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A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*

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Summary

Bacterial populations frequently act as a collective by secreting a wide range of compounds necessary for cell–cell communication, host colonization and virulence. How such behaviours avoid exploitation by spontaneous 'cheater' mutants that use but do not contribute to secretions remains unclear. We investigate this question using *Pseudomonas aeruginosa* swarming, a collective surface motility requiring massive secretions of rhamnolipid biosurfactants. We first show that swarming is immune to the evolution of *rhlA*$^-$ 'cheaters'. We then demonstrate that *P. aeruginosa* resists cheating through metabolic prudence: wild-type cells secrete biosurfactants only when the cost of their production and impact on individual fitness is low, therefore preventing non-secreting strains from gaining an evolutionary advantage. Metabolic prudence works because the carbon-rich biosurfactants are only produced when growth is limited by another growth limiting nutrient, the nitrogen source. By genetically manipulating a strain to produce the biosurfactants constitutively we show that swarming becomes cheatable: a non-producing strain rapidly outcompetes and replaces this obligate cooperator. We argue that metabolic prudence, which may first evolve as a direct response to cheating or simply to optimize growth, can explain the maintenance of massive secretions in many bacteria. More generally, prudent regulation is a mechanism to stabilize cooperation.

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Introduction

Rather than being isolated entities, bacteria communicate with each other (Bassler and Losick, 2006), form biofilms (Costerton *et al.*, 1999) and secrete many molecules during the course of an infection (Arvidson, 2000; Griffin *et al.*, 2004; Visca *et al.*, 2007). The realization that bacteria interact in many ways is challenging our traditional view of microbes, and may affect the way we treat infections caused by pathogenic bacteria (Foster, 2005). Specifically, bacteria secrete numerous substances necessary for nutrient scavenging, host colonization and pathogenesis (Arvidson, 2000; Cascales *et al.*, 2007; Visca *et al.*, 2007). Understanding the widespread evolution of secretions in microbes is a challenge. These substances can be costly to synthesize but, once released, will also benefit other cells within range (Crespi, 2001). Microbial secretions are therefore expected to be vulnerable to exploitation by 'cheater' mutants that do not contribute but still benefit from the secretions of others (West *et al.*, 2006). Empirical studies have demonstrated that 'cheating' can indeed occur for a number of microbial products including iron-scavenging molecules (Griffin *et al.*, 2004), digestive enzymes and quorum-sensing molecules (Diggle *et al.*, 2007) and antibiotic resistance factors (Dugatkin *et al.*, 2005; Chuang *et al.*, 2009).

Despite such predictions of the rise of non-secretors and the collapse of microbial secretions, bacteria do make use of a wide range of secretions both in nature and pathogenesis. How then are these systems maintained? Recent years have witnessed a surge in the application of social evolution theory to answer this question (Crespi, 2001; Foster *et al.*, 2006; West *et al.*, 2006; 2007a; Nadell *et al.*, 2009). One explanation is simply that secreting cells are unlikely to benefit non-secreting cells (Griffin *et al.*, 2004). For example, the spatial structure that is expected to naturally emerge within cell groups can keep secretors together and away from non-secretors that would exploit them (Nadell *et al.*, 2010). Pleiotropic constraints provide another candidate mechanism to stabilize cooperation in microbes (Foster *et al.*, 2004), and there is recent evidence that such constraints can limit the rise of 'cheaters' for the case of the secretion of iron-scavenging molecules (Harrison and Buckling, 2009). However, strong spatial structure and pleiotropy are unlikely to be

ubiquitous raising the question of whether other factors are needed to explain microbial cooperation, particularly for high abundance secretions for which potential costs are very high.

An important feature of bacterial lifestyles is that environments change constantly. It is therefore very likely that bacteria evolved mechanisms to regulate cooperative secretions, which may, in turn, affect their evolutionary costs and benefits (Perkins and Swain, 2009). Here we indentify a regulatory mechanism that stabilizes cooperative secretions against cheating competitors within microbial groups. We use swarming, a collective form of surface motility in the opportunistic pathogen *Pseudomonas aeruginosa* (Kohler *et al*., 2000; Rashid and Kornberg, 2000; Kearns, 2010). In order to swarm, individual bacteria must secrete rhamnolipid biosurfactants (Deziel *et al*., 2003; Caiazza *et al*., 2005) which are synthesized through a well-characterized pathway involving the *rhl* gene family (Zhu and Rock, 2008). Gene *rhlA* encodes for the enzyme RhlA which uses metabolic intermediates from fatty acid biosynthesis to produce 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the lipid precursors of rhamnolipids (Zhu and Rock, 2008). Two enzymes, RhlB and RhlC, are metabolically downstream of RhlA and each adds a single rhamnose to produce mono-rhamnolipids and di-rhamnolipids respectively (Zhu and Rock, 2008). The secreted biosurfactants are thus a mixture of HAAs, mono- and di-rhamnolipids. RhlA, RhlB and RhlC have no other known functions, and loss-of-function mutants in the gene *rhlA* are incapable of any biosurfactant production and, consequently, swarming (Deziel *et al*., 2003; Caiazza *et al*., 2005). RhlA expression is the only requirement for the initiation for rhamnolipid synthesis in *P. aeruginosa* (Zhu and Rock, 2008), which makes the genetic regulation of the *rhlAB* operon key to the use of rhamnolipids biosurfactants as cooperative secretions.

We show that *P. aeruginosa* lowers the cost of biosurfactant secretion by regulating *rhlAB* expression to ensure that biosurfactants are produced only when carbon source is in excess of that needed for growth. This mechanism, which we call 'metabolic prudence', makes swarming colonies less susceptible to exploitation by *rhlA*$^-$ cheaters, which lack biosurfactant secretion but can swarm using the secretions of others. This mechanism works by ensuring that cells only invest carbon into rhamnolipid synthesis when growth is limited by another nutrient, the nitrogen source. By constructing a strain where the *rhlAB* operon is regulated by an inducible promoter, we show that swarming can become susceptible to cheating. We end by discussing how metabolic prudence explains why certain pathogenic secretions are evolutionary stable, as well as the broader implications of prudence for the evolution of cooperative behaviours.

Results

Swarming motility in P. aeruginosa *is a cooperative trait that resists 'cheating'*

Swarming assays were initiated by inoculating *P. aeruginosa* at the centre of an agar Petri dish. A growing colony swarms towards the edges of the dish, covering the entire distance in less than 20 h to produce spectacular star-shaped patterns (Fig. 1A, Video S1). Swarming colonies consistently produced seven times more cells compared with those that were prevented from swarming ($P < 2 \times 10^{-9}$). We tested this both by placing cells on hard agar, which physically prevents swarming, and by removing the *rhlA* gene, which is essential for biosurfactant synthesis (Ochsner *et al*., 1994, Fig. 1B). Although the secreted biosurfactants are visible to the naked eye, the precise quantification of biosurfactants on swarming agar is difficult. However, we measured the biosurfactants produced in a shaking planktonic shaking culture with same medium composition and it amounted to 0.14–0.16 g l^{-1}, which corresponds to 19.8–22.5% of the total-cell dry mass (0.717 g l^{-1}, see *Experimental procedures*). We then tested whether the *rhlA*$^-$ mutant was capable of swarming using the secretions provided by wild-type (WT) cells. For this, we inoculated the *rhlA*$^-$ mutant and WT in different locations of the same Petri dish. The experiment showed that the *rhlA*$^-$, while unable to swarm alone, can indeed swarm in the presence of a biosurfactant-producing strain (Fig. 2A, Video S2) and thus that *rhlA*$^-$ cells can indeed use exogenous biosurfactants for swarming.

Following these initial results, our initial expectation was that *rhlA*$^-$ cells would behave as 'cheaters' when mixed with WT in the same swarming colony. Specifically, we expected the *rhlA*$^-$ to outcompete the WT since they can use the biosurfactants produced by others without contributing to their production. When we carried out competitions between *rhlA*$^-$ and WT cell types in 1:1 mixtures, we saw that the presence of the *rhlA*$^-$ mutant decreased mean fitness as the colonies at 24 h were smaller than those of the WT (Fig. 2B). However, we were surprised to not find any evidence of fitness differences between the two strains (Fig. 2B inset plots, $P > 0.3$). We carried out experiments at ratios of 100:1, 10:1, 1:10 and 1:100, which also showed no fitness difference (Fig. S1). To test this observation with greater accuracy, we extended the experiment over four cycles of swarming (Fig. 2C). At day 4, the two strains still had indistinguishable cell numbers ($P > 0.8$). Accordingly, the calculated ratio of the fitness of WT and *rhlA*$^-$ and was statistically indistinguishable from 1 (0.99 ± 0.05, where interval represents 95% confidence level). This lack of a measurable fitness difference was at odds with the copious amounts of biosurfactants secretions both measured in liquid (~20% of the biomass produced) and visible in swarming assays (Fig. 2D), which

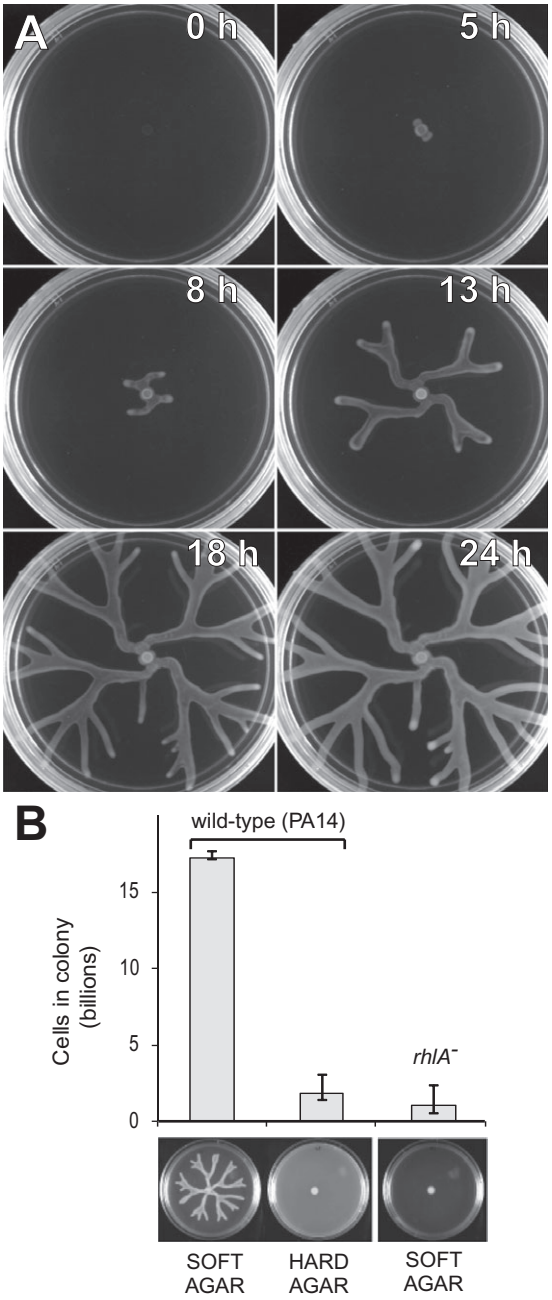

Fig. 1. Swarming is a collective form of surface motility in *Pseudomonas aeruginosa* that benefits the colony but requires individual cells to synthesize and secrete rhamnolipid biosurfactants.
A. Frames from time-lapse imaging of swarming. See Video S1.
B. Swarming colonies achieve much higher cell numbers than colonies grown on hard agar (1.5% agar, which prevents wild-type swarming, $P < 2 \times 10^{-9}$) or colonies of mutants lacking the *rhlA* gene necessary for biosurfactant synthesis ($P < 2 \times 10^{-9}$), showing that swarming benefits the colony.

we expected should translate into a major cost for the secreting cells.

Biosurfactant secretion occurs when cells have excess carbon

We sought to understand biosurfactant secretion further by turning to a liquid culture assay, where the medium is well mixed by constant shaking and cells are grown in their planktonic state. We used the same nutrient composition as in swarming assays except for the agar, which is the solidifying agent and therefore was omitted from liquid media. This method allowed the monitoring of growth and gene expression with high time resolution, together with the ability to do end-point measurements of rhamnolipids secreted. As mentioned above, the rhamnolipids secreted by WT amounted to 20% of their biomass, whereas as the *rhlA*$^-$ secreted no rhamnolipids as expected. Consistent with the lack of fitness difference on agar, experiments in liquid showed no evidence of a difference in growth rate or final density between the WT and *rhlA*$^-$ when both strains were grown separately (Fig. 3A). We also grew both strain in competition by mixing WT and *rhlA*$^-$ in the same medium. We still found no detectable change in the ratio of the two cell types, even after four daily passages ($P > 0.6$, Fig. 3A insert). We then studied the timing of biosurfactant synthesis expression by integrating a reporter fusion of the *rhlAB* promoter (P_{rhlAB}) and green florescent protein (GFP) (Boles *et al.*, 2005) into the WT genome. Time series of OD_{600} (cell density in liquid) and GFP (expression of *rhlAB*) measurements revealed that gene expression initiates only at high cell density, coinciding with the time that growth slows down and the bacteria enter stationary phase (Fig. 3B, see also Lequette and Greenberg, 2005). We then tested whether such a delay in the expression of *rhlAB* still occurs in swarming plates. We did this by comparing fluorescence by colonies of the strain carrying the P_{rhlAB}GFP fusion with that of a strain where GFP was under the regulation of a constitutive promoter (Fig. 3C). The time series of *rhlAB* expression confirmed that the delay also occurs in swarming assays (Fig. 3D). The observation that *rhlAB* expression is delayed provided a potential explanation for our findings: cell-density (quorum) sensing ensures that biosurfactant synthesis is delayed until cultures reach a high cell density, thus limiting its impact doubling timing (fitness).

We tested this idea by investigating the role of quorum sensing. The *rhlAB* operon is regulated by two hierarchical quorum-sensing systems: *lasI*/*lasR* and *rhlI*/*rhlR* (Latifi *et al.*, 1996; Fig. 4A). Each system has its own quorum-sensing signal. The signal HSL is produced by LasI and the signal C4HSL is produced by RhlI. We reasoned that if these systems were central to the delay in *rhlAB* expression, then exogenous supplementation of the two respec-

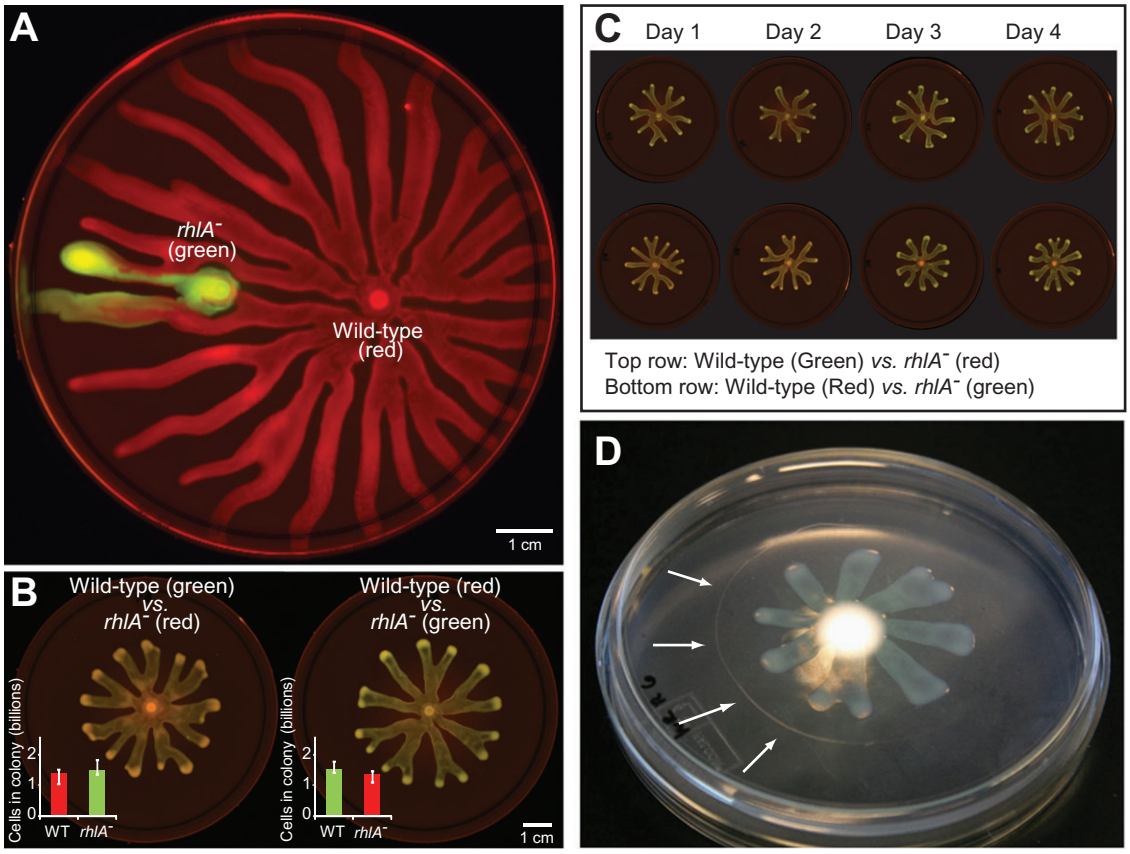

Fig. 2. The mutant *rhlA*⁻, lacking biosurfactant secretion, can use the secretions of others to swarm yet has no measurable competitive advantage.
A. Wild-type bacteria and *rhlA*⁻ were placed on the same plate in colonies initially separated by 2 cm. *rhlA*⁻ bacteria (labelled in green), incapable of swarming on their own, became motile once they became mixed with biosurfactant-secreting wild-type cells (labelled in red, see Video S2).
B. *rhlA*⁻ swarms equally as well as the wild type when mixed in the same colony at 1:1. The two genotypes show no fitness difference within the mix at 24 h, suggesting a low cost of biosurfactant secretion.
C. The wild type remains robustly at 1:1 frequency (24 h of growth) when mixed with the *rhlA*⁻ even over 4 days of consecutive passages ($P > 0.6$) with no visible loss in swarming over time.
D. The massive ring of biosurfactants secreted (indicated with white arrows) is visible by naked eye.

tive quorum-sensing signals should trigger early *rhlAB* expression. We first measured the concentrations of signal that restore WT levels of biosurfactant secretion in the signal negative mutants *lasI*⁻ and *rhlI*⁻ (Fig. 4B). Then, we tested the effect of adding signals at saturating levels to WT cultures at the beginning of growth. Surprisingly, we found that adding either or both signals did not detectably affect WT growth (Fig. 4C and D) nor did it induce over-secretion (Fig. 4F). We also confirmed that the signal negative mutants, *lasI*⁻ and *rhlI*⁻, are incapable of swarming but that swarming is recovered by complementing the medium with the appropriate autoinducer (Fig. 5A). Using the P_{rhlAB}GFP reporter fusion, we further confirmed that while autoinducers are necessary for *rhlAB* expression in swarming assays, autoinducer presence by itself is not enough to remove the observed delay (Fig. 5B and C). This is consistent with previous reports suggesting that additional regulatory elements can modulate *rhlAB* expression in addition to the hierarchical *lasI*/*lasR*–*rhlI*/*rhlR* quorum sensing (Medina *et al.*, 2003; Heurlier *et al.*, 2004; Yarwood *et al.*, 2005).

We next investigated the effect of nutrient on the timing of the expression of biosurfactant synthesis gene. The common minimal medium used for swarming uses casamino acids (5 g l^{-1}) as the sole source of carbon and nitrogen. The addition of more casamino acids, which increases both carbon and nitrogen source levels in the media, allowed growth to higher optical density (OD_{600}) levels (Fig. 6A). This showed, as expected, that entry into the stationary phase was regulated by nutrients. Importantly, we found that when plotting *rhlAB* expression against growth rate (Fig. 6B) across the entire range of casamino acids used all the data collapsed to a single curve. On the other hand, the same expression data did

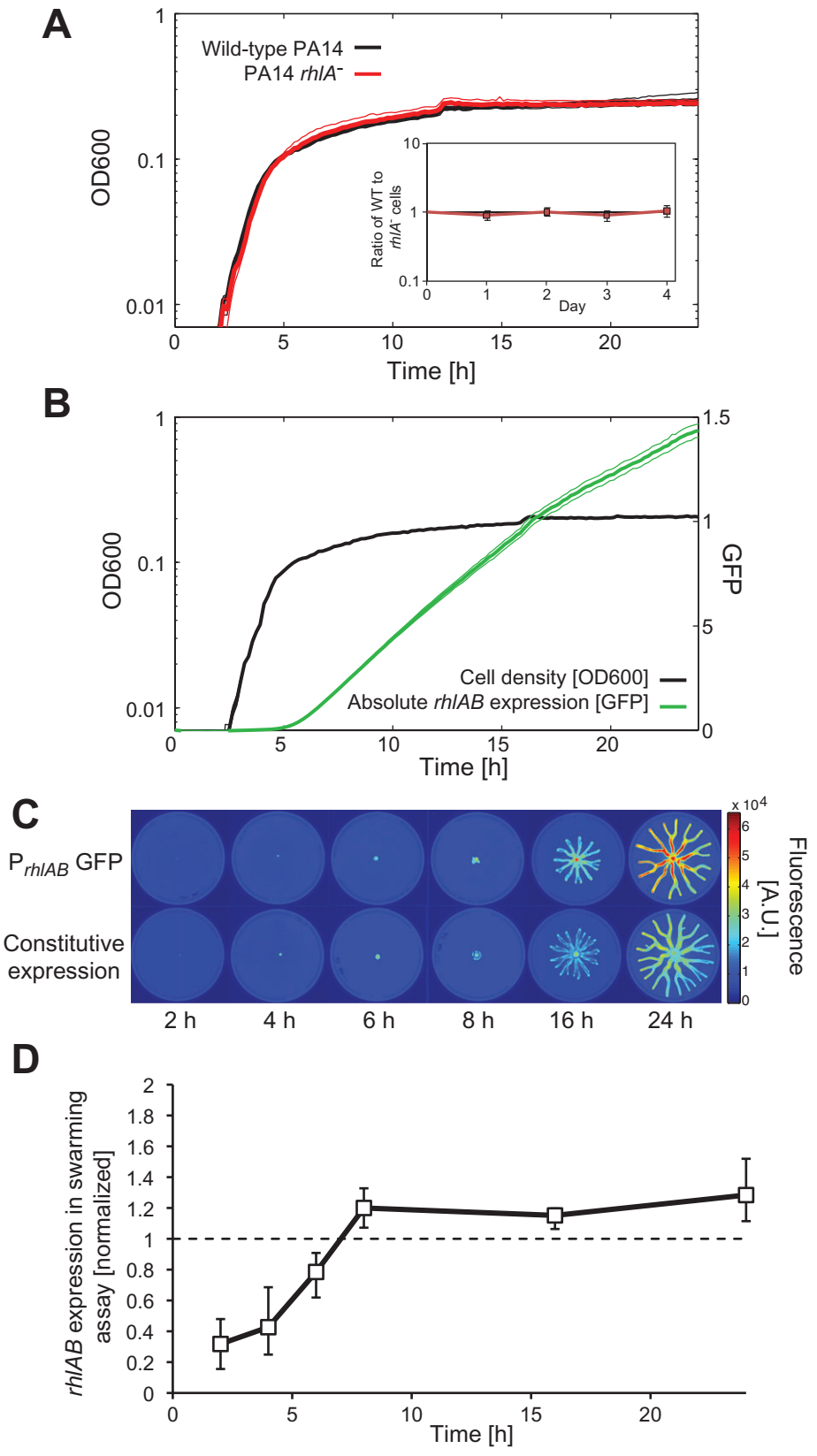
A
Wild-type PA14
PA14 rhlA-
OD600
Time [h]
Ratio of WT to rhlA- cells
Day
B
OD600
GFP
Cell density [OD600]
Absolute rhlAB expression [GFP]
Time [h]
C
PrhlAB GFP
Constitutive expression
2 h
4 h
6 h
8 h
16 h
24 h
x 10^4
Fluorescence [A.U.]
D
rhlAB expression in swarming assay [normalized]
Time [h]

Fig. 3. A. Growth curves in shaken liquid cultures show no difference between wild type (black) and *rhlA*⁻ (red), in spite of the large amounts of biosurfactants secreted by wild type. Inset: The ratio of wild type to *rhlA*⁻ cfu in a mixed culture grown in liquid (starting ratio of 1:1) is maintained even after four daily passages to fresh medium, again showing a lack of a fitness difference.
B. A GFP fusion to the *rhlAB* promoter in the WT shows that *rhlAB* expression is delayed until stationary phase. In (A) and (B) – thick lines are the median among six replicates with thinner lines showing the limits of 66 percentile among replicates. The same criterion was used in all following plots of growth curves.
C. Time-course of *rhlAB* expression was measured in swarming assays using the P_{rhlAB}GFP reporter fusion and compared with constitutive GFP expression. The pictures shown use pseudocolour to highlight the fluorescence level.
D. The P_{rhlAB}GFP expression was quantified by image analysis and normalized relatively to constitutive expression to confirm that the delay in *rhlAB* expression observed in liquid culture (B) occurs also in the swarming assay.

not collapse to a single curve when plotted against OD (Fig. 6B inset). This suggests that *rhlAB* expression is tightly coupled to the growth rate, but not cell density *per se*, and therefore that cells initiate biosurfactant synthesis when growth decreases.

Earlier studies aimed at optimizing industrial rhamnolipid production (Guerrasantos *et al.*, 1984) found that rhamnolipid secretion depends strongly on the ratio of carbon to other essential nutrients. Consistent with this, we supplemented our growth medium with a nitrogen source (ammonium sulphate) and found the same effect of increased OD (Fig. 6C) as obtained by increasing casamino acids. This result suggested that indeed growth in the casamino acids medium is limited by nitrogen source and suggested that nitrogen depletion is what triggers *rhlAB* expression in our assay. We then carried out additional growth experiments in ranges of carbon and nitrogen levels, which we could manipulate independently by using glycerol as the sole carbon source and ammonium sulphate as sole the nitrogen source. The results confirmed that nitrogen limitation induces *rhlAB* expression, but not carbon limitation (Fig. 6D).

Taken together, our data suggest that the cells delay expression of biosurfactant genes to only secrete when

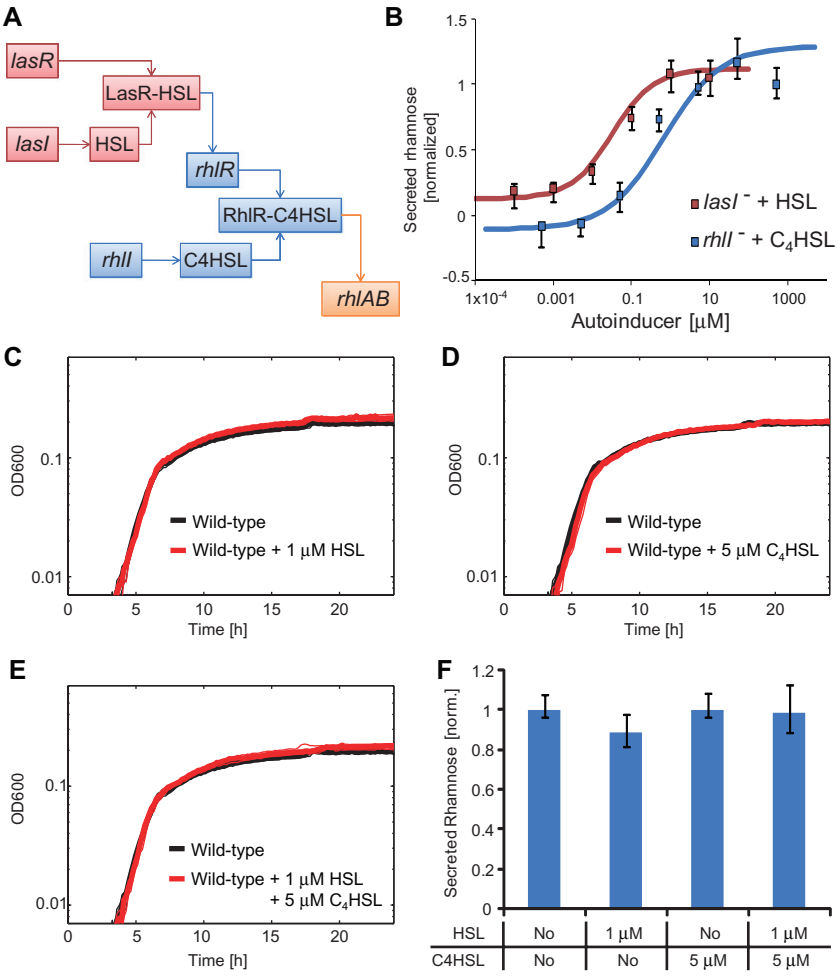

Fig. 4. The HSL and C4HSL quorum-sensing signals are required for biosurfactant secretion, yet their addition to the medium addition does not cause over-secretion.
A. Diagram of the hierarchical quorum-sensing system *lasR*/*lasI*–*rhlR*/*rhlI* regulating expression of the *rhlAB* operon.
B. Mutants that are unable to synthesize either of the two quorum-sensing signals (*lasI*⁻ lacks synthesis of HSL and *rhlI*⁻ lacks synthesis of C4HSL) do not secrete biosurfactants in the absence of exogenous signal. These signal dose–response curves allowed measuring the autoinducer concentrations needed to restitute WT level of rhamnolipid secretion: 1 μM for HSL and 5 μM for C4HSL.
C–E. Adding saturating amounts of either signal (C and D) or both together (E) to growth media did not visibly affect growth of the wild type.
F. The rhamnolipids measured at the end of growth also showed no significant difference ($P > 0.03$, $P > 0.3$ and $P > 0.1$, respectively, for HSL, C4HSL and both signals when compared with wild type grown without signal addition).

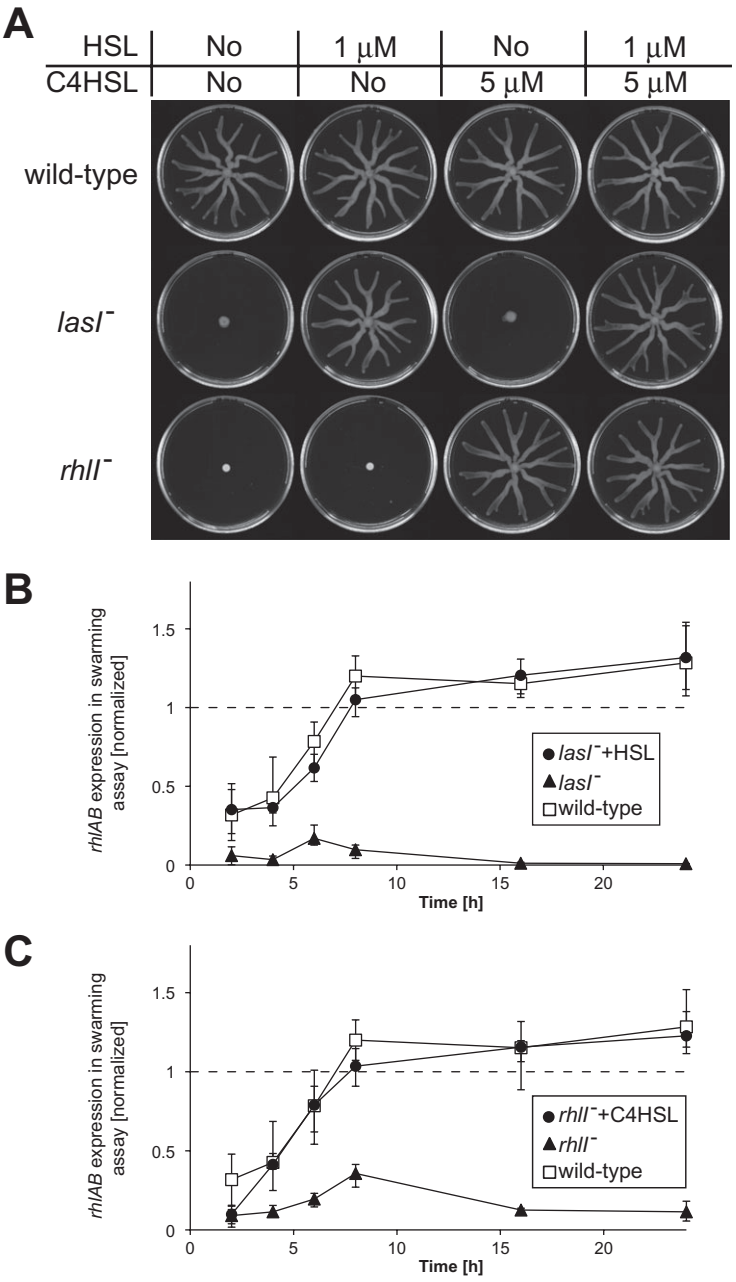

Fig. 5. The genes for synthesis of quorum-sensing autoinducers (*lasI* and *rhlI*) are necessary for *rhlAB* expression and swarming but adding the autoinducers does not anticipate *rhlA* expression.
A. *lasI*⁻ and *rhlI*⁻ lack swarming motility and swarming is rescued by adding the corresponding autoinducer, HSL and C4HSL, respectively, to the swarming medium.
B. The *lasI*⁻ mutant does not express *rhlAB* unless its product, HSL, is added to the medium. Nevertheless, HSL addition does not anticipate *rhlAB* expression relative to the wild type.
C. The *rhlI*⁻ mutant and its product, the autoinducer C4HSL, show the same effect – complementation by signal addition does not remove the delay in *rhlAB* expression.
In both (B) and (C), the P_{rhlAB}GFP fluorescence was normalized by GFP expression regulated by a constitutive promoter.

excess carbon is present, which can occur when growth becomes limited by other nutrients such as nitrogen. This model explains why there is little or no growth cost to biosurfactant production. Biosurfactants are only produced when the cells are not dividing, due to nitrogen limitation, and use carbon source that under these circumstances cannot be used for growth. Notably, secretion also depends on the hierarchical quorum-sensing systems regulating *rhlAB* (Latifi *et al.*, 1996, Figs 4 and 5). Therefore, *P. aeruginosa* appears to combine environmental information on the availability of excess carbon and quorum sensing to trigger the synthesis of biosurfactants.

Removing the native regulation of biosurfactants allows cheaters to invade

A key prediction of our model is that it would be costly for bacteria to produce biosurfactants during exponential growth. We therefore engineered a strain that produces biosurfactants even before reaching stationary phase by inserting *rhlAB* into the *rhlA*⁻ background under the regulation of the inducible P_{BAD} promoter (Boles *et al.*, 2005). This inducible strain (PA14 *rhlA*⁻ P_{BAD}*rhlAB*) expresses biosurfactant synthesis genes in the presence of an inducer (0.5% L-arabinose), thus bypassing their native

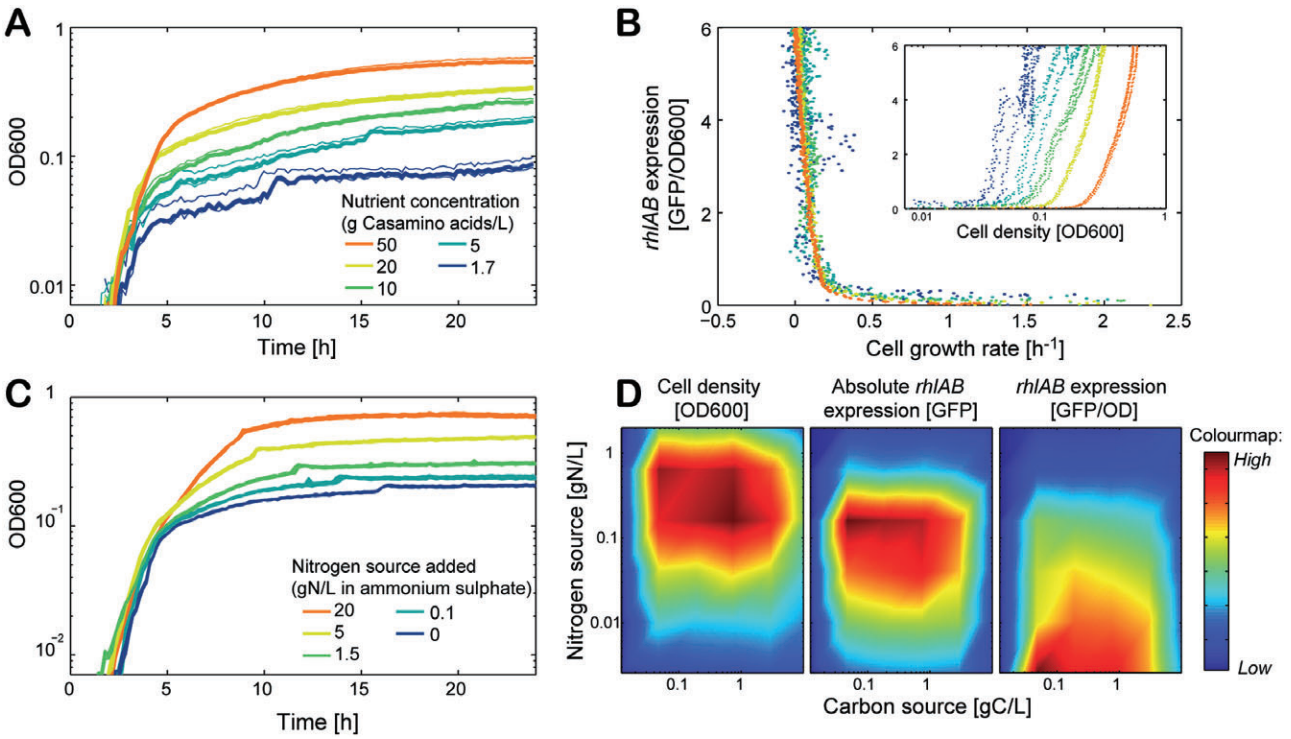

Fig. 6. The expression of genes for biosurfactant synthesis is growth rate dependent and is triggered by nitrogen limitation.
A. Increasing nutrients (casamino acids, the sole carbon and nitrogen source in the medium) increases growth, showing that entry into the stationary phase is due to nutrient depletion.
B. *rhlAB* expression data measured at all nutrient levels collapse to a single curve when plotted against growth rate – but not OD_{600} (inset) – revealing that expression is a function of growth rate, not cell density *per se*.
C. Complementing media with a nitrogen source (ammonium sulphate) has the same effect as increasing casamino acids, showing that growth was originally limited by nitrogen in the standard medium.
D. Two-dimensional matrices of OD, GFP expression and GFP/OD measured at 24 h for a range of carbon (glycerol) and nitrogen (ammonium sulphate) source levels. These data show that *rhlAB* expression is favoured at lower nitrogen/carbon ratios, when compared with the conditions producing optimal cell growth (highest OD).

transcriptional regulation. We measured growth curves in liquid and saw that induction caused a significant impact for this engineered strain in terms of its exponential growth rate measured in liquid (Fig. 7A) when compared with the same strain without the inducer, the WT or the *rhlA*$^-$ strains ($P < 0.002$). The induced strain secreted 3.6-fold the WT secretion levels by the end of the experiment (Fig. 7B, $P < 10^{-6}$), and therefore our manipulation is affecting both the timing of gene expression and the overall amount of secretions. However, the cost of this relative to the WT is only seen during exponential growth – when the WT does not express – showing the importance of timing (Fig. 7A). Returning to the swarming assay, we confirmed that the inducible strain could swarm when L-arabinose was added to the medium (Fig. 7C). Despite differences to the WT swarm morphology, likely to be derived from differences in biosurfactant secretion (Caiazza *et al.*, 2005), swarming motility still provided an enormous net benefit as shown by the final number of cells in swarming colonies (Fig. 7D). Next, we competed the inducible strain against the *rhlA*$^-$ mutant. For this we prepared mixed swarming assays where the inoculum was a 1:1 mix of the two strains. As expected, the inducible strain was both exploited and outcompeted in direct competition against *rhlA*$^-$ (Fig. 8A). Unlike the WT (Fig. 2C), the frequency of the inducible strain decreased in favour of *rhlA*$^-$ cells over daily passages (visible in colour shift in Fig. 8A and plotted in Fig. 8B). By the end of the fourth daily passage, the colonies showed no swarming motility (Fig. 8A) and the final number of cells in colonies decreased to the numbers of cells in a colony of pure *rhlA*$^-$ (Fig. 8C, $P > 0.6$).

We have shown that prudent regulation makes rhamnolipid secretion immune to *rhlA*$^-$ mutants, something that is not true of obligate secretion of rhamnolipids (Fig. 8). This provides a candidate evolutionary explanation for why *P. aeruginosa* regulates rhamnolipid secretion as it does, i.e. the frequent evolution of *rhlA* mutants favoured a molecular mechanism that prevented *rhlA* mutants from invading. However, an alternative explanation for the origin of prudent regulation is that it is simply more efficient, i.e. the WT is more efficient in its use of rhamnolipids than the obligate producer. There is no clear fitness difference

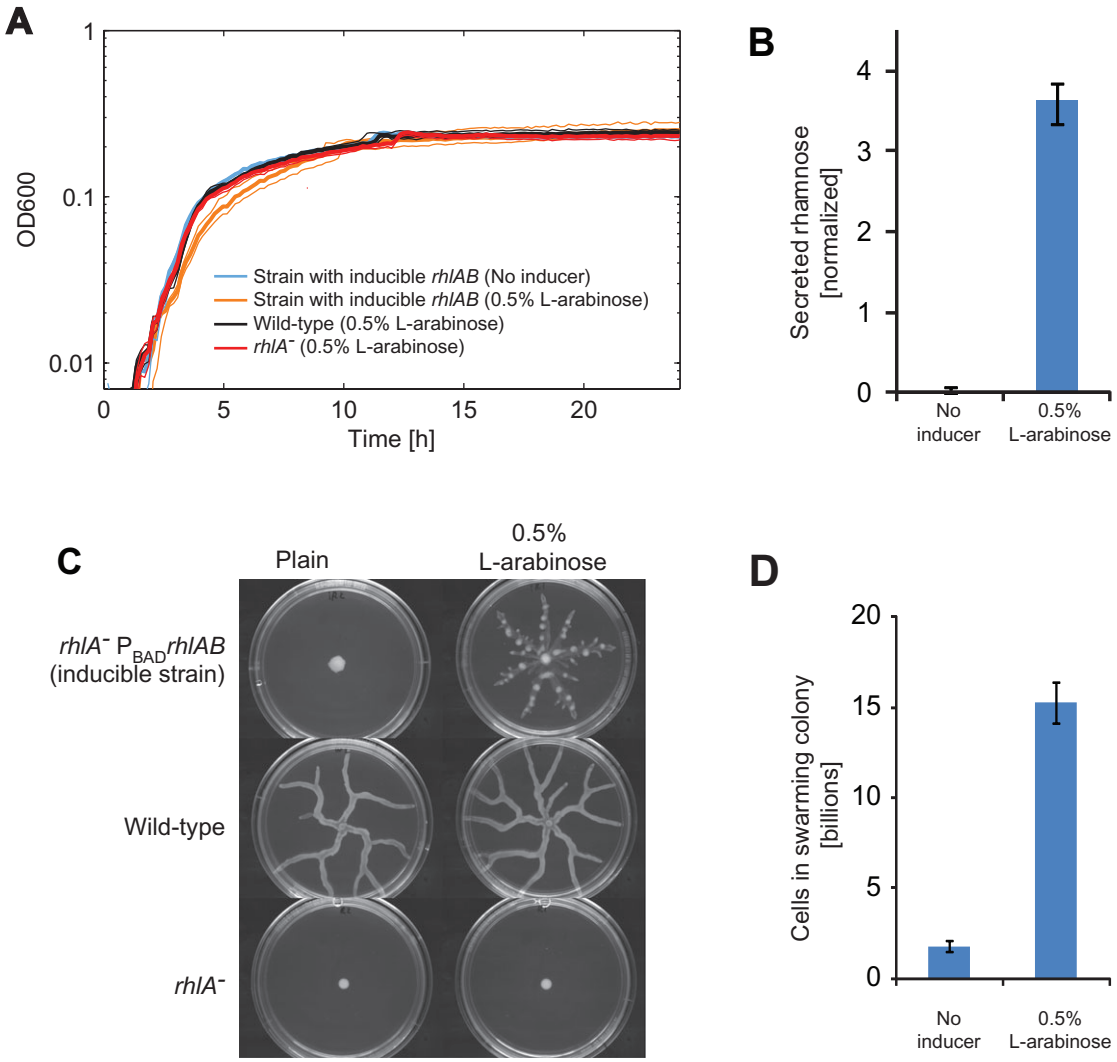

Fig. 7. Unregulated biosurfactant secretion has a detectable impact to growth in liquid culture but still provides a benefit in swarming assays.
A. The addition of 0.5% L-arabinose to the inducible strain (*rhlA*⁻ P_{BAD}*rhlAB*) results in constitutive biosurfactant secretion. Induction affects its growth in the exponential phase in contrast to the same strain in the absence of the inducer as well as the WT and *rhlA*⁻ strains in the presence of the inducer.
B. Rhamnolipids measured at 24 h of growth in liquid show that secretion by the inducible strain increases from virtually nil (without inducer) to 3.6× the wild-type secretion in medium with 0.5% L-arabinose (values normalized by wild-type secretion level).
C. The inducible strain swarms in medium complemented with 0.5% L-arabinose. The inducer does not affect the phenotypes of wild type and *rhlA*⁻.
D. Cell counts from colonies of the inducible strain with and without the inducer show that induced swarming is very beneficial to the population.

between the obligate producer and the WT when alone, with both reaching comparable cell densities (Figs 1B and 7D). In order to test their performance in direct competition, we mixed the WT with the inducible strain in 1:1 mixes in media containing 0.5% L-arabinose. Our data show that the WT initially increases in frequency above the initial 1:1 at 2 h ($P < 0.0016$) but then shows no further advantage (Fig. S2). This is consistent with there being an efficiency cost to obligate secretion, but only when cells are growing most rapidly. Overall, our data suggest that prudent secretion can evolve either due to efficiency or due to costs associated with cheater mutants. But either way, the outcome is evolutionarily stable cooperation.

Discussion

The evolution of cooperation is a fundamental problem in biology because selection for selfishness and cheating should undermine group behaviours (Pennisi, 2005). Secretions by bacteria are often viewed as an example of this problem (West *et al.*, 2006; 2007a; Diggle *et al.*, 2007; Sandoz *et al.*, 2007) since the secreted products of one

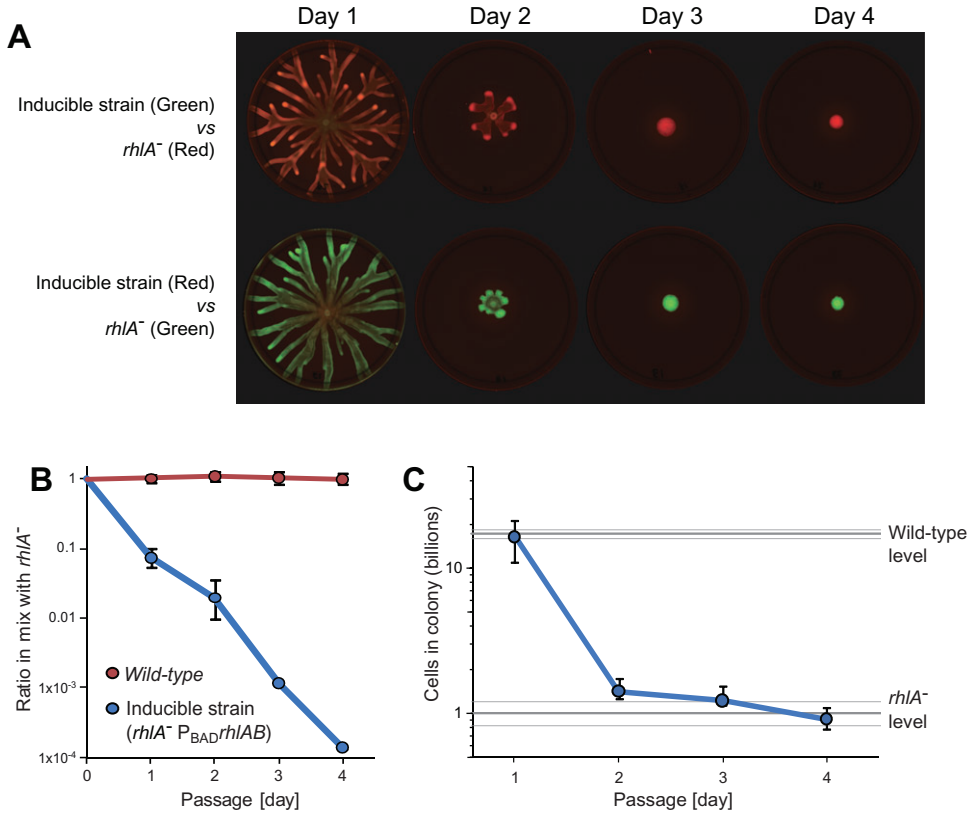

Fig. 8. Biosurfactant secretion becomes exploitable in a biosurfactant secreting strain that lacks the native *rhlAB* regulation (the inducible strain *rhlA*⁻ P_{BAD}*rhlAB*).
A. Unlike the wild type (Fig. 2C), *rhlA*⁻ P_{BAD}*rhlAB* is rapidly outcompeted by *rhlA*⁻ and colonies lose swarming over 4 days of consecutive passages. The experiment was also conducted by swapping fluorescent labels to show the outcome is independent of the fluorescent protein used.
B. Cell count ratios measured from the competition experiments confirm that strain *rhlA*⁻ P_{BAD}*rhlAB* has a strong competitive disadvantage against *rhlA*⁻ (blue), while wild-type cells do not (red).
C. The productivity of swarming colonies (number of cells in colony) comprising a mixture of *rhlA*⁻ P_{BAD}*rhlAB* and *rhlA*⁻ is comparable to that of the wild type at day 1 but decreases down to a level comparable to that of *rhlA*⁻ at day 4.

cell that can be used by another would seem an exploitable trait. Here we propose that one way bacteria can solve this problem is by prudent regulation of secretion genes (Fig. 9A). We showed that *P. aeruginosa* regulates *rhlAB* expression to ensure massive secretions of biosurfactants occur only when they will not severely impact on growth. The direct consequence of this is a lower cost of biosurfactant synthesis to individual cells. This explains why WT swarming motility is stable in 1:1 competition with non-producers, and even over several passages (Fig. 2C). By engineering a strain that secretes but lacks the native regulation, we confirmed that the reverse is also possible. Unregulated biosurfactant secretion becomes costly (Fig. 7A), making swarming exploitable and ultimately leading to a loss of swarming motility at a cost to the entire colony (Fig. 8B).

The regulation of biosurfactant synthesis in *P. aeruginosa* has proved puzzling (Medina *et al.*, 2003; Lequette and Greenberg, 2005) because it deviates from the established paradigm of quorum sensing where gene expression is simply a function of cell density. Our study helps to resolve this puzzle by showing that *rhlAB* expression in the presence of excess carbon can be tightly coupled to the growth rate, and not just cell density (Fig. 6B). Accordingly, the WT does not secrete biosurfactants, or swarm, unless both quorum-sensing and nutrient conditions are suitable. This suggests that *P. aeruginosa* has evolved to ensure biosurfactants are only secreted when both cell density is high enough for these secretions to be useful *and* there is an excess of carbon to minimize the impact of their synthesis (Fig. 9B).

The role of swarming and rhamnolipid secretion in nature

What is the role of swarming in natural systems? While often interpreted as an adaptation (Verstraeten *et al.*, 2008; Venturi *et al.*, 2010), the functional significance of

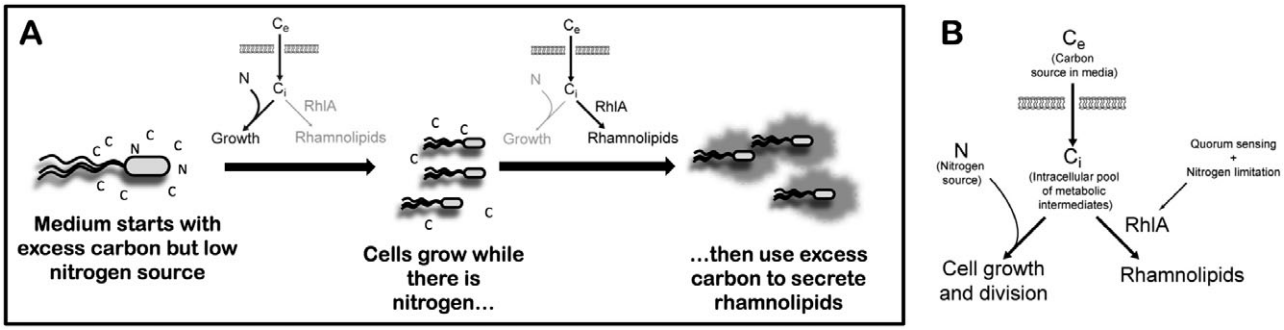

Fig. 9. Our data suggest that *Pseudomonas aeruginosa* regulates gene expression to secrete rhamnolipid biosurfactants only when carbon source is in excess – a mechanism we call 'metabolic prudence'.
A. Bacteria can grow and divide if both carbon and nitrogen source are available. In casamino acids media, carbon is initially in excess relative to nitrogen, and therefore bacteria grow exponentially until the nitrogen becomes limiting. Subsequently, cells express the biosurfactant synthesis genes and use the excess carbon at a low cost to their fitness (unlike cell biomass, rhamnolipid biosurfactants contain no nitrogen in their composition).
B. The enzyme RhlA plays a central role in biosurfactant synthesis. This enzyme is under the genetic regulation of *rhlAB*, the expression of which requires both quorum-sensing and adequate-nutrient conditions.

swarming in nature has yet to be established. The inverse regulation of biofilm formation and swarming motility suggests a close link between these two key surface behaviours (Caiazza *et al*., 2007) and supports that swarming is a genuine adaptation. Nevertheless, it remains possible that swarming as studied in the laboratory does not occur in exactly the same way in nature. Our conclusions, however, do not rest on the relevance of swarming under natural conditions because there is considerable evidence that rhamnolipid biosurfactants have additional functions such as the emulsification of water-insoluble substrates (Ochsner *et al*., 1994), promoting biofilm detachment (Boles *et al*., 2005), antimicrobial activity (Haba *et al*., 2003), virulence (Zulianello *et al*., 2006) and the disruption of host defences during infection (Read *et al*., 1992; Alhede *et al*., 2009).

An alternative explanation for maintenance of swarming might have been the discovery that *rhlA*$^-$ mutants are prevented from cheating due to a major pleiotropic cost to the loss of *rhlA* expression (Foster *et al*., 2004). However, there is no evidence in the literature of any pleiotropic effects in rhamnolipid synthesis genes (Zhu and Rock, 2008), and to be consistent with our data such a major pleiotropic cost would have to exactly match the cost of secretion to allow for growth curves of WT and *rhlA*$^-$ to match so closely as observed (Fig. 3A). Combined with our remaining data therefore we conclude that such a hidden cost is highly unlikely. It is still possible that small un-measurable cost exist, for example due to transcription and translation of the gene rather than its actual function, but is not critical to our arguments that biosurfactant secretion is absolutely cost free. What we can conclude, supported by competitions both in swarming (Fig. 2C) and in liquid (Fig. 3A inset), is that if any cost exists it is much smaller than simply expected from the large amounts of rhamnolipids secreted (~20% of biomass) and it is not enough to allow cheaters to increase in the population (Fig. 2C). We showed that this is possible because gene regulation ensures biosurfactant synthesis genes expression does not coincide with fast growth (Figs 3B and 6B).

Broader evolutionary implications

We propose that metabolic prudence is a molecular mechanism that renders bacterial secretions stable against 'cheaters', or at least greatly slows their evolution. This mechanism may play a key role in other microbial species as well. It is well accepted in microbial physiology that, when limited by a nutrient other than carbon and energy sources, cells will tend to redirect the non-limiting carbon flux to other functions, including secretions (Harder and Dijkhuizen, 1983). Recent mathematical models suggest that bacteria sense intracellular metabolites to perform systems-wide adjustments of metabolic fluxes (Kotte *et al*., 2010). Although the growth-limiting factor inducing rhamnolipid secretion was the nitrogen source in our experiments, a recent study supports our model by showing that iron limitation can also induce *rhlAB* expression in *P. aeruginosa* (Glick *et al*., 2010). A concrete example in another system is biofilm formation in *Salmonella typhimurium*. There, the synthesis of the cellulose, carbon-rich, matrix is positively regulated by *csgD*, a transcription regulator that is induced by depletion of phosphate, nitrogen and iron but not by depletion of carbon source (Gerstel and Romling, 2001).

It is important to distinguish between mechanistic (proximate) and evolutionary (ultimate) explanations for phenotypes (Tinbergen, 1963; West *et al*., 2007b). Most obviously, prudent regulation is a mechanistic phenomenon that involves responding to nutrient conditions and

limiting secretion to times where it has little or no effect on growth rate. However, this observation naturally feeds into ultimate considerations of why cooperation evolves. Namely, the existence of prudence can stabilize cooperation by altering its costs and benefits so as to reduce the fitness of cheating strategies (Fig. S2). But why would prudence itself be favoured by natural selection? Our data show that regulated biosurfactant secretion has the potential for large fitness returns (Fig. 1B) at effectively no cost (Fig. 2B and C). Therefore, so long as groups exist the tendency for a secreting cell to help itself and its genotype more than others in the population will allow prudent cooperation to evolve. It is important to emphasize that prudent cooperation may evolve without there ever being a cheating genotype, simply because prudence will tend to be an efficient strategy that allows minimum energy to be expended. It may also arise as a secondary modification to unregulated cooperation that is frequently disrupted by cheating genotypes, such as unregulated secretion (Fig. 8A). Either way, the outcome is stable cooperation that is resistant to any subsequent cheating genotypes.

There is a growing body of evidence to show that social organisms modulate social behaviours in response to changing costs and benefits (Korb and Heinze, 2008). Work on bacteria shows that reducing costs can increase cooperation over evolutionary timescales (Brockhurst *et al.*, 2008) and that some systems have phenotypic responses that only function to make cooperation more costly in the face of cheating (Brockhurst *et al.*, 2008; Kümmerli *et al.*, 2009). Closest to our findings is work showing that helping in social vertebrates can be increased by supplementary feeding (Clutton-Brock *et al.*, 1998; 1999). It is unclear in such cases whether the modulation of helping is sufficient to make it cost free and prevent the evolution of cheating, as demonstrated here. Nevertheless, the discovery of such responses in vertebrates suggests that prudence may play a role in diverse social species and provide a general explanation for the evolution of cooperation.

Experimental procedures

Strains

Pseudomonas aeruginosa PA14 (also called wild type or WT in the text) was donated by R. Kolter, Harvard Medical School. The *rhlA*$^-$ mutant strain was constructed from PA14 by deleting *rhlA* gene in its entirety using a suicide plasmid constructed by the gene splicing by overlap extension technique (Horton *et al.*, 1990). *rhlAB* exists as a monocistronic operon in this parent strain, and therefore the *rhlA*$^-$ mutant was constructed in a fashion that *rhlB* may remain functional and under the control of its native promoter. GFP and DsRedExpress constitutively labelled varieties of WT and *rhlA*$^-$ were prepared using the miniTn*7* transposon delivery plasmid, resulting in expression by the constitutive promoter $P_{A1/04/03}$GFP (Lambertsen *et al.*, 2004). Plasmids pYL122 (Lequette and Greenberg, 2005) (containing the P_{rhlAB}GFP fusion provided by E.P. Greenberg, University of Washington) and pEC16 (Boles *et al.*, 2005) (containing P_{BAD}*rhlAB*, provided by P.K. Singh, University of Washington) were used to integrate the constructs into the *P. aeruginosa* chromosome as a single copy. In the construction of the inducible strain PA14 *rhlA*$^-$ P_{BAD}*rhlAB*, although the *rhlA*$^-$ background strain keeps its functional *rhlB* under the control of its native promoter, the inclusion of *rhlAB* ensures that mono-rhamnolipid synthesis is completed in the inducible strain in the event that the native *rhlB* is inactive or its product stoichiometrically limiting thus allowing the measurement of secreted rhamnolipids.

Media and assays

The minimal medium for plate and liquid assays was prepared using the following recipe: 800 ml of Milipore water (with no agar for liquid assays, with 6.25% agar for swarming assays, with 1.857% agar for hard agar assays), 200 ml of 5× stock phosphate buffer, 1 ml of 1 M magnesium sulphate, 0.1 ml of magnesium sulphate, 25 ml of 200 g l^{-1} solution of casamino acids (Bacto™ from BD, Sparks, MD). One litre of 5× stock phosphate buffer was prepared by dissolving 12 g of Na_2HPO_4 (anhydrous), 15 of KH_2PO_4 (anhydrows) and 2.5 g of NaCl into 1 l of Milipore water. The final pH of medium was 6.7. When necessary, media composition was altered as described in the text. Autoinducers *N*-(3-Oxododecanoyl)-L-homoserine lactone (called HSL in the text) and *N*-Butyryl-DL-homoserine lactone (called C4HSL in the text) were acquired from Sigma-Aldrich (St. Louis, USA). Each swarming plate was prepared by pouring exactly 20 ml of medium onto a Petri dish and allowed to cool upright for 30 min. The plates were then turned upside down and left at room temperature to dry for 15 h. Inocula were prepared from 1 ml of overnight cultures washed twice with PBS. Plates inoculation was carried out by spotting a 2 μl drop of pre-washed culture at the centre of the swarming plate and allowed to dry. Plates were then placed upside down at 37°C for 24 h. Each swarming experiment was repeated nine times (three different days with three experimental replicates each). All liquid assays were carried out at 37°C with shaking, in 96-well microtitre plates using the Safire 2 (Tecan US) with OD_{600} and green fluorescence measured at 10 min intervals.

Imaging and quantification

Still pictures were taken with a gel doc imager (AlphaInnotech ChemiImager). Time-lapse videos were acquired using a Marshall electronics v-1070 surveillance camera, set up in a room acclimatized to 37°C. Swarming plates with fluorescently labelled strains were imaged using the Amersham Typhoon 9400 (GE Healthcare). Colony-forming units (cfu) were estimated by plating serial dilutions with different strains distinguished by fluorescent colour. Data points for number of cells in colony and rhamnose secreted measurements shown in plots represent the median value among all experimental replicates, with error bars representing the 95 and 5 percentile. Cell number ratios were determined by dividing the cfu number of one colour by the cfu number of the other colour. Error bars for such ratio measurements were estimated from binomial distribution fitting (Johnson *et al.*, 1993).

rhlA expression in swarming assays was assessed by quantifying the total GFP expression in swarming colonies of a strain containing the P_{rhlAB}GFP construct. The total colony fluorescence was measured in Photoshop and normalized by the fluorescence of a colony expressing GFP constitutively (promoter $P_{A1/04/03}$GFP). Secreted lipids were extracted from growth supernatants using a chloroform/methanol extraction protocol adapted from Caiazza *et al*. (2007). The rhamnolipids in the extract were measured using the anthrone colorimetric assay (Zhu and Rock, 2008). The amount of rhamnose in culture supernatant (47.4 mg l^{-1} for the WT grown for 24 h in the standard minimal medium) was calibrated using a rhamnose calibration curve. This was converted into rhamnolipid concentration applying a conversion factor of 3.0–3.2 (Camilios Neto *et al*., 2008), leading to the concentration of biosurfactants of 0.14–0.16 g l^{-1}. The concentration of dry mass of cells (0.717 g l^{-1}) was measured by gravimetry. Nalgene sterile analytical filter units (Thermo Fisher Scientific, Rochester, NY) with 0.2 μm pore size where pre-dried for 24 h at 65°C and used to filter 120 ml of culture. The filters where then dry for 48 h until mass became stable over time.

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