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rhlA is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids

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Pseudomonas aeruginosa produces extracellular glycolipids composed of L-rhamnose and 3-hydroxyalkanoic acid called rhamnolipids. Although these compounds are usually regarded as biosurfactants or haemolysins, their exact physiological function is not well understood. Rhamnolipids are synthesized by a rhamnosyltransferase, encoded by the *rhlAB* operon, which catalyses the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) moieties of various lengths. RhlB is the catalytic protein of the rhamnosyltransferase. *rhlA* is indispensable for rhamnolipid synthesis, but its function is unknown. Using a liquid chromatography/mass spectrometry method, the production of extracellular HAAs by *P. aeruginosa* was detected previously and it was demonstrated that they are the actual precursors of rhamnolipid biosynthesis. In this report, evidence is presented indicating that *rhlA* is required for production of HAAs and that these HAAs display potent surface-active properties. *P. aeruginosa* can colonize surfaces by swarming motility, a form of organized translocation requiring the production of wetting agents. Using *rhlA* and *rhlB* mutants it was observed that swarming requires the expression of the *rhlA* gene but does not necessitate rhamnolipid production, as HAAs act as surfactants. Finally, it was shown that the use of ammonium instead of nitrate as source of nitrogen and an excess of available iron both decrease *rhlA* expression and swarming motility.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous and versatile Gram-negative bacterium capable of three types of motility: flagellum-mediated swimming, type IV pilus-dependent twitching, and swarming, which was only recently reported in this species (Déziel *et al.*, 2001; Köhler *et al.*, 2000; Rashid & Kornberg, 2000). Swarming is a form of organized surface translocation allowing rapid colonization of surfaces that depends on extensive flagellation and cell-to-cell contact (Fraser & Hughes, 1999; Harshey, 1994). It is a multicellular phenomenon requiring cell differentiation and cooperation, two features also involved in biofilm formation and bacterial virulence (Harshey, 1994; Sharma & Anand, 2002).

Swarming cells need to overcome the strong surface tension of water surrounding the colony to efficiently colonize the surface (Matsuyama & Nakagawa, 1996). This surface conditioning is often achieved by the production of surface-active compounds, which act as wetting agents (Lindum *et al.*, 1998; Matsuyama *et al.*, 1992, 1995; Mendelson & Salhi, 1996; Toguchi *et al.*, 2000). Spreading growth of *Serratia marcescens* depends on the production of various extracellular lipopeptides named serrawetins (Matsuyama *et al.*, 1986, 1992, 1995). Synthesis of serrawettin W2, a cyclic lipodepsipentapeptide required for swarming motility of *Serratia liquefaciens*, is regulated in a cell-density-dependent manner by an acyl-homoserine lactone-based quorum sensing system (Lindum *et al.*, 1998). Swarming of *Bacillus subtilis* relies on the production of surfactin (Mendelson & Salhi, 1996), a cyclic lipopeptide biosurfactant whose synthesis is also controlled by quorum sensing via two pheromone signal peptides, ComX and CSF (Lazazzera *et al.*, 1997; Solomon *et al.*, 1996).

P. aeruginosa produces extracellular glycolipids composed of L-rhamnose and 3-hydroxyalkanoic acid (rhamnolipids) (Hauser & Karnovsky, 1957; Jarvis & Johnson, 1949). Although their exact physiological function is still unclear, these amphiphilic molecules are usually considered biosurfactants, acting as solubilizing agents promoting the uptake

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Abbreviations: HAA, 3-(3-hydroxyalkanoyloxy)alkanoic acid; LC/MS, liquid chromatography/mass spectrometry; MSM, mineral salts medium.

of hydrophobic substrates, especially *n*-alkanes (Beal & Betts, 2000; Itoh *et al.*, 1971). Moreover, rhamnolipids are virulence factors found in high concentrations in sputa of *P. aeruginosa*-colonized cystic fibrosis patients (Kownatzki *et al.*, 1987). Rhamnolipids interfere with the normal tracheal ciliary function (Read *et al.*, 1992), inhibit the phagocytic response of macrophages (McClure & Schiller, 1996) and act as heat-stable extracellular haemolysins (Johnson & Boese-Marrazzo, 1980). In liquid cultures, they are produced as a complex mixture of congeners containing one or two 3-hydroxy fatty acids of various length, linked to a mono- or dirhamnose moiety (Déziel *et al.*, 1999, 2000). In general, the two more abundant rhamnolipids are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Lang & Wullbrandt, 1999; Maier & Soberón-Chávez, 2000) (Fig. 1; *m*, *n*=6). According to the biosynthetic pathway proposed by Burger *et al.* (1963), rhamnolipid synthesis proceeds by two sequential glycosyl transfer reactions, each catalysed by a different rhamnosyltransferase. The first rhamnosyltransferase, which catalyses the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA; Fig. 1), is encoded by the *rhlAB* operon (Ochsner *et al.*, 1994a, b, 1995). Both genes, co-expressed from the same promoter, are essential for rhamnolipid synthesis but, whereas *rhlB* is known to encode the catalytic subunit of the rhamnosyltransferase, the function of *rhlA* is still unresolved. RhlA is probably an inner-membrane-bound protein (Rahim *et al.*, 2001), presumably involved in the synthesis or transport of rhamnosyltransferase precursor substrates or in the stabilization of the RhlB protein (Ochsner *et al.*, 1994a). Environmental factors, especially nutritional conditions, influence rhamnolipid production (Guerra-Santos *et al.*, 1986). Furthermore, cell-to-cell signalling regulates the expression of the *rhlAB* operon (Ochsner *et al.*, 1994b; Ochsner & Reiser, 1995; Pearson *et al.*, 1997; Pesci *et al.*, 1997). This quorum sensing system is composed of *rhlI*, the *N*-butyrylhomoserine lactone autoinducer synthase gene, and *rhlR*, which encodes the transcriptional activator (Ochsner *et al.*, 1994b; Ochsner & Reiser, 1995). The second rhamnosyltransferase, encoded by *rhlC*, has been characterized and its expression shown to be co-ordinately regulated with *rhlAB* by the same quorum sensing system (Rahim *et al.*, 2001). Köhler *et al.* (2000) reported that cell-to-cell signalling, and both flagella and type IV pili, are required for swarming motility of *P. aeruginosa*. They observed that an *rhlA* mutant was unable to swarm and therefore concluded that rhamnolipid production is required for swarming motility of *P. aeruginosa*.

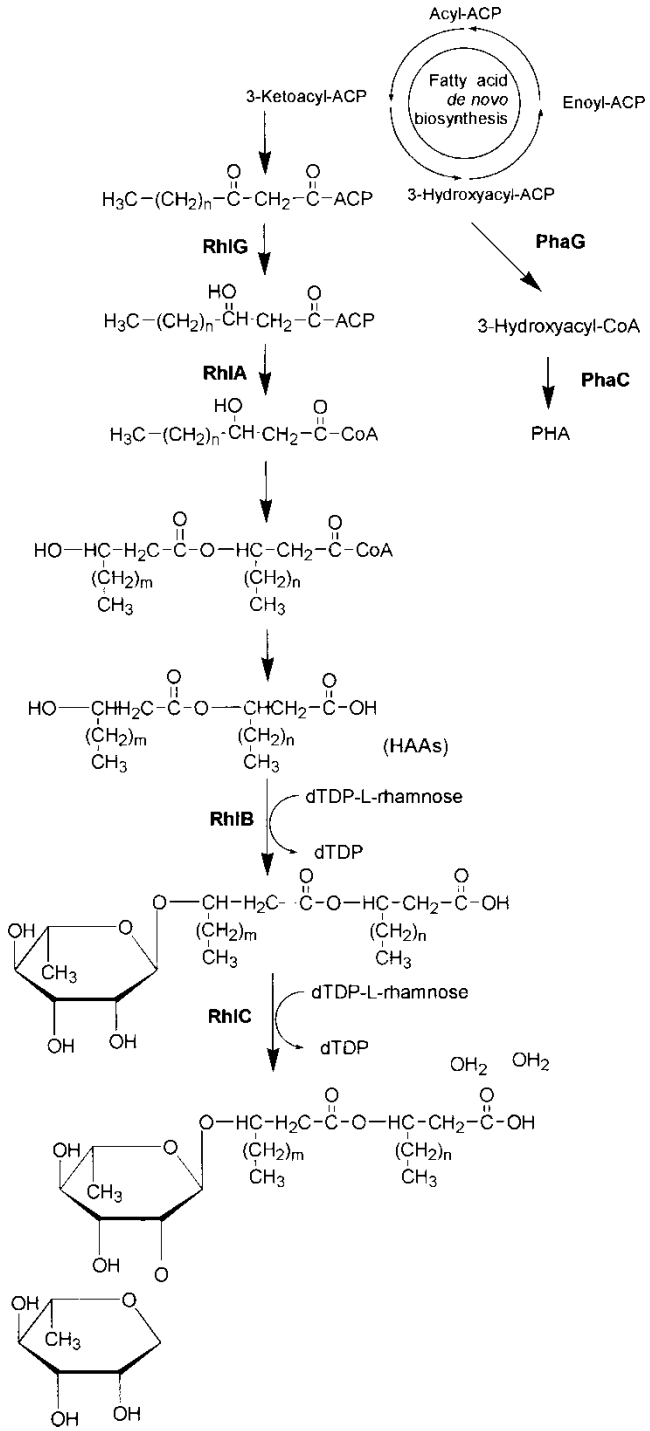


Fig. 1. Putative synthetic pathway of rhamnolipids in *P. aeruginosa*. The proposed position of RhlA is based on its homology with PhaG. *n*, *m*=4, 6 or 8. Pha, Poly-3-hydroxyalkanoate.

Using a liquid chromatography/mass spectrometry (LC/MS) method developed to directly analyse rhamnolipids in culture supernatants (Déziel *et al.*, 1999, 2000), we recently detected the production of extracellular HAAs by *P. aeruginosa* 57RP (Lépine *et al.*, 2002). We demonstrated that these compounds were not rhamnolipid degradation products, but the actual intermediates of rhamnolipid biosynthesis postulated by Burger *et al.* (1963). In this report, we present evidence indicating that *rhlA* is required for production of HAAs and that these HAAs display potent surface-active properties. We show that swarming motility is tightly linked to the expression of the *rhlA* gene,

but does not necessitate rhamnolipid production, as HAAs can act as wetting agents. Finally, we provide new insights about the influence of the nutritional environment of the cells on the expression of *rhlA* and swarming motility.

METHODS

Bacterial strains, plasmids and culture media. Three strains of *P. aeruginosa* used in this study were kindly provided by Dr Urs Ochsner (Department of Microbiology, University of Colorado, USA): wild-type strain PG201, and Tn*5*-Gm-induced mutants *rhlA*$^-$ UO299 and *rhlB*$^-$ UO287.

Expression of the *rhlAB* operon was investigated by monitoring β-galactosidase activity of the wild-type strain PG201 containing an *rhlA′-lacZ* translational fusion (plasmid pECP60) (Pesci *et al.*, 1997). pECP60 was introduced into strain PG201 by electroporation (Smith & Iglewski, 1989) and PG201(pECP60) transformants were selected with carbenicillin (200 μg ml^{-1}). X-Gal (40 μg ml^{-1}) was added to culture media when required.

Swarm plates were composed of 0·5 % Bacto-agar and 8 g nutrient broth l^{-1}, both from Difco, supplemented with 5 g glucose l^{-1} and dried overnight at room temperature before use (Rashid & Kornberg, 2000). Cells were point-inoculated with a sterile toothpick or 2 μl of an overnight culture and the plates were incubated at 30 °C for 24–48 h. For liquid swarm medium, agar was omitted.

An iron-limited mineral salts medium (MSM) designed to promote rhamnolipid production (Déziel *et al.*, 1996, 2000), supplemented with 2 % (w/v) carbon source, was also used. The composition was (g l^{-1}): KH_2PO_4, 0·7; Na_2HPO_4, 0·9; $NaNO_3$, 2·0; $MgSO_4.7H_2O$, 0·4; $CaCl_2.2H_2O$, 0·1; $FeSO_4.7H_2O$, 0·001. The final pH was 6·7.

The effects of the nitrogen source (25 μM NH_4Cl or $NaNO_3$, or 12·5 μM both) or addition of iron (0, 1, 5 or 10 μM $FeSO_4.7H_2O$) on growth and *rhlA* expression of PG201(pECP60) were studied on swarm agar plates containing 1·5 % agar and carbenicillin (300 μg ml^{-1}). The plates were spot-inoculated in triplicate with 2 μl from an overnight culture and incubated for 20 h at 30 °C. The colonies were then recovered and resuspended in 1 ml PBS. β-Galactosidase activity was assayed according to Miller (1972).

Biosurfactant production and analysis. Cultures were grown in 50 ml iron-limited MSM supplemented with 2 % (w/v) mannitol in 250 ml Erlenmeyer flasks and incubated at 30 °C with gyratory shaking at 200 r.p.m. (Déziel *et al.*, 1999, 2000). The cell-free supernatant was analysed after 6 days incubation at 30 °C and 200 r.p.m. Surface and wetting activities were qualitatively compared with the drop-collapsing test (Jain *et al.*, 1991) and surface tension was measured by the ring method with a du Nouy tensiometer (Fisher Scientific). LC/MS analyses were performed as described previously for rhamnolipids and HAAs (Déziel *et al.*, 2000; Lépine *et al.*, 2002). Culture samples were centrifuged at 16 000 ***g*** for 5 min to remove the bacteria and the supernatant was filtered through a 0·2 μm filter. An internal standard (16-hydroxyhexadecanoic acid) was added before injecting the sample into the mass spectrometer to allow quantitative measurements. All the analyses were performed in triplicate with a triple quadrupole mass spectrometer Quattro II (Micromass) equipped with a Z-spray interface using electrospray ionization in negative mode. The spectrometer was interfaced to an HP 1100 HPLC (Agilent Technologies) equipped with a 150 mm × 4 mm Zorbax C_8 reverse phase column (particle size 5 μm). Quantification was performed by integration of the pseudo-molecular and the proper fragment ions.

Determination of biosurfactant production on swarm plates was performed as follows. Each strain was inoculated on five MSM agar plates with 2 % mannitol and after 48 h of incubation the whole content was mixed with 100 ml 1 % $KHCO_3$ (pH 9), incubated overnight at 4 °C and centrifuged for 10 min at 1800 ***g*** to remove solids. The supernatant was then acidified to pH 4 with concentrated HCl and extracted three times with 40 ml ethyl acetate. The organic fractions were finally pooled, dried and evaporated. The residue was resuspended in an aqueous solution containing 36 % acetonitrile and 4 mM ammonium acetate.

RESULTS AND DISCUSSION

Production of HAAs requires the expression of *rhlA*

P. aeruginosa 57RP, a strain isolated from hydrocarbon-contaminated soil (Déziel *et al.*, 1996), excretes HAAs, the lipidic precursor of rhamnolipids, along with rhamnolipids (Lépine *et al.*, 2002). With the goal to increase the production of HAAs, we cultivated an *rhlA*$^-$ mutant (Δ*rhlA*::Km) of this strain. Such mutation also prevents *rhlB* transcription (Ochsner *et al.*, 1994a). Because *rhlA* is considered part of the rhamnosyltransferase 1 complex (Ochsner *et al.*, 1994a), we anticipated that inactivation of this gene would inhibit the glycosylation of HAAs and lead to an increase in the concentration of HAA intermediates in culture supernatants. This mutant did not produce rhamnolipids as expected but, surprisingly, did not produce HAAs either.

We further investigated this observation with the reference strain PG201, which was originally used to isolate the *rhlAB* operon, and the *rhlA*$^-$ UO299 and *rhlB*$^-$ UO287 mutants, both derived from PG201 (Ochsner *et al.*, 1994a). UO299 does not express both *rhlA* and *rhlB*, whereas *rhlA* is still expressed in UO287 (Ochsner *et al.*, 1994a). Cultures of the mutant UO299 contained no trace of HAAs or rhamnolipids, as observed with mutant 57RP (Δ*rhlA*::Km). However, UO287, while not producing rhamnolipids, was still secreting HAAs. These results indicate that *rhlA* is involved in the synthesis of HAAs, the lipidic precursors of rhamnolipids and substrate of the RhlB rhamnosyltransferase. Thus, dimers of 3-hydroxyalkanoic acids are directly excreted in the extracellular milieu or coupled to rhamnose by the rhamnosyltransferase encoded by *rhlB* to produce rhamnolipids.

The RhlB rhamnosyltransferase prefers longer chain and saturated HAAs

We determined the concentrations of free HAAs congeners, along with the mono- and dirhamnolipids, in the supernatant of a PG201 culture (Table 1). The free HAA profile in strain PG201 is similar to the one previously observed for strain 57RP (Lépine *et al.*, 2002). Notably, the supernatant was proportionally depleted in C_{10}-C_{10} HAA (23·6 %) relative to the mono- (64·4 %) and dirhamnolipids (60·8 %) and contained a large proportion of C_8-C_8 HAA (22·1 %), while no rhamnolipids containing C_8-C_8 were

Table 1. Concentration of HAAs, and mono- and dirhamnolipids in PG201 and UO287 culture supernatants after 169 h of incubation

Numbers in parentheses are the SD. Samples were analysed in triplicate. Numbers in brackets are the concentration percentage of a given congener within the same family of compounds. ND, Not detected.

HAA	Monorhamnolipid concn in PG201 (μM)	Dirhamnolipid concn in PG201 (μM)	HAA concn in PG201 (μM)	HAA concn in UO287 (μM)
C_8-C_8	ND	ND	18·5 (1·7) [22·1]	3·4 (0·2) [0·5]
C_8-C_{10}	81·2 (5·7) [13·5]	593·3 (63·0) [11·9]	6·6 (1·3) [7·9]	66·4 (3·7) [9·2]
C_{10}-C_8	37·8 (2·7) [6·3]	275·8 (29·3) [5·5]	6·5 (1·3) [7·8]	43·9 (2·4) [6·1]
C_8-C_{12}	3·1 (0·1) [0·5]	23·9 (2·4) [0·5]	ND	3·0 (0·2) [0·4]
C_{12}-C_8	ND	ND	ND	ND
C_{10}-C_{10}	388·8 (17·5) [64·4]	3039·3 (310·9) [60·8]	19·7 (1·0) [23·6]	409 (32·3) [56·6]
C_{10}-C_{12}	26·1 (1·2) [4·3]	434·5 (53·2) [8·7]	4·6 (0·4) [5·5]	102·7 (11·6) [14·2]
C_{12}-C_{10}	8·4 (0·4) [1·4]	140·3 (17·2) [2·8]	0·8 (0·1) [0·9]	23·3 (2·6) [3·2]
C_{12}-C_{12}	1·2 (0·2) [0·2]	23·2 (2·7) [0·5]	ND	2·7 (0·2) [0·4]
C_8-$C_{12:1}$	9·3 (0·6) [1·5]	47·6 (7·0) [1·0]	ND	(0·3) [0·3]
$C_{12:1}$-C_8	ND	ND	ND	ND
C_{10}-$C_{12:1}$	44·5 (4·2) [7·4]	392·8 (39·0) [7·9]	26·2 (1·3) [31·3]	62·0 (2·3) [8·6]
$C_{12:1}$-C_{10}	ND	ND	ND	ND
C_{12}-$C_{12:1}$	3·3 (0·6) [0·5]	(3·6) [0·6]	0·8 (0·2) [1·0]	(0·3) [0·5]
$C_{12:1}$-C_{12}	ND	ND	ND	ND
TOTAL	**603·6**	**5001·3**	**83·8**	**722·2**

detected. In contrast, the total free HAA concentration in the *rhlB*$^-$ mutant UO287 supernatant was almost nine times higher than in the wild-type strain PG201 supernatant (Table 1). Proportions of the various free HAAs differed between strains UO287 and PG201. Most notably, the proportion of free HAAs represented by the C_{10}-C_{10} congener goes from 23·6 % for PG201 to 56·6 % for strain UO287. This value is very close to the proportion of the C_{10}-C_{10} congener observed in mono- (64·4 %) and dirhamnolipids (60·8 %) in PG201. The percentage of free HAAs represented by the C_8-C_8 congener goes from 22·1 % for PG201, to only 0·5 % for strain UO287, while no C_8-C_8 congeners are observed in mono- and dirhamnolipids of PG201. The very high percentage of free HAAs represented by the C_{10}-$C_{12:1}$ congener (31·3 %) in PG201 becomes 8·6 % in UO287, close to the values observed for the C_{10}-$C_{12:1}$ congeners in mono- and dirhamnolipids of PG201. These results further strengthen our hypothesis that the RhlB rhamnosyltransferase has a preference for longer chain and saturated HAAs (Lépine *et al.*, 2002), leaving an HAA pool enriched in shorter chain and unsaturated congeners, as seen in the free HAAs of PG201. The residual free HAA pool of PG201 is small because the RhlB rhamnosyltranferase utilizes most of the available HAAs. The larger UO287 pool is therefore representative of the initial HAAs pool before rhamnolipid synthesis. This explains why the pool of free HAAs in UO287 cultures is very similar to the HAA profile of mono- and dirhamnolipids observed with PG201.

The biosynthetic pathway of HAAs is not completely elucidated. Campos-García *et al.* (1998) have described an NADPH-dependent ketoacyl reductase, RhlG, which is presumably responsible for draining the fatty acid precursors of HAAs from the general *de novo* biosynthetic pathway towards HAA synthesis (Fig. 1). Although it is essential for rhamnolipid production and presumed to be part of the rhamnosyltransferase 1 complex, the exact function of the *rhlA* gene product is unknown. A role for the RhlA protein in rhamnolipid precursor biosynthesis had previously been suggested, but not demonstrated (Ochsner *et al.*, 1994a). The amino acid sequence of the *rhlA* gene product exhibits 41–48 % identity and 60–65 % similarity with 3-hydroxyacyl ACP : CoA transferases (PhaG) identified in various members of the *Pseudomonas* genus (*Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas oleovorans*, *Pseudomonas syringae* and *Pseudomonas* sp. 61-3). PhaG directly links *de novo* fatty acid biosynthesis to poly-3-hydroxyalkanoate synthesis (Rehm *et al.*, 1998). Thus, based on its homology with PhaG, RhlA could potentially be an acyltransferase catalysing the transfer of the 3-hydroxyacyl moiety from the ACP thioester to CoA. HAAs would result from the condensation of two of these 3-hydroxacyl-CoA residues. It is important to note that the rhamnolipid and poly-3-hydroxyalkanoate biosynthesis pathways are competing for common intermediates (Fig. 1) (Rehm *et al.*, 2001). Future work will be required to determine the exact function of *rhlA* in HAA synthesis.

HAAs are surfactants

Besides the lack of concordance between the composition of the initial HAA pool and the preference of the RhlB rhamnosyltransferase, presence of HAAs in cultures of the wild-type strain indicates that their release by the *rhlB*$^-$

mutant is not simply the result of overproduction. The mixture of hydroxylated fatty acids is actually excreted under normal growth conditions, suggesting that free HAAs play a specific role of their own in the bacteria. The excretion of anionic biosurfactants, such as rhamnolipids, is revealed by the formation of a dark blue halo around colonies growing on SW Blue agar plates (Siegmund & Wagner, 1991). As expected, the *rhlA* mutant UO299 did not produce a halo on these plates. Interestingly, the *rhlB* mutant UO287 produced a small halo, indicating that the cells released anionic amphiphilic compounds other than rhamnolipids.

Accordingly, significant surface activity was detected in the extracellular fluids of strains PG201 and UO287 cultivated in a liquid medium designed to promote the production of rhamnolipids (Table 2). A positive drop-collapsing test and a surface tension below 45 mN m^{-1} usually denote the presence of surface-active agents. The surface tension of *rhlB*$^{-}$ mutant cultures was very low and consistently lower than wild-type cultures, suggesting that HAAs display a potent surface-tension-lowering activity. Total rhamnolipids and HAAs from PG201 and UO287 cultures, respectively, were purified, diluted in water over a range of concentrations and the surface tension of these solutions was measured. Fig. 2 shows that HAAs can decrease the surface tension to a lower value than rhamnolipids, confirming that HAAs have excellent tensioactive properties. Thus, HAAs represent a new class of biosurfactants released by *P. aeruginosa* in addition to rhamnolipids.

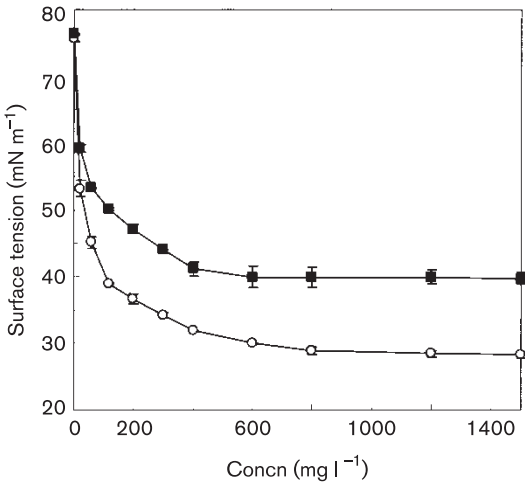


Fig. 2. Surface tension of the total mixtures of rhamnolipids or HAAs. Squares, rhamnolipid mixture purified from a PG201 culture; circles, HAA mixture extracted from a UO287 culture. Each data point shows the mean of triplicate measurements from two different experiments. Error bars represent the SD.

HAAs act as wetting agents for swarming motility

Bacterial species that are capable of swarming motility often require the production of an extracellular wetting agent (Sharma & Anand, 2002). Köhler *et al.* (2000) concluded that rhamnolipid production was required for swarming motility because an *rhlA*$^{-}$ mutant was unable to swarm. We investigated further the relationship between swarming motility and biosurfactant production in *P. aeruginosa* PG201. Swarming was observed with this strain at 21, 30 and 37 °C in the medium described by Rashid & Kornberg (2000), for Bacto-agar concentrations between 0·4 and 0·8 %. In comparison, swarming was completely abrogated in the *rhlA*$^{-}$ mutant, but the *rhlB*$^{-}$ mutant was still able to swarm (Fig. 3).

We then checked directly for biosurfactant release in swarm plates. Extraction of the whole content of MSM swarm agar plates, after removal of the cells, confirmed that HAAs were produced under these growth conditions by PG201 and UO287 (Table 3). These results indicated that RhlB rhamnosyltransferase activity, hence production of rhamnolipids, is not required for swarming of *P. aeruginosa*, but that *rhlA* is essential. It also suggests that HAAs act as a wetting agent promoting swarming motility. Although HAAs alone seemed sufficient, the wild-type strain always produced larger swarming zones than the *rhlB*$^{-}$ mutant (Fig. 3), indicating that rhamnolipids also contribute to the swarming process. *Serratia rubidaea*, which is capable of swarming motility, similarly produces concurrently two

Table 2. Properties of *P. aeruginosa* PG201 and its mutants UO299 and UO287

Strain	PG201 (wild-type)	UO299 (*rhlA*$^{-}$)	UO287 (*rhlB*$^{-}$)
Size of swarming zone	++	−	+
Halo on SW blue agar plates	+++	−	+
Foaming of liquid cultures*	+++	−	+
Cellular aggregation (clumps)*	−	+	−
Drop collapsing test on supernatant*	++	−	+++
Surface tension of supernatant (mN m^{-1})*	38	59	29

*Cells were cultivated for 6 days at 200 r.p.m. and 30 °C in MSM with 2 % mannitol.

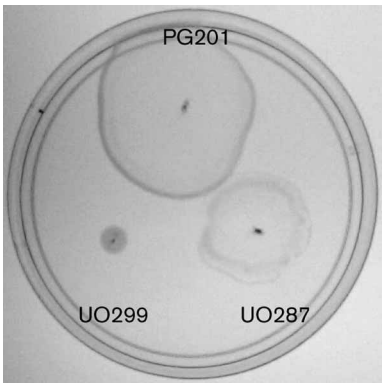


Fig. 3. Swarm plate incubated for 24 h at 30 °C with *P. aeruginosa* PG201 (wild-type strain), and with UO287 *rhlB*$^-$ and UO299 *rhlA*$^-$ mutants. Under these experimental conditions, the swarming colonies display a concentric pattern instead of the familiar dendritic pattern.

types of surface-active extracellular lipids: rubiwettin RG1, a mixture of glycolipids, and rubiwettin R1, a mixture of its glucose-free fatty acids counterpart displaying a potent wetting activity (Matsuyama *et al.*, 1990).

Production of biosurfactants correlates with swarming motility

Nitrate promotes, whereas ammonium and excess iron repress, rhamnolipid production (Guerra-Santos *et al.*, 1984; Mulligan & Gibbs, 1989). The effect on *rhlAB* expression and swarming of the addition of an equimolar concentration of $NaNO_3$ or NH_4Cl, or the addition of $FeSO_4.7H_2O$ was investigated. We monitored the *β*-galactosidase activity of strain PG201 containing an *rhlA′-lacZ* translational fusion. Addition of nitrate strongly stimulated *rhlA′-lacZ* expression and swarming (Fig. 4), whereas ammonium had the opposite effect. Interestingly, the stimulating effect of $NaNO_3$ seemed to supersede the repressing effect of NH_4Cl when they were added together at the same molar concentration (Fig. 4b). Like most bacteria (Merrick & Edwards, 1995), *P. aeruginosa* favours ammonium over nitrate as a source of nitrogen (Fig. 4c).

Table 3. Quantification of biosurfactant (rhamnolipids and HAAs) production in swarm agar plates by LC/MS

The whole agar content of five MSM swarm plates was extracted after 48 h growth.

Strain	Rhamnolipids (mg l^{-1})	HAAs (mg l^{-1})
PG201 (wild-type)	85	0·6
UO299 (*rhlA*$^-$)	0	0
UO287 (*rhlB*$^-$)	0	4·3

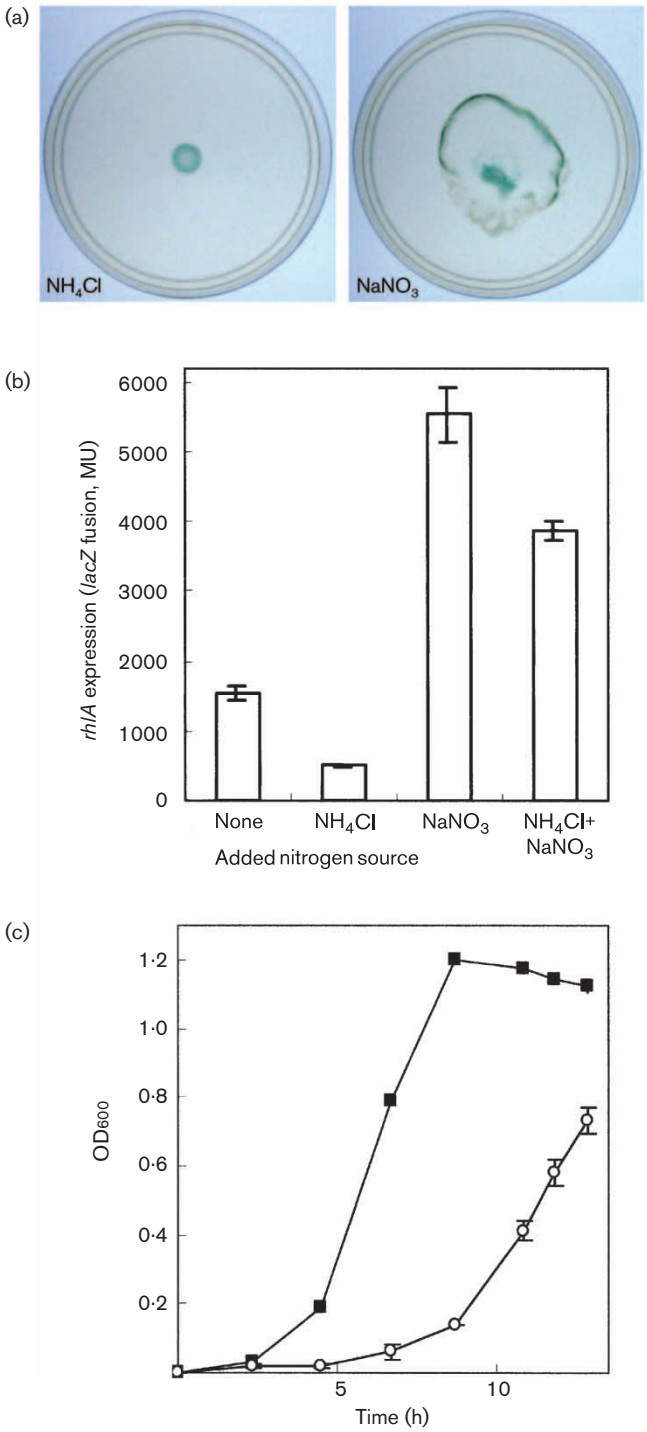
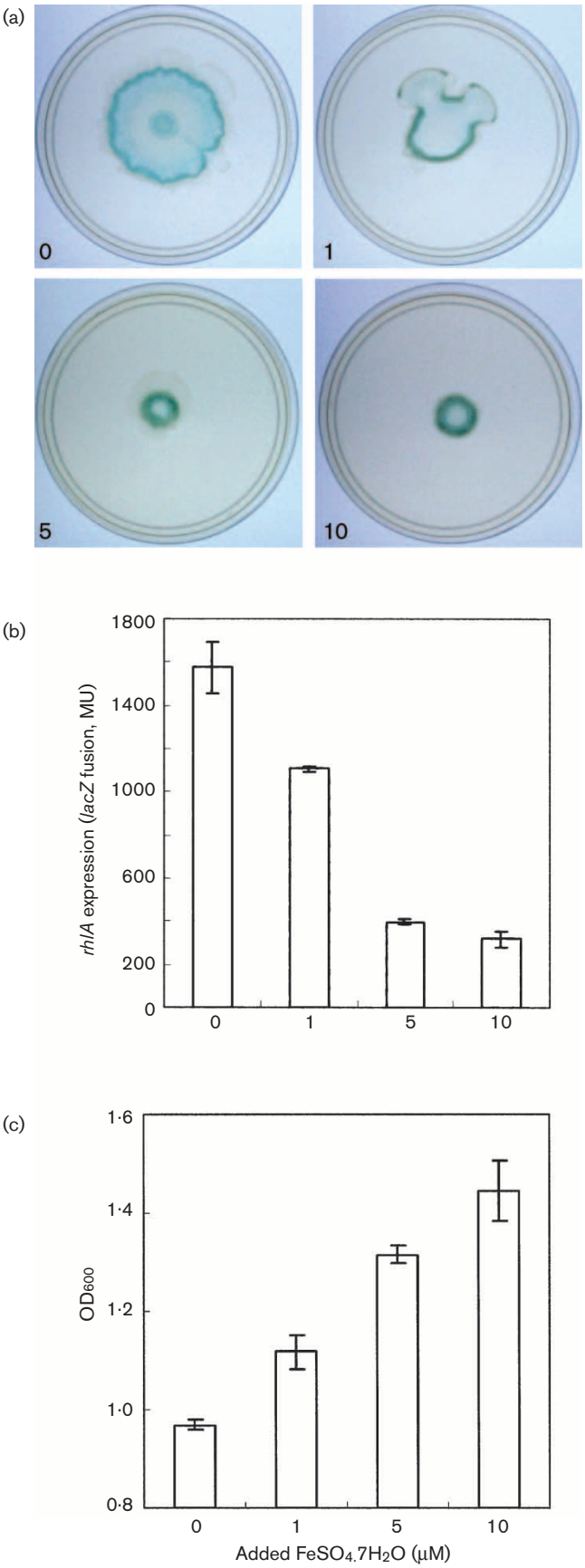


Fig. 4. Effect of various nitrogen sources on swarming motility and *rhlA* expression in *P. aeruginosa*. (a) Strain PG201 containing a *rhlA′-lacZ* translational fusion grown on swarm agar supplemented with $NaNO_3$ or NH_4Cl for 20 h at 30 °C. X-Gal allows the visual detection of *rhlA′-lacZ* expression. (b) Expression of *rhlA′-lacZ* in swarming zones by monitoring *β*-galactosidase activity (Miller units, MU). (c) Growth of strain PG201 in liquid swarm medium supplemented with $NaNO_3$ (circles) or NH_4Cl (squares).



Swarming motility was repressed by increasing concentrations of iron in the growth medium (Fig. 5a), and this also correlated with the downregulation of *rhlA′-lacZ* expression (Fig. 5b). On the other hand, supplemented iron promotes growth (Fig. 5c). These results demonstrate that growth conditions promoting biosurfactant production also favour swarming motility.

Since NH_4^+ is preferred over NO_3^- as a source of nitrogen, it appears that a less attractive nitrogen source promotes biosurfactant production and swarming. Furthermore, the observation that excess iron prevented swarming suggests that less favourable nutritional conditions in general may elicit swarming motility, presumably as a means to find a new niche with more propitious nutrient supplies. Mulligan & Gibbs (1989) have found a direct relationship between enhanced rhamnolipid production and increased glutamine synthetase activity. Synthesis of this enzyme, which is upregulated under nitrogen-limiting conditions, is controlled by the RpoN σ factor (σ^{54}; Totten *et al.*, 1990). It is noteworthy that σ^{54} is also required for transcription of *rhlAB* genes (Ochsner *et al.*, 1994a; Pearson *et al.*, 1997) and the synthesis of pili (Ishimoto & Lory, 1989) and flagella (Totten *et al.*, 1990), all features required for swarming motility (Köhler *et al.*, 2000). These observations offer further evidence that biosurfactant production in *P. aeruginosa* is closely associated with motility, apparently reflecting a general role for survival in the natural environment. The expression of the *rhlAB* operon and the production of rhamnolipids are regulated by quorum sensing (Ochsner & Reiser, 1995; Pearson *et al.*, 1997) and also by nutritional factors (Guerra-Santos *et al.*, 1986). Our results suggest that nutritional conditions supersede cell-to-cell communication and high cell density does not necessarily correlate with up-regulation of quorum-sensing-controlled genes such as *rhlAB*. Bollinger *et al.* (2001) reached a similar conclusion when the expression of *sodA*, a quorum-sensing-regulated gene encoding the manganese-cofactored superoxide dismutase, was induced by iron limitation independently of quorum sensing. Further studies will be required to elucidate the interplay between nutrition-based and cell-density-based gene regulation in *P. aeruginosa*.

Conclusion

The finding of a second class of biosurfactants produced by *P. aeruginosa* as well as the complex regulation of *rhlAB* expression indicate that swarming motility plays a critical

Fig. 5. Effect of increasing concentrations of iron on swarming motility and *rhlA* expression in *P. aeruginosa*. (a) Strain PG201 containing a *rhlA′-lacZ* translational fusion grown on swarm agar supplemented with various concentrations of $FeSO_4.7H_2O$ for 20 h at 30 °C. X-Gal allows the visual detection of *rhlA′-lacZ* expression. (b) Expression of *rhlA′-lacZ* in swarming zones by monitoring β-galactosidase activity (Miller units, MU). (c) Growth of strain PG201 in liquid swarm medium supplemented with various concentrations of $FeSO_4.7H_2O$.

role in the environmental adaptation of this bacterium. The capacity for surface-colonizing migration is increasingly recognized as a widespread trait in eubacteria (Fraser & Hughes, 1999; Harshey, 1994). The multicellular and cooperative nature of swarming motility clearly associates this phenomenon with the natural propensity of microorganisms to form biofilms (Eberl *et al.*, 1999; Mireles *et al.*, 2001; Sharma & Anand, 2002). Interestingly, Singh *et al.* (2002) recently reported that very low available iron concentrations, resulting from sequestering, markedly stimulated surface motility, thus preventing biofilm development by *P. aeruginosa*. It is therefore intriguing that we also observed increased surface motility when limiting iron conditions prevailed. These results support the hypothesis that biosurfactant production and surface motility are hyper-expressed under unfavourable nutritional conditions, presumably to prevent *P. aeruginosa* from settling and forming a biofilm. This hypothesis is strengthened by the very recent report that rhamnolipid production is involved in the maintenance of biofilm architecture (Davey *et al.*, 2003).

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