respectively. Fluorescence (RFU/OD_{600}) from a *rhlA-gfp* reporter was measured at 30 h. RhlR Active Independently of LasR (RAIL) strain E90 is included as a reference. Groups represent clusters based on a dendrogram elaborated with the same variables (data not shown). Data are listed in Table S3.

well as food products from public markets. A LasR-defective strain is one that is deficient in its ability to perform LasR-dependent regulatory activity. Since a LasR-defective profile is not necessarily the consequence of a mutation in the *lasR* gene itself but could also be caused by variations in regulatory elements, we hypothesized that this method would allow for a more accurate and less biased estimation. Indeed, although we did find a good correlation between our classification scheme and the presence of single nucleotide polyphormisms (SNPs) in the *lasR* gene (Table 1), a SNP was found in the sequence of the *lasR* gene of strain 34JS while this isolate nevertheless has a functional LasR. LasR variations were found in isolates with residual LasR activity in a previous study (Feltner *et al*., 2016). Unexpectedly, removing 3-oxo-C_{12}-HSL from the list of variables based on the PCA analysis was supported by the fact that analyses relying only on AHLs or on HAQs and pyocyanin led to dendrogram topologies like the ones obtained with all variables. The main difference was the uncovering of a cluster containing HAQ-negative isolates when HAQs are used among variables instead of only AHLs. Not surprisingly, considering the diversity of strains investigated, a few outliers remain. Isolate DCB62 changes from the 'LasR-functional' cluster to the 'LasR-deficient' cluster when AHLs are not used as variables (Fig. S1). Our hypothesis is that this isolate overproduces pyocyanin (which is more associated with LasR-deficient strains under these conditions). Since it produces significant 3-oxo-C_{12}-HSL, it is likely LasR is functional in this strain. This is possibly also the case for isolates DCB114 and DCB118, which were classified as LasR-deficient in Fig. S2 but produce significant 3-oxo-C_{12}-HSL (Table S3). Their pyocyanin production is like most LasR-deficient isolates but a functional LasR could be present in these isolates. Still, these exceptions do not detract from the main conclusion, the prevalence of a defective LasR function among environmental strains based on quantifiable phenotypic traits, regardless of the presence of significant modifications in the sequence of the *lasR* gene.

Based on our analyses, a high proportion of environmental isolates are defective in their ability to perform LasR-dependent functions. This is the first time that there is a characterization of such a collection of environmental strains for the prevalence of defective LasR activity. We know only two other studies reporting the presence of a few LasR-defective strains in environmental settings: swimming pools and rivers (Cabrol *et al*., 2003) and dental waterlines units (Vincent *et al*., 2017). Interestingly, we noted that 23% (9/40) of the strains from the International *Pseudomonas aeruginosa* reference panel are *lasR*- mutants, including three that are not from CF origin (Freschi *et al*., 2018). Importantly, the percentage of first cultured *P. aeruginosa* isolates from CF patients that are *lasR* mutant is around 20%–25% (Mayer-Hamblett *et al*., 2014a; 2014b; Feltner *et al*., 2016). The high prevalence of environmental *lasR*-isolates found here raises a daring question: do these strains really mostly evolve *in vivo* (e.g. CF lungs) as currently thought or could have they been initially acquired by patients directly from the environment?

Very few studies investigated the production profile of HAQs by clinical isolates, but our work shows that it is a relevant indicator of LasR activity. Indeed, LasR is responsible for the activation of the *pqs* operon early during growth but it also controls the transcription of the *pqsH* and *pqsL* genes, which encodes for the enzymes responsible for the production of PQS and HQNO respectively. Interestingly, we uncovered the presence of several environmental *P. aeruginosa* isolates defective in the third QS system, mediated by the PQS signal. We did not find variations in the coding sequence of the *pqs* operon or in the *mvfR* (*pqsR*) gene in these strains. Using HAQs as variables to identify LasR-deficient isolates does not consider the presence of these HAQ-negative isolates. This was an unexpected outcome of this method. We were surprised to find this many in our collection, and the available literature would not have predicted such finding. Using only AHL quantification would not have allowed us to identify the presence of such isolates and studying how this impacts their QS regulation is of great interest. Looking at the production of 3-oxo-C_{12}-HSL becomes then the evident way to confirm the functionality of LasR in these special isolates. This can then be done on a smaller set of isolates (only the HAQ-negative ones) after the first screen using our global approach rather than measuring all variables, for example, on the whole set of 176 strains. Investigations on why these isolates are unable to produce HAQs under our conditions are on-going. Of note, some of these isolates produced significant levels of pyocyanin in King's A media, which is surprising since a defect in the *pqs* operon should affect the function of PqsE, a protein essential for full activity of RhlR, the principal activator of pyocyanin production (Groleau *et al*., 2020). We previously observed a few such strains producing no HAQs but still able to produce pyocyanin, among CF isolates (Feltner *et al*., 2016). It will be interesting to investigate whether there is an active PqsE in these strains.

RhlR activity is required by *P. aeruginosa* to upregulate functions important for survival in various environments. Our identification of several RAIL strains in our environmental collection highlights the relevance of recent studies where such strains with a rewired QS circuitry have been investigated (Feltner *et al*., 2016; Chen *et al*., 2019; Cruz *et al*., 2020). This can be visualized, for instance, in the early production of 3-oxo-C_{12}-HSL in isolate 78RV, which we identified as a RAIL strain that we presume

may be the result of an RhlR-driven activation of *lasI* transcription (Fig. 1).

The expression of RhlR independent of prior LasR activation is forcing us to reconsider the archetypal hierarchy of the *P. aeruginosa* QS cascade. Since full regulation of RhlR-dependent QS depends on PqsE in reference strains such as PA14 (Groleau *et al*., 2020), it will be interesting to study this interaction in RAIL and HAQ-negative isolates from various environments. The presence of these RhlR-active isolates might explain why some phenotypes such as protease production might not be consistent between LasR-defective strains. For example, many isolates produced no AHL but were still able to grow on casein agar (Tables S2 and S3).

The prevalence of LasR-deficient isolates was high. While we cannot exclude the possibility that a few isolates coming from the same environment are clonal, which would lead to some over-evaluation, this does not alter our conclusion that LasR-defective strains are neither atypical nor restricted to clinical isolates. Studies on the actual prevalence of LasR-deficient strains depending on the type of environments will be interesting to uncover the selective pressure for the loss of this regulatory function. Clearly, our understanding of *P. aeruginosa*, after all a saprophytic species, is biased by an emphasis on studying clinical isolates. For instance, *P. aeruginosa* also forms small colony variants under specific conditions using phase variation as an adaptation mechanism. This ability was long associated with clinical isolates, but it was found to be conserved among environmental strains as well (Besse *et al*., 2021).

Loss of LasR function and HAQ production, as well as independent upregulation of RhlR activity, appear to be features that are beneficial to *P. aeruginosa* as it adapts to many environmental conditions, not just those of chronic infections. This challenges our traditional view of the QS circuitry of this versatile bacterium and should be kept in mind when devising approaches to control the virulence and response of this opportunistic pathogen.

Experimental procedures

Strains and growth conditions

The 174 isolates included in the analysis were collected in Canada and Ivory Coast. In Canada, samples were collected from an oil-contaminated sand pit (17 isolates) (Déziel *et al*., 1996) and from the environment (tap aerator, drain and sink surface) of 18 sinks from nine different rooms in a hospital (37 isolates) (Lalancette *et al*., 2017), both in the Montreal region. In Ivory Coast, samples were collected from bovine meats, fresh or smoked fish and their direct environment (stalls and cutting boards) in five different public markets in Abidjan (120 isolates) (Benie *et al*., 2017). We also included isolates PUPa3 and PG201 isolated from soils in India and Switzerland respectively (Guerra-Santos *et al*., 1984; Kumar *et al*., 2005). A detailed list of the isolates and their origin is presented in Table S1. Bacteria were routinely grown in Lysogeny broth (LB) (AlphaBiosciences, Baltimore, MD, USA) or TSB (BD Difco) at 37°C in a TC-7 Roller Drum (New Brunswick) at 150 r.p.m. Reference strains were PA14 (Rahme *et al*., 1995), its isogenic *lasR*::Gm mutant (Déziel *et al*., 2004) and clinical *lasR* mutant isolate E90 (Feltner *et al*., 2016).

Phenotypical assays

For pyocyanin measurements, bacteria were grown in King's A broth with 10 μM of FeCl3 (King et al., 1954) for 18 h at 37°C. Briefly, an overnight culture in TSB was diluted to an OD_{600} of 0.01. At selected times during growth, a sample of the culture was centrifuged (10,000 × *g*, 15 min). The supernatant was transferred to a 96-well plate and the OD_{695} was measured using a Cytation microplate reader (BioTek, Winooski, VT, USA). Since clumping of cells was apparent in cultures of many strains, we assessed growth using total protein quantification of the cell pellet harvested from the whole culture at the time of sampling. The cell pellets were suspended in 0.1 N NaOH and heated at 70°C for 1 h with frequent vortexing. Protein concentrations were determined using the Bradford quantification assay (Bio-Rad, Montreal, Canada) with bovine serum albumin as a standard.

To detect iridescence and autolysis of colonies, bacteria were grown on LB agar plates for 24 h at 37°C and colonies were visually inspected for the appearance of a metallic sheen (iridescence) and the presence of autolysis zones.

Protease production was ascribed to strains displaying the ability to grow on casein as a sole carbon source. Bacteria were grown overnight in LB broth. Five microliters of culture were deposited on the surface of M9 media agar containing 0.1% sodium caseinate as sole carbon source. The plates were incubated at 37°C for 24–48 h before visual inspection.

Quantification of QS signalling molecules

Concentrations of HAQs and AHLs in cultures were measured for bacteria grown in King's A medium. Based on their production profile, concentrations of 3-oxo-C_{12}-HSL, C_4-HSL were measured at 3 h (1) and 6 h (2) time points. The main C_7 HAQ congeners HHQ, HQNO and PQS were measured at 6 h (1) and 24 h (2). Briefly, an equal volume of methanol containing internal standard tetradeuterated 4-hydroxy-2-heptylquinoline (HHQ-d4) was added to the culture sample. The suspension was

vortexed and centrifuged for 5 min at maximum speed to remove bacteria. The resulting supernatant was transferred in vials for liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analyses, as previously reported (Lépine *et al*., 2018) For quantifications of AHLs, ethyl acetate extracts were analysed by LC/MS/MS as described (Lépine *et al*., 2018). The cell pellets were suspended in 0.1 N NaOH and heated at 70°C for 1 h with frequent vortexing. Protein concentrations were obtained using the Bradford quantification assay (Bio-Rad). Production (mg l^{-1}) is presented as ratios over biomass (μg ml^{-1} of proteins).

Measurement of the expression of rhlA-gfp

Bacteria were transformed by electroporation with plasmid pJF01 containing the promoter region of the *rhlA* gene in front of the *gfp* reporter gene (Feltner *et al*., 2016). Transformants were selected on 30 μg ml^{-1} of gentamicin. To evaluate the expression of the *rhlA-gfp* reporter, bacteria were grown overnight in TSB containing 30 μg ml^{-1} gentamicin. After three washes with phosphate-buffered saline (PBS), cells were suspended in King's A media and transferred in a 96-well microplate. The plate was incubated for 30 h at 37°C with shaking. Fluorescence was measured (excitation: 489 nm and emission: 520 nm) using a Cytation microplate reader (BioTek). Relative fluorescence units data were normalized by the OD_{600}.

Sequence analyses

The genomic sequences of 45 of our environmental *P. aeruginosa* strains available in GenBank (Table S4) were compared to the reference strain UCBPP-PA14 (GenBank: CP000438.1) using snippy version 4.6.0 (https://github.com/tseemann/snippy).

Statistical analyses

Statistical analyses were performed using the software R (Team, 2018). Pairwise comparison of phenotypic profiles was expressed under the Euclidean distance units computed on Hellinger-transformed molecule concentrations matrix. Distance matrix was represented with dendrogram elaborated with agglomerative hierarchical clustering based on the Ward's linkage method. Cluster stability was assessed by bootstrap resampling method (Hennig, 2007) implemented in the package 'fcp'. The heatmap, the dendrogram were done with the package 'ggplot2' (Wickham, 2009). The most distinctive variables among stains were identified with a PCA. All raw data used for clustering analyses are found in Table S3.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of isolates used in this study and their origins.

Table S2. Phenotypical characteristics of isolates and reference strains.

Table S3. AHLs, HHQ, HQNO and PQS and pyocyanin quantifications. All data is concentration in mg l^{-1} normalized by total protein concentration (μg ml^{-1}) in sample. Expression of *rhlA-gfp* reporter (RFU/OD600).

Fig. S1. Clustering of LasR-defective isolates using only AHL variables. A cluster dendrogram was performed on a subset of isolates using the following variables: C4-HSL_1 and 2 and 3-oxo-C12-HSL_1 and 2 were sampled at 3 and 6 h. Values

are listed in Table S3. Robustness (R) represents the proportion of clustering runs in which a pair of isolates appeared together in some cluster, given that they were clustered together in at least one run, averaged over all such pairs. R: ****> 90%, ***80%–89%, **70%–79%.

Fig. S2. Clustering of LasR-defective isolates. A cluster dendrogram was performed on 176 isolates using chosen variables HHQ_1 and 2, HQNO_1 and 2, PQS_1 and 2 and pyocyanin (PYOKA_2). Concentrations of HHQ_1 and 2, HQNO_1 and 2, PQS_1 and 2 sampled at 6 and 24 h. Pyocyanin (PYOKA_2) was sampled at 24 h. Values are listed in Table S3. Robustness (R) represents the proportion of clustering runs in which a pair of isolates appeared together in some cluster, given that they were clustered together in at least one run, averaged over all such pairs. R: ****> 90%, ***80%–89%, **70%–79%.