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Implementation of an adaptive laboratory evolution strategy for improved production of valuable microbial secondary metabolites

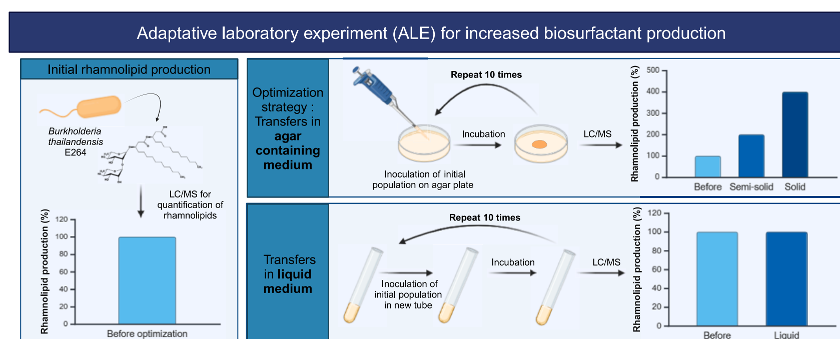
Sarah Martinez, David N. Bernard, Marie-Christine Groleau, Mylène C. Trottier, Eric Déziel *

Centre Armand-Frappier Santé Biotechnologie, Institut National de la recherche Scientifique (INRS), Laval, Québec, Canada

HIGHLIGHTS

- Biosurfactants are coveted products, but optimization is needed, as yields remain low.
- Low rhamnolipid biosurfactant production by promising *Burkholderia thailandensis.*
- Challenging to promote higher production of non-essential secondary metabolites.
- Establishment of a directed evolution strategy to select overproducing mutants.
- Convergent evolution reveals QsmR as a repressor of rhamnolipids production.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial surface-active agents, such as rhamnolipids, represent an attractive substitute for synthetic surfactants. However, current production bioprocesses are generally inefficient. Adaptive laboratory evolution strategies could offer a promising avenue to improve secondary metabolites production. In the bacterium *Burkholderia thailandensis*, the social behaviour called swarming motility relies on biosynthesis of rhamnolipids. Since experimental swarming requires lower agar concentrations, we hypothesized that augmenting the agar concentration would constrain the cells to produce more rhamnolipids. Consecutive rounds of *B. thailandensis* cultivation on swarming media performed with increasing agar concentrations enhanced rhamnolipid production by the evolved populations, with a correlation between rhamnolipid production and agar concentrations. Whole-genome sequencing of superior producing evoluants revealed inactivating mutations in *qsmR*, which codes for a transcriptional regulator not known to influence rhamnolipid production. Results indicate that QsmR represses rhamnolipid biosynthetic genes transcription. The developed directed evolution strategy could be used to improve biosurfactant yields with other producing bacteria.

* Corresponding author at: INRS - Armand-Frappier Santé Biotechnologie, 531, Boul. des Prairies, Laval, Québec, H7V 1B7, Canada.
E-mail address: eric.deziel@inrs.ca (E. Déziel).

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1. Introduction

A successful bioeconomy relies on efficient bioprocesses to provide sustainable alternatives to the production of needed chemicals. Surfactants are widely used in several fields, such as pharmaceutical, personal care, agricultural and food industries (Singh et al., 2007). Typically derived from petrochemical processes, synthetic surfactants are of concern because of their poor biodegradability, significant environmental toxicity, and non-sustainable production. Therefore, intense investigations are focused on surface-active molecules of biological origin, which represent an interesting and greener alternative to synthetic ones. Microbial surfactants have many advantages due to their biodegradability, low toxicity, and ability to be produced from renewable resources of raw material (Dias & Nitschke, 2023; Pardhi et al., 2022; Santos et al., 2016). Identified for the first time in 1949 from cultures of the bacterium *Pseudomonas aeruginosa* (Jarvis & Johnson, 1949), rhamnolipids are among the best-studied and more widely used biosurfactants (Abdel-Mawgoud et al., 2010; Nitschke et al., 2005). However, since *P. aeruginosa* is an important opportunistic pathogen, alternatives based on non-pathogenic bacteria are actively sought to develop new industrial processes for rhamnolipid production. We previously reported the capacity of the β-proteobacteria *Burkholderia thailandensis* to produce rhamnolipids (Dubeau et al., 2009). This non-pathogenic bacterium is increasingly considered a promising candidate for the commercial production of rhamnolipids (Blunt et al., 2023; Funston et al., 2016; Irorere et al., 2018; Kourmentza et al., 2018; Kumar et al., 2023). However, current processes for rhamnolipid production using this microorganism require important improvements to provide better yields.

Besides traditional approaches such as culture conditions optimization methodologies and screenings for more productive strains, modification of the genetic makeup of microorganisms can immensely increase the production of secondary metabolites through altered regulation of biosynthetic genes or redirection of metabolic fluxes. Random mutagenesis methodologies are frequently used to obtain derived strains with a desired phenotype and learn more about genetic factors involved in the production of specific metabolites (Barquist et al., 2013). For instance, we have exploited a transposon mutagenesis approach to isolate mutants of *B. thailandensis* with increased rhamnolipid production and identified the transcriptional regulator ScmR as a repressor of rhamnolipid biosynthesis (Martinez et al., 2020). However, such an approach remains conservative and labor-intensive. An emerging approach is the use of directed evolution, a process by which natural selection is applied through experimental pressure to improve specifically a phenotype of choice, linked to genomic alterations (Chou & Keasling, 2013; Cooper, 2018). While adaptive laboratory evolution has great potential to optimize the productivity of metabolites of interest, it is challenging to identify selective conditions for non-essential functions, such as production of secondary metabolites, because increased fitness of evolved mutants is indispensable (Dragosits & Mattanovich, 2013; Lee & Kim, 2020; Mavrommati et al., 2022).

Like *P. aeruginosa*, *B. thailandensis* can perform swarming motility (Dubeau et al., 2009), a social surface behaviour requiring both a functional flagellum and production of a surface-wetting agent, such as a surfactant (Kearns, 2010). Swarming motility is an important trait expressed by some bacteria under specific conditions, typically culture media solidified with lower than usual agar concentrations. In a past study, we showed that a swarming-impaired mutant of *P. aeruginosa* rapidly accumulated compensatory mutations that allowed for recovery of the swarming phenotype after being subjected to directed evolution (Robitaille et al., 2020). Also, repeated transfer rounds of swarming *P. aeruginosa* cells drove remarkable parallel evolution towards a hyperswarmer phenotype (Van Ditmarsch et al., 2013).

Production of a wetting agent such as rhamnolipids reduces friction between the surface and the bacteria, promoting spreading over the semi-solid agar surface (Déziel et al., 2003; Dubeau et al., 2009; Nickzad et al., 2015). We hypothesized that surfactant-dependant swarming motility could be used to identify key factors controlling rhamnolipid production.

Higher hardness, i.e. agar concentration, of the surface has a negative impact on swarming motility (Deforet, 2023; Kamatkar & Shrout, 2011; Tremblay & Déziel, 2008). Under laboratory conditions, swarming is typically seen on plates solidified with only 0.5 % of agar. Based on the importance of swarming motility for colony spreading on a surface and the need for rhamnolipid production to maintain this trait, we postulated that gradually increasing the agar concentration would constitute a selective pressure constraining the cells to produce more rhamnolipids to achieve surface wetting, and thus effective swarming colony spreading. In this study, we show that this strategy successfully allowed to select rhamnolipid-overproducing mutants and constitutes a promising novel approach to select bacteria with augmented production of secondary metabolites, such as biosurfactants.

2. Materials and Methods

2.1. Bacterial strains and plasmids

The whole-genome sequenced type strain *B. thailandensis* E264 was used for this work. Bacterial strains and plasmids used in this study are presented in Table 1. All strains were stored in 15 % glycerol at −80 °C and plasmids were stored at −20 °C.

2.2. Media and growth conditions

Bacteria were routinely grown from −80 °C frozen stocks by culturing in tryptic soy broth (TSB) (BD Difco, Mississauga, Ont., Canada). Cultures were incubated at 37 °C under rotation at 240 rpm in a TC-7 roller drum (New Brunswick, Canada), or on TSB plates solidified with 1.5 % agar. For rhamnolipid production, nutrient broth (NB) medium (BD Difco, Mississauga, Ont., Canada) supplemented with 4 % (w/v) glycerol was used. When necessary, antibiotics were included at the following concentrations: 10 μg ml^{-1} tetracycline (Tc), and 100 μg ml^{-1}

Table 1
Strains and plasmids used in this study.

Strains	Characteristic	Reference
Burkholderia thailandensis E264	Wild-type strain	(Brett et al., 1998)
qsmR-1 mutant BT09539	Transposon mutant in BTH_II2865 in *B. thailandensis*, Tp^R E264 qsmR-135::ISlacZ-PrhaBo-Tp/FRT	(Gallagher et al., 2013)
BTH_II2866 mutant BT02189	Transposon mutant in BTH_II2866 in *B. thailandensis* E264 BTH_II2866-177:: ISlacZ/hah-Tc	(Gallagher et al., 2013)
BTH_II2867 mutant BT11163	Transposon mutant in BTH_II2867 in *B. thailandensis* E264 BTH_II2267-155::ISlacZ-PrhaBo-Tp/FRT	(Gallagher et al., 2013)
BTH_II2868 mutant BT08158	Transposon mutant in BTH_II2868 in *B. thailandensis* E264 BTH_II2268-194::ISlacZ-PrhaBo-Tp/FRT	(Gallagher et al., 2013)
Plasmids		
pME6000	Broad-host-range cloning vector; Tc^R	(Maurhofer et al., 1998)
pSM1	*qsmR* gene inserted in *Bam*HI-*Hin*dIII sites in pME6000; Tc^R	This study
pAH5	P*rhlA1-lacZ* transcriptional reporter; Tp^R	(Martinez et al., 2020)
pAH8	P*rhlA2-lacZ* transcriptional reporter; Tp^R	(Martinez et al., 2020)

Tp: trimethoprim; Tc: tetracycline.

trimethoprim (Tp) for *B. thailandensis* and 15 μg ml^{-1} tetracycline (Tc), and 100 μg ml^{-1} trimethoprim (Tp) for *Escherichia coli.*

2.3. Directed evolution experiments

Plates used for the experimental evolution experiments were prepared as follows: 20 mL of freshly autoclaved medium consisting of NB supplemented with 0.5 % dextrose (Fisher) and one of three different concentrations (0.5 %, 1.5 % or 2.5 %) of Bacto-agar (Difco) were poured into standard 100 mm x 15 mm polystyrene Petri dishes and then dried in a laminar flow hood for 30 min (Lai et al. 2009). Immediately following the drying period, plates were inoculated at their center with 5 μL of bacterial culture at an $OD_{600} = 3.0$, then incubated at 30 °C for 16 h in plastic bags, to prevent drying. This initial culture was obtained by growing E264 in 3 mL NB + 4 % glycerol medium overnight. After 16 h, the culture was diluted at $OD_{600} = 0.1$ in new tubes containing the same medium and cultured until the OD_{600} reached 3.0 (Nanodrop ND-1000, Thermo Fisher Scientific).

Every 16 h, each colony that had grown on the various plates was completely harvested with a swab and resuspended in phosphate-buffered saline (PBS). OD_{600} was adjusted to 3.0 and new swarming plates were inoculated as specified above. Identical parallel passages were also performed as liquid controls in culture tubes under the same conditions but without agar. This procedure was repeated daily for 10 days (Fig. 1). Samples of cell suspensions from each passage were stored in 15 % glycerol at −80 °C. Three replicates (lineages) were performed for every assay and the experiments were performed three times, independently.

2.4. Rhamnolipid quantification

Rhamnolipid were quantified in culture supernatants after 5 days by liquid chromatography coupled to tandem mass spectrometry, as previously described (Dubeau et al., 2009), with some modifications (Martinez et al., 2020).

2.5. DNA extraction and whole genome sequencing

Genomic DNA of rhamnolipid-overproducing bacteria was extracted with the Purelink genomic DNA kit (Invitrogen, Life Technologies, Burlington, Ont., Canada). Purity controls were confirmed by UV spectrophotometric analyses and DNA quantification was verified on a Corbett Life Science Rotor-Gene 6000 thermal cycler using the Quant-iT™ PicoGreenR dsDNA Assay Kit (Invitrogen, Life Technologies, Burlington, Ont., Canada), according to the manufacturer's protocol. Samples and reference strains were sent to McGill University and Génome Québec Innovation Centre (Montreal, Qc, Canada) for whole genome sequencing. Libraries were created and modified using the Illumina Paired-End Sample Prep Kit and sequenced on the Illumina MiSeq platform (Illumina, Inc.).

2.6. Data analysis

Quality of raw data was assessed, reads were quality trimmed and downstream analysis was carried out using the Galaxy platform (Goecks et al., 2010). Reads were assembled and mapped to the E264 wild-type strain reference genome, and SNPs, insertions and deletions were determined using Lasergene (DNASTAR).

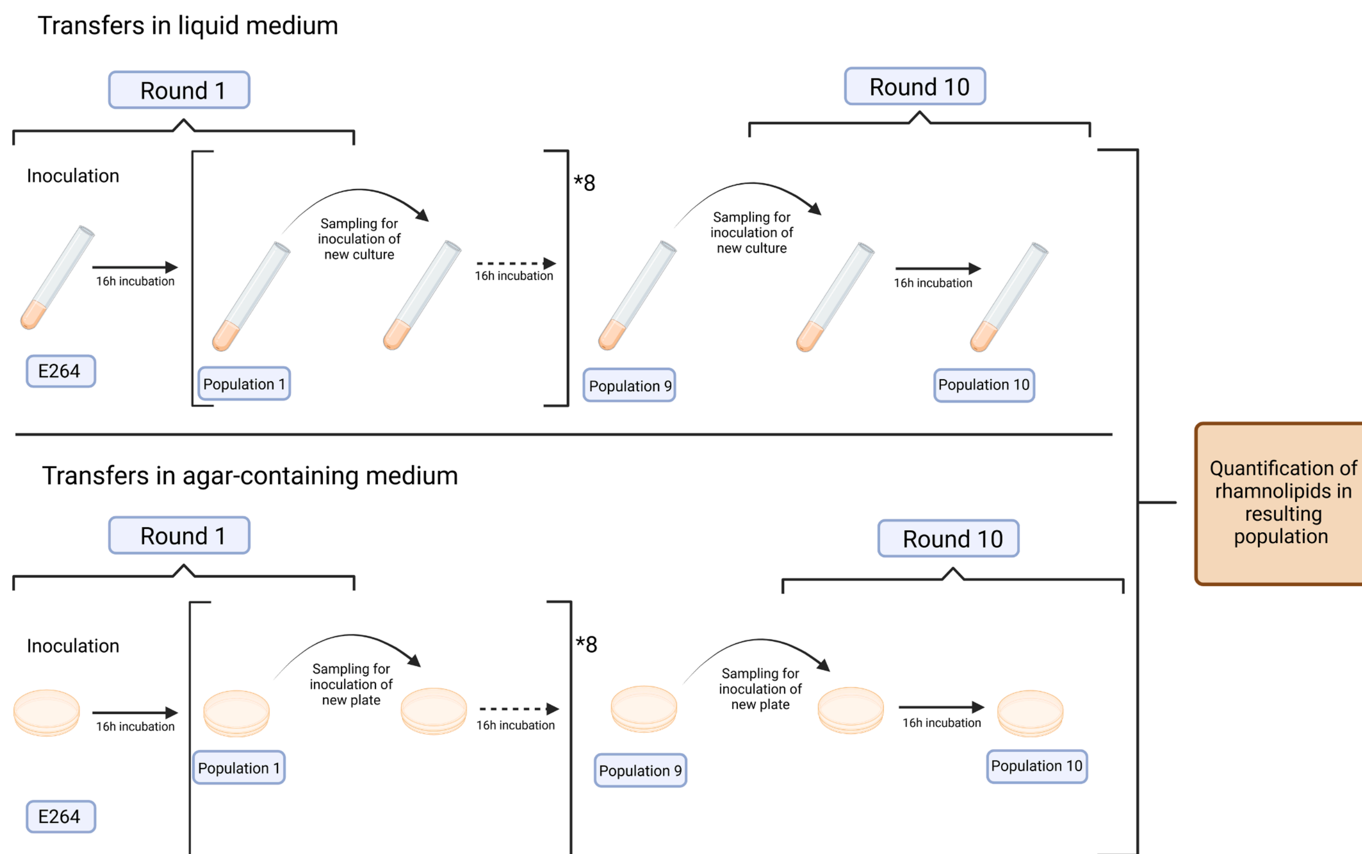


Fig. 1. Experimental strategy for transfers in liquid medium or agar containing medium. Different agar concentrations were tested: 0.5%, corresponding to the standard condition for swarming motility assays, 1.5% and 2.5%. Liquid transfers were performed in parallel as non-swarming conditions controls. After the 10 transfers, resulting populations were recovered and cultured in liquid media for quantification of rhamnolipid production.

2.7. Vector construction

For construction of the *qsmR* complementation plasmid pSM1, the *qsmR* gene (locus BTH_I2865) was amplified by PCR from *B. thailandensis* E264 genomic DNA using primers Thai-complementation_qsmR-F (5′- CGGGGTACCCAAAACGCGCA-CACTTTTCC −3′) and Thai-Complementation_qsmR-R (5′-CGCGGATCCTATTTCACCGGGCGCGATTC −3′) and cloned inside the predigested *Bam*HI and *Hin*dIII sites of the pME6000 vector (Table 1). The construct was transformed in *E. coli* DH5α and tetracycline was used for selection. The pSM1 plasmid was transferred in the *qsmR-1* mutant (BT09539) by electroporation.

2.8. β-galactosidase activity assays

Plasmids carrying *lacZ* transcriptional fusions to both *rhl* operons promoter regions pAH5 (promoter region upstream of BTH_II1075 [*rhlA1*]) and pAH8 (promoter region upstream of BTH_II1881 [*rhlA2*]) were independently transferred in the *qsmR-1* mutant (BT01082) by electroporation (1,800 V). β-galactosidase assays were performed as described (Miller, 1972), with the following modification: normalization of the activity was achieved by correcting for viable bacterial cells (as CFU/mL) instead of OD_{600}, as we have shown that cell density measurements are sometimes misleading with *B. thailandensis* (Martinez & Déziel, 2020).

2.9. RT-qPCR assays

Total RNA of *B. thailandensis* E264 and *qsmR* mutant L2C1 (from directed evolution) cultures at 24 h, 72 h and 96 h was extracted as previously described (Le Guillouzer et al., 2017). cDNA synthesis was performed using the iScript reverse transcription supermix (Bio-Rad Laboratories), and amplification was accomplished as described (Le Guillouzer et al., 2017). The reference gene was *ndh* (Subsin et al., 2007). The *ndh* gene displayed stable expression under the different tested genetic contexts. All primers used for cDNA amplification are presented in Table 2. Differences in gene expression between *B. thailandensis* E264 strains were calculated using the $2^{-\Delta\Delta CT}$ formula (Livak & Schmittgen, 2001). A threshold of 0.5 was chosen as statistically significant.

3. Results

3.1. Surface tension favors rhamnolipid production of a Burkholderia thailandensis population

Innovative strategies are needed to develop bacteria with an improved biosurfactant productivity. A directed evolution strategy was established to obtain evolved clones of *B. thailandensis* producing more rhamnolipids than the parental strain E264. Three independent lineages initiated from the common ancestor were treated to 10 consecutive cultivation rounds on swarming plates with higher agar concentrations. After ten rounds, rhamnolipid production by the evolved whole populations was quantified from liquid cultures using liquid chromatography coupled to mass spectrometry (LC/MS) analysis. Results were compared to rhamnolipid production from the ancestral E264 strain and to control populations parallelly transferred ten times in liquid cultures of the same media. Fig. 2 shows that the control population obtained after 10 passages in liquid media produced concentrations of rhamnolipids similar to the ancestral wild-type strain. In contrast, rhamnolipid production was increased in cultures from the evolved populations developed following the transfers on agar plates. The higher the agar concentration used for the selective plates, the greater the rhamnolipid production from the respective evolved populations was, until a plateau was reached at 1.5 % agar. These results suggest that mutants producing more rhamnolipids than the ancestral strains were enriched in the evolved populations.

3.2. Rhamnolipid-overproducing mutants selected in the evolved population

The whole evolved populations from each lineage, obtained after 10 rounds on 1.5 % agar plates, were plated to obtain isolated colonies. Thirty clones, ten from each lineage, were randomly selected and tested for their rhamnolipid productivity by LC/MS. Several of these clones indeed produced significantly more rhamnolipids than the ancestral wild-type strain (Fig. 3). Out of the 30 clones characterized, 13 produced at least three times more rhamnolipids than the wild-type E264, culminating with clone C5 from lineage 3, which produced seven times more rhamnolipids than the ancestral strain. These results support our hypothesis that using the ability to swarm on higher concentrations of

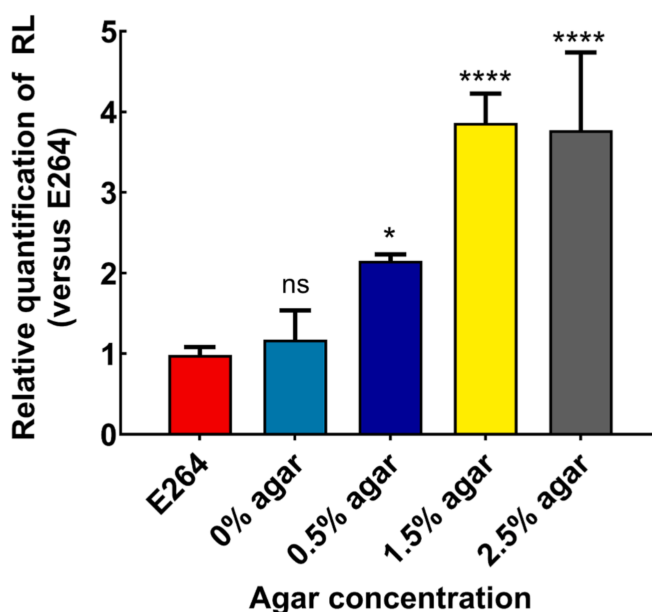


Fig. 2. *Burkholderia thailandensis* populations adapted to grow and migrate by swarming motility on culture plates with higher agar concentrations produce superior levels of rhamnolipids. Ancestral wild-type E264 strain and resulting evolved populations from the tenth round of selection on each concentration of agar contained in the medium were cultured for 5 days in NB with 4 % glycerol to assess rhamnolipid production. The error bars represent standard deviation from the mean ($n = 3$ independent cultures, each derived from one of the three independent lineages). Data analysed using a one-way ANOVA with post hoc. Dunnett's multiple comparisons tests (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns = not significant).

Table 2
Primers used for qRT-PCR.

Genes	Oligonucleotides	Sequences (5′to 3′)	Reference
ndh	SLG_qRT-PCR_ndh_F	ACCAGGGCGAATTGATCTC	(Le Guillouzer et al., 2017)
	SLG_qRT-PCR_ndh_R	GATGACGAGCGTGTCGTATT	
BTH_I2866	SM_qrtPCR_2866_fwd	CTCGACCTGAAGCGCTATCA	This study
	SM_qrtPCR_2866_rev	GTAGGCGAGATTGAGCTTCG	

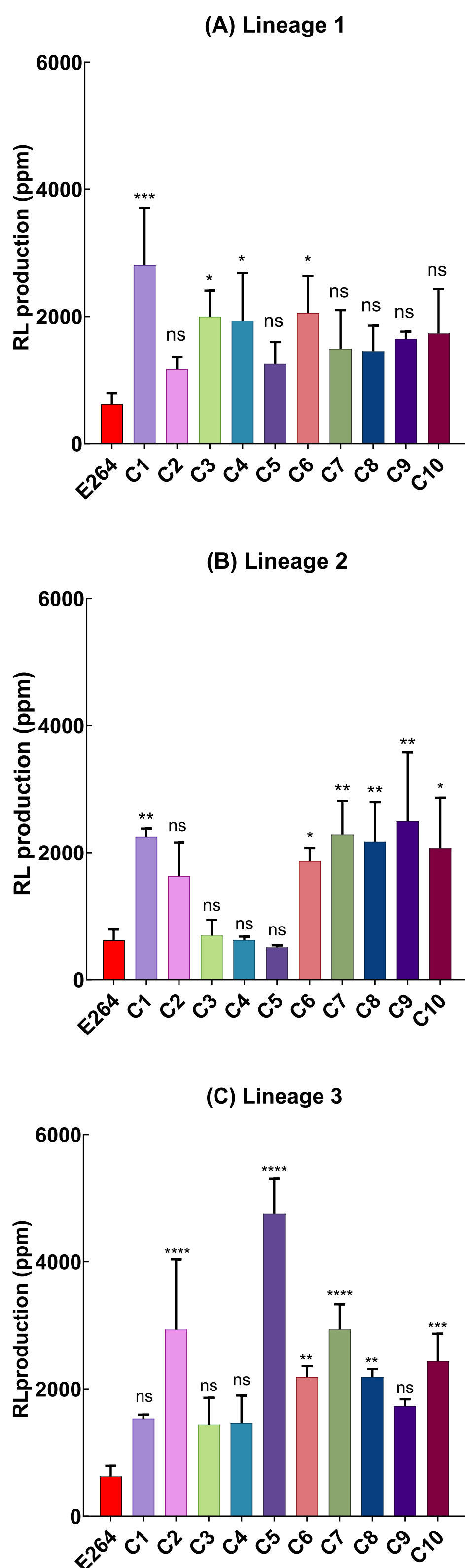


Fig. 3. Rhamnolipid (RL) production by clones selected from *B. thailandensis* populations evolved on 1.5 % agar plates. (A) lineage 1, (B) lineage 2, (C) lineage 3. Rhamnolipid production was determined by growing the clones for 5 days in NB with 4 % glycerol. Experiments were performed in triplicates. The error bars represent standard deviation from the mean ($n = 3$ independent cultures), Data analysed using a one-way ANOVA with post hoc. Dunnett's multiple comparisons tests (****$p < 0.0001$, *** $p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns = not significant).

agar as a selective pressure forced the bacteria to adapt to maintain this trait by producing more rhamnolipids.

3.3. Whole genome sequencing reveals selection for mutations in the qsmR gene

Among the above 30 isolated clones, we selected two of the best-producing clones from each lineage for whole genome sequencing. To identify genetic changes linked with the adaptive phenotype, we compared sequences with the ancestral wildtype E264 strain. Interestingly, three of the six sequenced clones carried mutations into the BTH_I2865 locus (Table 3). In fact, clones C1 and C7 from lineage 2 carry the same single nucleotide polymorphism (SNP) at position 230 resulting in an L77P amino acid substitution, and clone 7 from lineage 3 (L3-C7) carries several alterations (SNP, deletions) resulting in a frameshift starting from amino acid 168. This gene codes for an IclR-type transcriptional regulator orthologous to the QsmR regulator of *Burkholderia glumae* (Kim et al., 2007). This regulator was previously studied in *B. thailandensis* as well (Goo et al., 2012; Majerczyk et al., 2014). Sequencing also revealed clones with mutations into the BTH_II0885 locus encoding a putative TetR family transcriptional regulator and into locus BTH_I2820 encoding a predicted dioxygenase. Finally, L3-C7 carrying a large mutation into *qsmR* also harbors a mutation into the BTH_II0864 locus, corresponding to the gene coding for the CplV-5 ATPase, part of the type VI secretion system. No relation with rhamnolipid production was previously noted for any of these genes. We concluded that the frequency of mutations in *qsmR* was evidence of strong selection for acquisition of an adaptive trait, presumably increased rhamnolipid production.

3.4. QsmR controls the production of rhamnolipids

Sequencing results indicated that three out of six clones presented alterations in the BTH_II2865 locus, coding for the QsmR transcriptional regulator. A BTH_II2865 mutant was acquired from the *B. thailandensis* transposon mutant library (Gallagher et al., 2013) and tested for swarming motility and rhamnolipid production. For this *qsmR-1* mutant, a hyperswarmer phenotype was obtained, as was also observed for the three *qsmR* mutants obtained from the evolution experiment (Fig. 4**A**). Accordingly, rhamnolipid production was increased in cultures of the *qsmR-1* mutant (Fig. 4**B**). Furthermore, rhamnolipid concentrations in *qsmR-1* mutant cultures were restored to wild-type levels when complemented with a *qsmR* expression vector, confirming results were indeed caused by the inactivating mutation in the *qsmR* gene (Fig. 4**B**).

To explore the impact of the evolved mutations on the integrity of the QsmR protein, we performed an alignment of the deduced protein sequences. Both C1 and C7 clones from lineage 2 carry the same single SNP (Table 1 and Fig. 5**A**). This mutation is located into a helix-turn-helix motif, a DNA-binding domain of ~ 60 amino acids typically present in IclR-type regulators (Molina-Henares et al., 2006). The replacement of a leucine by a proline (at position 77) into an alpha helix would prevent the formation of a hydrogen bond, destabilizing the DNA-binding site and thus potentially decreasing the affinity between the regulator and the target DNA (Fig. 5**B**). Clone 7 from lineage 3 harbors several mutations in the *qsmR* gene, including two nucleotide deletions which shift the reading frame during protein translation, thus causing massive disruption in the effector binding domain (Fig. 5**A**), involved in the binding of small effectors or signal molecules (Huang et al., 2012).

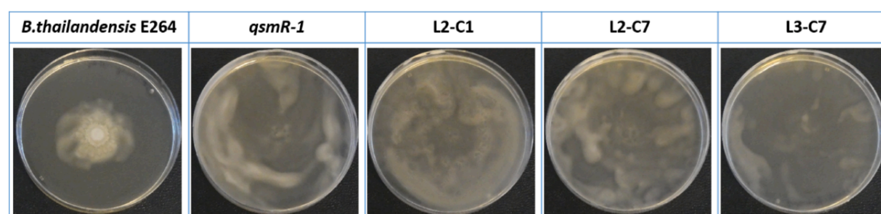
Goo et al. (Goo et al., 2012) showed that QsmR is required for oxalate production, which affects the pH of cultures. To verify if rhamnolipid overproduction is indirectly due to an effect of pH change, we monitored the pH in cultures of both the *qsmR-1* mutant and the wild-type strain under our culture conditions; pH profiles were similar for both strains (data not shown), confirming that overproduction of rhamnolipids was not related to a pH change but due to another mechanism regulated by QsmR.

Table 3
Mutations found in evolved clones of *B. thailandensis* E264 displaying increased rhamnolipid production.

Clone	Gene	Mutation type	Amino acid	Function
Lineage 1				
C1	BTH_I2820	Deletion (c.381_389delGCTCAACCG)	p.P127_R130delinsP	Carotenoid 9,10–9,10 cleavage dioxygenase
C3	BTH_I2820	Deletion (c.381_389delGCTCAACCG)	p.P127_R130delinsP	Carotenoid 9,10–9,10 cleavage dioxygenase
Lineage 2				
C1	BTH_I2865	SNP (c.230 T > C)	p.L77P	Transcriptional regulator QsmR
C7	BTH_I2865	SNP (c.230 T > C)	p.L77P	Transcriptional regulator QsmR
Lineage 3				
C5	BTH_II0885	Deletion (c.471_472delGC)	p.E157fs	TetR family transcriptional regulator
C7	BTH_I2865	SNP (c.495_496CC > TA) Deletion (c.498_500delCCG) Deletion (c.504delG)	P166T p.P166_R167delinsP p.S168fs	Transcriptional regulator QsmR
	BTH_II0864	Insertion (c.2872_2873insCCAACGCGC)	p.R958delinsPNAR	ClpV-5

*SNP = Single Nucleotide Polymorphism.

A



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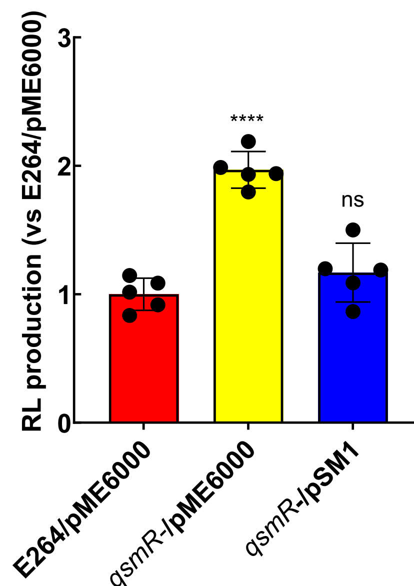

Fig. 4. Inactivation of QsmR leads to a hyperswarmer phenotype and overproduction of rhamnolipids (RLs). (A) Swarming assays were realized with the three *qsmR-* mutants obtained from the directed evolution experiment and the *qsmR-1* transposon mutant, compared to the wildtype E264 strain. (B) Rhamnolipids production was quantified in cultures of the *qsmR-1* mutant compared to the production in wildtype strain E264 confirming increased production by inactivating *qsmR*. Rhamnolipid production of the *qsmR-1* mutant restored to wildtype levels when complemented with the pME6000 plasmid expressing the *qsmR* gene (pSM1). E264 and the *qsmR-1* mutant contained the empty pME6000. The error bars represent standard deviation from the mean ($n = 5$ independent cultures). Data analysed using a one-way ANOVA with post hoc. Dunnett's multiple comparisons tests (****$p < 0.0001$, ns = not significant).

In *B. thailandensis* the *rhlA*, *rhlB* and *rhlC* rhamnolipid biosynthetic genes are clustered together, along with other genes of still unknown functions (Dubeau et al., 2009). Furthermore, *B. thailandensis* carries two such *rhl* operons, that are orthologous but differently regulated (Dubeau et al., 2009; Martinez et al., 2020). To understand how QsmR is involved in rhamnolipid production, we verified the levels of transcription from the promoters of the two *rhl* operons, using expression reporters. Since rhamnolipid production is maximal after 3 days under

A

c	VKESGKTRTL	FRIRLTIDKN	LKSSFEKREI	SVTTRTNAQA	VSREREATTD	EITALARGLA	60
L2	VKESGKTRTL	FRIRLTIDKN	LKSSFEKREI	SVTTRTNAQA	VSREREATTD	EITALARGLA	60
L3	VKESGKTRTL	FRIRLTIDKN	LKSSFEKREI	SVTTRTNAQA	VSREREATTD	EITALARGLA	60
WT	VLRRIATSDA	PVSNRELAEL	TGIPKPTVSR	ITATLVSAGF	LFQLPDSERF	VLTASVLELS	120
L2	VLRRIATSDA	PVSNREPAEL	TGIPKPTVSR	ITATLVSAGF	LFQLPDSERF	VLTASVLELS	120
L3	VLRRIATSDA	PVSNRELAEL	TGIPKPTVSR	ITATLVSAGF	LFQLPDSERF	VLTASVLELS	120
WT	NGFLRNFDIR	ARSRPFLVEL	AEKTSLSVHL	AVRDRLDMVA	IDVIRPRSAV	LVTRLETGSR	180
L2	NGFLRNFDIR	ARSRPFLVEL	AEKTSLSVHL	AVRDRLDMVA	IDVIRPRSAV	LVTRLETGSR	180
L3	NGFLRNFDIR	ARSRPFLVEL	AEKTSLSVHL	AVRDRLDMVA	IDVIRTSPRR	GSTRGSKRAR	180
WT	M---DIARTA	VG--------	RAYLAALEDD	ARRELIGALQ	AAAGDDWPFV	VSRLNAAL	229
L2	M---DIARTA	VG--------	RAYLAALEDD	ARRELIGALQ	AAAGDDWPFV	VSRLNAAL	229
L3	GWTSRARRSA	VRTSPRSRTT	RAAS-SAHCR	PRRATTGRSS	--YRG-MPRS	PTSRNTATRS	236
WT	IAQHGYAIAI	GE----WREE	LNAIAAGFVA	P-----TGQC	YAVNCGGSAH	QCTPDFLRSV	280
L2	IAQHGYAIAI	GE----WREE	LNAIAAGFVA	P-----TGQC	YAVNCGGSAH	QCTPDFLRSV	280
L3	RSANGAKN-M	RSRRASSRRR	ANATRSIAAA	PRTSARPTSC	ARSRCPR---	-CASASQRSR	291
WT	AVPALRECIA	KITREIGAAA	WPNRAR----				306
L2	AVPALRECIA	KITREIGAAA	WPNRAR----				306
L3	A---------	--------RS	APPRGRIAPG				304

B

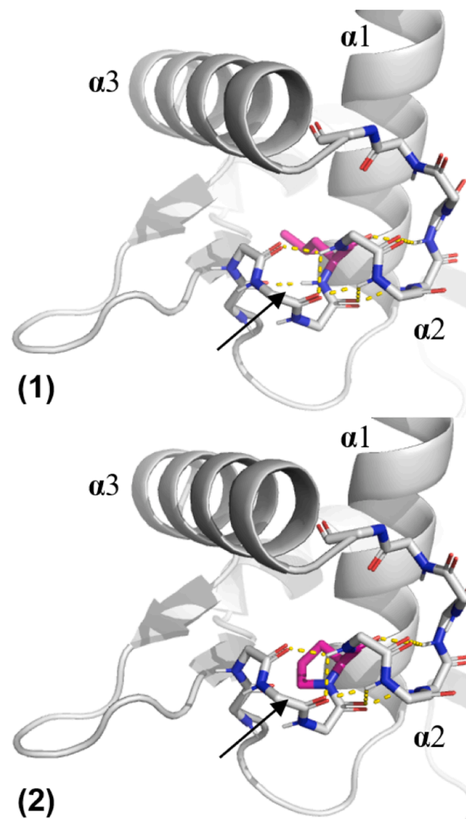

Fig. 5. Sequence alignment and structural predictions of QsmR mutant proteins compared to the wild type protein. (A) Sequence alignment between wildtype QsmR and the two mutant types identified by whole-genome sequencing of clones of interest. Sequence alignments were realized using NCBI BLAST. Differences in protein sequences are highlighted in red. (B) *Burkholderia glumae* QsmR AlphaFold predicted structure (AlphaFold DB: AF-A4KCQ2-F1-v4), the closest analogous structure available of *B. thailandensis* QsmR. The backbone of helix α2 is represented as sticks, hydrogen bonds are depicted as dashed lines, and the mutation site is represented in magenta. (1) shows the wildtype protein and (2) shows the mutant from lineage2 L2C1/C7. Introduction of a proline destabilizes helix α2 by removing a hydrogen bond, emphasized by an arrow in (2).

our cultivation conditions (Martinez et al., 2020), the bacteria were incubated in flasks cultures with sampling every 8 h for 72 h. Fig. 6 shows that the transcription of both operons is higher in the *qsmR-1* mutant background compared to the wildtype, although the effect is stronger on the expression of operon *rhl2*. Taken together, these results indicate that mutations in the evolved clones alleviated a repressing activity by QsmR on the transcription of both rhamnolipid biosynthetic operons, explaining the overproduction.

4. Discussion

Microbially-produced surface-active agents represent an attractive alternative to synthetic surfactants and an asset in our transition to a more sustainable bio-based economy. However, the productivity of most currently known biosurfactant-producing microbes is too low to achieve economic sustainability (Banat et al., 2014). Optimization of bioprocesses is essential, but strain selection and improvement are also required. In this regard, targeted strategies to genetically enhance productivity generally offer insufficient improvements. The specific aim of this study was to develop a directed evolution strategy to increase the production of biosurfactants by promising microorganisms, using rhamnolipids from *B. thailandensis* as a proof of concept. We successfully implemented a methodology using swarming motility as a selective pressure to identify spontaneous mutants producing up to seven times more rhamnolipids than the ancestral strain *B. thailandensis* E264.

Our hypothesis was based on the fact that swarming motility requires the production of a wetting agent and that this social behaviour is an important trait for the colonisation of semi-solid surfaces (Dubeau et al., 2009). We had previously used directed evolution experiments with *P. aeruginosa* to isolate gain-of-function mutants restoring the swarming motility of a non-swarmer mutant (Robitaille et al., 2020). Under laboratory conditions, the agar concentration (usually in the range 0.5 %–2 %) used to solidify the medium is a critical factor to achieve swarming motility. Indeed, a minimal level of surface wetness is required, typically achieved by lowering the concentration of agar (Harshey, 2003). In *P. aeruginosa*, drying longer, increasing the concentration of agar, or adding extra salt or sugar in the agar matrix can all lead to a decrease in, or inhibition of, swarming motility, supporting the model that this surface behavior is restricted by the surface tension at the swarm front (Tremblay & Déziel, 2010; Yang et al., 2017). The results we obtained support our hypothesis that an augmentation in surface tension *via* restrictive, higher agar concentration, constrained the bacterial population to produce more rhamnolipids, thus reducing surface tension to levels allowing swarming motility to occur. Evolution experiments have been used in the past to increase the production of metabolites by adapting microorganism to a stress or other growth-limiting conditions (Portnoy et al., 2011); that is, survival was always the selective factor. However, our report is among the first adaptive laboratory evolution methodology to select bacteria producing more of a non-essential, secondary metabolite, here, surface-active rhamnolipids.

Three of the six sequenced clones carried loss-of-function mutations in the gene encoding the QsmR transcriptional regulator. Parallel mutations in the same gene represent compelling evidence of selection for a beneficial adaptive trait (Cooper, 2018). The two *qsmR*- mutants from lineage 2 carried the same mutation, corresponding to a single SNP located in an alpha helix that might destabilise the total domain structure and decrease the affinity between the regulator and the target DNA. The *qsmR* mutant from lineage 3 harbored several mutations, both indels and substitutions, all located in the effector binding domain. Disruption in the effector binding domain could affect the protein–ligand liaison and thus disable full regulator activity. The fact that these evolved clones produce the same levels of rhamnolipids than the *qsmR-1* transposon mutant concurs with a complete loss of QsmR activity. Using transposon-mediated mutagenesis, we previously found that mutants in the ScmR regulator also produce higher levels of rhamnolipid than the E264 wild-type strain (Martinez et al., 2020). Next, both strategies might be combined where the mutants obtained using transposon-mediated mutagenesis could be submitted to directed evolution, or vice-versa.

We also demonstrated that loss of QsmR resulted in higher transcription of both *rhl* biosynthetic operons, with the impact being more important on the *rhlA2* operon. We previously reported that both *rhlA* alleles are functional and necessary for maximal rhamnolipid production (Dubeau et al., 2009). However, the *rhlA1*- mutant produces more rhamnolipids than the *rhlA2*- mutant (Dubeau et al., 2009), indicating that the two operons are differently regulated. Our recent data with the ScmR transcriptional repressor confirm this model, where both operons were differently regulated (Martinez et al., 2020). We demonstrated here that QsmR plays a greater role into the regulation of the *rhlA2* operon compared to the *rhlA1* operon.

How exactly does the inactivation of QsmR result in increased rhamnolipid titers remains to be fully determined. While we do not know whether the repression on transcription of *rhl* genes is direct or indirect, we also noted that *qsmR* (BTH_I2865) may form an operon with three other genes, BTH_I2866, BTH_I2867 and BTH_I2868. The latter are predicted to encode homologs of the *mdtABC* genes belonging to the

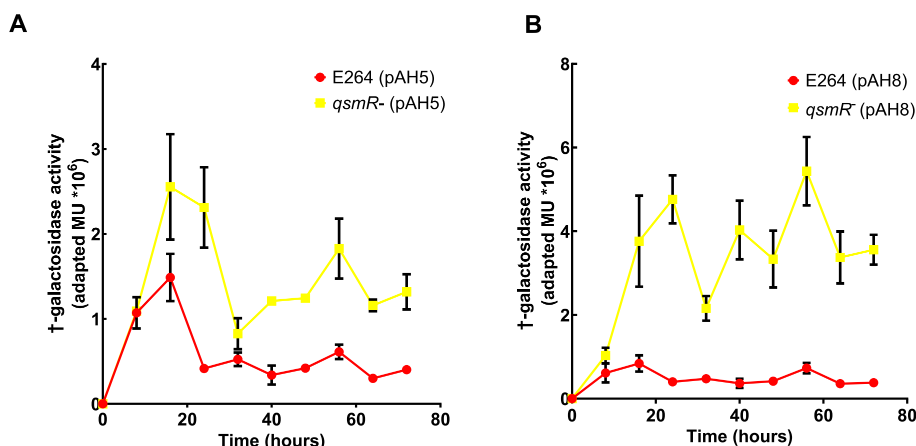

Fig. 6. Both *rhl* operons of *B. thailandensis* E264 are upregulated in a *qsmR* mutant. β-galactosidase assays were performed in samples from cultures in NB medium supplemented with 4 % glycerol during 72 h for the wildtype and the *qsmR-1* mutant strains, carrying one of two transcriptional *lacZ* fusion reporters for each *rhl* operon. Kinetics of (A) pAH5 (promoter region upstream of BTH_II1075 [*rhlA1*]) and (B) pAH8 (promoter region upstream of BTH_II1881 [*rhlA2*]) in *B. thailandensis* E264 and its isogenic *qsmR-1* mutant are presented. Data measured in adapted Miller Units (MU). The values are means ± standard deviations (error bars) for three replicates.

Resistance Nodulation and cell Division (RND) transporter family, according to the Burkholderia.com database. Indeed, BTH_I2866 presents a 72 % identity with the *mdtA* homolog found in *Ralstonia taiwanensis* (RALTA_RS02645), BTH_I2867 presents 77 % identity with the *mdtB* homolog found in *R. taiwanensis* (RALTA_RS02640) and BTH_I2868 74 % with the *mdtC* homolog in *R. taiwanensis* (RALTA_RS02635). Since very little is known on how rhamnolipids are exported outside producing bacterial cells, we considered that the overproduction in the *qsmR* mutant might be explained by an upregulation of the putative efflux system encoded by *mdtABC*. RND transporters have wide substrate specificity and require TolC for their function (Fralick, 1996; Nishino et al., 2003; Sharff et al., 2001). To investigate that possibility, BTH_I2866 transcription was assessed in the *qsmR-1* mutant by RT-qPCR. As shown in **Supplementary Material**, BTH_I2866 is expressed five times more when QsmR is absent, suggesting that it represses the transcription of this operon. However, when looking at rhamnolipid production profiles in the BTH_I2866, BTH_I2867 and BTH_I2868 transposon mutants, there was no difference with the wild-type strain **(see Supplementary Material)**, excluding an effect of this putative efflux system on rhamnolipid production.

QsmR has been mostly investigated in *B. glumae*, where it acts in conjunction with quorum sensing to regulate positively or negatively several functions and factors, such as flagellum motility and production of toxoflavin toxin and exoprotease (Chen et al., 2015; Chun et al., 2009; Goo et al., 2012; Lelis et al., 2019). Quorum sensing positively regulates rhamnolipid biosynthesis in *P. aeruginosa* (Abdel-Mawgoud et al., 2010), and in *B. glumae* (Nickzad et al., 2015), while it appears to repress rhamnolipid production in *B. thailandensis*, likely through the ScmR regulator (Irorere et al., 2019; Martinez et al., 2020). Since *B. thailandensis* quorum sensing is intricately regulated (Le Guillouzer et al., 2017), and there are indications it can repress *qsmR* (Majerczyk et al., 2014), there is no doubt the respective roles of QsmR, ScmR and of the various quorum sensing transcriptional regulators (BtaR1, BtaR2, BtaR3) in regulating the production of rhamnolipids is very complex, especially considering that two differently regulated *rhl* biosynthetic operons are involved. Overall, our findings open new perspectives about unknown regulation systems directly or indirectly involved in rhamnolipid biosynthesis in *B. thailandensis* and could be of interest for the development of metabolic engineering tools for large-scale rhamnolipid production.

5. Conclusion

Microbial surfactants are highly sought-after replacements for synthetic compounds. This is the first report of an adaptive laboratory evolution approach to identify mutants producing higher levels of a microbial surface-active metabolite. We believe this strategy is very promising and could be successfully applied to other biosurfactant-producing microorganisms.

CRediT authorship contribution statement

Sarah Martinez: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **David N. Bernard:** Visualization, Software, Investigation. **Marie-Christine Groleau:** Writing – review & editing, Visualization, Investigation. **Mylène C. Trottier:** Visualization, Validation, Investigation, Data curation. **Eric Déziel:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132255.

Data availability

Data will be made available on request.

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