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Minimal increase in genetic diversity enhances predation resistance

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Abstract

The importance of species diversity to emergent, ecological properties of communities is increasingly appreciated, but the importance of within-species genetic diversity for analogous emergent properties of populations is only just becoming apparent. Here, the properties and effects of genetic variation on predation resistance in populations were assessed and the molecular mechanism underlying these emergent effects was investigated. Using biofilms of the ubiquitous bacterium *Serratia marcescens*, we tested the importance of genetic diversity in defending biofilms against protozoan grazing, a main source of mortality for bacteria in all natural ecosystems. *S. marcescens* biofilms established from wild-type cells produce heritable, stable variants, which when experimentally combined, persist as a diverse assemblage and are significantly more resistant to grazing than either wild type or variant biofilms grown in monoculture. This diversity effect is biofilm-specific, a result of either facilitation or resource partitioning among variants, with equivalent experiments using planktonic cultures and grazers resulting in dominance by a single resistant strain. The variants studied are all the result of single nucleotide polymorphisms in one regulatory gene suggesting that the benefits of genetic diversity in clonal biofilms can occur through remarkably minimal genetic change. The findings presented here provide a new insight on the integration of genetics and population ecology, in which diversity arising through minimal changes in genotype can have major ecological implications for natural populations.

Keywords: bacterial biofilms, community resilience, diversity, ecological complementarity, genetic variation, protozoan grazing

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Introduction

Understanding the relationship between species diversity and emergent properties of communities or ecosystems—stability, resilience, ecosystem functioning, invasibility—has been a central theme of ecology for over 50 years (Elton 1958), and it is now clear that species diversity affects the dynamics of communities across a wide variety of organisms (Loreau & Hector 2001; Cardinale *et al.* 2002, 2011; Folke *et al.* 2004; Hooper *et al.* 2005). These consequences of diversity suggest that communities are not simply the sum of their parts, but instead, multispecies interactions generate community-level properties independently of individual species traits alone. Recent evidence indicates that genetic

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diversity in populations may have an analogous role to that of species diversity in communities and play a critical role in the dynamics of populations (Hughes & Stachowcz 2004), their productivity and their resilience to disturbance (Fox 2005; Reusch *et al.* 2005; Crutsinger *et al.* 2006).

Fundamental questions remain about the importance of genetic diversity for emergent properties of populations. Foremost among these is that the precise mechanisms underlying genetic changes may well determine the strength of any ecological effect of diversity, but such molecular mechanisms are unknown for any organism. Second, most studies (Hughes *et al.* 2008) compare large differences in genetic diversity (e.g. populations of nonvarying clones against populations of diverse genotypes), which may not reflect typical differences among natural populations. Thus, the minimal level of genetic diversity necessary to generate emergent ecological effects is unclear. Finally, the generality of the phenomenon across the major domains of life is unclear. Although bacteria represent the major source of biomass and diversity on Earth, studies on the emergent ecological effects of genetic diversity have been performed almost exclusively on eukaryotes (Hughes *et al.* 2008).

Serratia marcescens is a broadly distributed bacterium which occurs in habitats from soils to the ocean (Grimont & Grimont 1978) and is a human, plant and animal pathogen (Hejazi & Falkiner 1997). As is the case for almost all bacteria, *S. marcescens* forms biofilms, structured, surface aggregations of cells embedded in an extracellular matrix, distinctly different from the planktonic state (free-living), which are highly unstructured and often motile. Such bacterial systems are genetically tractable relative to most eukaryotes, facilitating the identification of specific genetic characters responsible for ecological properties. Because of this and their essentially sessile and colonial (modular) nature, biofilms are an ideal system both for studying the consequences of genetic diversity on the emergent properties of modular organisms as well as for comparing these effects between biofilms and more complex eukaryotic systems.

Bacterial biofilms are found in every environment. Monospecies bacterial biofilms are colonial (Andrews 1998), in the sense that they are comprised of large numbers of more or less genetically identical cells, and are thus similar to clonal eukaryotes which have been the focus of studies on the ecological consequences of genetic diversity (Hughes *et al.* 2008). While such biofilms are colonial, arising via repeated replication of nominally identical cells, genetic variation and substantial phenotypic diversification still often arises among cells in architecturally complex bacterial biofilms. This is the case for *S. marcescens* which produces a range of heritable variants when grown as a biofilm. These biofilm-unique variants are phenotypically distinct from the parental wild type (WT) and are not generated in planktonically grown populations (Koh *et al.* 2007).

Using these biofilm-derived genetic variants of *S. marcescens*, we addressed the ecological consequences of intraspecies genetic diversity in a protozoan predator—bacterial biofilm system. Protozoan grazing is one of the major environmental challenges confronted by bacteria (Jürgens & Matz 2002; Matz *et al.* 2004) and is thus an ideal system in which to explore the ecological consequences of genetic variation. We predicted that such genetic variation, by affecting the structure and complexity of biofilms, can contribute to the emergent ecological effects of genetic diversity, i.e. biofilm resilience to disturbances from predation. The findings presented here also provide a rare insight into the genetic mechanisms underlying emergent population-level phenomenon.

Material and methods

Bacterial strains, biofilm variants of Serratia marcescens *and culture conditions*

Serratia marcescens and *Escherichia coli* strains used in this study are shown in Table 1. Biofilms formed by *S. marcescens* consistently produce five colony variants that are phenotypically distinct from the parental wild type (WT) and are not generated in planktonically grown populations (Koh *et al.* 2007). Based on colony morphology and texture, the morphotypic variants have been characterized as: sticky smooth variant (SSV), sticky rough variant (SRV), sticky rough umbonate variant (SRUV), smooth ultramucoid variant (SUMV) and nonsticky small colony variant (NSCV). These biofilm-unique variants are heritable; subculturing of colonies results in persistence of the morphotype and associated functional traits (Koh *et al.* 2007).

The *S. marcescens* strains were routinely grown at 30 °C in M9 minimal medium supplemented with 0.1% (wt/vol) glucose or Luria-Bertani (LB) medium, and *E. coli* strains were grown at 37 °C in LB medium. Streptomycin (200 μg/mL), kanamycin (50 μg/mL), chloramphenicol (20 μg/mL), gentamicin (20 μg/mL) and tetracycline (12 μg/mL) were added as required, to select for genetically modified bacterial strains used for the study of genetic mechanisms underlying variant diversity. For *trans*-complementation experiments, bacterial cultures were supplemented with 0.02% (wt/vol) arabinose to induce expression of the cloned *etk* gene, where indicated.

Table 1 List of bacterial strains used in this study

Strains	Phenotypic and genotypic characteristics*	Source or reference
Serratia marcescens		
MG1	Wild type, (Amp^R) (Tet^R); rough colony surface and 'nonsticky'	(Givskov *et al*. 1988)
SSV	MG1; spontaneous smooth and 'sticky' (with wet elastic texture) derivative isolated from MG1 biofilm	(Koh *et al*. 2007)
SRUV	MG1; spontaneous rough, umbonated and 'sticky' (with wet elastic texture) derivative isolated from MG1 biofilm	(Koh *et al*. 2007)
SRV	MG1; spontaneous rough and sticky (with dry elastic texture) derivative isolated from MG1 biofilm	(Koh *et al*. 2007)
SUMV	MG1; spontaneous smooth, mucoid and 'sticky' (with wet elastic texture) derivative isolated from MG1 biofilm	(Koh *et al*. 2007)
NSCV	MG1; spontaneous small colony and 'nonsticky' derivative isolated from MG1 biofilm	(Koh *et al*. 2007)
BMG1001	MG1; WT derivative isolated from MG1 biofilm	(Koh *et al*. 2007)
STK01–STK15	MG1; spontaneous 'sticky' (or mucoid) variant derivatives isolated from MG1 biofilm	This study
BMG1002–BMG1016	MG1; WT derivative isolated from MG1 biofilm	This study
NSCV001–NSCV005	MG1; spontaneous small colony and 'nonsticky' derivative isolated from MG1 biofilm	This study
Escherichia coli		
JM109	F′, *e14*$^-$ (*mcrA*$^-$), *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^- m_K^-), *sup*E44, *rel*A1, λ$^-$, Δ(lac-proAB), [F′*tra*D36, *pro*AB, *lacI*^{q}ZΔ M15]	(Koh *et al*. 2007)
HB101	F$^-$, *hsd*S20 (rb$^-$, mb$^-$), *sup*E44, *ara*14, *gal*K2, *lac*Y1, *pro*A2, *rps*L20 (Str^R), *xyl*-5, *mtl*-1, l-, *rec*A13, *mcr*A$^-$, *mcr*B$^-$	(Raleigh & Wilson 1986)
TOP10	F$^-$, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15 Δ*lac*X74, *deo*R, *rec*A1, *ara*D139 Δ(*ara-leu*)7697, *gal*K, *rps*L (Str^R), *end*A1, *nup*G	Invitrogen Inc.
S17-1λ*pir*	*E. coli* K-12, *rec*A *thi hsd*RM$^+$, RP4::2-Tet::Mu::Km Tn7, λ*pir* phage lysogen, (Tet^R) (Str^R)	(de Lorenzo & Timmis 1994)
MG3617	*E. coli* S17-1λ*pir*, pJMS10, (Tet^R) (Str^R) (Km^R)	(Kristensen *et al*. 1995)
Acanthamoeba castellanii	Wild-type, ATCC 30234	American Type Culture Collection
Tetrahymena sp.	Wild-type	(Weitere *et al*. 2005)

*Amp^R, Resistance to ampicillin; Km^R, Resistance to kanamycin; Str^R, Resistance to streptomycin; Tet^R, Resistance to tetracycline.

Biofilm grazing experiments

To address the effects of genetic diversity and potential emergent properties generated at the population level, the performance of biofilms comprised of a mixture of the five different variant genotypes, with and without the WT (high diversity), were compared to the average performance of biofilms formed by individual genotypes (low diversity) in the presence of the amoeba biofilm-predator. Biofilm formation and predator growth were assessed over twelve days. Grazing experiments with single and mixed biofilms populations were performed in 24-well tissue culture plates. Overnight cultures of *Serratia marcescens* WT and biofilm-derived morphological variants were diluted to an $OD_{600\ nm}$ of 0.7 in 0.5 × M9 complete medium. For the mixed-culture biofilm grazing experiments, the inocula were prepared by mixing equal proportions of the $OD_{600\ nm}$-adjusted *S. marcescens* monocultures. Biofilms were pre-established in the tissue culture plates for 24 h by inoculating 10 μL of *S. marcescens* seeding cultures into each well containing 1 mL of 0.5 × M9 complete medium. After 24-h incubation, 75% of the spent medium was carefully removed and replaced with fresh 0.5 × M9 complete medium containing approximately 1×10^4 axenic *Acanthamoeba castellanii*. The cultures were replenished with fresh media every second day by replacing 75% of the spent medium with 0.5 × M9 complete medium. The protozoan grazing experiments were conducted at room temperature with constant agitation at 60 rpm.

The number of *A. castellanii* cells, biofilm biomass and the frequencies of colony morphotypes in plankton and biofilms were followed over 12 days, at 'Day 0', 'Day 6' and 'Day 12'. Day 0 refers to biofilms that had been pre-established for 24 h prior to inoculation with the *A. castellanii*. Biofilms in the 24-well tissue culture plates were quantified by staining with 0.3% (wt/vol)

crystal violet as described previously by Koh *et al.* (2007). Crystal violet-quantified biofilm biomass was corrected to include the contribution of crystal violet stain by different concentrations of *A. castellanii* to the overall $OD_{540\ nm}$ readings. Plating of bacterial biofilm onto LB10 agar, from samples collected from each treatment, was also performed to quantify bacterial biomass and the frequencies of each colony morphotypes that are present in each treatment. Each genotype was differentiated by the unique physical characteristics of its colony as described in Table 1. In addition, random checks were performed by sequencing the *etk* of the corresponding colony morphotypes to ensure that the colony morphotypes correspond to their respective genotypes. The abundance of *A. castellanii* present in each treatment was determined by direct cell counting using an inverted microscope.

Planktonic grazing experiments

Seeding bacterial cultures was prepared as described for the biofilm grazing experiments. Approximately 1×10^3 axenic *Tetrahymena pyriformis* were co-inoculated together with the seeding bacterial cultures into the 24-well tissue culture plates. The cultures were replenished with fresh media every second day by centrifuging at 500 ***g*** and then carefully replacing 75% of the spent medium with fresh $0.5 \times$ M9 complete medium. All protozoan grazing experiments were conducted at room temperature with constant agitation at 60 rpm.

The number of *T. pyriformis* cells, planktonic biomass and the frequencies of colony morphotypes in planktonic fraction were followed daily over 6 days. Day 0 refers to the start of the experiment where the bacterial cultures were co-inoculated with *T. pyriformis*. Samples from the planktonic fraction were collected by carefully removing 0.5 mL of the supernatant from the wells of each treatment and plating onto LB10 agar to quantify the bacterial population in each treatment and the frequencies of the colony morphotypes present. The abundance of *T. pyriformis* was determined by analysing five samples of 5 μL aliquots from each treatment under the microscope.

Diversity indices and statistical analysis

The diversity of colonial morphotypes in the *Serratia marcescens* biofilm population was measured by differentiating at least 800 colonies per sample for each time point. The Simpson's reciprocal index (1/D), richness (S) and evenness (E) were calculated as described previously (Simpson 1949; Magurran 1988). Statistical analyses of data were performed by one-way ANOVA, repeated measures ANOVA or *t*-tests where appropriate, unless stated otherwise, using the SYSTAT® version 12 (SYSTAT® Software Inc.). Tukey tests provided post hoc comparisons of means for one-way ANOVA. The effects of complementarity *vs.* selection in mixed variant biofilms were analysed using the methods of Loreau & Hector (2001). Briefly, this approach uses a modified version of the Price selection equation to estimate how much variation in performance in mixtures is owing to the overrepresentation of a single genotype (selection effects) and how much is owing to an increase in the average performance of each genotype (complementarity effects).

Gene knockout library, genetic screens and construction of etk *expression vector and complementation studies*

The phenotypes of the *Serratia marcescens* morphological biofilm variants persisted with subculturing, and thus suggest that these biofilm variants have a genetic basis. By deciphering the molecular mechanisms of these genetic differences, we attempted to better understand the ecological and evolutionary consequences of diversity in *S. marcescens* biofilms.

Construction of the gene knockout library of *S. marcescens* biofilm-unique variants using mini-Tn5-Km, a mini-Tn5 derivative carrying a kanamycin resistance marker, for the transposon mutagenesis experiments was performed by tri-parental mating as described previously (de Lorenzo & Timmis 1994). The transconjugant colonies with wild-type (WT) colony morphology were selected for mapping of the transposon insertion sites, which was performed using an adaptor ligation PCR protocol described previously by Siebert *et al.* (1995). Genes that are important for the variant colony morphotypes were selected for validation via complementation studies.

The *etk* gene, encoding for exopolysaccharide tyrosine kinase, was identified from the gene knockout library and selected for validation. Along with its native promoter, the *etk* gene was cloned into the pBAD-TOPO® vector (Invitrogen Inc.) containing an arabinose-inducible promoter. The plasmid, pBAD-CMPGm, was first constructed by ligating an 830 bp-fragment of *Nde*I-digested *aac*C1 (encoding 3-N-aminoglycoside acetyltransferase conferring resistance to gentamicin) (Hoang *et al.* 1998) derived from pGEM-Gm, to the *Nde*I-digested and dephosphorylated vector pBAD-CMP (Table S1, Supporting information). The *Nco*I/*Pme*I fragment carrying the chloramphenicol resistance marker, *cat*, located downstream of the inducible Pbad promoter in the plasmid pBAD-CMPGm, was then replaced with the *Nco*I/*Pme*I-digested PCR fragment of etk_{MG1} amplified from *S. marcescens* MG1 WT, to construct pBAD-GmETK$_{WT}$. All gene constructs used were amplified

with proofreading AccuPrime™ *Pfx* DNA polymerase (Invitrogen Inc.) and performed in accordance to the manufacturer's instruction. All recombinant plasmids were confirmed by capillary sequencing. Plasmids carrying the different variant *etk* genes (i.e. pBAD-GmETK$_{SSV}$, pBAD-GmETK$_{SRUV}$, pBAD-GmETK$_{SRV}$ and pBAD-GmETK$_{SUMV}$) were constructed using the strategy described above. *Trans*-complementation of *S. marcescens* MG1 strains was performed by electroporation with highly electrocompetent cells prepared by a rapid microcentrifuge-based method described by Choi *et al.* (2006). The transformants were selected on LB agar supplemented with gentamicin (20 μg/mL), incubated at 30 °C for not more than 24 h. All plasmids, DNA oligonucleotides and adaptors used in this study are listed in Table S1 (Supporting information).

Detection of phosphorylated tyrosine residues on the Etk by immunoblot

Detection of phosphorylated Etk was performed using total cell lysate of the different *Serratia marcescens* strains which was separated on NuPAGE® 4–12% Bis-Tris gradient gels (Invitrogen Inc.) and electro-transferred onto an Immobilon-P polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were stained with Ponceau S to determine transfer efficiency and as a loading control. The phosphorylated Etk was detected by immunoblotting using PY20 antiphosphotyrosine primary antibody (Sigma-Aldrich Inc.) and secondary horseradish peroxidase (HRP)-conjugated antimouse IgG antibody (Amersham Bioscience). As a loading control, the same membrane was reprobed using anti-GroEL primary antibody (Sigma-Aldrich Inc.) and detected using HRP-conjugated antirabbit IgG antibody (Amersham Biosciences).

Quantification of exopolysaccharide by uronic acid determination

Isolates were cultured on M9 minimal agar supplemented with 0.5% (wt/vol) glucose or LB10 agar (grown for 3 days at 30 °C) were resuspended in 20 mL of 0.15 M NaCl solution. The bacterial suspension of each strain was adjusted to achieve a final optical density of 1.000 ± 0.020 at 600 nm. The bacterial suspensions were vortexed vigorously for 30 s and a 10 mL aliquot of the bacterial suspension was centrifuged at 10 000 *g* for 20 min at 4 °C. The resulting supernatant was carefully transferred to a fresh tube and kept on wet ice. Exopolysaccharide (EPS) was quantified with the uronic acid determination of Blumenkrantz & Asboe-Hansen (1973) method, using D-glucuronic acid as the standard.

Results

Biofilms of individual genotypes do not display a dominant phenotype against predation by protozoan

Morphological variants of *Serratia marcescens* showed variation in a range of phenotypes (Fig. 1a), particularly for biofilm-associated traits (attachment, swarming and biofilm development). While there were no significant differences between intrinsic growth rates of the WT and the derived variants (Table S2, Supporting information), the variants differed significantly in their ability to form biofilms compared to the WT (Fig. 1b). Biofilms of individual variant genotypes were then tested for resistance to predation by the biofilm-feeding amoeba *Acanthamoeba castellanii*. The biofilms of these variant genotypes were equally or more susceptible to protozoan grazing compared to the parental WT clone (Fig. 1c). None of the variant genotypes formed biofilms with both high biofilm-forming capacity and increased grazing resistance, relative to the WT (Fig. 1b,c).

Genetic diversity was maintained and resistance to grazing was observed when biofilm predators were introduced

Multivariant biofilms (with or without the WT) were significantly more resistant to protozoan grazing than the average performance of any of the biofilms formed by individual genotypes (Fig. 2). The multivariant biofilms reduced predator growth threefold and accumulated significantly greater biofilm biomass (Fig. 2 and Table S3, Supporting information) compared to the WT. All variants persisted in the mixed biofilms for the duration of the experiment (Fig. S1, Supporting information), where the mean CFU/ml for the multivariant + WT biofilms for each strain after 12 days were, $n = 3$: WT, $2.5 \times 10^7 \pm 2.5 \times 10^6$; SSV, $2.0 \times 10^8 \pm 1.9 \times 10^7$; SRUV, $4.2 \times 10^6 \pm 2.9 \times 10^6$; SRV, $2.3 \times 10^7 \pm 1.1 \times 10^7$; SUMV, $6.0 \times 10^7 \pm 3.1 \times 10^7$; NSCV, $4.2 \times 10^6 \pm 2.7 \times 10^6$.

The effects of diversity in these biofilms may be owing to dominance by one or a few strains (selection effects) or to complementarity arising from resource partitioning or facilitation across the multiple strains. We used standard formulae to calculate the relative contributions of complementarity effects *vs.* selection effects (Loreau & Hector 2001) for this system. These analyses showed that there was a strong complementarity effect of variant diversity for grazing resistance, suggesting that the performance of each genotype was better in the mixture relative to their individual performance (*t*-test, $t = 5.73$, d.f. = 3, $P = 0.0105$). A strong positive selection effect (*t*-test,

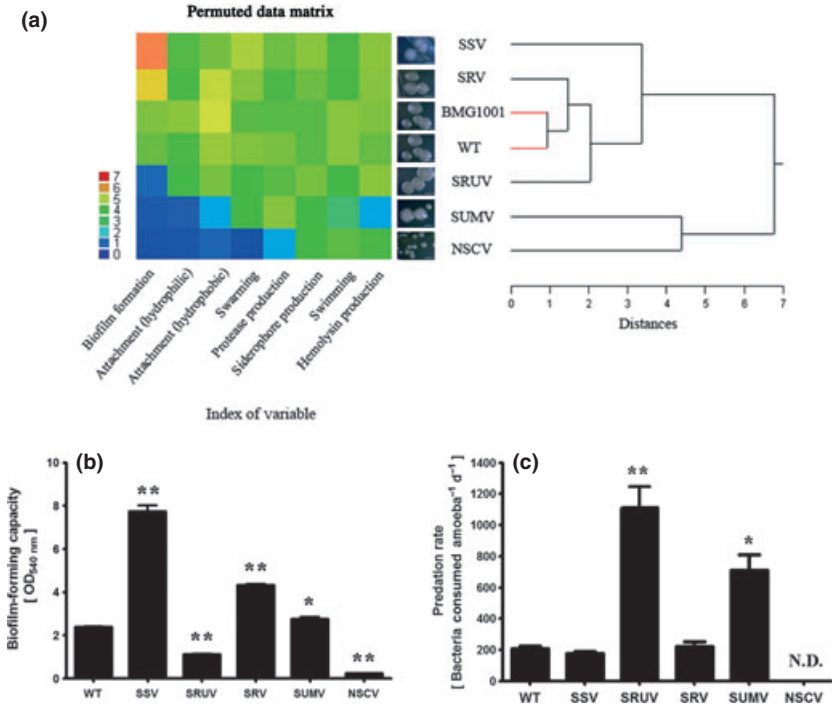


Fig. 1 Phenotypic analysis of the parental WT and biofilm variants. Cluster analysis of the phenotypic characterization of the parental wild-type and the biofilm-derived variants, based on colonization, biofilm formation, motility and virulence traits (a). The permuted data of the phenotypes exhibited by each of the isolates are presented as a matrix plot (left) and are grouped according to the similarities among the isolates in the level of phenotypic integration (right, cluster analysis dendrogram), as measured by matrix correlation tests (SYSTAT® Software Inc.). The matrix plot was generated using hierarchical clustering of the permutated raw data and the colour scheme represents the profile distribution of the phenotypes exhibited by each isolate, where red (blue) denotes strong (weak) expression of the phenotypes indicated at the bottom of each column. Isolates that were analysed for phenotypic characteristics were the parental wild-type (WT), biofilm-derived wild-type (BMG1001), 'nonsticky' variant (NSCV) and the 'sticky' variants comprising of SSV, SRUV, SRV and SUMV. The horizontal axis on the cluster analysis dendrogram (right) indicates increasing distance and dissimilarity. The horizontal red lines for the WT and the BMG1001 isolate indicate no dissimilarity in phenotypic traits between the organisms. Biofilm-derived variants exhibit varying biofilm-forming capacity (b) and their susceptibility to grazing by biofilm-feeding amoeba, *Acanthamoeba castellanii* (c). Statistical significance was determined by analysis of variance; *, $P < 0.05$; **, $P < 0.001$. Values shown are Mean ± SE. Predation rate was not detectable (N.D.) in NSCV biofilms owing to poor biofilm formation.

$t = 7.05$, d.f. = 3, $P = 0.0059$) was also observed, indicating that not all genotypes performed equally well. This was because the best performing genotype grown individually (SSV) was also the most abundant in the mixture.

Genetic diversity in the planktonic phase was lost in the presence of predator. The effects of genetic diversity on resistance against grazing in *Serratia marcescens* biofilms raised the question as to whether the consequences of genetic diversification are unique to biofilms. To address this, planktonic grazing experiments were performed by co-inoculating planktonic monovariant and multivariant bacterial cultures with the suspension-feeding predator *Tetrahymena pyriformis*, a protozoan that feeds specifically on planktonic bacteria. During the course of the 6 day experiment in which the predator was grown in the presence of a mixture of genotypes or individual genotypes, the predator coexisted with the bacterial population at relatively high numbers, with the numbers of *T. pyriformis* ranging between 10^3 and 10^4 per well. Planktonic cells of the parental genotype (i.e. WT) were readily consumed, and cell numbers were about two orders of magnitude lower than in the predator-free control treatment (mean numbers of $1.2 \times 10^6 \pm 2.2 \times 10^5$ CFU vs. $5.8 \times 10^8 \pm 4.6 \times 10^7$ CFU/well; $P < 0.0001$, *t*-test, $n = 3$) (Fig S2, Supporting information). Conversely, the number of cells in the mixed genotype bacterial assemblage ($3.4 \times 10^8 \pm 4.6 \times 10^7$ CFU/well) was more than half that of the ungrazed control wells on Day 6. The number of predators was about 50% lower in the mixed genotype cultures than in the presence of the parental strain alone ($P < 0.0001$, *t*-test, $n = 3$). The exposure of planktonic cultures to grazers resulted in low diversity, consisting almost exclusively of a single genotype (Table 2 and Fig. 3), in stark contrast to the mainte-

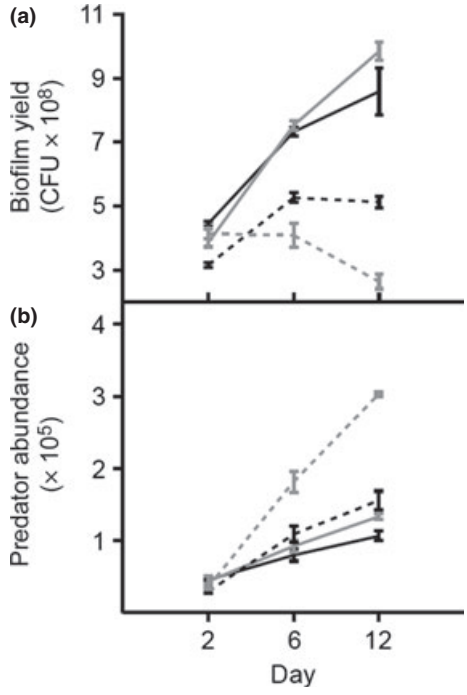


Fig. 2 Effects of biofilm diversity on bacterial population abundance against predation. Panel (a) shows yield of biofilms in the presence of predators, while panel (b) shows abundance of biofilm-feeding amoeba, *Acanthamoeba castellanii*, in different biofilms. In both panels, dashed lines indicate the average yield of monovariant biofilms and solid lines indicate yield of multivariant biofilms and grey lines indicate the presence of the WT strain and black lines indicate the absence of the WT. Values shown are Mean ± SE.

nance of the high diversity biofilm cultures in the presence of grazers.

Polymorphism within a tyrosine kinase gene, etk, *involved in the regulation of exopolysaccharide synthesis, is the key to phenotypic diversification in* Serratia marcescens *biofilms*

To better understand the ecological and evolutionary consequences of diversity in *Serratia marcescens* biofilms, the molecular mechanisms of these genetic differences were investigated. Comparative genomic DNA fingerprinting of the biofilm-derived variants by infrequent restriction site (IRS)-PCR indicated that there were no major gene rearrangements when compared to the WT (Fig. S3, Supporting information). However, transposon mutagenesis of the mucoid variant SUMV identified an autophosphorylating tyrosine protein kinase, designated here as exopolysaccharide tyrosine kinase (*etk*), that is important for exopolysaccharide production and the consequent variant colony morphotypes (Fig. 4). More importantly, sequence analysis revealed that all of the 'sticky' variant phenotypes (SSV, SRV, SRUV, SUMV) carried a single, nonsynonymous single-base mutation in critical regions of the *etk* (Fig. 5). These mutations affected the ATP-dependent tyrosine autophosphorylation of the kinase (Fig. 4b) and the protein function in EPS biosynthesis (Wugeditsch *et al*. 2001). No mutations were observed in a selected pool of 'nonsticky' isolates (biofilm-derived WTs and variant NSCVs). *Trans*-complementation of the variant *etk*s, containing

Table 2 The influence of predation on the diversity, evenness and species richness of the mixed biofilm and planktonic populations

		Mean ± SE‡					
		Simpson's reciprocal index		Evenness		Species richness	
Population composition	Population type†	NON-GRAZ	GRAZ	NON-GRAZ	GRAZ	NON-GRAZ	GRAZ
WT only§	Biofilm	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.0 ± 0.0	1.0 ± 0.0
	Planktonic	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.0 ± 0.0	1.0 ± 0.0
Multivariant	Biofilm	1.85 ± 0.08	1.99 ± 0.12	0.37 ± 0.02	0.40 ± 0.02	5.0 ± 0.0	5.0 ± 0.0
	Planktonic	4.01 ± 0.17	1.00 ± 0.00*	0.80 ± 0.03	0.20 ± 0.00*	5.0 ± 0.0	1.3 ± 0.3*
Multivariant + WT	Biofilm	2.30 ± 0.19	2.20 ± 0.07	0.38 ± 0.03	0.36 ± 0.01	6.0 ± 0.0	6.0 ± 0.0
	Planktonic	4.23 ± 0.17	1.01 ± 0.01*	0.71 ± 0.03	0.17 ± 0.00*	6.0 ± 0.0	1.3 ± 0.3*

*Significant differences between microbial populations that are grown in the presence (GRAZ) and absence (NON-GRAZ) of protozoa ($P < 0.001$, ANOVA).

†1×10^3 *Tetrahymena pyriformis* was inoculated into each well for planktonic grazing experiments, while 1×10^4 *Acanthamoeba castellanii* was seeded into each well for biofilm grazing experiments.

‡Data are means of three independent replicates in one experiment. The diversity data for each population represent the end-point measurement of diversity in the experiment.

§Spontaneous mutants that were morphologically indifferent from those observed in overnight planktonic cultures (in the absence of protozoan) were disregarded.

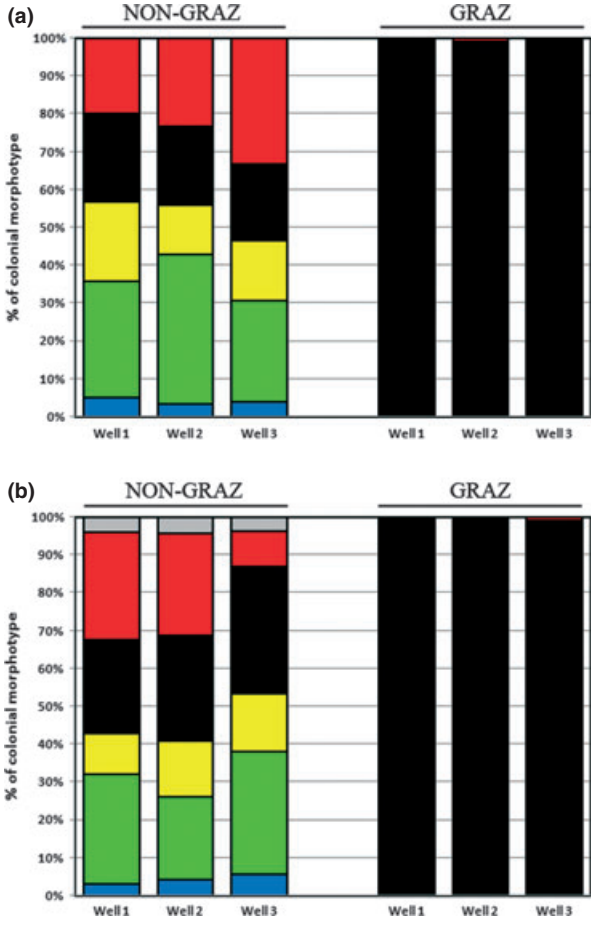


Fig. 3 Evolution of the planktonic populations over 6 days in the absence (NON-GRAZ) and presence (GRAZ) of grazing pressure by *Tetrahymena pyriformis*. The bar chart shows the genotypes of *Serratia marcescens* present in the planktonic fraction of the multivariant populations (a) and the genotypes of *S. marcescens* present in the planktonic fraction of the multivariant + WT populations (b). Colony morphotypes of WT (), biofilm-derived variants SSV (), SRUV (), SRV (), SUMV () and NSCV () were counted and expressed as percentage of total colony counts for all colonial morphotypes.

SNPs, into the 'nonsticky' parental WT strain in independent allelic replacement experiments showed that the 'sticky' phenotypes can be induced in the WT (Fig. S4, Supporting information). This confirmed that SNPs in the *etk* were responsible for the 'sticky' variant phenotypes obtained from the *S. marcescens* biofilms.

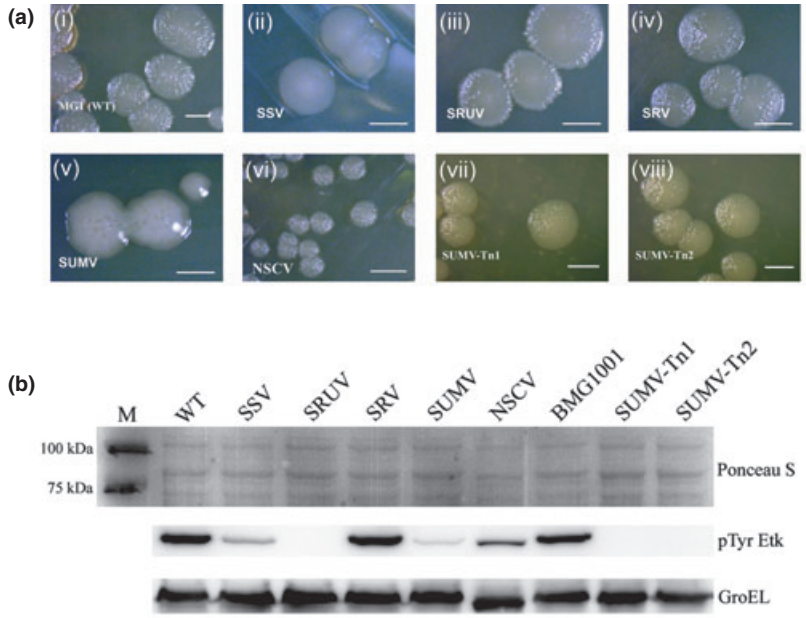
Discussion

The genetic basis and effects of diversity on emergent ecological properties of populations are unresolved challenges in biology. In this study, we tested the ecological implications of cellular diversity on populations of *Serratia marcescens*, utilizing a protozoan predator—bacterial biofilm system—and demonstrated strong emergent effects of genetic diversity in multivariant biofilms, manifested as enhanced resistance against predation. Similar effects of diversity were not observed in planktonic cultures, in which one resistant strain came to dominate. Remarkably, the generation of the variants—and thus a key ecological consequence of diversity in these biofilms can largely be attributed to a series of single nucleotide polymorphisms (SNPs) at one tyrosine kinase gene, *etk*.

Increased genetic diversity within a population has previously been proposed to increase the performance of the population via two main mechanisms: selection effects and complementarity effects (Seger & Brockman 1987). The former occurs through the overrepresentation of the best genotype(s), while the latter is an emergent property of the population and reflects resource partitioning or facilitation (Stachowicz *et al.* 2007). We did observe a selection effect in the mixed variant biofilms, with the best performing monovariant genotype (SSV) also the most abundant in mixed biofilms. However, analysis of our results also indicated a strong complementarity effect, with the consequence that phenotypic diversity not only provided the multivariant biofilm overall with the emergent property of greater resilience against grazing, but also provided enhanced protection to susceptible genotypes.

Previous studies (Reusch *et al.* 2005) have found that resource partitioning drives the emergent effects of genetic diversity. In our study, because of the nature of biofilms, resource partitioning as well as facilitation (whereby the presence of one genotype increases the performance of another) may also be responsible for enhanced resistance against predators. Indeed, the distinction between the two is not necessarily clear for biofilms, or for other systems more generally (Fox 2005; Cardinale *et al.* 2007). Resource partitioning among species in multispecies biofilms is common in bacterial systems and the different genotypes in these *S. marcescens* monovariant biofilms may also use different components of the resource pool. This could reflect utilization of different nutrients or metabolites, or equally may reflect spatial partitioning within the biofilm. Confocal laser scanning microscopy suggests that the complex architecture of a fully differentiated biofilm of *S. marcescens* is a result of spatial localization and differentiation of cells in micro-environments (Koh *et al.* 2007).

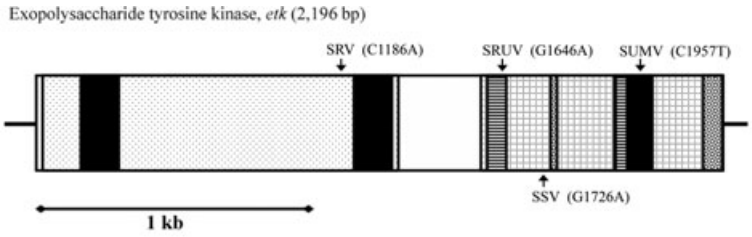
Equally, spatial differentiation and partitioning within *S. marcescens* biofilms may drive facilitation. This is suggested by the observation that predators were less abundant in the high diversity populations, indicating that the more genetically diverse populations were more resistant to predators than monovariant populations. If resource partitioning alone explained our



Isolates	Mean ± SE [a]		Stick-to-touch [c]
	% of tyrosine phosphorylation relative to WT Etk	Uronic acid content [b] (μg/mL)	
WT	100.00	0.12 ± 0.12	No
SSV	17.35 ± 6.43**	3.29 ± 0.18***	Yes
SRUV	0.00***	1.05 ± 0.04*	Yes
SRV	141.24 ± 30.20	1.12 ± 0.15*	Yes
SUMV	12.27 ± 6.02***	16.98 ± 0.37***	Yes
NSCV	46.18 ± 8.91	- 0.15 ± 0.02	No
SUMV-Tn1	0.00***	0.02 ± 0.17	No
SUMV-Tn2	0.00***	- 0.36 ± 0.22	No

Fig. 4 Phosphorylation of the Etk inversely regulates EPS production and the sticky/mucoid colony phenotype. Panel (a) illustrates the colony morphotype of (i) the parental WT MG1 strain, (ii–vi) the biofilm dispersal variants and (vii–viii) the Etk-deficient mutants of the mucoid variant (owing to mini-Tn5 insertion in the *etk*). Bar, 2.5 mm. Mutation in the *etk* of the phenotypic variants affects tyrosine phosphorylation of the Etk (b), as demonstrated by data obtained from western immunoblots of whole-cell lysate from *Serratia marcescens* MG1 parental WT, and the biofilm-derived variants SSV, SRUV, SRV, SUMV and NSCV, which were probed with the PY20 antiphosphotyrosine antibody, and anti-GroEL antibody as loading control (b, top). SUMV-Tn1 and SUMV-Tn2, both of which contains a transposon (mini-Tn5) insertion in the C-terminal region of the *etk*, were used as negative control for the immunoblot assay. M represents the Precision Plus protein molecular weight ladder (Bio-Rad Laboratories). The extent of phosphorylation at the tyrosine residues on the Etk and the amount of EPS produced by each strain were also quantified (b, bottom). Tyrosine phosphorylation on the Etk was normalized against GroEL and expressed as a percentage of tyrosine phosphorylation of the Etk relative to the parental WT (MG1). [a]Data are means of three replicates, while *, ** and *** represent significant differences at $P < 0.5$, $P < 0.01$ and $P < 0.001$ (analysis of variance), respectively, compared to the parental WT. [b]Uronic acid quantification was used to evaluate the amount of EPS produced by each strain. [c]Indicates that the colonies demonstrate some extent of elasticity when touched and then stretched by an inoculating loop.

findings, we would have expected to see higher numbers of predators in the high diversity populations because the increased abundance of prey should have promoted the growth of the predators. Spatial and structural diversity play an important role at an ecosystem level for complex communities such as coral reefs, rainforests and kelp communities, where structural diversity of ecosystem engineers has important consequences for community resilience to disturbance (Done *et al.* 1996; Stachowcz *et al.* 1999; Hughes & Stachowcz 2004). In a similar fashion, spatial resource partitioning within biofilms may result in complex, resistant architectures, facilitating an increase in resistance of the biofilm to stress factors such as grazing.



Position	Specific nucleotide	Amino acid change	Isolates	Predicted effect on function
1	C1186A	Q396K	SRV	Unknown. Retains ATPase activity
2	G1640A	G546D	STK006	Loss of ATPase activity (mutation within Walker A motif)
3	C1646A	T549N	SRUV, STK010, STK014	Loss of ATPase activity (mutation within Walker A motif)
4	G1726A	G576R	SSV, STK012, STK013	Loss of phosphorylation 727Y residue (conformation change)
4a	G1726T	G576W	STK003	Loss of phosphorylation 727Y residue (conformation change)
5	C1955A	P652Q	STK011	Reduced ATPase activity (mutation next to the Walker B motif)
6	C1957A	P653S	STK007, STK015	Reduced ATPase activity (mutation next to the Walker B motif)
6a	C1957T	P653T	SUMV*, STK001, STK002, STK008	Reduced ATPase activity (mutation next to the Walker B motif)
7	C2021A	A674E	STK004	Reduced ATPase activity
8	A2110T	N704Y	STK005, STK009	Reduced ATPase activity

Fig. 5 Summary of mutations observed in the *etk* of biofilm-derived 'sticky' variants where the diagrammatic representation of the structural features of *etk* and the mutations identified are indicated by the arrows (top). The EPS biosynthesis domain (▭), catalytic domain of the ATPase (▭), three potential transmembrane α-helices (▬), the Walker ATP-binding motifs (▭) and tyrosine phosphorylation sites (▭) are shown. Details of specific mutations and their predicted effects on gene function are also indicated (bottom). Isolates that are underlined indicate variants that have been characterized in detail previously (Koh *et al.* 2007). STK001 to STK015 represent additional isolates with the 'sticky' morphotype that were isolated from the effluent of the *Serratia marcescens* microcolony biofilms. **Trans*-complementation studies of the WT with pBAD-GmETK$_{SUMV}$ confirmed that mutation at position 6a gives rise to the smooth (SSV) phenotype, instead of the expected mucoid (SUMV) phenotype. In addition, complementation of the mucoid transposon insertion mutants (SUMV-Tn1 and SUMV-Tn2) with either pBAD-GmETK$_{SSV}$ or pBAD-GmETK$_{SUMV}$ resulted in the mucoid phenotype (see Fig. S4 in Supporting Information), confirming that a second mutation is required for the mucoid phenotype. This result is consistent with previous finding that mucoid dispersal variants (SUMV) can be consistently isolated from a smooth variant (SSV) maternal biofilm (Koh *et al.* 2007).

In striking contrast to the observations made for biofilms, grazed planktonic multivariant populations were eventually dominated by the morphotypic variant SUMV at the expense of all other genotypes, including the parental WT (Fig. 3). Similarly, Vogwill *et al.* (2011) noted that a naturally associated parasite, a phage, of *Pseudomonas fluorescens* was able to reduce the diversity of variant morphotypes and resulted in the selection of a subpopulation of phage-resistant variants. In this study, all variant morphotypes, except SUMV, were effectively consumed by the predators when grown as individual genotypes (Fig. S2, Supporting information). Resistance of the variant SUMV to predation may be owing to its higher production of extracellular materials relative to other genotypes, resulting in clumping of cells, which can increase effective cell size and thus resistance of bacteria to size-selective protozoan feeding (Matz *et al.* 2002). The presence of SUMV did not provide a benefit to the other variants and thus, in contrast to the biofilm cultures, there was no evidence of complementarity in the multivariant planktonic cultures. However, the success of variant SUMV against predators in the planktonic environment confers an additional benefit of the survival of *S. marcescens*, reflecting a 'portfolio' effect (Seger & Brockman 1987); that is, the success of one such genotype increases the likelihood that at least some cells from the parental biofilm will survive upon dispersal into the planktonic phase. Interestingly, studies conducted over evolutionary timescales in bacteria (100's of generations) suggest that predation drives prey diversification and divergence (Friman *et al.* 2008, 2011), whereas our study over ecological

timescales found that predation reduced the diversity of prey. It would be interesting to determine whether our experiments, left longer, would result in similar diversification following an initial drop in diversity.

Our finding that selection effects dominated in planktonic cultures supports our hypothesis that complementarity effects primarily occur in surface-associated biofilms as a result of spatial structuring, which should be much reduced in planktonic cultures. It should be noted that because different predators were used for the biofilm (amoebae are surface-associated predators) and planktonic (ciliates are only effective predators in the plankton) experiments, it is not possible to rule out predator-specific effects. In the absence of additional axenic cultures of relevant surface-associated and planktonic predators, this issue cannot yet be resolved. However, at the system level (biofilms vs. plankton), the differences in the consequences of diversity in the biofilms and planktonic systems were clear; when biofilms were exposed to the amoeba predator, complementarity effects maintain the diversity of the genotypes whereas in the plankton, predation reduces the genetic diversity in the population to a single genotype, the grazing-resistant variant.

This study has enabled the identification of the precise mechanism underlying genetic changes that determines the strength of the ecological consequences of genetic diversity, a link that is increasingly a focus in ecological studies (e.g. Whitham *et al.* 2006). The findings that a single nucleotide polymorphism within a single gene locus resulted in significant phenotypic variation and diversification within the *S. marcescens* biofilm is an important breakthrough as the fundamental question on the minimal level of diversity necessary to generate emergent ecological effects was unclear until now (Fig. 5). The pleiotropic effects of mutations in the tyrosine protein kinase can be extensive (Grangeasse *et al*. 2007), and thus these small changes in the genome of *S. marcescens* (SNP mutations in the *etk*) have significant ecological consequences owing to their effects on traits such as grazing resistance and biofilm formation.

For some eukaryotes, genetic diversity can enhance productivity or provide protection against disturbance and perturbation by increasing the range of potential responses to variable (Reusch *et al.* 2005). In stark contrast, little is known about comparable emergent ecological effects of genetic diversity for bacteria, the most diverse group of organisms on the planet. Moreover, even though modular organisms (single species biofilms, corals, and many plants) are the ecologically dominant organisms in many systems, the effects of self-generated genetic diversity within colonies of such modular organisms are also largely unstudied. The effects of enhanced diversity within colonies for such organisms may be just as broadly important as its effects at the more commonly studied levels of organization of communities.

This study also supports the uniqueness of the biofilm mode of life for bacteria. Here, biofilm-based diversification played an important role in the resistance to predation. For less structured planktonic cultures, however, phenotypic diversification allowed persistence owing to a portfolio effect (Bolnick *et al.* 2011), such that the production of many phenotypes resulted in selection of at least one variant that was resistant to predation. Such portfolio effects are likely to be particularly important at the dispersal stage, as the habitats colonized by dispersal cells are unpredictable. This highlights the potential for multiple, emergent effects of diversity for biofilms. More generally, the ability to diversify with minimum genetic changes and to generate multiple emergent effects of diversity under different modes of life may well highlight how bacteria are able to survive and adapt to the ever changing conditions on Earth for at least 3.5 billion years.

Acknowledgements

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K.S.K.'s research interests include the ecology and dispersal biology of bacterial biofilms, and population ecology and developmental biology of biogranulation in wastewater treat-

ment processes. C.M.'s research focuses on the interactions of bacteria and eukaryotes, primarily on predator-prey interactions. C.H.T.'s research focuses on the role of cellular signaling on biogranulation and biological nutrient removal in wastewater. D.J.M.'s research interest is in role of phenotypic links among life-history stages in population dynamics, sexual selection and quantitative genetics in marine invertebrates. S.A.R. current research focus is in the mechanisms that drive biofilm development and dispersal. P.D.S.'s research interests include marine chemical ecology, the impact of climate change on seaweed communities, the ecology and biology of bacterial biofilms, and environmental biotechnology. S.K.'s research focuses on the means by which bacteria form biofilms, the ecology of biofilm consortia in bacteria–eukaryote interactions, and applications and control of biofilm processes.

Data accessibility

All sequences of the *etk* genes determined in this study have been deposited in GenBank™, accession numbers EU371820 to EU371861. Values of phenotypic assays, cell counts, biomass and morphotype frequency that were used for Figs 1–4 are presented in Data S1–S4, respectively, in the Supplementary Information.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Evolution of colonial morphotypes over 12 days in the absence (NON-GRAZ) and presence (GRAZ) of grazing pressure by *Acanthamoeba castellanii*.

Fig. S2 Monoculture of *Serratia marcescens* dispersal variants exhibiting varying degree of susceptibility to grazing by *Tetrahymena pyriformis* (GRAZ).

Fig. S3 Comparative genomic DNA fingerprinting of biofilm-derived *Serratia marcescens* MG1 morphological variants by IRS-PCR.

Fig. S4 Colony morphotypes and the quantification of EPS of the parental wild-type MG1 and Δ*etk* strains of variant SUMV, complemented with different *etk*s.

Table S1 List of plasmids and oligonucleotides used in this study.

Table S2 Assessment of the growth rates of *Serratia marcescens* parental WT and biofilm-derived variants in M9 minimal medium supplemented with 0.1% (wt/vol) glucose.

Table S3 ANOVA testing the effects of diversity on biofilm growth rates in the presence of *Acanthamoeba castellanii* over 12 days (a) and predator abundance over the same period (a).

Data S1 Phenotypic assays of the biofilm isolates of *Serratia marcescens* MG1.

Data S2 Biofilm yield and abundance of biofilm-feeding *Acanthamoeba castellanii* grown in the presence of different *Serratia marcescens* genotypes.

Data S3 Frequencies of the different *Serratia marcescens* genotypes that are present in the mixed planktonic *S. marcescens* populations (with or without the WT) grown in the presence of the planktonic-feeding *Tetrahymena pyriformis*.

Data S4 Quantification of tyrosine phosphorylation of the Etk and EPS uronic acid content of the *Serratia marcescens* isolates.

Method S1 Growth Studies.

Method S2 Motility assays.

Method S3 Attachment and biofilm formation assays.

Method S4 Assay for protease activity.

Method S5 Assay for haemolytic activity.

Method S6 Assay for siderophore production.

Method S7 DNA fingerprinting by infrequent restriction site (IRS)-PCR.