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SHORT COMMUNICATION

Siderophore production and biofilm formation as linked social traits

Freya Harrison and Angus Buckling
Department of Zoology, University of Oxford, Oxford, UK

The virulence of pathogenic microbes can depend on individual cells cooperating in the concerted production of molecules that facilitate host colonization or exploitation. However, cooperating groups can be exploited by social defectors or 'cheats'. Understanding the ecology and evolution of cooperation is therefore relevant to clinical microbiology. We studied two genetically linked cooperative traits involved in host exploitation by the opportunistic human pathogen *Pseudomonas aeruginosa*. Clones that defected from cooperative production of iron-scavenging siderophores were deficient in biofilm formation. The presence of such clones in mixed biofilms with a wild-type clone led to reduced biofilm mass. The fitness advantage of siderophore-deficient mutants in the presence of wild-type bacteria was no greater in biofilm than in planktonic culture, suggesting that these mutants did not gain an additional advantage by exploiting wild-type biofilm polymer. Reduced biofilm formation therefore represents a pleiotropic cost of defection from siderophore production.
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Bacteria cooperate in numerous ways, including public goods production, food scavenging and niche provision (West *et al.*, 2006). Cooperative traits can be linked by correlated expression or by their effects (Velicer, 2003; Banin *et al.*, 2005; Juhas *et al.*, 2005). Understanding selective pressures on cooperation is therefore likely to require consideration of multiple traits simultaneously. We investigated two clinically relevant cooperative traits: production of iron-scavenging siderophores (Griffin *et al.*, 2004; Harrison *et al.*, 2006) and biofilm formation (Costerton *et al.*, 2003; Brockhurst *et al.*, 2006; Moreau-Marquis *et al.*, 2008). Both can be exploited by 'cheats': cells that cease producing costly public goods (siderophores or biofilm matrix polymers) but benefit from the efforts of their neighbours. Cheats decrease total public good availability and hence population growth (Griffin *et al.*, 2004; Brockhurst *et al.*, 2006), potentially causing reduced virulence (Harrison *et al.*, 2006).

In *Pseudomonas aeruginosa*, biofilm formation requires iron (Singh *et al.*, 2002) and siderophore-deficient mutants show a pleiotropic reduction in biofilm-forming ability (Banin *et al.*, 2005). This could impose an additional cost on mutants with reduced siderophore production. However, siderophore-deficient mutants might be 'supercheats' within biofilms, exploiting two public goods whereas paying the costs of neither. If so, siderophore-deficient cells should not only invade cooperator biofilms, but also enjoy a greater growth advantage in biofilms than under planktonic growth.

To investigate this hypothesis, we grew biofilms using six siderophore-deficient clones, singly and in combination with the wild-type (cooperator) strain PA01. One clone was an isogenic transposon-bearing PA01 mutant (Jacobs *et al.*, 2003) with an insertion in *pvdF*, a gene required for synthesis of the primary siderophore pyoverdine (Visca *et al.*, 2007). The remaining clones evolved from the methionine auxotroph PA06049 (a transposon mutant of PA01: Rella *et al.*, 1985)) under selection for siderophore cheats (Harrison and Buckling, 2005; Supplementary information). Biofilms were grown using a microplate/peg lid system (Moskowitz *et al.*, 2004), in iron-limited or iron-supplemented medium (Full methods in Supplementary information).

Although biofilm formation by the *pvdF* mutant was enhanced by iron supplementation ($P=0.004$; Figure 1b), the mutant had lower total biofilm mass than its ancestor PA01 in both iron regimes (*t*-tests, $P<0.001$; Figure 1; Supplementary Figure S1). This is consistent with previous results (Banin *et al.*, 2005). In contrast, planktonic growth of the mutant

Correspondence. Current address: F Harrison, Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK.
E-mail: freya.andersdottir@gmail.com
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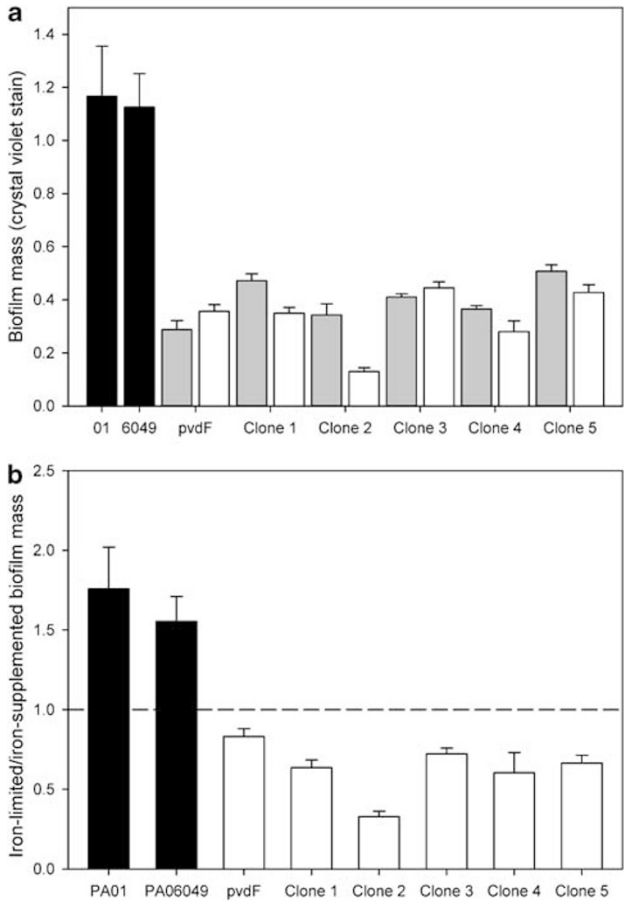

Figure 1 (**a**) Single-strain and mixed-strain biofilm mass in iron-limited growth medium. *y* Axis shows A_{590} of crystal violet-stained biofilms. White bars represent single-strain biofilms and grey bars represent PA01 + cheat mixtures; bars show mean ± standard error of the mean ($N = 10$ in each case). Pure cheat biofilms had lower mass than pure ancestral wild-type biofilms (*pvdF* versus PA01, $T_{15} = 7.08$, $P < 0.001$; clones 1–5 versus PA06049, $T \geqslant 7.39$, $P < 0.001$). Mixed PA01 + siderophore mutant cultures produced less biofilm than pure PA01 cultures ($T \geqslant 5.05$, $P < 0.001$). (**b**) Ratios of biofilm mass in iron-limited versus iron-supplemented growth medium for strains grown singly. *y* Axis shows A_{590} of crystal violet-stained iron-limited biofilms/A_{590} of crystal violet-stained iron-supplemented biofilms; bars show mean ± standard error of the mean ($N = 10$ in each case). Ratios were <1 for siderophore-deficient clones ($T \geqslant 2.81$, $P \leqslant 0.010$) but >1 for wild types (PA01 $T_9 = 3.88$, $P = 0.002$, PA06049 $T_9 = 4.34$, $P = 0.001$).

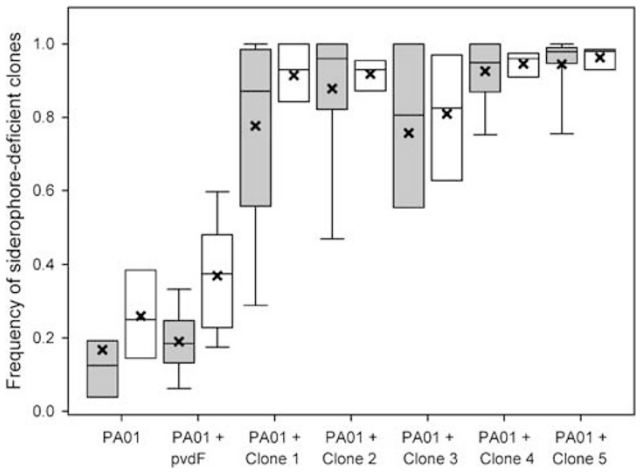

Figure 2 Box plots showing frequencies of siderophore-deficient clones recovered from PA01 and PA01 + cheat biofilm (grey boxes) and planktonic (white boxes) subpopulations in iron-limited growth medium. Boxes show median, 10th, 25th, 75th and 90th percentile, crosses show mean. For pure PA01 and PA01 + evolved clone mixtures, siderophore-deficient cells reached comparable frequencies in biofilm and planktonic subpopulations (Wilcoxon signed-rank tests, $P \geqslant 0.25$). In PA01 + *pvdF* populations, cells of the *pvdF* mutant reached lower frequencies in biofilm ($P = 0.014$), though this was not significant once α was corrected for false discovery rate.

was reduced relative to PA01 only under iron limitation (iron-limited $P = 0.01$, iron-supplemented $P = 0.727$; Supplementary Figure S2). This demonstrates a greater cost to siderophore deficiency in biofilms relative to planktonic cultures. Further, in mixed culture the mutant reached lower frequencies in biofilm than in plankton (Wilcoxon signed-rank test, $P = 0.014$; Figure 2). Mixed cultures produced biofilms of a comparable mass to pure *pvdF* cultures (*t*-test, $P = 0.38$; Figure 1), which was less than expected, given the proportions of wild-type and *pvdF* cells present (*t*-test, $P < 0.001$). As decreased biofilm mass presumably has disadvantages in isolation, but confers no additional cheating benefit in the presence of the wild type, this probably represents a pleiotropic fitness cost of siderophore cheating. The mutant is certainly not a 'supercheat'.

PA06049 produced a comparable level of biofilm to PA01 when iron was limiting (*t*-tests, $P > 0.9$). All five evolved clones produced less biofilm than PA06049 under iron limitation (*t*-tests, $P < 0.001$) and under iron supplementation (*t*-tests, $P \leqslant 0.021$). Iron limitation reduced biofilm formation by siderophore-deficient clones (*t*-tests, $P < 0.02$). This supports the link between siderophores, iron and biofilms in clones that could carry mutations in traits other than siderophore production. In contrast with the *pvdF* mutant, these results cannot be explained by poor growth of mutants in iron-limited medium: whereas one mutant showed reduced planktonic growth relative to PA06049 (clone 2, $P = 0.003$), one showed increased growth (clone 3, $P = 0.002$) and the remainder showed similar growth to PA06049 ($P > 0.4$) (Supplementary Figure S2). This apparently contradicts findings that siderophore deficiency decreases iron-limited monoculture growth (Griffin *et al.*, 2004). However, our clones had adapted to laboratory conditions for approximately 200 generations and other, growth-enhancing, mutations would have outweighed the costs of reduced siderophore production.

Mixed PA01 + evolved clone cultures produced less biofilm than pure PA01 cultures in all but one case (Figure 1a; Supplementary Figure S1; *t*-tests, $P < 0.001$ except iron-supplemented PA01 + clone 5 $P = 0.351$). Iron-limited mixtures formed biofilms whose mass did not differ from that expected given

the proportions of wild-type and siderophore-deficient cells present (*t*-tests, $P \geqslant 0.369$), except for PA01 + clone 3, which formed less biofilm than expected ($P < 0.001$).

Under iron limitation, evolved siderophore cheats did not gain an additional advantage in biofilm: in no case did the frequency of cheats recovered from the biofilm and liquid subpopulations differ (Figure 2, Wilcoxon signed-rank tests, $P \geqslant 0.25$). Moreover, although siderophore-deficient clones appeared *de novo* in pure PA01 populations, their biofilm and planktonic frequencies did not differ ($P = 0.299$). Like the *pvdF* mutant, these clones are not 'supercheats', and biofilm deficiency represents a pleiotropic fitness cost of siderophore cheating. Pleiotropic costs of cheating are known (Foster *et al.*, 2004), but we are unaware of any other report that cheating incurs a cost by disrupting another social trait.

Defective biofilms could impose a severe fitness cost on siderophore-deficient subpopulations. Rare siderophore-deficient mutants in a mainly wild-type biofilm may enjoy a fitness advantage due to exploitation of their neighbours, but increased mixing (or more frequent mutation to this phenotype) will compromise biofilm formation and this could prevent siderophore-deficient clones spreading. Conversely, if selection favours reduced siderophore production, this could have the knock-on effect of reducing biofilm, even if biofilms aid persistence. Experiments using mutants arising *de novo* in PA01 biofilms will clarify this area.

Siderophore production and biofilm-forming ability decrease over time in chronic *P. aeruginosa* infections of patients with cystic fibrosis (De Vos *et al.*, 2001; Lee *et al.*, 2005; Smith *et al.*, 2006). Do both changes represent adaptation to the airways? Or might selection for siderophore mutants explain the reduction in biofilm? This study emphasizes the importance of studying selection on a given cooperative trait in the context of other relevant social traits.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)