

Phenotypic plasticity of a cooperative behaviour in bacteria

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Abstract

There is strong evidence that natural selection can favour phenotypic plasticity as a mechanism to maximize fitness in animals. Here, we aim to investigate phenotypic plasticity of a cooperative trait in bacteria – the production of an iron-scavenging molecule (pyoverdin) by *Pseudomonas aeruginosa*. Pyoverdin production is metabolically costly to the individual cell, but provides a benefit to the local group and can potentially be exploited by nonpyoverdin-producing cheats. Here, we subject bacteria to changes in the social environment in media with different iron availabilities and test whether cells can adjust pyoverdin production in response to these changes. We found that pyoverdin production per cell significantly decreased at higher cell densities and increased in the presence of cheats. This phenotypic plasticity significantly influenced the costs and benefits of cooperation. Specifically, the investment of resources into pyoverdin production was reduced in iron-rich environments and at high cell densities, but increased under iron limitation, and when pyoverdin was exploited by cheats. Our study demonstrates that phenotypic plasticity in a cooperative trait as a response to changes in the environment occurs in even the simplest of organisms, a bacterium.

Introduction

Over the last 40 years, evolutionary biology has revolutionized our understanding of animal behaviour (Krebs & Davies, 1997). Evolutionary theory predicts that natural selection will favour individuals that maximize their inclusive fitness (Hamilton, 1964; Grafen, 2006). A key factor affecting fitness of an individual is the ability to adjust behaviour in response to changes in the environment (phenotypic plasticity), rather than have behavioural strategies fixed, depending on genotype. There is a convincing body of evidence that natural selection has favoured phenotypic plasticity in a variety of vertebrate and invertebrate species (Charnov, 1992; Stearns, 1992; Scheiner, 1993; Pigliucci, 1996; Agrawal, 2001; West-Eberhard, 2003).

It has recently been suggested that micro-organisms perform a number of cooperative behaviours, including the formation of fruiting bodies and the production of

extracellular factors that benefit the local group (Crespi, 2001; Velicer, 2003; West *et al.*, 2006, 2007a; Foster *et al.*, 2007). Cooperative behaviours add a new level of complexity to phenotypic plasticity, as they can vary in response to both the ecological and social environment. For example, in a cooperatively breeding bird, the decision to help or to breed can vary in response to the number of empty breeding sites in the population (an ecological factor) and/or to the number of helpers or the kin relationship on a given nest (social factors). Conditional adjustment of cooperative behaviour, depending upon the social environment, has been shown in a range of animals, including birds, mammals and social insects (Abbot *et al.*, 2001; Russell & Hatchwell, 2001; Clutton-Brock, 2002; Griffin & West, 2003; Field *et al.*, 2006; Komdeur, 2006; Ratnieks *et al.*, 2006). By contrast, relatively little is known about the extent to which microbes exhibit phenotypic plasticity with their social behaviours; adjusting them in response to variation in social conditions (Bassler & Losick, 2006; Keller & Surette, 2006; Diggle *et al.*, 2007a; West *et al.*, 2007a).

We test whether *Pseudomonas aeruginosa* cells adjust production of iron-scavenging siderophore molecules in response to variations in the social environment. Iron is a

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major limiting factor for bacterial growth because most iron in the environment is in the insoluble Fe(III) form and is actively withheld by hosts during opportunistic *P. aeruginosa* infections (Guerinot, 1994; Ratledge & Dover, 2000; Wandersman & Delepelaire, 2004; Miethke & Marahiel, 2007). In response to iron deficiency, *P. aeruginosa* releases pyoverdinin, the primary siderophore of this species, into the local environment (Budzikiewicz, 2001; Visca *et al.*, 2007). Pyoverdinin production is a cooperative behaviour (West *et al.*, 2007b) as it can provide a fitness benefit to neighbouring cells, which can take up iron bound to pyoverdinin produced by others. Individuals that do not produce pyoverdinin can avoid the metabolic cost of its production, but potentially still exploit the pyoverdinin produced by others (West & Buckling, 2003; Griffin *et al.*, 2004; Harrison *et al.*, 2006; Buckling *et al.*, 2007; Ross-Gillespie *et al.*, 2007; Kümmelerli *et al.*, 2008). Thus, pyoverdinin defective mutants, which have been isolated in natural populations, are potential cheats (De Vos *et al.*, 2001; Visca *et al.*, 2007).

Previous studies have demonstrated the underlying regulatory pathways controlling pyoverdinin production. At the proximate level, pyoverdinin production is regulated by the sigma factor PvdS, which in turn is regulated by the ferric uptake regulator (Fur) (Escobar *et al.*, 1999; Hantke, 2001; Visca *et al.*, 2002; Ravel & Cornelis, 2003; Wandersman & Delepelaire, 2004). In response to high intracellular iron concentration, the Fur protein forms a complex with iron and binds to the *pvdS* promoter, which represses pyoverdinin synthesis (Barton *et al.*, 1996; Leoni *et al.*, 1996; Visca *et al.*, 2002). Facultative expression of genes of the pyoverdinin synthesis pathway has since been experimentally demonstrated in media supplemented with differing amounts of iron (Tiburzi *et al.*, 2008). In contrast to this understanding at the genetic level, there is a lack of information on what happens at the phenotypic level, and the fitness consequences. In addition, it is not clear how pyoverdinin production is adjusted in response to the social environment, and how this may interact with iron availability.

In this paper, we first show that pyoverdinin production is extremely fine tuned in response to the amount of extracellular iron available confirming previous findings (Tiburzi *et al.*, 2008). Moreover, such facultative pyoverdinin production is essential, as shown by our experiments with constitutive pyoverdinin-producing mutants (i.e. strains that maintain high levels of pyoverdinin synthesis, irrespective of iron availability, due to mutations in the *fur* gene), which revert back to facultative production under high iron concentrations. We then test whether there is facultative adjustment in pyoverdinin production in response to cell density and the proportion of cheats within a population – two social factors that can cause changes in extracellular iron availability and in the efficiency of pyoverdinin use under natural conditions. Based on our knowledge of proximate mechanisms regulating pyoverdinin production, we

predict: (1) a decrease in pyoverdinin production per cell with increasing cell density, as pyoverdinin molecules diffuse freely in the media and are therefore shared more efficiently in the local environment at higher cell densities (Greig & Travisano, 2004; MacLean & Gudelj, 2006); (2) an increase in pyoverdinin production per cooperative cell with increasing proportions of cheats, to compensate for a higher proportion of exploitative neighbours; and (3) that iron availability influences both costs and benefits of pyoverdinin production, as well as the relative fitness of cooperators and cheats in mixed cultures.

Materials and methods

Bacteria strains

We used *P. aeruginosa* strain PAO1 (ATCC 15692), obtained from Pierre Cornelis' laboratory (Vrije University, Brussels, Belgium; Ghysels *et al.*, 2004), as the pyoverdinin-producing wild type (*wt*). This strain also produces pyochelin, which is a secondary siderophore that has a significantly lower affinity for iron (Ankenbauer *et al.*, 1985; Budzikiewicz, 2001). We also used three different deletion mutants directly derived from the specific PAO1 strain used (Ghysels *et al.*, 2004): strain PAO1 Δ *pvdD*, which is unable to produce pyoverdinin (*pvd*⁻) as the pyoverdinin synthetase *pvdD* is knocked out; strain PAO1 Δ *pchEF*, which is unable to produce pyochelin (*pch*⁻) due to deletions of pyochelin synthetases *pchE* and *pchF*; and PAO1 Δ *pvdD pchEF*, which is a double knockout defective for both pyoverdinin and pyochelin production (*pvd*⁻/*pch*⁻). Finally, we used two strains (C6 and A4) that constitutively produce pyoverdinin due to point mutations in the *fur* gene (Barton *et al.*, 1996). A4 was derived from PAO1, whereas C6 was derived from PA6261, a PAO1 mutant with a deletion in the *anr* gene, which controls anaerobic respiration in *P. aeruginosa* (Barton *et al.*, 1996).

We carried out all experiments in static 96-well microtitre plates in 200 μ L volumes of minimal-iron media (CAA): 5 g casamino acids, 1.18 g K₂HPO₄·3H₂O, 0.25 g MgSO₄·7H₂O, per litre; supplemented with 20 mM NaHCO₃ (sodium bicarbonate) and 100 μ g mL⁻¹ human apo-transferrin (Sigma, Gillingham, UK) (Meyer *et al.*, 1996; Griffin *et al.*, 2004). Apo-transferrin is a powerful natural iron chelator that binds free Fe(III) in the presence of bicarbonate (Schlabach & Bates, 1975) and prevents siderophore-mediated uptake of iron by bacteria. To prevent evaporation, we filled the outer wells of each microtitre plate with sterile water and only used the inner wells for experiments. In experiments with *wt*, *pvd*⁻, *pch*⁻ and *pvd*⁻/*pch*⁻, strains were cultured from freezer stock for 24 h prior to experimentation in 30-mL glass universals containing 6 mL of standard King's medium B (KB) in an orbital shaker (200 rpm) at 37 °C. We measured optical density (OD) at 600 nm

and diluted denser cultures prior to experimentation because strains differ slightly in their OD after a 24h growth period in KB (*wt*: 1.213 ± 0.006 , *pvd*⁻: 1.247 ± 0.007 , *pch*⁻: 1.174 ± 0.014 ; or *pvd*⁻/*pch*⁻: 1.116 ± 0.005). If experiments included the C6 and A4 strains, all cultures were grown from freezer stock for 24 h at 37 °C in 30-mL shaken glass universals containing 6 mL of minimal iron media (CAA). This was performed to prevent C6 and A4 reverting back to facultative pyoverdinin production in iron-rich media prior to experimentation.

Unless stated otherwise, we inoculated 10^5 bacteria from KB cultures into CAA media and let them grow under experimental conditions for 24 h at 37 °C in a static incubator. We then measured OD at 600 nm. Pyoverdinin fluoresces green and can be quantified in solution using a fluorimeter (SPECTRAMAX M2; Molecular Devices (Workingham, UK); excitation: 400 nm, emission: 460 nm, cut off: 475 nm) (Ankenbauer *et al.*, 1985; Cox & Adams, 1985; Prince *et al.*, 1993). Pyoverdinin is the only component measured in the culture using these excitation and emission parameters: nonpyoverdinin-producing strains give readings of zero. We used the ratio RFU/OD as a quantitative measure of pyoverdinin produced per unit of bacteria (i.e. per cell).

Manipulating iron availability

To test the effect of iron availability on pyoverdinin production and bacterial growth, we added different quantities (range 0–500 μM) of Fe(III)Cl₃ (ferric chloride; Sigma) to the CAA media. We subjected all four bacterial strains to nine different iron supplementation treatments in 12-fold replication.

To assess fitness consequences of constitutive pyoverdinin production, we subjected the *fur* mutants A4 and C6 to nine different iron supplementation treatments, as described above, in ninefold replication and compared pyoverdinin production and population growth to the *wt*. The *fur* gene is essential in *P. aeruginosa* (Barton *et al.*, 1996; Banin *et al.*, 2005), such that high iron conditions are lethal for *fur* mutants, and select for restoration of *fur* function and reversion to facultative pyoverdinin production. Indeed, we found that cultures inoculated with *fur* mutants showed decreased levels of pyoverdinin production with increased iron supplementation (see Fig. A1). Using chrome azurol S (CAS) assays (Schwyn & Neilands, 1987; Shin *et al.*, 2001), we could qualitatively demonstrate that high iron concentrations resulted in high reversion rates to facultative pyoverdinin production, presumably due to restoration of *fur* function. For C6, reversion was complete in treatments with FeCl₃ ($\geq 2 \mu\text{M}$) such that no constitutive pyoverdinin producers were detectable after a 24-h growth phase, whereas for A4 some constitutive pyoverdinin producers could persist under all iron concentrations (see Fig. A2). Despite reversion to facultative pyoverdinin production, our data

show that pyoverdinin was significantly overproduced by C6 and A4 compared with the *wt* in treatments with relatively low iron supplementations (0–5 μM FeCl₃). Pyoverdinin overproduction and processes involved in the restoration of *fur* function were associated with significantly lower bacterial densities and therefore reduced fitness in cultures with C6 and A4 and their revertants compared with *wt* cultures (see Fig. A3).

Manipulating cell density

To test whether cell density influences the quantity of pyoverdinin produced per cell, we varied inoculum size by adding approximately 10^3 , 10^4 , 10^5 or 10^6 cells from *wt* cultures grown in KB to the CAA media. We carried out experiments using three different iron supplementation regimes (0, 0.5 or 50 μM FeCl₃) in 24-fold replication.

If pyoverdinin production per cell is density dependent, we expected a change in the pyoverdinin concentration, relative to cell density, at different time points during growth. To test this, we inoculated 10^5 bacteria from *wt* cultures grown in KB into CAA media and recorded cell density and pyoverdinin concentration over time (0–54 h). This also allowed us to gather information on the durability of pyoverdinin molecules, which can be reused multiple times and accumulate in the media (Faraldo-Gómez & Sansom, 2003). We carried out the experiment with three different iron supplementation regimes (0, 0.5 or 50 μM FeCl₃) in eightfold replication.

Manipulating wild type vs. mutant density

We competed the *wt* strain with the *pvd*⁻/*pch*⁻ strain to test whether *wt* cells (cooperators) upregulate pyoverdinin production in the presence of *pvd*⁻/*pch*⁻ cells (cheats). We mixed *wt* and *pvd*⁻/*pch*⁻ from KB cultures in the following proportions of *wt* culture volumes: 0.17, 0.33, 0.5, 0.67, 0.83 and 1; and inoculated 10^5 cells into CAA media under three different iron supplementation regimes (0, 0.5 or 50 μM FeCl₃) in 16- to 20-fold replication. Volume ratios match cell ratios quite well, although *wt* cultures grow to slightly higher densities than *pvd*⁻/*pch*⁻ in KB (volume mixing of 1 : 1 resulted in a proportion of *pvd*⁻/*pch*⁻ cells = 0.48). We tested whether pyoverdinin production per *wt* cell is a function of the proportion of *wt* and *pvd*⁻/*pch*⁻ cells in the media. To account for changes in the proportion of *wt* cells, due to competition with *pvd*⁻/*pch*⁻, we used the average proportion of *wt* cells before and after the competition as the independent variable. We determined the initial and final ratios accurately by plating out dilutions of mixed cultures on KB agar and counting the number of colony-forming units (CFUs). The two strains were distinguished by their colour difference: *wt* colonies are green, whereas *pvd*⁻/*pch*⁻ colonies are white. We then calculated the relative fitness (*v*) of *pvd*⁻/*pch*⁻ as

$$v = \frac{x_2(1 - x_1)}{x_1(1 - x_2)}$$

where x_1 is the initial proportion of *pvd*-/*pch*- and x_2 is their final proportion. The fitness value of v therefore signifies whether *pvd*-/*pch*- increased in frequency ($v > 1$), decreased in frequency ($v < 1$) or remained at the same frequency ($v = 1$) over the competitive period.

Statistical analysis

We used regression models to test for significant relationships between iron availability, cell density, time, *pvd*-/*pch*- density (independent variables) and the pyoverdinin production per cell (dependent variable). We logarithmically transformed the values of pyoverdinin production per cell as well as iron concentration and bacterial relative inoculation densities prior to analysis. We performed linear and polynomial (quadratic and cubic) regressions for all data sets and determined the model that best fit our data following the procedure of Crawley (2007). We used R 2.7.0 (<http://www.r-project.org/>) for all statistical computations.

Results

Cells adjust pyoverdinin production in response to iron availability

The pyoverdinin-producing strains (*wt* and *pch*-) increased pyoverdinin production as iron was increasingly limited (Fig. 1a, linear regression on log-transformed values for *wt*: $r^2 = 0.964$, $F_{1,106} = 2888$, $P < 0.00001$; for *pch*-: $r^2 = 0.965$, $F_{1,106} = 2922$, $P < 0.00001$). Under high iron supplementation ($\text{FeCl}_3 \geq 50 \mu\text{M}$), pyoverdinin production ceased completely. These results are consistent with a similar pattern of facultative pyoverdinin production in response to iron supplementation described by Tiburzi *et al.*, (2008).

Pyoverdinin production had a dramatic effect on population growth and absolute fitness (Fig. 1b). At relatively low levels of iron supplementation (0–5 μM), the *wt* and *pch*- strain grew to significantly higher densities than pyoverdinin-defective strains (*pvd*- and *pvd*-/*pch*-) (contrast comparisons following ANOVA: $10.8 < t_{232} < 21.1$, all $P < 0.0001$). Under these conditions, strain *pvd*-/*pch*- hardly grew at all, whereas strain *pvd*- that is able to produce pyochelin grew to intermediate densities, illustrating that pyochelin can only partly compensate for the lack of pyoverdinin. Population growth of pyoverdinin-defective strains (*pvd*- and *pvd*-/*pch*-) increased at relatively high levels of iron supplementation (20–500 μM), although they grew to significantly lower densities than pyoverdinin-producing strains (contrast comparisons following ANOVA: $5.6 < t_{185} < 7.1$, all $P < 0.0001$).

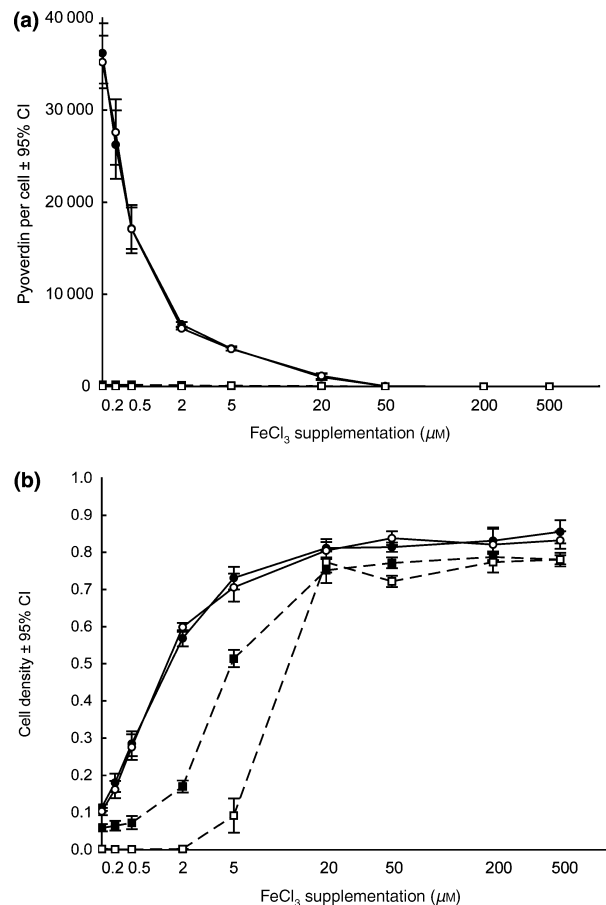


Fig. 1 (a) Pyoverdinin production per cell (RFU/OD₆₀₀) and (b) cell density (OD₆₀₀) after 24-h growth in iron-depleted media (CAA + 20 mM NaHCO₃ + 100 $\mu\text{g mL}^{-1}$ apo-transferrin) supplemented with various quantities of FeCl₃ (in μM). Values are given as mean \pm 95% confidence interval (CI) across 12 replicates. Pyoverdinin-producing strains (solid lines): *wt* (filled circle) and *pch*- (open circle). Pyoverdinin-defective strains (dashed lines): *pvd*- (filled square) and *pvd*-/*pch*- (open square).

Cells adjust pyoverdinin production in response to cell density

In iron-poor environments (0 and 0.5 μM FeCl₃), pyoverdinin production per cell decreased significantly with an increasing density of inoculum (Fig. 2, quadratic regression for 0 μM FeCl₃: $r^2 = 0.593$, $F_{2,92} = 69.5$, $P < 0.00001$; linear regression for 0.5 μM FeCl₃: $r^2 = 0.624$, $F_{1,94} = 158.6$, $P < 0.00001$). In the iron-rich environment (50 μM FeCl₃), bacteria produced no or very low amounts of pyoverdinin.

Our time-series analysis revealed that the amount of pyoverdinin per cell in the media varied significantly over the growth period (Fig. 3a) and therefore as a function of cell density (Fig. 3b). The relationship between the amount of pyoverdinin in the media and time (length of

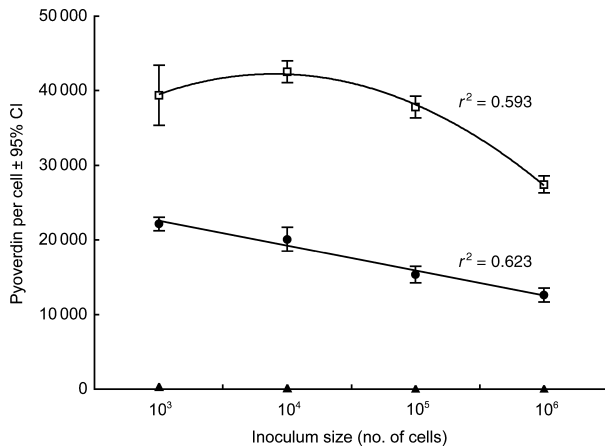


Fig. 2 Pyoverdinin production per *wt* cell (RFU/OD₆₀₀) as a function of the bacteria inoculum size. Values are given as mean \pm 95% confidence interval (CI) across 24 replicates after 24-h growth in iron-depleted media supplemented with 0 μ M FeCl₃ (squares), 0.5 μ M FeCl₃ (circles) or 50 μ M FeCl₃ (triangles).

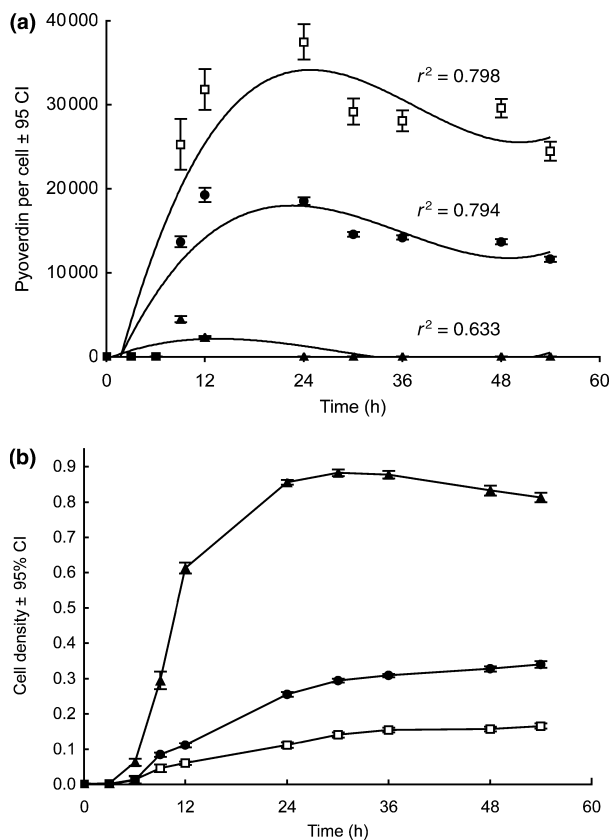


Fig. 3 (a) Quantities of pyoverdinin per *wt* cell in the media and (b) cell density (OD₆₀₀) at different time points during the growth period. Values are given as mean \pm 95% confidence interval (CI) across eight replicates after growth in iron-depleted media supplemented with 0 μ M FeCl₃ (squares), 0.5 μ M FeCl₃ (circles) or 50 μ M FeCl₃ (triangles).

growth period) was best described by cubic regressions (Fig. 3a, 0 μ M FeCl₃: $r^2 = 0.798$, $F_{3,76} = 105.3$, $P < 0.00001$; 0.5 μ M FeCl₃: $r^2 = 0.794$, $F_{3,76} = 102.2$, $P < 0.00001$; 50 μ M FeCl₃: $r^2 = 0.633$, $F_{3,76} = 46.3$, $P < 0.00001$). Hence, the amounts of pyoverdinin per cell increased rapidly after the lag phase (after 6 h) and peaked at 9 h (50 μ M FeCl₃), at 12 h (0.5 μ M FeCl₃) or at 24 h (0 μ M FeCl₃) and decreased thereafter. Pyoverdinin molecules can be reused multiple times (Faraldo-Gómez & Sansom, 2003); however, our time-series analysis shows that pyoverdinin detected at 12 h had completely disappeared after 24 h in the iron-rich environment (50 μ M FeCl₃), suggesting a limited durability.

Cells adjust pyoverdinin production in response to the presence of cheats

In iron-limited environments (0 and 0.5 μ M FeCl₃), *wt* cells (cooperators) significantly increased pyoverdinin production in the presence of higher proportions of nonpyoverdinin-producing cheats (*pvd*-/*pch*-) (Fig. 4a, linear regression for 0 μ M FeCl₃: $r^2 = 0.461$, $F_{1,108} = 94.2$, $P < 0.00001$; for 0.5 μ M FeCl₃: $r^2 = 0.871$, $F_{1,88} = 594.4$, $P < 0.00001$). A negative relationship might be expected simply due to the density effect observed in Fig. 2 (i.e. higher proportions of *pvd*-/*pch*- mean lower *wt* densities). However, the effect of *pvd*-/*pch*- cheat frequency on pyoverdinin production was four times (0 μ M FeCl₃) and three times (0.5 μ M FeCl₃) stronger (measured as the maximal difference in pyoverdinin production per cell between treatments) than the effect seen in the density experiment, and occurred at a cell density range 1000 times smaller than in the density experiment. In the iron-rich environment (50 μ M FeCl₃), pyoverdinin production was close to zero for all cooperator-to-cheat ratios.

The nonsiderophore-producing *pvd*-/*pch*- strain (cheat) exploited *wt* cooperators efficiently in the environment where no iron was added: cheat fitness values were significantly higher than one (Fig. 4b, two-tailed *t*-tests: $2.25 < t_{15-19} < 5.56$, all $P < 0.05$). This fitness advantage disappeared in the environments where iron was added (0.5 and 50 μ M FeCl₃), where *pvd*-/*pch*- fitness was either not significantly different from one or significantly lower than one, depending on the cooperator-to-cheat ratio (Fig. 4b). The proportion of cheats had a marginally significant effect on relative cheater fitness only in the 0 μ M FeCl₃ treatment (ANOVA on log-transformed data: $F_{4,89} = 2.50$, $P = 0.048$), whereas there was no significant effect in the 0.5 μ M FeCl₃ (ANOVA: $F_{4,73} = 2.25$, $P = 0.072$) and 50 μ M FeCl₃ (ANOVA: $F_{4,73} = 0.84$, $P = 0.50$) treatments. In the 0 μ M FeCl₃ treatment, cheats with a proportion of 67% cooperators had significantly lower fitness values than cheats in cultures with 50% (contrast comparisons: $P = 0.013$) and 83% ($P = 0.028$) cooperators. This observation does not have an obvious biological explanation.

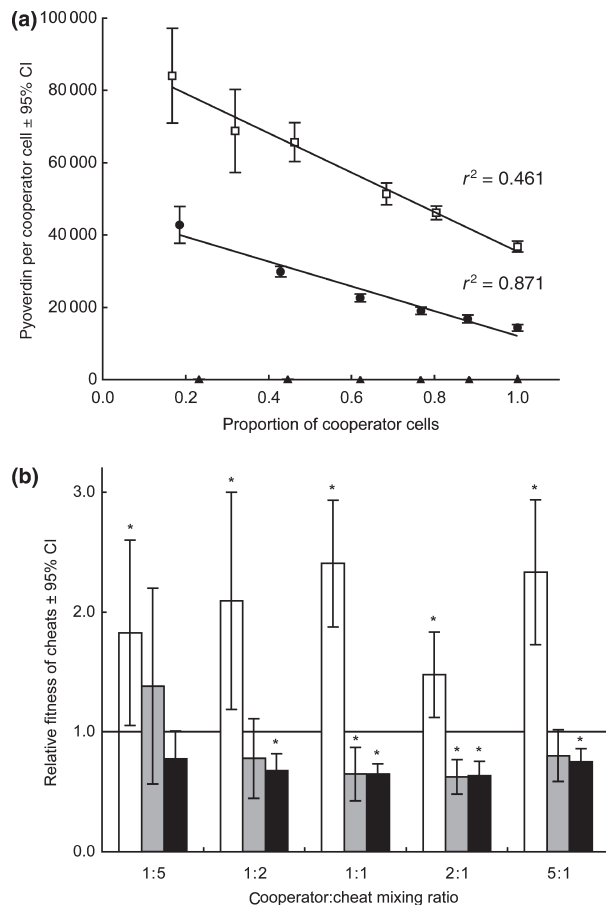


Fig. 4 Competition between nonpyoverdinin-producing *pvd*-/*pch*- (cheats) and pyoverdinin-producing *wt* (cooperators) in various mixing ratios and its effect on (a) the pyoverdinin production per cooperator cell, and (b) relative fitness of cheats compared with that of cooperators. Values with an asterisk are significantly different from 1 ($P < 0.05$). Competitions took place over 24 h in iron-depleted media supplemented with 0 μM FeCl₃ (squares and open bars), 0.5 μM FeCl₃ (circles and grey bars) or 50 μM FeCl₃ (triangles and black bars).

Discussion

We have demonstrated that the bacterium *P. aeruginosa* facultatively adjusts the production of its primary siderophore molecule, pyoverdinin, depending upon the social environment. Specifically, we have shown that wild-type cells produce more pyoverdinin at lower population densities and when a high proportion of the population is composed of individuals that do not produce pyoverdinin (cheats). Furthermore, facultative adjustment of pyoverdinin production in response to iron availability significantly affected the competitive dynamics between cheats and wild-type cells, such that the relative fitness advantage that cheats experienced in iron-depleted media disappeared in iron-rich environments as pyoverdinin production ceased.

We have shown that pyoverdinin production per wild-type cell decreases with cell density (Fig. 2). We predicted this because, at higher densities, pyoverdinin molecules are shared more efficiently, as fewer molecules are lost due to random diffusion. Consequently, investment in pyoverdinin production can be reduced because fewer molecules per cell are required to guarantee a sufficient supply of iron. This finding further demonstrates that pyoverdinin production has social consequences for both the focal cell that produces pyoverdinin and other, neighbouring cells and selection for pyoverdinin production can therefore be influenced by both direct and indirect fitness consequences (Griffin *et al.*, 2004; Kümmerli *et al.*, 2008). At higher cell densities, the relative importance of indirect fitness consequences increases (Greig & Travisano, 2004). In addition, the observation that *P. aeruginosa* downregulates pyoverdinin production in the presence of higher numbers of cooperative conspecifics contrasts with observations from between-species competitive interactions, where *P. aeruginosa* cells upregulated pyoverdinin production in the presence of competing *Staphylococcus aureus* bacteria (Harrison *et al.*, 2008).

The reduction of pyoverdinin production with increased cell density contrasts with the production of a number of extracellular products that are controlled by quorum sensing (QS) and only released above a certain cell density threshold (Diggle *et al.*, 2007a, Bassler & Losick 2006). There is some controversy over whether or not the regulatory pathways of QS and siderophores are interlinked. Although some studies showed evidence for such links (Stintzi *et al.*, 1998; Whiteley *et al.*, 1999; Cornelis & Aendekerk, 2004; Juhas *et al.*, 2004; Oglesby *et al.*, 2008), others could not confirm them (Schuster *et al.*, 2003; Wagner *et al.*, 2003) or provided alternative explanations (Bredenbruch *et al.*, 2006; Diggle *et al.*, 2007c; Dubern & Diggle, 2008). It has been suggested that QS coordinates release of extracellular products at high population densities, when their production will provide the greatest benefit (Diggle *et al.*, 2007b; Sandoz *et al.*, 2007). We suggest that variation in the fitness consequences of producing different extracellular products could account for the differences in their production in response to cell density. If a product is essential for growth, then it should be released at all population densities, and hence less will be required per cell at higher population densities due to more efficient sharing. By contrast, costly extracellular products that are beneficial, but not essential for growth, should only be produced at higher population densities when their production cost is diminished (Brown & Johnstone, 2001). Formal theory addressing this difference would be extremely useful (Nadell *et al.*, 2008).

How does facultative adjustment of cooperative behaviour affect the relative fitness of cooperators and cheats? Facultative adjustment of pyoverdinin production, as opposed to fixed levels of production, benefits cheats in low iron concentrations because cooperators are

stimulated into increasing production of pyoverdinin to compensate for exploitation (Fig. 4a). However, as iron supplementation is increased, we have shown that wild-type cooperator cells reduced ($0.5 \mu\text{M}$) or switched off ($50 \mu\text{M FeCl}_3$) pyoverdinin production (Fig. 1a). Thus, the total cost of pyoverdinin production was reduced when it was not needed, which increased the fitness of the cooperators relative to the cheats when grown in mixed populations (Fig. 4b). The overall effect of phenotypic plasticity in environments with fluctuating iron availabilities is therefore hard to predict, because, in mixed populations, it provides a relative advantage to cheats at low iron concentrations, but a disadvantage at high iron concentrations. This emphasizes the importance of examining both the ecological and social structure of natural populations. Additionally, a mechanism to avoid exploitation by cheats, and more generally by other strains, would be to make pyoverdinin molecules more specific such that their uptake is limited to close relatives (e.g. clonemates). In agreement with this idea, evidence for diversifying selection at the pyoverdinin locus has been described (Fuchs *et al.*, 2001; Smith *et al.*, 2005).

Monocultures of pyoverdinin-defective strains (*pvd-* and *pvd-/pch-*) grew to significantly lower densities than wild-type cultures under high iron availability (Fig. 1b) suggesting that the knocked out genes (coding for nonribosomal peptide synthetases) have other fitness-related functions. This seems plausible because nonribosomal peptide synthetases are multifunctional enzymes generally involved in the syntheses of a variety of exoproducts (Finking & Marahiel, 2004; Grünwald & Marahiel, 2006).

To conclude, our results illustrate two points. First, we have demonstrated phenotypic plasticity in response to changes in the social environment in a cooperative trait in bacteria. Phenotypic plasticity is also likely to affect selection on the production of other extracellular bacterial products that have been proposed as potential cooperative public goods (West *et al.*, 2007a). Secondly, the production of extracellular molecules such as siderophores is often associated with virulence effects in the host (Meyer *et al.*, 1996; Rumbaugh *et al.*, 1999; Takase *et al.*, 2000; West & Buckling, 2003; Harrison *et al.*, 2006; Harrison, 2007; Miethke & Marahiel, 2007). Thus, the consequences of phenotypic plasticity and the relative fitness of mutants compared with wild types may play a key role in their within-host evolution.

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Appendix

We used two strains (C6 and A4) of *Pseudomonas aeruginosa* that constitutively produce pyoverdine due to point mutations in the *fur* gene (Barton *et al.*, 1996) to compare their performance with that of facultatively pyoverdine-producing *wt* bacteria (PAO1). The *fur* gene is essential in *P. aeruginosa* (Barton *et al.*, 1996; Banin *et al.*, 2005), such that high iron conditions are lethal for *fur* mutants, and selects for restoration of *fur* function and reversion to facultative pyoverdine production.

Indeed, we found that pyoverdine production in cultures inoculated with C6 and A4 significantly decreased with increasing quantities of iron supplementation – a pattern that is very similar to the facultative pyoverdine production pattern shown by the *wt* strain (Fig. A1, linear regression on log-transformed values, for C6: $r^2 = 0.953$, $F_{1,79} = 1616$, $P < 0.00001$; for A4: $r^2 = 0.945$, $F_{1,79} = 1351$, $P < 0.00001$; for *wt*: $r^2 = 0.958$, $F_{1,79} = 1846$, $P < 0.00001$).

To determine whether the facultative pyoverdine-production patterns by C6 and A4 were due to bacteria strains having restored *fur* function, we compared phenotypes of cultures prior to and after the experiment on CAS plates (Schwyn & Neilands, 1987; Shin *et al.*, 2001). The production of pyoverdine causes a colour change from blue to orange in the CAS reagent, which leads to an orange halo forming around the colonies on the agar plate. Accordingly, colonies of a facultative pyoverdine producer should have no halos on CAS plates supplemented with iron (100 μM FeCl_3), whereas halos should appear on CAS plates with no iron supplementation. By contrast, colonies of constitutive pyoverdine producers

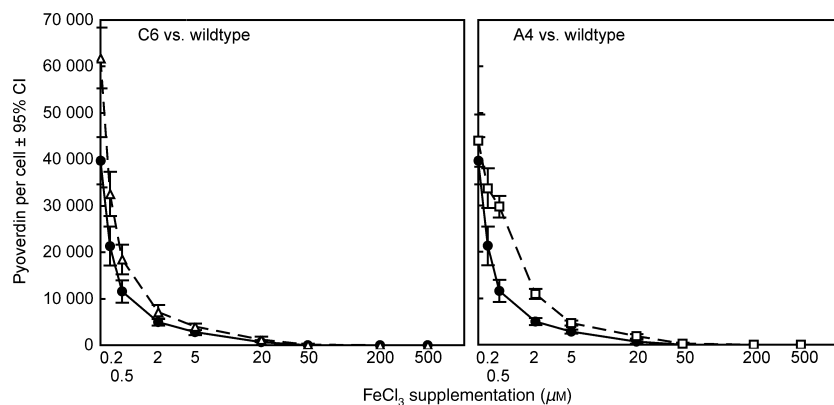


Fig. A1 Mean pyoverdine production per cell (RFU/OD₆₀₀) in iron-depleted media supplemented with various quantities of FeCl_3 (in μM). Comparisons between wild type (filled circles) and (a) C6 *fur* mutant (open triangles) and (b) A4 *fur* mutant (open squares).

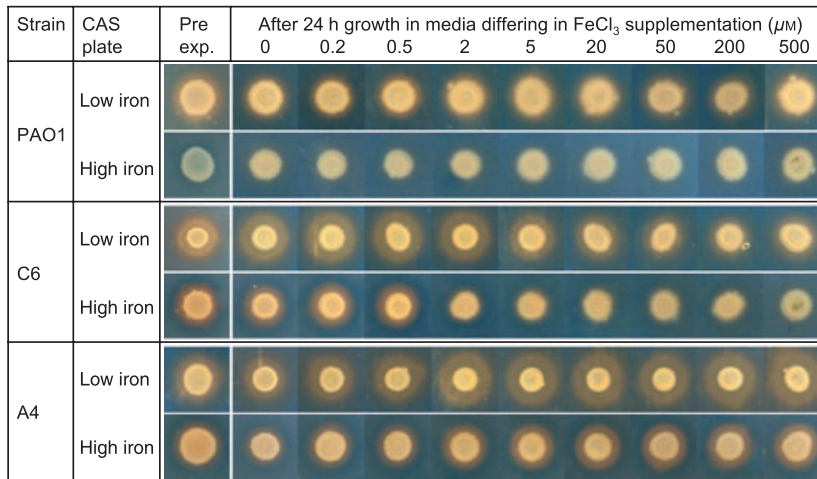


Fig. A2 The effect of experimental treatments (FeCl₃ supplementation in μM) on whether or not colonies of *Pseudomonas aeruginosa* produce pyoverdinin on chrome azurol S (CAS) plates supplemented with no or high iron (100 μM FeCl₃). Pyoverdinin production causes a colour change from blue to orange in the CAS reagent, which leads to the formation of orange halos around colonies. Facultative pyoverdinin producers (PAO1) should have halos on low iron but not on high iron plates, whereas constitutive pyoverdinin producers (C6 and A4) should have halos on both low and high iron plates. The absence of halos around C6 colonies from experimental treatments supplemented with FeCl₃ ≥ 2 μM suggests that restoration of facultative pyoverdinin production occurred.

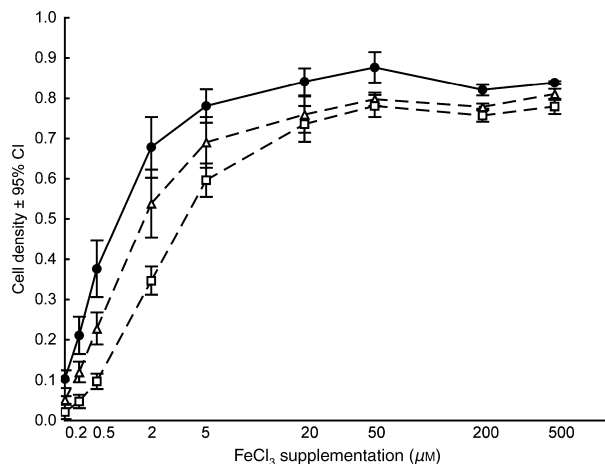


Fig. A3 Mean cell density (OD600) after 24-h growth in iron-depleted media supplemented with various quantities of FeCl₃ (in μM). *wt* (closed circles), C6 *fur* mutant (open triangles), A4 *fur* mutant (open squares).

should have halos, both on iron-rich and iron-poor CAS plates. As expected, *wt* cultures plated onto iron-rich and iron-poor CAS plates showed facultative pyoverdinin production patterns no matter whether cultures were plated out prior to or after the experiment (Fig. A2). Moreover, as expected, cultures of C6 and A4 developed halos, both on iron-rich and iron-poor plates when plated out prior to experimentation (Fig. A2). However, for C6 this

pattern changed when cultures were plated out after the experiment. On iron-rich CAS plates, there were no longer halos around colonies from cultures supplemented with FeCl₃ (≥ 2 μM) illustrating that in these experimental treatments only bacteria with mutations restoring *fur* function could survive and grow (Fig. A2). For A4, halos appeared on iron-rich CAS plates around colonies from all iron supplementation treatments. This indicates that some proportion of constitutive pyoverdinin producers survived in all treatments (Fig. A2). However, the strong similarity of pyoverdinin production patterns between C6 and A4 in response to iron supplementation (Fig. A1) suggests that mutations restoring *fur* function emerged and spread in A4 as well.

Despite reversions to facultative pyoverdinin production, our data show that pyoverdinin was significantly overproduced by C6 and A4 when compared with the *wt* in treatments with relatively low iron supplementations (0–5 μM FeCl₃, contrast comparisons following ANOVA, C6 vs. *wt*: $t_{126} = 7.3$, $P < 0.00001$, A4 vs. *wt*: $t_{126} = 10.6$, $P < 0.00001$). Pyoverdinin overproduction and restoration of *fur* function were associated with significantly lower bacterial densities, and therefore reduced fitness, in cultures with C6 and A4 and their revertants when compared with *wt* cultures (contrast comparisons following ANOVA: C6 vs. *wt*: $t_{232} = 7.4$, $P < 0.00001$, A4 vs. *wt*: $t_{232} = 13.8$, $P < 0.00001$, Fig. A3).

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