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Minimal increase in genetic diversity enhances predation
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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.

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 (Cardinale *et al.* 2011; Cardinale *et al.* 2002; Folke *et al.* 2004; Hooper *et al.* 2005; Loreau & Hector 2001). 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 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 & Stachowicz 2004), their productivity and their resilience to disturbance (Crutsinger *et al.* 2006; Fox 2005; Reusch *et al.* 2005).

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 non-varying 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 that 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 non-sticky 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⁻¹) glucose or Luria-Bertani (LB) medium, and *E. coli* strains were grown at 37°C in LB medium. Streptomycin (200 µg mL⁻¹), kanamycin (50 µg mL⁻¹), chloramphenicol (20 µg mL⁻¹), gentamicin (20 µg mL⁻¹), and tetracycline (12 µg mL⁻¹) 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⁻¹) arabinose to induce expression of the cloned *etk* gene, where indicated.

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 *S. marcescens* WT and biofilm-derived morphological variants were diluted to an OD_{600nm} 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_{600nm}-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 x 10⁴ 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⁻¹) 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_{540nm} 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 corresponds 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 $500 \times g$ and then carefully replacing 75% of the spent medium with fresh 0.5× 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 analyzing five samples of 5µL aliquots from each treatment under the microscope.

Diversity indices and statistical analysis. The diversity of colonial morphotypes in the *S. 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 (Magurran 1988; Simpson 1949). 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 posthoc comparisons of means for one-way ANOVA. The effects of complementarity vs. selection in mixed variant biofilms were analyzed using the methods of Loreau and Hector (2001). Briefly, this approach uses a modified version of the Price selection equation to estimate how much variation in performance in mixtures is due to the overrepresentation of a single genotype (sampling effects) and how much is due to an increase in the average performance of each genotype.

Gene knock-out library, genetic screens and construction of *etk* expression vector and complementation studies. The phenotypes of the *S. 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 attempt to better understand the ecological and evolutionary consequences of diversity in *S. marcescens* biofilms.

Construction of the gene knock-out 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 knock-out 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 *aacC1* (encoding 3-N-

aminoglycoside acetyltransferase conferring resistance to gentamicin) (Hoang *et al.* 1998) derived from pGEM-Gm, to the *NdeI*-digested and dephosphorylated vector pBAD-CMP (Table 1, Supporting information). The *NcoI/PmeI* fragment carrying the chloramphenicol resistance marker, *cat*, located downstream of the inducible Pbad promoter in the plasmid pBAD-CMPGm, was then replaced with the *NcoI/PmeI*-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⁻¹), 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 *S. marcescens* strains which was separated on NuPAGE® 4-12% Bis-Tris gradient gels (Invitrogen Inc.) and the 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 anti-phosphotyrosine primary antibody (Sigma-Aldrich Inc.) and secondary horseradish peroxidase (HRP)-conjugated anti-mouse 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 anti-rabbit IgG antibody (Amersham Biosciences).

Quantification of exopolysaccharide (EPS) by uronic acid determination. Isolates were cultured on M9 minimal agar supplemented with 0.5% (wt vol⁻¹) 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 $10,000 \times g$ for 20 min at 4°C. The resulting supernatant was carefully transferred to a fresh tube and kept on wet ice. EPS was quantified with the uronic acid determination of Blumenkrantz and Asboe-Hansen method (1973), 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 three-fold 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 due 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, $t = 7.05$, 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 *S. 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 six 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 versus $5.8 \times 10^8 \pm 4.6 \times 10^7$ CFU per 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 per 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 maintenance 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 *S. marcescens* biofilms. To better understand the ecological and evolutionary consequences of diversity in *S. 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, non-synonymous 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 “non-sticky” isolates (biofilm-derived WTs and variant NSCVs). *Trans*-complementation of the variant *etks*, containing SNPs, into the “non-sticky” 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 (Seeger & Brockman 1987). The former occurs through the over-representation 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 (Cardinale *et al.* 2007; Fox 2005). 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 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; Hughes & Stachowcz 2004; Stachowcz *et al.* 1999). 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.

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 due 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; Friman *et al.* 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 due 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, 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 due 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, since 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.

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377 **TABLES AND FIGURES**

378 **Table 1** List of bacterial strains used in this study.

Strains	Phenotypic and genotypic characteristics ^a	Source or reference
<i>Serratia marcescens</i>		
MG1	wild type, (Amp ^R) (Tet ^R); rough colony surface and “non-sticky”	(Givskov <i>et al.</i> 1988)
SSV	MG1; spontaneous smooth and “sticky” (with wet elastic texture) derivative isolated from MG1 biofilm	(Koh <i>et al.</i> 2007)
SRUV	MG1; spontaneous rough, umbonated, and “sticky” (with wet elastic texture) derivative isolated from MG1 biofilm	(Koh <i>et al.</i> 2007)
SRV	MG1; spontaneous rough and sticky (with dry elastic texture) derivative isolated from MG1 biofilm	(Koh <i>et al.</i> 2007)
SUMV	MG1; spontaneous smooth, mucoid, and “sticky” (with wet elastic texture) derivative isolated from MG1 biofilm	(Koh <i>et al.</i> 2007)
NSCV	MG1; spontaneous small colony and “non-sticky” derivative isolated from MG1 biofilm	(Koh <i>et al.</i> 2007)
BMG1001	MG1; WT derivative isolated from MG1 biofilm	(Koh <i>et al.</i> 2007)
STK01 to STK15	MG1; spontaneous “sticky” (or mucoid) variant derivatives isolated from MG1 biofilm	This study
BMG1002 to BMG1016	MG1; WT derivative isolated from MG1 biofilm	This study
NSCV001 to NSCV005	MG1; spontaneous small colony and “non-sticky” derivative isolated from MG1 biofilm	This study
<i>Escherichia coli</i>		
JM109	F ⁺ , <i>e14</i> ⁺ (<i>mcrA</i> ⁺), <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> (<i>rK</i> ⁺ <i>mK</i> ⁺), <i>supE44</i> , <i>relA1</i> , λ , Δ (<i>lac-proAB</i>), [<i>F</i> <i>traD36</i> , <i>proAB</i> , <i>lacI</i> ^q Δ M15]	(Koh <i>et al.</i> 2007)
HB101	F ⁺ , <i>hsdS20</i> (<i>rb</i> ⁺ , <i>mb</i> ⁺), <i>supE44</i> , <i>ara14</i> , <i>galK2</i> , <i>lacY1</i> , <i>proA2</i> , <i>rpsL20</i> (Str ^R), <i>xyl-5</i> , <i>mtl-1</i> , <i>l-</i> , <i>recA13</i> , <i>mcrA</i> ⁺ , <i>mcrB</i> ⁺	(Raleigh & Wilson 1986)
TOP10	F ⁺ , <i>mcrA</i> , <i>mcr-hsdRMS-mcrBC</i> , <i>lacZ</i> , <i>lacY</i> , <i>lacA</i> , <i>recA1</i> , <i>araD139</i> , <i>arg-leu</i> 7697, <i>galK</i> , <i>rpsL</i> (Str ^R), <i>endA1</i> , <i>nupG</i>	Invitrogen Inc.
S17-1 λ <i>pir</i>	<i>E. coli</i> K-12, <i>recA</i> <i>thi</i> <i>hsdRM</i> ⁺ , RP4::2-Tet::Mu::Km Tn7, <i>λpir</i> phage lysogen, (Tet ^R) (Str ^R)	(de Lorenzo & Timmis 1994)
MG3617	<i>E. coli</i> S17-1 λ <i>pir</i> , pJMS10, (Tet ^R) (Str ^R) (Km ^R)	(Kristensen <i>et al.</i> 1995)
<i>Acanthamoeba castellanii</i>	wild-type, ATCC 30234	American Type Culture Collection
<i>Tetrahymena</i> sp.	wild-type	(Weitere <i>et al.</i> 2005)

379 ^a Amp^R – Resistance to ampicillin; Km^R – Resistance to kanamycin; Str^R – Resistance to streptomycin; Tet^R – Resistance to
380 tetracycline.

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

Population composition	Population type ^b	Mean ± SE ^a					
		Simpson's reciprocal index		Evenness		Species richness	
		NON-GRAZ	GRAZ	NON-GRAZ	GRAZ	NON-GRAZ	GRAZ
WT only ^c	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*

^a 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. ^b 1×10^3 *T. pyriformis* was inoculated into each well for planktonic grazing experiments, while 1×10^4 *A. castellanii* was seeded into each well for biofilm grazing experiments. ^c Spontaneous mutants that were morphologically indifferent from those observed in overnight planktonic cultures (in the absence of protozoan) were disregarded. * Significant differences between microbial populations that are grown in the presence (GRAZ) and absence (NON-GRAZ) of protozoa ($P < 0.001$, ANOVA).

FIGURE LEGENDS

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 permuted raw data and the color 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 analyzed for phenotypic characteristics were the parental wild-type (WT), biofilm-derived wild-type (BMG1001), “non-sticky” 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, *A. castellanii* (c). Statistical significance was determined by analysis of variance; *, $P < 0.05$; **, $P < 0.001$. Values shown are Mean $\pm$ SE. Predation rate was not detectable (N.D.) in NSCV biofilms due to poor biofilm formation.

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, *A. 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 $\pm$ SE.

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 *S. 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.

Fig. 4 Phosphorylation of the Etk inversely regulates EPS production and the sticky/mucoid colony phenotype. Panel (a) illustrate 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 (due 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 *S. marcescens* MG1 parental WT, and the biofilm-derived variants SSV, SRUV, SRV, SUMV, and NSCV, which were probed with the PY20 anti-phosphotyrosine 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.

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 ( ), 3 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 *S. 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).

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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 Figures 1 – 4 are presented in Data 1 – 4 respectively in the Supplementary Information.

554 **SUPPORTING INFORMATION** Additional supporting information may be found in the online
555 version of this article.

556 **Method S1** Growth Studies.

557 **Method S2** Motility assays

558 **Method S3** Attachment and biofilm formation assays

559 **Method S4** Assay for protease activity

560 **Method S5** Assay for hemolytic activity

561 **Method S6** Assay for siderophore production

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

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

564 **Table S2** Assessment of the growth rates of *S. marcescens* parental WT and biofilm-derived variants in
565 M9 minimal medium supplemented with 0.1% (wt vol⁻¹) glucose.

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

568 **Fig. S1** Evolution of colonial morphotypes over 12 days in the absence (NON-GRAZ) and presence
569 (GRAZ) of grazing pressure by *Acanthamoeba castellanii*. The biofilm fraction of the microcosm, seeded
570 with multivariants (a) and multivariants + wild-type (b), were collected and plated onto LB10 agar.
571 Colony morphotypes of wild-type ( ) and the biofilm-derived variants SSV ( ), SRUV ( ), SRV ( ), SUMV ( ), and NSCV ( ), were counted and expressed as
572 percentage of total colony counts for all colonial morphotypes.
573

Fig. S2 Monoculture of *S. marcescens* dispersal variants exhibiting varying degree of susceptibility to grazing by *Tetrahymena pyriformis* (GRAZ). Bacterial cell densities were determined on the 6th day of co-incubation with the planktonic-feeding protozoa by plating an aliquot of the planktonic fraction onto LB10 agar. Statistical significance was determined by student *t*-test (*n* = 3) with reference to the respective controls without protozoa (NON-GRAZ); *, *P* < 0.005; **, *P* < 0.001. Values shown are Mean ± SE.

Fig. S3 Comparative genomic DNA fingerprinting of biofilm-derived *S. marcescens* MG1 morphological variants by IRS-PCR. No differences between the biofilm-derived variants (lanes 6 to 10) from the parental WT (lanes 3) were observed. (a – e), Genomic DNA of *S. marcescens* isolates was digested with *Xba*I and *Cfo*I restriction endonucleases and amplified by PCR using IRS-AC1 and PX-G primers (a), IRS-AC1 and PX-T primers (b), IRS-AC1 and PX-C primers (c), IRS-AC1 and PX-A primers (d), and IRS-AC1 and PX primers (e). (f – j), Genomic DNA of *S. marcescens* isolates digested with *Xba*I and *Pst*I restriction endonucleases and amplified by PCR using IRS-PS1 and PX-G primers (f), IRS-PS1 and PX-C primers (g), IRS-PS1 and PX-T primers (h), IRS-PS1 and PX-A primers (i), IRS-PS1 and PX primers (j). Lanes: 1, No genomic DNA control; 2, Undigested genomic DNA control; 3, *S. marcescens* MG1 parental WT; 4, *E. coli* K12; 5, *E. coli* DH5α; 6 to 10, biofilm-derived variants SSV, SRUV, SRV, SUMV, and NSCV, respectively; 11, biofilm-derived WT BMG1001; 12, *S. marcescens* MG1 *ompX* mutant that had a 312 bp region in the *ompX* gene replaced by a 0.9 kb gentamicin gene cassette. M is the molecular weight marker with band sizes as indicated.

Fig. S4 Colony morphotypes and the quantification of EPS of the parental wild-type MG1 and Δetk strains of variant SUMV, complemented with different *etks*. Panels (a) illustrate the parental wild-type MG1 strain (i), wild-type harbouring pBAD-GmETK_{WT} (ii), wild-type harbouring pBAD-GmETK_{SSV} (iii), wild-type harbouring pBAD-GmETK_{SRUV} (iv), wild-type harbouring pBAD-GmETK_{SRV} (v), wild-type harbouring pBAD-GmETK_{SUMV} (vi), SUMV-Tn1 (Δetk strains of variant SUMV) (vii), SUMV-Tn1 harbouring pBAD-GmETK_{SUMV} (viii), SUMV-Tn1 harbouring pBAD-GmETK_{SUMV} (ix), SUMV-Tn2 (Δetk strains of variant SUMV) (x), SUMV-Tn2 harbouring pBAD-GmETK_{SUMV} (xi), and SUMV-Tn2 harbouring pBAD-GmETK_{SUMV} (xii). Bar, 2.5 mm. The amount of EPS produced by each strain is presented (b) and was quantified as described in Materials and Methods. ^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

603 the amount of EPS produced by each strain. ^c Indicates that the colonies demonstrate some extent of
604 elasticity when touched and then stretched by an inoculating loop.

605 **Data 1** Phenotypic assays of the biofilm isolates of *S. marcescens* MG1.

606 **Data 2** Biofilm yield and abundance of biofilm-feeding *A. castellanii* grown in the presence of different
607 *S. marcescens* genotypes.

608 **Data 3** Frequencies of the different *S. marcescens* genotypes that are present in the mixed planktonic *S.*
609 *marcescens* populations (with or without the WT) grown in the presence of the planktonic-feeding
610 *Tetrahymena pyriformis*.

611 **Data 4** Quantification of tyrosine phosphorylation of the Etk and EPS uronic acid content of the *S.*
612 *marcescens* isolates.

613

Fig. 2

(a)

(b)

(a)

(b)

Isolates	Mean $\pm$ SE ^a		Stick-to-touch ^c
	% of tyrosine phosphorylation relative to WT Etk	Uronic acid content ^b ($\mu\text{g/mL}$)	
WT	100.00	0.12 ± 0.12	No
SSV	$17.35 \pm 6.43^{**}$	$3.29 \pm 0.18^{***}$	Yes
SRUV	0.00^{***}	$1.05 \pm 0.04^*$	Yes
SRV	141.24 ± 30.20	$1.12 \pm 0.15^*$	Yes
SUMV	$12.27 \pm 6.02^{***}$	$16.98 \pm 0.37^{***}$	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. 5

Exopolysaccharide tyrosine kinase, *etk* (2,196 bp)

Position	Specific nucleotide	Amino acid change	Isolates	Predicted effect on function
1	C1186A	Q396K	<u>SRV</u>	Unknown. Retains ATPase activity
2	G1640A	G546D	STK006	Loss of ATPase activity (mutation within Walker A motif)
3	C1646A	T549N	<u>SRUV</u> , STK010, STK014	Loss of ATPase activity (mutation within Walker A motif)
4	G1726A	G576R	<u>SSV</u> , 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

SUPPLEMENTARY INFORMATION

MINIMAL INCREASE IN GENETIC DIVERSITY ENHANCES PREDATION RESISTANCE

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Method S1. Growth studies

Growth studies of *Serratia marcescens* MG1 and its biofilm-derived variants were conducted in side-arm flasks containing 20 mL of M9 minimal medium supplemented with 0.1% (wt vol⁻¹) glucose (M9 complete medium). Two hundred microliters of overnight bacterial culture were inoculated into each flask and were incubated at room temperature with constant agitation at 200 rpm. Hourly spectrophotometric readings were taken at 600 nm.

Method S2. Motility assays

Swimming and swarming motility assays were performed using minimal broth Davis medium without dextrose, supplemented with 0.5% (wt vol⁻¹) casamino acids, 0.2% (wt vol⁻¹) glucose, and 0.4% (wt vol⁻¹) or 0.7% (wt vol⁻¹) Bacto purified agar, respectively. Overnight cultures of *S. marcescens* were stabbed into the center of the agar in 90 mm petri dishes and incubated at 30°C for 24 h. Swimming and swarming motility were assessed quantitatively by measuring the diameter of the swimming or swarming colony (growth) of each strain.

Method S3. Attachment and biofilm formation assays

Attachment and biofilm formation assays were performed by inoculating 100 µL of overnight cultures, normalized to 0.7 OD_{600nm}, into 96-well tissue culture plates and 24-well tissue culture plates containing 0.9 mL M9 complete medium, respectively. For attachment assays, cultures were incubated at room temperature for 2 h without agitation, while for biofilm assays cultures were incubated at room temperature for 24 h with constant agitation at 150 rpm. Quantification of the bacterial biomass in both assays were performed by carefully removing the aqueous phase from each well, gently rinsing three times with phosphate-buffered saline (pH 7.2), and then staining with 1% (wt vol⁻¹) solution of filtered crystal violet for 20 min at room temperature. The wells were subsequently rinsed three times with PBS

to remove excess crystal violet and the bacterial biomass was quantified by solubilizing the crystal violet that was retained by the biomass using 95% ethanol. Spectrophotometric readings of the ethanol-dissolved crystal violet were taken at 545 nm.

Method S4. Assay for protease activity

Protease activity was quantified using the azocasein proteolytic degradation method as described by Tomarelli *et al.* (1949), with some minor modifications. Briefly, bacterial culture supernatant was removed by centrifugation and filtered through a 0.22 μm cellulose-acetate filter. The filtered supernatant (100 μL) was incubated at 30°C with 100 μL of 1% (wt vol⁻¹) azocasein solution, which was prepared by dissolving azocasein in 0.1M Tris-HCl buffer (pH 8.0). The azocasein reaction was terminated after 2 h by adding 400 μL of ice-cold 10% (wt vol⁻¹) trichloroacetic acid and then incubated at 4°C for 15 min. Unreacted azocasein was removed by centrifugation at 15,000 $\times$ g for 10 min at 4°C. The supernatant (500 μL) containing azopeptide was resuspended in 500 μL of 0.5M NaOH, and the azopeptide (orange coloration) was measured at 440 nm using a spectrophotometer. Protease activity units were calculated by normalizing azopeptide measurements (at OD_{440nm}) against the bacterial cell density (OD_{600nm}) of the *S. marcescens* overnight culture.

Method S5. Assay for hemolytic activity

Liquid hemolysis assays were performed by mixing 200 μL of *S. marcescens* culture supernatant with 600 μL of assay buffer [10mM Tris-HCl pH 7.2, 150 mM NaCl, 10 mM MgCl₂, 10 mM CaCl₂, and 0.2% (wt vol⁻¹) gelatin] and 200 μL of sheep erythrocytes, that were prepared from heparinized blood of sheep. The mixture was incubated in a 37°C water bath for 30 min and intact sheep erythrocytes were removed by centrifugation at 120 $\times$ g for 10 min. The clear supernatant containing lysed sheep erythrocytes was measured at 545 nm using a spectrophotometer. Hemolysin activity units were calculated by normalizing

measurements of lysed sheep erythrocytes (OD_{545nm}) against the cell density (OD_{600nm}) of the *S. marcescens* overnight cultures used for the hemolysin assay.

Method S6. Assay for siderophore production

The production of siderophore was determined as described by Schwyn and Neilands (1987) using the chrome-azuroil-S (CAS) agar. The assay was performed by spotting 10 µL of overnight bacterial culture, normalized to 0.7 OD_{600nm}, onto CAS agar. Siderophore production by the *S. marcescens* isolates was recorded as the diameter of the orange halo produced by the colony and was expressed as siderophore units, calculated using the formula: (diameter of the orange halo) ÷ (diameter of the colony).

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

DNA fingerprinting by IRS-PCR is based on the principal of amplification by PCR of DNA sequences flanking infrequent restriction sites as described previously (Mazurek *et al.* 1996) with some modifications. The sequences of the oligonucleotides and adaptors that were used in IRS-PCR analysis are listed in Table S1 (Supporting information). DNA templates for IRS-PCR were prepared by digesting 500 ng of genomic DNA with 20 U of *Cfo*I and *Xba*I restriction endonucleases. The mixture was incubated at 37°C for 2 h and adaptors (IRS-AC or IRS-AX adaptors) were ligated to the digested genomic DNA fragments in a 20 µL reaction mix with the following conditions: 1× ligase buffer, 625 nM ATP, 1 µM IRS-AX adaptor, 1 µM IRS-AC adaptor, and 1.5 U T4 DNA ligase. Ligation reactions were performed at 4°C overnight and T4 DNA ligase was deactivated at 65°C for 20 min. Preparation of the IRS-PCR DNA template was completed by restriction endonuclease digestion with 5 U of *Xba*I and *Cfo*I at 37°C for 20 min. Preparation of IRS-PCR templates was also performed with other restriction endonucleases (*Pst*I and *Xba*I) and adaptors (IRS-AP or IRS-AX adaptors), to increase the genomic coverage for the DNA fingerprinting analysis.

113 IRS-PCR was carried out in a 50 μ L mixture comprised of: 1 $\times$ PCR buffer, 1.5 μ M MgCl₂, 1 μ M IRS-
114 AC1 primer (or IRS-AP1 primer for *Pst*I/*Xba*I digests), 1 μ M PX primer, 800 nM dNTPs, 0.5 μ L of “IRS-
115 PCR-ready” template DNA, 0.5 U of AmpliTaq[®] DNA polymerase (Applied Biosystems). PCR
116 amplification was also performed with a PX primer modified by the addition of an extra nucleotide at the
117 3' end, i.e., PX-T, PX-A, PX-G, and PX-C (Table S1, Supporting information), to generate unique
118 banding patterns. Amplification mixtures were subjected to 5 min denaturation at 95°C; 30 cycles of 95°C
119 for 60 s, 60°C for 30 s, and 72°C for 120 s; followed by a final refrigeration step. PCR-amplified DNA
120 fragments were analyzed on a 6.5% polyacrylamide gel in 1 $\times$ TBE buffer. Electrophoresis was performed
121 with a constant voltage at 200 V for 2 h. The polyacrylamide gels were stained with ethidium bromide.

122

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

Designation	Genotypic characteristics ^a or sequence	Source or reference
Plasmids		
pRK600	Helper plasmid for conjugative transfer, ColE1, <i>oriV</i> , RK4 <i>tra</i> ⁺ RP4 <i>oriT</i> , (Cmp ^R)	(Kessler <i>et al.</i> 1992)
pJMS10	Transposon delivery vector for mini-Tn5 <i>luxAB res-npt-xyIE-res</i> , (Amp ^R) (Km ^R)	(Kristensen <i>et al.</i> 1995)
pGEM [®] -T Easy	<i>lacZα</i> , TA cloning vector, (Amp ^R)	Promega Co.
pBAD-TOPO [®]	<i>araBAD</i> , TA expression vector, (Amp ^R)	Invitrogen Inc.
pGEM-Gm	pGEM [®] -3Zf(+) (Amp ^R), <i>aacC1</i> (Gm ^R) cloned into TA cloning site	This study
pBAD-CMP	pBAD-TOPO [®] (Amp ^R), <i>cat</i> (Cmp ^R) cloned into TA cloning site	This study
pBAD-CMPGm	pBAD-CMP (Amp ^R) (Cmp ^R), <i>aacC1</i> (Gm ^R) cloned into <i>NdeI</i> site	This study
pBAD-GmETK _{MGI}	pBAD-GM (Amp ^R) (Gm ^R), <i>etk_{MGI}</i> cloned into <i>PmeI</i> and <i>NcoI</i> site	This study
pBAD-GmETK _{SSV}	pBAD-GM (Amp ^R) (Gm ^R), <i>etk_{SSV}</i> cloned into <i>PmeI</i> and <i>NcoI</i> site	This study
pBAD-GmETK _{SRUV}	pBAD-GM (Amp ^R) (Gm ^R), <i>etk_{SRUV}</i> cloned into <i>PmeI</i> and <i>NcoI</i> site	This study
pBAD-GmETK _{SRV}	pBAD-GM (Amp ^R) (Gm ^R), <i>etk_{SRV}</i> cloned into <i>PmeI</i> and <i>NcoI</i> site	This study
pBAD-GmETK _{SUMV}	pBAD-GM (Amp ^R) (Gm ^R), <i>etk_{SUMV}</i> cloned into <i>PmeI</i> and <i>NcoI</i> site	This study
Oligonucleotides – PCR Primers^b		
PX	AGA GTC TGC CAG TAC TAG A	(Mazurek <i>et al.</i> 1996)
PX-G	AGA GTC TGC CAG TAC TAG AG	(Mazurek <i>et al.</i> 1996)
PX-C	AGA GTC TGC CAG TAC TAG AC	(Mazurek <i>et al.</i> 1996)
PX-T	AGA GTC TGC CAG TAC TAG AT	(Mazurek <i>et al.</i> 1996)
PX-A	AGA GTC TGC CAG TAC TAG AA	(Mazurek <i>et al.</i> 1996)
AP1	GGA TCC TAA TAC GAC TCA CTA TAG GGC	(Siebert <i>et al.</i> 1995)
AP2	AAT AGG GCT CGA GCG GC	(Siebert <i>et al.</i> 1995)
P6	GCC CGG TCG CAT TAC ACC TT	(Siebert <i>et al.</i> 1995)
P7	CGC TTC ATC ACT TCG GTC TGA GA	(Siebert <i>et al.</i> 1995)
NdeGm _{(38)F} (+)	CTA <u>TCA TAT</u> GGA ATT GAC ATA AGC CTG TTC GT	This study
NdeGm _{60R} (–)	CTA <u>TCA TAT</u> GTT GTG ACA ATT TAC CGA ACA AC	This study
NcoEtk _{(171)F} (+)	CTA <u>TCC ATG</u> GTG AAT TCG TTT ACG GCC TTC	This study
PmeEtk _{2410R} (–)	CTA <u>TGT TTA AAC</u> CCA ACG CAT TTC TTT CCT GT	This study
Etk _{(171)F} (+)	TGA ATT CGT TTA CGG CCT TC	This study
Etk _{2410R} (–)	CCA ACG CAT TTC TTT CCT GT	This study

Etk_525F (+)	GTG ACC GCT TTG AGG ATG TT	This study
Etk_1081F (+)	AAG CAA ATC TCT GCA ATG CC	This study
Etk_471R (-)	CGG GAT GCT TAA ATG GCT AA	This study
Etk_1081R (-)	GGC ATT GCA GAG ATT TGC TT	This study
Etk_1536R (-)	TTT GTG TAC CTG CCC CTT TC	This study
pBAD (+)	ATG CCA TAG CAT TTT TAT CC	Invitrogen Inc.
pBAD (-)	GAT TTA ATC TGT ATC AGG	Invitrogen Inc.

Oligonucleotides – Adaptors

IRS-AC1	AGA ACT GAC CTC GAC TCG CAC G	(Mazurek <i>et al.</i> 1996)
IRS-AC2	TGC GAG A	(Mazurek <i>et al.</i> 1996)
IRS-AX1	PO ₄ – CTA GTA CTG GCA GAC TCT	(Mazurek <i>et al.</i> 1996)
IRS-AX2	GCC AGT A	(Mazurek <i>et al.</i> 1996)
IRS-API	PO ₄ – GAC TCG ACT CGC ATG CA	(Riffard <i>et al.</i> 1998)
IRS-AP2	TGC GAG T	(Riffard <i>et al.</i> 1998)
AD1	CTA ATA CGA CTC ACT ATA GGG CTC GAG CGC CCG CCC GGG CAG GT	(Siebert <i>et al.</i> 1995)
AD2	PO ₄ – ACC TGC CC – NH ₂	(Siebert <i>et al.</i> 1995)

^a Amp^R – Resistance to ampicillin; Km^R – Resistance to kanamycin; Cmp^R – Resistance to chloramphenicol; Gm^R – Resistance to gentamicin.

^b Nucleotides that are underlined indicate restriction sites that are specifically designed and introduced for cloning purposes; (+) indicates forward primer; (-) indicates reverse primer.

Table S2. Assessment of the growth rates of *S. marcescens* parental WT and biofilm-derived variants in M9 minimal medium supplemented with 0.1% (wt vol⁻¹) glucose.

Strains	Mean $\pm$ SE ^a
	Average exponential growth rate (OD ₆₀₀ hr ⁻¹) ^b
WT	0.1493 $\pm$ 0.01466
SSV	0.1440 $\pm$ 0.01434
SRUV	0.1448 $\pm$ 0.01505
SRV	0.1452 $\pm$ 0.01556
SUMV	0.1424 $\pm$ 0.01476
NSCV	0.1509 $\pm$ 0.01186

^a Data are means of three replicates. ^b No significant differences ($P > 0.1$, analysis of variance) in growth rates relative to the parental WT.

Table S3. ANOVA analysis of 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 (b).

Source	df	MS	F	P
(a)		Biofilm abundance		
Treatment	3	346.47	157.2	<0.0001
Time	2	316.06	143.4	<0.0001
Time x Treatment	6	104.05	47.2	<0.0001
Error	36	2.20		
(b)		Amoeba abundance		
Treatment	8	517.37	266.3	<0.0001
Time	3	398.39	205.1	<0.0001
Time x Treatment	24	52.15	26.8	<0.0001
Error	108	1.94		

138 Fig. S1

Fig. S1. Evolution of colonial morphotypes over 12 days in the absence (NON-GRAZ) and presence (GRAZ) of grazing pressure by *Acanthamoeba castellanii*. The biofilm fraction of the microcosm, seeded with multivariants (a) and multivariants + wild-type (b), were collected and plated onto LB10 agar. Colony morphotypes of wild-type ( ), and the biofilm-derived variants SSV ( ), SRUV ( ), SRV ( ), SUMV ( ), and NSCV ( ), were counted and expressed as percentage of total colony counts for all colonial morphotypes.

149 Fig. S2

150
 151 **Fig. S2. Monoculture of *S. marcescens* dispersal variants exhibiting varying degree of susceptibility**
 152 **to grazing by *Tetrahymena pyriformis* (GRAZ).** Bacterial cell densities were determined on the 6th day
 153 of co-incubation with the planktonic-feeding protozoa by plating an aliquot of the planktonic fraction onto
 154 LB10 agar. Statistical significance was determined by student *t*-test ($n = 3$) with reference to the respective
 155 controls without protozoa (NON-GRAZ); *, $P < 0.005$; **, $P < 0.001$. Values shown are Mean ± SE.

156

Fig. S3

(a)

(b)

Fig. S3 (continued)

Fig. S3 (continued)

Fig. S3 (continued)

(g)

(h)

Fig. S3 (continued)

(i)

(j)

Fig. S3 (continued). Comparative genomic DNA fingerprinting of biofilm-derived *S. marcescens* MG1 morphological variants by IRS-PCR. No differences between the biofilm-derived variants (lanes 6 to 10) from the parental WT (lanes 3) were observed. (a – e), Genomic DNA of *S. marcescens* isolates digested with *Xba*I and *Cfo*I restriction endonucleases and amplified by PCR using IRS-AC1 and PX-G primers (a), IRS-AC1 and PX-T primers (b), IRS-AC1 and PX-C primers (c), IRS-AC1 and PX-A primers (d), and IRS-AC1 and PX primers (e). (f – j), Genomic DNA of *S. marcescens* isolates digested with *Xba*I and *Pst*I restriction endonucleases and amplified by PCR using IRS-PS1 and X-G primers (f), IRS-PS1 and PX-C primers (g), IRS-PS1 and PX-T primers (h), IRS-PS1 and PX-A primers (i), IRS-PS1 and PX primers (j). Lanes: 1, No genomic DNA control; 2, Undigested genomic DNA control; 3, *S. marcescens* MG1 parental WT; 4, *E. coli* K12; 5, *E. coli* DH5 α ; 6 to 10, biofilm-derived variants SSV, SRUV, SRV, SUMV, and NSCV, respectively; 11, biofilm-derived WT BMG1001; 12, *S. marcescens* MG1 *ompX* mutant that had a 312 bp region in the *ompX* gene replaced by a 0.9 kb gentamicin gene cassette. M is the molecular weight marker with band sizes as indicated.

Fig. S4

(b)

Isolates	Mean $\pm$ SE ^a	Stick-to-touch ^c
	Uronic acid content ^b (μ g/mL)	
WT	0.23 $\pm$ 0.15	No
WT + pBAD-Gm	0.34 $\pm$ 0.06	No
WT + pBAD-GmETK _{WT}	0.66 $\pm$ 0.19	No
WT + pBAD-GmETK _{SSV}	3.69 $\pm$ 0.81 ^{**}	Yes
WT + pBAD-GmETK _{SRUV}	2.14 $\pm$ 0.34 [*]	Yes
WT + pBAD-GmETK _{SRV}	1.43 $\pm$ 0.28	No
WT + pBAD-GmETK _{SUMV}	4.07 $\pm$ 0.28 ^{***}	Yes
SUMV-Tn1	-0.01 $\pm$ 0.12	No
SUMV-Tn1 + pBAD-Gm	0.28 $\pm$ 0.26	No
SUMV-Tn1 + pBAD-GmETK _{SSV}	13.50 $\pm$ 1.18 ^{***}	Yes
SUMV-Tn1 + pBAD-GmETK _{SUMV}	5.92 $\pm$ 1.20 ^{***}	Yes
SUMV-Tn2	0.04 $\pm$ 0.23	No
SUMV-Tn2 + pBAD-Gm	-0.16 $\pm$ 0.12	No
SUMV-Tn2 + pBAD-GmETK _{SSV}	11.60 $\pm$ 0.27 ^{***}	Yes
SUMV-Tn2 + pBAD-GmETK _{SUMV}	3.64 $\pm$ 0.43 [*]	Yes

Fig. S4 (continued). Colony morphotypes and the quantification of EPS of the parental wild-type MG1 and Δetk strains of variant SUMV, complemented with different *etks*. Panels (a) illustrate the parental wild-type MG1 strain (i), wild-type harbouring pBAD-GmETK_{WT} (ii), wild-type harbouring pBAD-GmETK_{SSV} (iii), wild-type harbouring pBAD-GmETK_{SRUV} (iv), wild-type harbouring pBAD-GmETK_{SRV} (v), wild-type harbouring pBAD-GmETK_{SUMV} (vi), SUMV-Tn1 (Δetk strains of variant SUMV) (vii), SUMV-Tn1 harbouring pBAD-GmETK_{SUMV} (viii), SUMV-Tn1 harbouring pBAD-GmETK_{SUMV} (ix), SUMV-Tn2 (Δetk strains of variant SUMV) (x), SUMV-Tn2 harbouring pBAD-GmETK_{SUMV} (xi), and SUMV-Tn2 harbouring pBAD-GmETK_{SUMV} (xii). Bar, 2.5 mm. The amount of EPS produced by each strain is presented (b) and was quantified as described in Materials and Methods.^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.

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

Assay for protease activity

Isolates	Azo peptide measurements (OD _{440nm})						
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001
Replicate 1	0.947	0.922	0.877	0.842	0.988	0.297	0.914
Replicate 2	1.008	0.98	0.921	0.866	1.004	0.372	0.832
Replicate 3	0.947	0.922	0.877	0.842	0.988	0.297	0.914

Assay for siderophore production

Isolates	Siderophore production (units)						
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001
Replicate 1	1.78	2.07	2.00	1.89	1.57	1.50	1.79
Replicate 2	1.77	2.00	1.90	1.85	1.64	1.48	1.76
Replicate 3	1.79	1.96	1.89	1.89	1.50	1.44	1.77

Assay for hemolysin production

Isolates	Lyzed sheep erythrocytes measurement (OD _{545nm})						
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001
Replicate 1	2.998	3.075	3.501	3.108	1.012	3.233	3.523
Replicate 2	3.234	3.252	3.423	3.723	0.822	2.730	3.284
Replicate 3	3.052	3.723	3.103	3.123	1.423	2.434	3.011

Assay for attachment on hydrophilic surfaces

Isolates	Measurement of crystal violet stain biomass (OD _{545nm})							
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001	Blank
Replicate 1	0.065	0.084	0.060	0.062	0.059	0.055	0.066	0.058
Replicate 2	0.066	0.076	0.061	0.067	0.059	0.061	0.072	0.057
Replicate 3	0.062	0.074	0.061	0.064	0.058	0.055	0.073	0.056
Replicate 4	0.071	0.082	0.067	0.063	0.057	0.057	0.078	0.056

Assay for attachment on hydrophobic surfaces

Isolates	Measurement of crystal violet stain biomass (OD _{545nm})							
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001	Blank
Replicate 1	0.123	0.121	0.091	0.101	0.061	0.085	0.138	0.055
Replicate 2	0.139	0.123	0.100	0.109	0.061	0.084	0.139	0.053
Replicate 3	0.134	0.126	0.106	0.107	0.063	0.094	0.134	0.052
Replicate 4	0.142	0.108	0.119	0.114	0.065	0.086	0.124	0.089

223

Assay for biofilm formation on surfaces

Isolates	Measurement of crystal violet stain biomass (OD _{545nm})							
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001	Blank
Replicate 1	0.657	2.413	0.823	1.705	0.469	0.303	0.771	0.184
Replicate 2	0.757	2.47	0.85	1.694	0.486	0.327	0.777	0.172
Replicate 3	0.755	2.428	0.792	1.823	0.467	0.335	0.792	0.186

224

Assay for swimming motility

Isolates	Measurement of crystal violet stain biomass (OD _{545nm})						
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001
Replicate 1	50	42	37	39	29	25	50
Replicate 2	50	40	45	38	32	36	50
Replicate 3	48	31	29	30	27	29	50
Replicate 4	46	26	30	30	26	25	45
Replicate 5	50	32	37	40	18	13	42

225

Assay for swarming motility

Isolates	Measurement of the diameter of the swarming colony (mm)						
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001
Replicate 1	40	47	50	54	56	3	79
Replicate 2	46	90	54	70	51	4	32
Replicate 3	46	76	30	56	33	3	38
Replicate 4	81	68	46	57	36	4	60
Replicate 5	90	56	38	76	44	2	58

226

Number of *Acanthamoeba castellanii* present in the grazed biofilms of *Serratia marcescens* isolates

Isolates	No. of <i>A. castellanii</i> per well					
	WT	SSV	SRUV	SRV	SUMV	NSCV
Replicate 1	3.08E+05	2.91E+05	1.20E+04	2.96E+05	1.01E+04	3.07E+03
Replicate 2	2.97E+05	2.52E+05	1.59E+04	2.48E+05	8.38E+03	2.79E+03
Replicate 3	3.04E+05	2.90E+05	1.26E+04	2.56E+05	6.42E+03	4.75E+03
Replicate 4	3.09E+05	2.94E+05	1.56E+05	3.43E+05	1.17E+04	6.98E+03

227

228

Amount of biofilm present in the grazed biofilm of *S. marcescens* isolates

Isolates	OD _{545nm} of crystal violet stained biofilms					
	WT	SSV	SRUV	SRV	SUMV	NSCV
Replicate 1	1.0665	4.3565	0.7765	2.4815	2.7015	0.5165
Replicate 2	1.3465	3.9115	0.6315	3.0315	2.6315	0.5215
Replicate 3	1.3765	3.9265	0.5565	2.5915	2.5365	0.6465
Replicate 4	1.2365	4.3065	0.4715	1.7815	2.3565	0.3615

Data 2. Biofilm yield and abundance of biofilm-feeding *A. castellanii* grown in the presence of different *S. marcescens* variant genotypes.

Quantification of biofilms grown from the different *S. marcescens* variant genotypes

Isolates	OD _{545nm} of crystal violet stained biofilms					
	WT	SSV	SRUV	SRV	SUMV	NSCV
Replicate 1	2.45875	8.55875	1.14875	4.47375	2.96375	0.21375
Replicate 2	2.45875	7.20875	0.98875	4.24375	2.87875	0.29375
Replicate 3	2.36375	7.75875	1.16875	4.30875	2.67875	0.23875
Replicate 4	2.26375	7.40875	1.16875	4.28875	2.46875	0.20875

Number of *Acanthamoeba castellanii* present in the grazed biofilms of *S. marcescens* isolates

Isolates	No. of <i>A. castellanii</i> per well					
	WT	SSV	SRUV	SRV	SUMV	NSCV
Replicate 1	3.08E+05	2.91E+05	1.20E+04	2.96E+05	1.01E+04	3.07E+03
Replicate 2	2.97E+05	2.52E+05	1.59E+04	2.48E+05	8.38E+03	2.79E+03
Replicate 3	3.04E+05	2.90E+05	1.26E+04	2.56E+05	6.42E+03	4.75E+03
Replicate 4	3.09E+05	2.94E+05	1.56E+05	3.43E+05	1.17E+04	6.98E+03

Amount of biofilm present in the grazed biofilm of *S. marcescens* isolates

Isolates	OD _{545nm} of crystal violet stained biofilms					
	WT	SSV	SRUV	SRV	SUMV	NSCV
Replicate 1	1.0665	4.3565	0.7765	2.4815	2.7015	0.5165
Replicate 2	1.3465	3.9115	0.6315	3.0315	2.6315	0.5215
Replicate 3	1.3765	3.9265	0.5565	2.5915	2.5365	0.6465
Replicate 4	1.2365	4.3065	0.4715	1.7815	2.3565	0.3615

Data 3. Frequencies of the different *S. 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*.

Proportion of morphotypes of *S. marcescens* present in the planktonic fraction of the MultiVariant populations grown in the presence of *Tetrahymena pyriformis*

Morphotypes present	Non-grazed			Grazed		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
SSV	4.75E+07	3.25E+07	4.25E+07	0.00E+00	0.00E+00	0.00E+00
SRUV	2.88E+08	3.78E+08	2.95E+08	0.00E+00	0.00E+00	0.00E+00
SRV	1.98E+08	1.25E+08	1.78E+08	0.00E+00	0.00E+00	0.00E+00
SUMV	2.18E+08	2.00E+08	2.23E+08	1.88E+08	3.45E+08	1.53E+08
NSCV	1.85E+08	2.23E+08	3.68E+08	0.00E+00	2.50E+05	0.00E+00
Total Counts	9.35E+08	9.58E+08	1.11E+09	1.88E+08	3.48E+08	1.53E+08

Proportion of morphotypes of *S. marcescens* present in the planktonic fraction of the MultiVariant + WT populations grown in the presence of *Tetrahymena pyriformis*

Morphotypes present	Non-grazed			Grazed		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
SSV	3.00E+07	4.50E+07	6.25E+07	0.00E+00	0.00E+00	0.00E+00
SRUV	2.75E+08	2.23E+08	3.50E+08	0.00E+00	0.00E+00	0.00E+00
SRV	1.03E+08	1.53E+08	1.65E+08	0.00E+00	0.00E+00	0.00E+00
SUMV	2.35E+08	2.85E+08	3.65E+08	4.18E+08	4.35E+08	5.58E+08
NSCV	2.70E+08	2.78E+08	1.00E+08	0.00E+00	0.00E+00	0.00E+00
WT	3.75E+07	4.50E+07	4.00E+07	0.00E+00	0.00E+00	0.00E+00
Total Counts	9.13E+08	9.83E+08	1.04E+09	4.18E+08	4.35E+08	5.58E+08

Data 4. Quantification of tyrosine phosphorylation of the Etk and EPS uronic acid content of the *S. marcescens* isolates.

Level of phosphorylation of the tyrosine kinase, expressed as a percentage relative to the WT.

Isolates	pTyr/GroEL (%)		
	Replicate 1	Replicate 2	Replicate 3
WT	100.00	100.00	100.00
SSV	14.02	8.27	29.77
SRUV	0.00	0.00	0.00
SRV	83.55	185.57	154.59
SUMV	6.70	5.82	24.30
NSCV	29.40	49.34	59.79
BMG1001	76.11	83.35	120.80
SUMVTn01	0.00	0.00	0.00
SUMVTn02	0.00	0.00	0.00

Quantification of uronic acid content of different *S. marcescens* isolates

Isolates	Measurement of the uronic acid content (ug/mL)								
	WT	SSV	SRUV	SRV	SUMV	NSCV	BMG1001	SUMV-Tn1	SUMV-Tn2
Replicate 1	0.136	3.335	0.749	1.293	16.609	-0.340	0.000	-0.476	-0.408
Replicate 2	-0.204	3.131	1.021	0.749	16.133	-0.613	0.000	0.272	-0.953
Replicate 3	0.244	3.176	1.012	1.431	17.764	0.175	0.524	0.244	-0.035
Replicate 4	0.314	3.525	1.463	1.012	17.415	0.175	0.454	0.035	-0.035

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