ORIGINAL ARTICLE

Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters

Alexandre Jousset¹, Laureène Rochat², Maria Péchy-Tarr², Christoph Keel², Stefan Scheu^{1,3,4} and Michael Bonkowski^{1,3,5}

¹Institute of Zoology, Darmstadt University of Technology, Darmstadt, Germany and ²Département de Microbiologie Fondamentale, Université de Lausanne, Lausanne, Switzerland

Soil pseudomonads increase their competitiveness by producing toxic secondary metabolites, which inhibit competitors and repel predators. Toxin production is regulated by cell–cell signalling and efficiently protects the bacterial population. However, cell communication is unstable, and natural populations often contain signal blind mutants displaying an altered phenotype defective in exoproduct synthesis. Such mutants are weak competitors, and we hypothesized that their fitness depends on natural communities on the exoproducts of wild-type bacteria, especially defence toxins. We established mixed populations of wild-type and signal blind, non-toxic *gacS*-deficient mutants of *Pseudomonas fluorescens* CHA0 in batch and rhizosphere systems. Bacteria were grazed by representatives of the most important bacterial predators in soil, nematodes (*Caenorhabditis elegans*) and protozoa (*Acanthamoeba castellanii*). The *gacS* mutants showed a negative frequency-dependent fitness and could reach up to one-third of the population, suggesting that they rely on the exoproducts of the wild-type bacteria. Both predators preferentially consumed the mutant strain, but populations with a low mutant load were resistant to predation, allowing the mutant to remain competitive at low relative density. The results suggest that signal blind *Pseudomonas* increase their fitness by exploiting the toxins produced by wild-type bacteria, and that predation promotes the production of bacterial defence compounds by selectively eliminating non-toxic mutants. Therefore, predators not only regulate population dynamics of soil bacteria but also structure the genetic and phenotypic constitution of bacterial communities.

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Introduction

Fluorescent pseudomonads are ubiquitous rhizosphere bacteria, and many species produce toxic exoproducts that increase their resistance to microfaunal predators and improve their competitiveness against other bacteria (Jousset *et al.*, 2008). These toxins often inhibit plant pathogens, making pseudomonads as potent biological control agents in agricultural systems (Haas and Defago, 2005). Toxin

production in *Pseudomonas* is controlled at the post-transcriptional level by the *gac/rsm* cascade (Lapouge *et al.*, 2008). Activation of the GacS/GacA two-component system by unknown signals leads to the production of small RNAs, which remove the translational inhibition of secondary metabolites. This cell-to-cell signalling allows the bacteria to coordinate their behaviour, activating the secondary metabolism at high bacterial density (Haas and Keel, 2003; Dubuis *et al.*, 2007).

Pseudomonas fluorescens CHA0 is an effective colonizer of the rhizosphere of mono- and dicotyledonous plants possessing remarkable biocontrol efficiency (Voisard *et al.*, 1994) and high resistance to predation (Jousset *et al.*, 2006) due to the production of an array of secondary metabolites including toxins, such as hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (Haas *et al.*, 2002).

Initially, rhizosphere bacteria originating from a single cell contribute equally to toxin production (de Werra *et al.*, 2008), but cell signalling is

Correspondence: A Jousset, Institute of Zoology, Darmstadt University of Technology, Schnitzpahnstr. 3, D-64287 Darmstadt, Germany.

E-mail: alexandre.jousset@gmx.net

³These authors contributed equally to this study.

⁴Current address: Terrestrial Ecology and Rhizosphere Research, Institute of Zoology, University of Cologne, Weyertal 119, 50931 Köln, Germany

⁵Current address: JF Blumenbach Institute of Zoology and Anthropology, Georg August University of Göttingen, Berliner Street 28, 37073 Göttingen, Germany

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genetically unstable, with *gacS/gacA* genes being frequently mutated and inactivated (Martinez-Granero *et al.*, 2005; van den Broek *et al.*, 2005b). The resulting mutants are impaired in cooperative group traits, such as extracellular enzymes and toxin production (Lapouge *et al.*, 2008), but under laboratory conditions show an improved growth rate compared to their wild-type ancestors due to the cessation of secondary metabolism (Bull *et al.*, 2001). Despite being weak competitors when inoculated alone (Natsch *et al.*, 1994), *gacS/gacA* mutants multiply rapidly within soil pseudomonad populations (Chancey *et al.*, 2002; Sanchez-Contreras *et al.*, 2002; Martinez-Granero *et al.*, 2005). Consequently, we hypothesized that these mutants gain advantage by exploiting the exoproducts of the wild-type population, with their competitiveness being the highest at low frequency in a dense wild-type population (Velicer *et al.*, 2000). In particular, mutants may benefit from the production of defence toxins that protect them from predators.

Predation significantly affects bacterial communities (Rønn *et al.*, 2002; Blanc *et al.*, 2006), and many bacteria evolved defence mechanisms such as the production of toxins. Toxic exoproducts protect bacteria by repelling predators, resulting in prey-switching towards more palatable prey (Jezbera *et al.*, 2006; Liu *et al.*, 2006), and at high toxin concentrations inhibit or even kill the predators (Matz *et al.*, 2004). Predators, therefore, affect the fitness of bacteria at the individual level by selective feeding on non-toxic cells, and at the population level by preferentially consuming bacteria from populations that are little protected by toxins, that is, those containing few toxin-producing bacteria. Therefore, signal blind, non-toxic *gacS/gacA* mutants should be preferentially consumed by predators, resulting in a reduced fitness. Mutants in mixed populations neither contribute to autoinducer signals nor to toxin production (Dubuis and Haas, 2007), and high mutant load has been shown to reduce toxin-mediated biocontrol activity of *P. fluorescens* (Duffy and Defago, 2000). Consequently, increasing frequency of *gacS/gacA* mutants may result in a lower predation resistance of the whole population.

We established populations of *P. fluorescens* CHA0 containing an increasing proportion of signal blind non-toxic *gacS* mutants in batch and rhizosphere systems. We exposed these populations to representatives of the two major groups of bacterial predators in soil, naked amoebae and nematodes, to investigate whether toxins differentially affect bacterial predators (Weitere *et al.*, 2005). If *gacS* mutants exploit the exoproducts of the wild type, they should perform better at low density, and we tested whether this strain is subject to negative frequency-dependent selection. However, the lack of defence compounds should make bacteria vulnerable to predation, and we tested whether predators of different functional groups (amoebae and nema-

todes) preferentially consume non-toxic mutants. As *gacS* mutants do not contribute to the defence of the population, we tested whether populations with higher loads of *gacS* mutants suffer from increased predation pressure by the different predators.

Materials and methods

Organisms

The wild-type strains *P. fluorescens* CHA0 (Voisard *et al.*, 1994) and its isogenic *gacS*-deficient mutant CHA19 (Zuber *et al.*, 2003) were tagged with two different fluorescent proteins allowing easy detection. A DsRed-tagged derivative CHA0-*r* was constructed as follows: A 0.7-kb *SmaI*-*HindIII* fragment from pDsRed.T3_S4T, containing the *dsred.T4_S4T* gene, which encodes a rapidly maturing red fluorescent protein variant with strongly enhanced brightness (Sorensen *et al.*, 2003), was cloned under the control of the constitutive P_{tac} promoter in pME6552. A 1.5-kb *MluI*-*HindIII* fragment from the resulting plasmid carrying the P_{tac} -*dsred.T3_S4T* fusion was then cloned into the mini-Tn7-Gm carrier plasmid, pME3280b (Zuber *et al.*, 2003). The construct obtained, pME7160, and the Tn7 transposition helper plasmid pUX-BF13 (Bao *et al.*, 1991) were coelectroporated (Baehler *et al.*, 2006) into the recipient strain CHA0. The *gacS* mutant CHA19 (Zuber *et al.*, 2003) was tagged with a green fluorescent protein as follows: a mini-Tn7-*gfp2* cassette carrying the *gfpmut3* gene controlled by a modified P_{tac} promoter (Koch *et al.*, 2001) was introduced into the chromosome of strain CHA19 using the transposition helper pUX-BF13, giving CHA19-*g*. The strains were kept routinely on nutrient agar plates (blood agar base 40 g l⁻¹, yeast extract 5 g l⁻¹) containing 8 µg ml⁻¹ gentamycin sulphate (Sigma-Aldrich, Munich, Germany). Preliminary experiments under batch experiment conditions showed that labelling did not affect the growth characteristics of the two strains, and that no spontaneous *gac* mutants of the DsRed-tagged wild-type strain could be detected in mixed communities in the tested time frame (data not shown).

Before inoculation, bacteria were grown in nutrient broth-yeast extract (NBY) medium (nutrient broth 25 g l⁻¹, yeast extract 5 g l⁻¹) at 30 °C with agitation. Late exponential phase bacteria were harvested by centrifugation (13 000 r.p.m. for 2 min), and washed three times in Neff's modified amoeba saline (AS; Page, 1988). The concentration of bacteria was determined by measuring optical density at 600 nm (OD₆₀₀) and checked under an Axioscope 2 (Zeiss, Jena, Germany) epifluorescence microscope at × 400 magnification.

The naked amoebae (*Acanthamoeba castellanii*) isolated from a woodland soil (Bonkowski and Brandt, 2002) were grown axenically on peptone-yeast extract-glucose (PYG) medium (peptone 20 g l⁻¹, yeast extract 5 g l⁻¹, glucose 10 g l⁻¹). Cells

were harvested by centrifugation (100 g, 10 min) and washed twice in AS before inoculation.

The bacterivorous nematode *Caenorhabditis elegans* was grown axenically in liquid medium (peptone 30 g l⁻¹, yeast extract 30 g l⁻¹, haemoglobin 500 mg l⁻¹ and cholesterol 1 mg l⁻¹). Before inoculation, juveniles and adults were collected on a 5-µm mesh, washed with sterile phosphate-buffered saline and resuspended in AS.

Experimental systems and setup

Two experimental systems of different complexity were established. In a simple batch system, the relative fitness of non-toxin-producing *P. fluorescens* mutant bacteria was investigated at six levels of mutant frequency. In complex rhizosphere systems, the fitness of wild-type and non-toxin-producing bacteria affected by both protozoan and nematode predators was investigated at three levels of mutant frequency.

Batch experiment. The experiment was set up in 96-well microtitre plates with mixed populations of *P. fluorescens* containing an initial frequency of 1, 10, 33, 66, 90 and 99% *gacS* mutants. Bacteria were inoculated at an initial concentration of 5×10^7 ml⁻¹ in AS containing 300 mg l⁻¹ PYG medium, in the presence or absence of *A. castellanii* (5000 individuals per ml). Plates were grown at 22 °C under agitation (150 r.p.m.). Preliminary experiments indicated no biofilm formation on the walls of the plate under the tested conditions (data not shown).

Rhizosphere experiment. The experiments were set up in microcosms filled with quartz sand and planted with rice. Quartz sand (grain size 100 µm–1.4 mm) was dried (72 h, 60 °C) and rewetted with 10% (w/w) of a modified Yoshida hydroponic nutrient solution (pH 6.0) containing 1.43 mM NH₄NO₃, 0.37 mM NaH₂PO₄ × 2 H₂O, 0.5 mM K₂SO₄, 1 mM CaCl₂ and 1.6 mM MgSO₄ × 7 H₂O (Walia *et al.*, 2005). The microcosms consisted of 30 × 200 mm glass tubes (Schott, Mainz, Germany) filled with 50 g of the wet autoclaved sand (30 min, 121 °C).

Rice seeds (*Oryza sativa* cv. Zhonghua11) were dehusked by careful grinding with a pestle in a mortar, and surface sterilized by soaking in 96% ethanol for 1 min and for 30 min in a solution containing 30 g l⁻¹ NaCl, 13 g l⁻¹ NaClO, 1 g l⁻¹ Na₂CO₃ and 1.5 g l⁻¹ NaOH (Hurek *et al.*, 1994). Seeds were separately germinated in 96-wells microtitre plates containing 100 µl AS. After 6 days, sterility was checked under a Diaphot inverted microscope (Nikon, Tokyo, Japan) at ×100 magnification. Sterile seedlings were aseptically transferred to the microcosms, and the plants were grown at a constant temperature of 22 °C and a 16 h photoperiod (500 µmol s⁻¹ m⁻²).

Three different mixtures of the bacterial strains *P. fluorescens* CHA0-r (wild type) and CHA19-g

(*gacS* mutant) were set up, with an initial frequency of 10%, 50% and 90% *gacS* mutants, and inoculated at a total concentration of 10⁶ bacteria per g sand (dry weight). Three predator treatments were established: a control without predators (Ctrl), addition of the bacterivorous amoeba *A. castellanii* (Amo) and addition of the bacterivorous nematode *C. elegans* (Nem). Amoebae were inoculated at a density of 10⁴ g⁻¹ sand and nematodes at a density of 50 g⁻¹ sand that correspond to their natural densities in the soil. Seven replicates were set up per treatment. Negative controls were consisted of non-inoculated sterile rice seedlings.

Measurements

In the batch experiment, 100 µl aliquots were collected after 48 h and fixed in 3% formaldehyde. The rhizosphere experiment was terminated after 12 days. Roots were removed from the sand, fixed in 8 ml of ice-cold phosphate-buffered saline containing 3% formaldehyde. Rhizosphere bacteria were detached by vortexing the roots for 2 min, sonicating two times for 1 min and vortexing again for 2 min.

To enumerate bacteria 100 µl aliquots of bacterial suspensions were filtered on a 0.2 µm polycarbonate membrane and counted under a Zeiss Axioscop 2 plus epifluorescence microscope at ×400 magnification. The filter settings for green fluorescent protein and DsRed counts were, respectively, an excitation filter of 470 and 546 nm, a dichroic filter of 493 and 580 nm, and an emission filter of 505–530 and 590 nm. Images were taken in triplicate using a Powershot G5 (Canon, Tokyo, Japan) digital camera, and bacteria were automatically counted using the ImageJ software (<http://rsb.info.nih.gov/ij/>). Red and green bacteria were counted separately. Unspecific excitation of the fluorescent proteins was assayed first with pure cultures of the two strains and did not interfere with the counts (data not shown).

Active nematodes were extracted in Baermann funnels, fixed in a 6% formaldehyde solution and enumerated at ×25 magnification. Amoebae were enumerated at the end of the experiment using a modified most probable number method (Darbyshire *et al.*, 1974). Briefly, 5 g of sand were gently shaken for 15 min in 20 ml AS, and 100 µl aliquots (four replicates per sample) were successively threefold diluted in a suspension of 10⁷ *Escherichia coli* per ml in AS in 96-well microtitre plates (Greiner Bio-one, Frickenhausen, Germany) and incubated in the dark at 15 °C. Wells were checked for the presence of active and encysted amoebae after 3, 7 and 14 days using an inverted microscope at ×100 magnification.

Statistical analyses and modelling of population dynamics

The relative fitness *v* of the *gacS* mutant was calculated by comparing its frequency at the

beginning and end of the experiment as described by Ross-Gillespie *et al.* (2007):

$$v = X_2 \times (1 - X_1) / X_1 \times (1 - X_2)$$

with X_1 , the initial and X_2 , the final frequencies of the strain. The stable frequency of *gacS* mutants in the population was defined according to the regression slope as $v = 1$.

The predation pressure p on the roots was estimated as

$$p = (Y_c - Y_p) / Y_c$$

where Y_c is the bacterial density in the control treatment, and Y_p is the bacterial density in the tested sample. The food selectivity index s of predators was calculated by dividing the percentages of *gacS* mutants in the diet of the predators and in the population. These two proportions were defined as the contribution of the *gacS* mutants to the total predation pressure ponderated by the percentage of mutants in non-grazed populations

$$s = (Z_c - Z_p) / (Y_c - Y_p) \times X_c$$

where X_c is the proportion of *gacS* mutants, and Z_p and Z_c are the total number of *gacS* mutants in the predator and control treatment, respectively. An index of 1 indicates the absence of preference for one of the two strains, an index > 1 preference for the *gacS* mutant.

The relative fitness of the *gacS* mutant, the predation pressure and the predator selectivity were analysed with a general linear model (type III sum of squares) investigating the effect of predator treatment (factor) and the original mutant frequency (linear descriptor) in a full factorial design. Data were analysed using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Before statistical analyses, data were checked for homogeneity of variances and log transformed if necessary; percentage values were arcsin-square root transformed before analysis to meet assumptions of homoscedasticity.

Results

Fitness of the *gacS* mutant

The *gacS*-deficient mutant was subject to negative frequency-dependent selection both in batch and rhizosphere systems (Figures 1 and 2). Its relative fitness (v) decreased in parallel with its frequency in the population, suggesting that the mutant was not able to displace wild-type populations, but instead required an elevated wild-type density for maximum fitness. In the batch system, pure cultures of *gacS* mutant grew better than the wild type in the exponential phase, but reached lower densities (see Supplementary Figure S1). In mixed cultures, the relative fitness of the mutant was negatively correlated with its initial frequency, showing a strong decline at high initial densities ($r^2 = 0.96$, $P < 0.001$; Figure 1). In absence of amoebae, the *gacS* mutant was fitter than the wild type at frequencies below

Figure 1 Relative fitness of the *gacS* mutant of *Pseudomonas fluorescens* CHA0 in the batch experiment after 48 h as a function of its initial frequency (initial *gacS* mutant-to-wild-type ratio) in the presence (black circles) or absence (open circles) of the predator *Acanthamoeba castellanii*. The relative fitness of the *gacS* mutant is expressed as ratio between its final and initial frequency; the two strains are at equilibrium at a relative fitness of 1.

Figure 2 Relative fitness of the *gacS* mutant of *Pseudomonas fluorescens* CHA0 in the rhizosphere of rice in relation to the initial *gacS* mutant-to-wild-type ratio without predators (a) with *Caenorhabditis elegans* (b) or *Acanthamoeba castellanii* (c). The vertical dashed lines indicate the frequency at which both strains are at equilibrium.

Figure 3 Predicted frequency at which the wild-type and *GacS* mutants of *Pseudomonas fluorescens* CHA0 are at equilibrium in bacterial populations without predator (Ctr), with *Caenorhabditis elegans* (Nem) or *Acanthamoeba castellanii* (Amo). Error bars indicate $\pm$ s.e. ($N=6$) for the intercept of the regression slopes (see Figure 2); different letters indicate significant differences between the intercept of the regression slopes (Tukey's HSD test, $\alpha=0.05$).

35%, where both strains were at equilibrium ($v=1$). Amoebae rapidly reduced the fitness of the mutant strain (see Supplementary Figure S2), especially at low mutant frequency ($F_{1,44}=34.7$, $P<0.001$ for the interaction between predator and mutant frequency; Figure 1), suggesting food preference for the non-toxic strain. Predators thus shifted the relationship between frequency and relative fitness of the *gacS* mutant, reducing its equilibrium frequency to only 20% ($F_{1,44}=6.37$, $P=0.015$). Similar shifts occurred in the rhizosphere (Figure 2); likewise the fitness of the *gacS* mutant was negatively correlated with its initial frequency ($F_{2,49}=295.4$, $P<0.001$). Both predators reduced the fitness of the *gacS* mutant strain. They significantly shifted the intercept of the regression lines between *gacS* mutant frequency and relative fitness lowering the equilibrium mutant frequency from 37% in the control treatment to 26% and 18% in the nematode and amoebae treatments, respectively ($F_{2,49}=7.2$, $P=0.0015$, Figure 3). Linear regressions explained well the observed changes in mutant frequency ($r^2=0.84$, 0.88 and 0.87 , $P<0.001$ for control, nematode and amoeba treatments, respectively).

Prey selection

Both predators preferentially consumed *gacS* mutants in the rhizosphere, with preferences being more pronounced at low mutant frequency ($F_{2,32}=9.5$, $P=0.001$), suggesting active food selection. However, food selectivity strongly differed between predators ($F_{1,32}=4.2$, $P=0.025$). The nematodes were less selective (Figure 4), and their diet composition was only marginally influenced by the frequency of the two bacterial strains. In contrast, prey selection of amoebae decreased parallel to the increase in mutant frequency. The preference index indicates that amoebae preferentially consumed the mutant strain when present at low frequency ($s=2.4$), but discriminated little between bacterial strains at the initial mutant frequency of 90% ($s=1.16$, Figure 5).

Figure 4 Food selectivity of *Acanthamoeba castellanii* (black circles) and *Caenorhabditis elegans* (white circles) at different frequencies of *gacS* mutants of *Pseudomonas fluorescens* CHA0. Values above 1 indicate preference for *gacS* mutants. Error bars indicate $\pm$ s.e.; different letters indicate significant differences between means at $P<0.05$ (Tukey's HSD test).

Figure 5 Predation intensity on the total bacterial population by *Acanthamoeba castellanii* (black circles) and *Caenorhabditis elegans* (white circles) at different initial frequencies of *gacS* mutants of *Pseudomonas fluorescens* CHA0. Predation intensity is expressed as percentage reduction in bacterial numbers compared with the respective treatment without predators at the end of the experiment. Error bars indicate $\pm$ s.e.; different letters indicate significant differences between means at $P<0.05$ (Tukey's HSD test).

Predation intensity

In the absence of predators bacteria reached in the rhizosphere, an average total density of 5.5×10^6 cells (root system) $^{-1}$ irrespective of the initial mutant frequency ($F_{2,48}=1.14$, $P=0.328$), suggesting that the carrying capacity of the system was independent of the initial mutant frequency. Nematodes and amoebae strongly reduced total bacterial numbers at the end of the experiment ($F_{2,48}=24.4$, $P<0.001$), with the effect of nematodes exceeding that of amoebae. Increasing *gacS* mutant frequency differently affected the predation pressure of the two predators ($F_{2,32}=3.7$, $P=0.035$ for the interaction between predator type and initial mutant frequency). Nematodes grazed on bacterial populations at all densities, thereby reducing bacterial numbers by 56%, 58% and 69% in 10%, 50% and 90% mutant treatments. In contrast, predation by amoebae

increased with the frequency of *gacS* mutants, reducing bacterial numbers by 11%, 36% and 56% in 10%, 50% and 90% mutant treatments (Figure 5). This suggests that bacterial toxins efficiently protected the population against amoebae, but less against nematodes. Increasing mutant frequency resulted in a loss of this protection, confirming that the *gacS* mutant did not contribute to defence at the population level.

Predator response

The density of amoebae at the end of the experiment was independent of the initial frequency of *gacS* mutants and reached on average $1.34 \pm 0.89 \times 10^4$ individuals per g sand ($F_{2,16} = 0.40$, $P = 0.700$), suggesting low mortality even at high frequency of toxic bacteria, although more amoebae may have been encysted due to increased toxin concentrations. In contrast, nematode density tended to increase with initial mutant frequency ($F_{2,16} = 2.90$, $P = 0.082$), with actively swimming nematode numbers of 2.9, 4.5 and 6.5 individuals per g sand in 10%, 50% and 90% mutant treatments, respectively, suggesting an inhibitory effect of the toxic wild-type bacteria on nematodes.

Discussion

Frequency-dependent fitness of *gacS* mutant bacteria

Our results show that spontaneous *gacS* mutation in rhizosphere bacteria is an effective strategy to increase fitness by exploiting secondary metabolism of wild-type neighbours. Both in batch and rhizosphere systems, the fitness of *gacS*-deficient mutants proved to be negatively frequency dependent. This suggests that mutant bacteria are favoured within the wild-type populations, where they may contribute up to 40% of the population.

In microorganisms, the coordinated production of common goods, such as siderophores (Ross-Gillespie *et al.*, 2007), extracellular enzymes (Greig and Travisano, 2004) or multicellular structures (Velicer *et al.*, 2000), may strongly improve bacterial fitness, but is vulnerable to cheating. Typically, signal blind mutants rapidly evolve inside cooperating populations (Sandoz *et al.*, 2007), reaping the benefits of the shared trait while sparing the associated costs. Cell signalling in *P. fluorescens* coordinates the production of exoproducts such as enzymes and toxins (Haas and Keel, 2003). Extracellular proteases, chitinases and lipases (van den Broek *et al.*, 2003) contribute to the breakdown and assimilation of organic material. Moreover, our results show that secondary metabolites protect bacteria from predation (*A. castellanii*) or significantly reduce the fitness of the predators (*C. elegans*). Secondary metabolites, including enzymes and toxins, therefore, can be considered as a common good beneficial to the whole population.

The negative frequency-dependent fitness of the *gacS* mutant suggests that this strain exploits the exoproducts of the wild-type strain. Cell signaling-deficient mutants indeed may have higher maximum growth rates, but in pure culture reach lower density than the wild-type strain, lack extracellular enzymes such as phospholipases or proteases (Sacherer *et al.*, 1994) and are more vulnerable to predation. However, the latter is not true in dense wild-type populations, indicating that the benefits of exploiting the exoproducts of neighbouring cells may outweigh the disadvantage of the altered phenotype.

Interestingly, a range of random and spontaneous mutations in the *gacS/gacA* genes, such as deletions, insertions and inversions, frequently switch off the response to population density signals (Martinez-Granero *et al.*, 2005; van den Broek *et al.*, 2005a). We propose that these high mutation rates result from the advantage of individual bacteria switching of cell signalling first. As the fitness of signal blind mutants depends on the frequency of wild-type bacteria, mutants evolving inside of an otherwise wild-type population receive greater benefit than bacteria mutating later when the relative frequency of wild-type bacteria has decreased. Remarkably, most mutations are reversible and cell signalling can be restored later (van den Broek *et al.*, 2005b), suggesting increased fitness when reverting to the original phenotype in populations loaded with mutants.

Selective feeding on *gacS* mutant bacteria

Predation exerts a major selective pressure on free living bacteria (Pernthaler, 2005) and plays a key role in driving bacterial microevolution (Wildschutte *et al.*, 2004; Meyer and Kassen, 2007). Consequently, bacteria evolved sophisticated defence strategies, including the production of toxins that repel or inhibit their predators (Andersen and Winding, 2004; Matz *et al.*, 2004; Jousset *et al.*, 2006). The production of these toxins often is density dependent resulting in increased protection of related neighbouring cells (Matz and Kjelleberg, 2005).

Both predators preferentially consumed *gacS* mutant bacteria, documenting that amoebae and nematodes can select their prey and that extracellular toxins act as repellent. Prey selection is crucial for bacterivores, such as protozoa and nematodes, as the quality of ingested bacteria directly affects their fitness (Zubkov and Sleight, 2000; Blanc *et al.*, 2006; Jousset *et al.*, 2006). In particular, amoebae were highly selective and preferentially consumed the non-toxic mutant in both the batch and rhizosphere experiment. Despite being less selective, nematodes also negatively affected the *gacS*-deficient mutant. Grazing by nematodes reduced total bacterial density, and thereby decreasing the size of the wild-type population. As the production of secondary meta-

bolites is density dependent, this likely impairs the protection of the mutant strain. Generally, the results confirm the vulnerability of *gacS* mutant bacteria to predation and support our hypotheses that they gain protection by toxins from wild-type bacteria.

Predation and population level defence

Predation was markedly reduced in populations with low *gacS* mutant frequency, suggesting that in addition to the individual level, bacteria benefit from the dominance of wild-type bacteria at the population level. Vulnerability to predation at high mutant frequency is in agreement with past observations that the biocontrol function of *P. fluorescens* collapses with increasing frequencies of *gacS* mutants (Duffy and Defago, 2000). The *gacS* mutant bacteria produce neither toxins nor autoinducers (Lapouge *et al.*, 2008), thus interfering with toxin production of wild-type bacteria. Consequently, proliferation of the mutant compromises the population level traits from which this strain benefits. If bacterial populations become overloaded with mutants, as is the case in the presence of hypermutable strains (Harrison and Buckling, 2005), the whole population may be eliminated by predators, suggesting that in spatially structured habitats predation may enforce cooperation, that is, toxin production, at the group level by counter selecting defenceless populations. Overall, toxin-mediated attenuation of predation by wild-type bacteria may help explain why keeping the ability to produce secondary metabolites in *P. fluorescens* is evolutionary stable despite the associated costs and the frequent loss of this function.

Conclusions

Results of this study suggest that by selectively feeding on non-toxic bacteria and reducing predation on bacterial populations loaded with mutants, predators reinforce toxin production of wild-type bacteria at both the individual and population levels. The study, therefore, provides an ecological and evolutionary explanation for the spontaneous deactivation of cell signalling by pseudomonads. At low frequency, signal blind toxin-deficient mutants profit from the exoproducts of coexisting wild-type bacteria, suggesting that they improve their fitness by cheating. Predators, especially amoebae, reduced the frequency of non-toxic phenotypes than nematodes, suggesting that protozoa efficiently reinforce toxicity of bacterial communities. Intriguing bacterial traits, such as the spontaneous deactivation of the GacS/GacA-mediated toxin production in pseudomonads, likely evolved in the context of predation pressure. Predator–prey interactions, therefore, not only allow a mechanistic understanding of the factors driving population dynamics and evolution of cooperating and cheating in *Pseudomonas* strains

but also may allow developing strategies for the successful establishment of biocontrol bacteria in the rhizosphere of plants.

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