Adaptive Divergence in Experimental Populations of *Pseudomonas fluorescens*.

V. Insight into the niche specialist ‘Fuzzy Spreader’ compels revision of the model 

_Pseudomonas Radiation._

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*Pseudomonas fluorescens* is a model for the study of adaptive radiation. When propagated in a spatially structured environment the bacterium rapidly diversifies into a range of niche specialist genotypes. Here we present a genetic dissection and phenotypic characterization of the “fuzzy spreader” (FS) morphotype — a type that arises repeatedly during the course of the *P. fluorescens* radiation and which appears to colonize the bottom of static broth microcosms. The causal mutation is located within gene *fuzY* (*pflu0478*) — the fourth gene of the five gene *fuzVWXYZ* operon. *fuzY* encodes a β-glycosyltransferase that is predicted to modify lipopolysaccharide (LPS) O-antigens. The effect of the mutation is to cause cell flocculation. Analysis of 92 independent FS genotypes showed each to have arisen as the result of a loss-of-function mutation in *fuzY*, although different mutations have subtly different phenotypic and fitness effects. Mutations within *fuzY* were previously shown to suppress the phenotype of mat-forming “wrinkly spreader” (WS) types. This prompted a re-investigation of FS niche preference. Time-lapse photography showed that FS colonizes the meniscus of broth microcosms forming cellular rafts that, being too flimsy to form a mat, collapse to the vial bottom, and then repeatably reform only to collapse. This led to a reassessment of the ecology of the *P. fluorescens* radiation. Finally, we show that ecological interactions between the three dominant emergent types (SM, WS and FS), combined with the interdependence of FS and WS on *fuzY* can, at least in part, underpin an evolutionary arms race with bacteriophage SBW25Φ2, to which mutation in *fuzY* confers resistance.
Adaptive radiation – the rapid emergence of phenotypic and ecological diversity within an expanding lineage – is among the most striking of evolutionary phenomena (Darwin 1859; Lack 1947; Dobzhansky 1951; Simpson 1953; Schluter 2000; Kassen 2009; Losos 2010). Fuelled by competition and facilitated by ecological opportunity, successive radiations have shaped much of life’s diversity. Of central importance are the phenotypic innovations that fashion the fit between organism and environment (Schluter 2000).

Understanding the nature of these innovations and the pathways by which they emerge and rise to prominence – often in parallel across estranged populations experiencing similar environments – is a central issue (Colosimo et al. 2005; Bantinaki et al. 2007; Conte et al. 2012). How mutational processes generate the variation presented to selection (McDonald et al. 2009; Braendle et al. 2010); how genetic architecture underpinning extant phenotypes determines the capacity of lineages to generate new and adaptive phenotypes (Poole et al. 2003; Wagner and Zhang 2011); and how ecological factors drive phenotypic divergence (Schluter 2009), are questions of seminal interest.

The relative simplicity of microbial systems, their capacity for rapid evolutionary change, and advances in technology that enable detailed genotypic traceability offer a unique opportunity to log moment-by-moment change in experimental populations, with the potential to contribute to understanding of how genotype shapes phenotype, how phenotype interplays with the environment, and how environment may shape genotype (Mortlock 1982; Lenski et al. 1998; Rainey et al. 2000; Buckling et al. 2009; Beaumont et al. 2009; Blount et al. 2012; Hindre et al. 2012). In the context of adaptive radiation, the bacterium Pseudomonas fluorescens SBW25 has served as a useful model (reviewed within MacLean 2005 and Kassen 2009). When propagated in a spatially structured environment, SBW25 rapidly diversifies into a range of niche specialist genotypes.

“Smooth” (SM) genotypes colonize the broth of static broth microcosms; “wrinkly spreader” (WS) genotypes colonize the air-liquid (AL) interface in the form of a self-supporting biofilm (or ‘mat’),
via the over-expression of an acetylated cellulose-like polymer (ACP); and “fuzzy spreaders” (FS) form large, spreading colonies and appear to occupy the microcosm floor (RAINEY and TRAVISANO 1998). These three dominant types and their variants are maintained over time in serially propagated microcosms by the virtue of negative frequency-dependent interactions: each genotype is fittest when rare and fallible when common.

Previous work has defined the underlying mutational bases of WS genotypes (SPIERS et al. 2002; GOYMER et al. 2006; BANTINAKI et al. 2007; McDONALD et al. 2009), identified a set of mutational pathways that evolution repeatedly follows to generate this adaptive type (RAINEY and RAINEY 2003), and explained why evolution follows a subset of all possible pathways (McDONALD et al. 2009). But what of ‘fuzzy spreader’? These morphotypes take longer to appear, but like WS, they arise repeatedly (RAINEY and TRAVISANO 1998). While WS is well understood, little is known about the ecology and genetics of FS. Here we describe the mutational causes of the FS phenotype, unravel its underlying architecture, delineate the genetic route from genotype to phenotype to fitness, and unravel the links between FS physiology and the causes of its ecological specialization.
MATERIALS AND METHODS

Bacterial strains, growth conditions, and manipulation: The ancestral (wild-type) “smooth” (SM) strain is *P. fluorescens* SBW25 (PBR368) that was isolated from the leaf of a sugar beet plant grown at the University Farm, Wytham, Oxford, in 1989 (RAINEY and BAILEY 1996). The niche-specialist archetypal “fuzzy spreader” (FS) genotype (PBR367) was derived from the ancestral genotype after 7 days of selection in a spatially structured microcosm (RAINEY and TRAVISANO 1998). The niche-specialist wrinkly spreader (WS) genotype (PBR366; *wspF*_{236insGACCGTC}) was derived from the ancestral genotype after 3 days of selection in a spatially structured microcosm (RAINEY and TRAVISANO 1998). The *awsX* mutant WS_T (PBR656; *awsX*_{Δ229-261}) and the *mwsR* mutant WS_Z (PBR662; *mwsR*_{G3055A}) were similarly derived (MCDONALD et al. 2009). SBW25-Tn7-*lacZ* (PBR760), FS-Tn7-*lacZ* (PBR761), SBW25-*fuzY*_{T443G}::Tn7-*lacZ* (PBR762), SBW25Δ*fuzY*::Tn7-*lacZ* (PBR763) and FS-*fuzY*_{wt}::Tn7-*lacZ* (PBR764) derivatives used in Figure 2 competition assays carry a chromosomal insertion of mini-Tn7-*lacZ* (CHOI et al. 2005). The strain FS-*lacZ* (PBR742) used in competition assays has a promoterless ‘*lacZ* inserted within the defective prophage locus of the chromosome (ZHANG and RAINEY 2007). Both the ‘*lacZ* and Tn7-*lacZ* markers are neutral with respect to fitness. A full list of strains and plasmids used in this study are given in Table S1. The 112 independent FS genotypes FS3–FS214b were obtained by propagating the ancestral SM genotype (PBR368) in 214 independent spatially structured microcosms (Table S2). Microcosms were diluted and plated onto KB agar after 5 days incubation and (except where otherwise indicated) a single FS genotype was selected at random from each microcosm. These genotypes were streaked for single colonies to check purity and then stored at -80°. In cases where two or more distinct fuzzy-like morphotypes were apparent, representatives of each genotype were selected and stored; these are indicated by ‘a’, ‘b’, etc. (Table S2).

*P. fluorescens* strains were cultured at 28° in King’s medium B [KB; (KING et al. 1954)]. *E. coli* strains were grown in LB at 37°. Antibiotics and supplements were used at the following
concentrations: ampicillin, 100 µg · ml⁻¹; cycloserine, 800 µg · ml⁻¹; gentamicin, 10 µg · ml⁻¹; kanamycin, 100 µg · ml⁻¹; spectinomycin, 100 µg · ml⁻¹; and tetracycline, 20 µg · ml⁻¹. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at a concentration of 60 µg · ml⁻¹. N-[5-nitro-2-furfurylidene]-1-arminutosohy-dantoin was used to counterselect E. coli (100 µg · ml⁻¹ in agar plates). Plasmid DNA was introduced to E. coli by transformation and P. fluorescens by transformation or conjugation, following standard procedures. The helper plasmid pRK2013 was used to facilitate conjugative transfer between E. coli and P. fluorescens (FIGURSKI and HELINSKI 1979).

Molecular biology techniques: Standard molecular biology techniques were used throughout (SAMBROOK et al. 1989). Gateway technology (vector pCR8; Invitrogen) was used to clone PCR fragments. The fidelity of all cloned fragments was checked by DNA sequencing (Macrogen Inc., South Korea). Oligonucleotide primers for allelic replacements and reconstructions were designed on the basis of the SBW25 genome sequence (SILBY et al. 2009).

Construction of deletion mutants and allelic replacements: A two-step allelic exchange strategy was used as described previously (RAINEY 1999; BANTINAKI et al. 2007). For the generation of deletion mutants, oligonucleotide primers were used to amplify ~1000 nucleotide regions flanking the gene(s) of interest. A third PCR reaction was used to SOE flanking sequences together (HORTON et al. 1989). The product was cloned into pCR8 (and sequenced to check for errors); the resulting fragment was excised using Bgl II or Spe I and introduced into the suicide vector pUIC3 (RAINEY 1999). Deletion constructs were mobilized into P. fluorescens by conjugation and allowed to recombine with the chromosome by homologous recombination. Recombinants from which pUIC3 had been lost were identified from LB agar plates after a brief period of nonselective growth in KB broth (KITTEN et al. 1998). To generate allelic replacements, PCR fragments (~3 kb) centered upon the allele of interest were cloned as above and introduced into pUIC3, and the
replacement was made by recombination. Deletion and allelic exchange mutants were confirmed by PCR; allelic exchange mutants were further checked by DNA sequencing.

Gene complementation: A DNA fragment encoding fuzY (pflu0478) flanked by Nde I and Bam HI restriction sites was amplified by PCR and ligated with pCR8. The resultant plasmid was purified from E. coli transformants and the integrity of the insert confirmed by Sanger sequencing (Macrogen Inc., South Korea). For expression in P. fluorescens, fuzY was excised from pCR8 and ligated between the Nde I and Bam HI sites of expression vector pSX encoding lacIq and an IPTG-inducible tac promoter (Owen and Ackerley 2011). P. fluorescens transformants were purified by streaking to single colonies and the presence of the desired construct was confirmed by PCR.

Fitness of genotypes: Competitive fitness of FS genotypes was determined by direct competition between each FS genotype and FS (PBR367), FS-lacZ (PBR742) or FS-Tn7-lacZ (PBR761). Competitive fitness of SM genotypes was determined by direct competition between each SM genotype and SM (PBR368), SBW25-lacZ (PBR743) or SBW25-Tn7-lacZ (PBR760). Figure 2 fitness assays comprised of six independent replicate contrasts using reciprocal strain marking as follows: in three replicate assays the reference strain (SBW25 or FS) was marked with mini-Tn7-lacZ, and in three replicate assays the reconstructed mutant strain was marked with mini-Tn7-lacZ, thus nullifying any effect of the marker on competition. Competitive fitness of WS genotypes was determined by direct competition between each WS genotype and WS mutant LSWS-Tn7-lacZ (wspFA901C; PBR741). All strains were grown overnight in shaken KB broth before introduction into spatially structured or unstructured microcosms (~10^5 cells of each competitor) at a ratio of 1:1. Relative fitness was calculated as the ratio of Malthusian parameters of the genotypes being compared (Lenski et al. 1991).
**Experimental evolution:** Cells from -80 °C glycerol stocks were streaked onto KB agar plates to produce isolated single colonies. Populations were founded by inoculation of 6 ml KB broth in glass microcosms with a single representative colony from these KB plates; replicate populations were derived from independent single colonies. Populations were incubated at 28° in structured (static) microcosms. Propagation of populations occurred at regular intervals (of duration stated in ‘Results’) by transfer of a mixed sample (6 µl) to a fresh microcosm. At the time of each transfer, samples were spread onto KB plates to screen for colonies (~500-1500) with a different morphology (for example, WS or FS or SM, as stated in ‘Results’). Representative colonies of new emergent types were streaked to single colonies on KB plates to confirm heritability and purity prior to propagation in liquid culture and storage at -80 °C.

**Sequencing and sequence analysis:** whole genome re-sequencing of the entire 6.7-megabase-pair genome of the archetypal FS isolate (PBR367) was achieved using Illumina Solexa technology. PE sequencing was performed in two channels for 76 cycles on an Illumina GAIIx with PE module. The data was analyzed using the Illumina OLB pipeline v1.8: the total cluster number was 58,919,811 with 38,328,187 PF clusters or 65%. Reads were aligned to the *P. fluorescens* SBW25 genome sequence (SILBY *et al.* 2009) using SOAP2; a single SNP (T443G) was identified after extracting all SNPs from regions covered by more than 10 sequence reads with a PHRED quality higher than 20 and where 95% of the aligned nucleotides agreed. The presence of the T443G SNP in FS and its absence in SBW25 was confirmed by Sanger sequencing (Macrogen Inc., South Korea). Analyses of regions upstream and downstream of *fuzY*, including the identification of orthologous genes in Pseudomonas species, were performed using Artemis 14.0.0 (RUTHERFORD *et al.* 2000) and the Generic Genome Browser (GBrowse) version 2.37 (STEIN *et al.* 2002) within the Pseudomonas Genome Database (WINSOR *et al.* 2009).
fuzY from 112 independently isolated FS isolates was amplified by PCR and sequenced by the Sanger method (Macrogen, South Korea). DNA sequences were analyzed using Sequencher 4.10.1 (Gene Codes Corporation) or Geneious 5.5.6 (Biomatters Ltd) software.

**Phage resistance assays:** Sensitivity of SBW25 mutants to bacteriophage SBW25Φ2 was determined by pipetting 5 µl droplets of diluted phage lysate onto lawns of bacterial cells overlaid onto KB plates. Bacterial overlays were prepared by mixing 200 µl of bacterial cells from an overnight culture with 3 ml of molten top agar (KB with 0.8% agar). Phage sensitivity was scored as the presence of clear zones or individual plaques on bacterial lawns after 24h incubation at 28°.

**Imaging:** *Niche preference in structured microcosms* - 6 ml KB broth in glass microcosms was inoculated with ~ 5 × 10^5 bacteria and incubated at 28° without shaking for 48 hours (unless otherwise indicated). Structured microcosms were photographed with a Canon PowerShot G7 camera.

*Movies of bacterial growth in structured microcosms over time were produced as follows* - 6 ml KB broth in glass microcosms was inoculated with ~ 5 × 10^5 bacteria and incubated at 28° without shaking. Bacterial growth was recorded by time-lapse photography using a Canon PowerShot G7 camera and PSRemote (Version 1.9.1) Camera Control software (Breeze Systems Ltd, UK): photographs were taken at 5 or 10 minute intervals over 24-72h periods and compiled into movies using Microsoft Movie Maker (Version 5.1).

*Colony morphology* - bacteria from a 10^6-fold dilution of an overnight KB culture were spread onto KB plates and incubated at 28° for 48 hours. Colony morphology was examined and photographed using a Zeiss Stemi 2000-C dissection microscope fitted with a Canon PowerShot A640 digital camera.
RESULTS

The ‘fuzzy spreader’ phenotype is caused by a single non-synonymous mutation in a putative LPS modification gene: The FS morphotype has proved intractable to standard genetic analysis. Repeated attempts to locate the causal loci by suppressor analysis have failed. We therefore sequenced the genome of an archetypal FS mutant (PBR367). Comparison of the FS genome with the SBW25 reference genome identified a single nucleotide polymorphism within pflu0478 (a T to G transversion at position 443), a gene predicted to encode a putative glycosyltransferase involved in modification of surface lipopolysaccharides (LPS). The SNP caused the substitution of valine for glycine at position 148 (Pflu0478V148G). pflu0478 is located in a predicted operon of five genes (pflu0475-pflu0479) that are likely transcribed as a single unit (Figure 1). These genes are hereafter designated as fuzVWXYZ for ‘fuzzy spreader locus’. The predicted functions of these gene products relate to LPS modification: fuzV (537073-538830) encodes a predicted carbamoyl transferase (NodU family), fuzW (538831-539727) encodes a hypothetical protein with a predicted acetyltransferase domain (NAT_SF superfamily, E-value: 2.31e-10) with significant similarity to the Mig-14 family of antimicrobial resistance proteins (E-value: 2.03e-150), fuzX (539697-541124) encodes a predicted deacetylase, fuzY (541184-542332) encodes a predicted β-glycosyltransferase (GT1 group), and fuzZ (542329-543210) encodes a predicted α-glycosyltransferase (GT2 group).

The fuz locus was previously identified in a suppressor analysis of WS types and shown to be required for expression of the WS phenotype (McDonald et al. 2009), indicating a genetic link between WS and FS phenotypes. A summary of the diguanylate cyclase (DGC)-encoding pathways underpinning the evolution of the WS phenotype is given in Figure S1.

Orthologues of fuzVWXYZ were identified across a range of Pseudomonas species (Figure 1). The structure of the 5’ end of the operon (fuzVWX) is highly conserved across all species, bar the more distantly related P. stutzeri. It is likely that the ancestral operon included an additional GT1-group glycosyltransferase between fuzV and fuzW. In contrast, the 3’ end of the operon is
more fluid: for most species, orthologues of \textit{fuzVWX} are coupled with an assortment of glycosyltransferases.

The region upstream of \textit{fuzV} is poorly conserved. Orthologues of the upstream genes are present in many pseudomonads but only in \textit{P. fluorescens} SBW25 and Pf0-1 is there juxtaposition of \textit{pflu0474} — encoding a short hypothetical protein of unknown function — and \textit{fuzV} (Figure 1); in most other species \textit{pflu0474} is linked to the permease component of a putative ABC-type multidrug transport system. This indicates that \textit{pflu0474} is not a component of the ‘fuzzy spreader’ operon.

\textbf{Loss of function mutations in gene \textit{fuzY} generate ‘fuzzy spreaders’}: To test whether the T443G substitution is alone sufficient to cause the FS phenotype, the mutation was reconstructed in a naïve SBW25 background. The resultant mutant — SBW25-\textit{fuzY}T443G (PBR744) — expressed a ‘fuzzy’ colony morphology on KB plates and exhibited the archetypal fuzzy spreader growth pattern within structured microcosms (Figure 2ABC). Similarly, reconstruction of the wild-type sequence in FS (FS-\textit{fuzY}\textsubscript{wt}, PBR752) generated a mutant with a smooth colony morphology that grew within the broth phase (Figure 2E). In order to determine whether the FS phenotype is a consequence of loss of function, \textit{fuzY} was cleanly deleted, taking care to retain the first codon of \textit{fuzZ}: SBW25\textDelta\textit{fuzY} (PBR745) also displayed the typical FS colony morphology and pattern of growth in structured microcosms (Figure 2D). Both SBW25-\textit{fuzY}T443G and SBW25\textDelta\textit{fuzY} were comparable to FS in fitness when competed against a marked FS derivative in both shaken (unstructured) and static (spatially structured) microcosms (Figure 2F & G).

If the FS phenotype is the simple consequence of a loss of function mutation in \textit{fuzY}, restoration of the ancestral phenotype should be achieved by expression of \textit{fuzY in trans}. Indeed, complementation of \textit{fuzY}T443G by over-expression of \textit{fuzY}\textsubscript{wt} from vector pSX (PBR753) resulted in restoration of the ancestral phenotype (Figure 2JK). Over-expression of \textit{fuzY}\textsubscript{wt} in ancestral SBW25 (PBR751) produced no discernible effect on either phenotype or growth (Figure 2HI).
A single genetic route confers the ‘fuzzy spreader’ phenotype through a diverse range of fuzY mutations: To determine whether mutation of fuzY is the sole cause of all ‘fuzzy’ morphotypes we isolated 112 FS and FS-like morphotypes from independent spatially structured microcosms and classified them on the basis of colony morphology. By visual inspection, two distinct classes of spreading colonies, ‘fuzzy’ in appearance, were discernible. ‘Typical’ fuzzy spreaders (FS; 88 isolates) were rough-textured and opaque in appearance, with irregular colony margins. ‘Atypical’ fuzzy spreaders (aFS; 24 isolates) were of a similar form, but differed from the ‘typical’ class in colony opacity and/or texture. Similarly, an inspection of niche occupancy revealed differences between the classes: FS types failed to grow within the broth phase, appearing as a ‘volcano-like’ biomass on the microcosm floor (as in Figure 2BC). In contrast, aFS types grew within the broth phase with many producing weak mats.

For each of the 112 isolates, we sequenced fuzY: 92 isolates harbored mutations; of these, 88 were of the ‘typical’ class, and 4 were of the aFS class. The remaining 20 genetically uncharacterized isolates were of the aFS class and phenotypically distinct from FS. These could be further partitioned into two groups on the basis of their susceptibility to bacteriophage SBW25Φ2 (Table S2). Thus, aFS aside, the mutational basis of the FS phenotype appears to be simple, with a diverse range of mutations from SNPs causing non-synonymous substitutions or nonsense mutations, to insertions and deletions (INDELs) of various lengths causing frame-shifts throughout the length of the gene (Table 1, Table S2).

Of 92 independent fuzY causal mutations, 54 were SNPs at 23 unique positions (Table 1). Of the 23 unique SNPs, 10 were transitions (predominantly GC > AT) and 13 were transversions (predominantly GC > TA and GC > CG); 16 were missense mutations and 7 were nonsense. Interestingly, more than a quarter of all the independently isolated fuzzy spreaders were caused by an identical SNP — the same T443G transversion carried by the re-sequenced ‘archetypal’ isolate — causing valine to be substituted for glycine at position 148 in the protein (V148G). Similar
single-site mutation biases have been reported elsewhere and explained by replication template
switching within imperfect repeat (quasipalindromic) sequences during DNA replication (De Boer
and Ripley 1984; Mo et al. 1991; Demarini et al. 1998; Viswanathan et al. 2000; Lovett
2004). A comparison of the DNA sequence surrounding the T443G ‘hotspot’ with nearby
sequences failed to identify any imperfect palindromic matches likely to template a T>G
substitution at position 443.

Of the 92 mutations, 28 were small INDELs at 27 unique positions. The majority of these
can be explained by nascent strand misalignment (slipped-strand mispairing, Levinson and
Gutman 1987) within monotonic nucleotide ‘runs’ (14 of 28), by duplication of adjacent sequence
(2 of 28), or by deletion of a directly repeated sequence (2 of 28). How the remaining 12 small
INDELs were created is not clear but, notably, one mutant (FS126a; Table S2) had acquired a
single bp insertion producing a perfect 5 bp inverted repeat of the adjacent sequence, potentially a
consequence of intramolecular template switching (Lovett 2004). Five of 92 mutants were caused
by large deletions within fuzY [Δ629-760, Δ775-896, Δ862-885, and Δ882-947 (which occurred
twice)]; two of 92 mutants had deletions extending from the 3’ end of fuzY into fuzZ; and one
mutant had a deletion extending from the 3’ end of fuzW to the 5’ half of fuzZ. The remaining
mutant was caused by the insertion of a transposon encoded elsewhere in the genome.

Typical FS isolates are indistinguishable from each other in terms of colony morphology;
however, subtle differences in phenotype are conferred by different mutations within fuzY. When
the independent FS isolates were screened for niche preference in spatially structured microcosms
most isolates were indistinguishable from the archetypal FS (fuzYT443G; PBR367). However, a
subset of seven FS isolates exhibited a small amount of growth within the broth phase in addition to
producing biomass on the microcosm floor. The mutations responsible for these ‘subtle’ isolates
(sFS) were located in two or three distinct regions of the gene: codons 90-144, codon 221, and
codon 271 — four were missense substitutions and three were insertions of 1, 8 and 126
nucleotides, respectively. Three of the four aFS isolates with mutations within fuzY carried
missense substitutions — two located close together (A316E and G318D) and a third (K213R) located nearby sFS mutant A221E (Table S2). The fourth aFS mutant had a large deletion extending from *fuzW* into *fuzZ*.

To further explore the possible phenotypic differences among *fuzY* mutants, we carried out fitness assays on a selection of independent FS mutations (Table 2), including the seven sFS genotypes and three of the aFS genotypes. Small fitness differences were detected between some pairs of *fuzY* mutants when competed in spatially structured microcosms; however, the variance between replicates — attributable to the tendency of the competitors to diversify into a range of niche specialists over the 3-day period of the assay — was large (Figure 3A). Competition assays performed in unstructured microcosms, by way of a comparison, revealed a greater number of statistically significant fitness differences (Figure 3B). In general, mutants differing in fitness to archetypal FS generally corresponded to those with sFS or aFS phenotypes. However, fitness measured in unstructured microcosms mapped poorly to that measured in structured microcosms. Surprisingly, we observed significant differences in the ability of some fuzzy isolates to diversify (predominantly into a range of WS types) during static incubation (Figure 3C); this subset of rapidly diversifying types belong exclusively to the aFS and sFS classes and in some instances these correspond to those with significantly different fitness as measured under shaken incubation (Figure 3B). When fitness and diversity measures were pooled by mutant class (FS vs sFS vs aFS), the trends were marked: for competitions in structured microcosms, aFS and sFS were equally fit and significantly fitter than FS (Figure 3D). For competitions in unstructured microcosms, aFS were significantly fitter than sFS, which were significantly fitter than FS (Figure 3E). Similarly, when the propensity of isolates to diversify was compared by mutant class, the sFS and aFS groups diversified to a significantly greater extent than the FS group (Figure 3F).

Taken together, the data suggest that although the majority of mutations within *fuzY* confer phenotypically indistinguishable ‘fuzzy’ morphologies – most likely the consequence of loss-of-function of the enzyme – some *fuzY* mutations confer a more subtle phenotype. It is likely that the
aFS and sFS groups express partially functional FuzY enzymes and consequently display cell surface properties intermediate to that of FS and SM. The greater propensity of aFS and sFS types to diversify in structured microcosms is likely attributable to the fact that the gene retains some functionality and that this functionality is required for expression of other niche specialist types, in particular WS.

Genes *fuzVWXYZ* encode phenotypically related functions, contributing to the modification of cell surface structures: Having mapped the full spectrum of FS mutations exclusively to *fuzY*, we sought to determine the contribution of flanking genes. Deletion of the entire operon (*fuzVWXYZ*) generated a small-colony variant (Table 3, Figure 4). With the exception of *fuzX*, all single gene deletion mutants gave rise to colonies with distinct phenotypes (Table 3, Figure 4); however, only deletion of *fuzY* generated the FS phenotype. Notable was the *fuzW* mutant, which reproducibly generated colonies of both ‘rough’ (PBR746-R) and ‘smooth’ morphotypes (PBR746-S) (Figure 4).

Revision of the FS niche preference: The explanatory model for the maintenance of niche specialist types arising during the course of the Pseudomonas radiation involves negative frequency-dependent interactions between SM, WS and FS arising from the emergence of tradeoffs due to diversifying selection. This model is based on data from reciprocal “invasion from rare” experiments combined with observation of the niche preference of the three dominant types: SM colonizes the broth phase, WS the AL-interface and FS, the microcosm floor (RAINEY and TRAVISANO 1998). While the existence of frequency-dependent interactions among the primary types is firmly established, there is reason to re-examine the niche preference of FS. This stems from the fact that oxygen is removed from the broth phase by the founding genotype within the first 12 hours of growth (B. IBELINGS and P. B. RAINEY unpublished; KOZA et al. 2010) leading to the seemingly contradictory observation that FS, evolved from the obligate aerobe SBW25, apparently grows in an environment with no oxygen. It is difficult to imagine how LPS modification might
confer the property of anaerobic growth upon FS. To further investigate the connection between genotype and phenotype we revisited the question of FS niche occupancy. Structured microcosms were inoculated with FS cells and their growth recorded by time-lapse photography (File S1). As expected, no substantial growth of FS took place in the broth phase, unlike that observed in microcosms seeded with ancestral SBW25 (File S2). Instead, the first visually discernible growth took place at the meniscus, with small rafts of cells observed to propagate both inwards towards the center of the AL-interface, and downwards in a stalactite-like fashion (File S1). As these rafts increase in size, they fall from the interface to the floor of the microcosm as snowflakes, but repeatedly reform. No substantial biofilm ever forms at the surface, but growth clearly occurs. Thus FS, like WS, specializes in colonizing the AL-interface, albeit by a distinctively different strategy. Rather than forming a mat via the production of adhesive polymers (SPIERS et al. 2002; 2003), FS forms transient rafts due to changes in a cell surface component.

To explore the basis of the fuzY-dependent raft-forming behavior the flocculating ability of FS cells was examined by growing cells for 3 days in structured microcosms (SM and WS were included as controls). Cells were then dispersed into suspension by vortex mixing and incubated statically for a further 24 hours, with the addition of tetracycline to prevent further growth. Time-lapse photography revealed rapid flocculation of FS cells with an almost complete clearing of the broth phase within 12 hours (File S3). In contrast, SBW25 and WS cells remained largely in suspension. From this we conclude that clumping is mediated by cell-cell attraction and is not a consequence of failure of mother and daughter cells to disperse following cell division. That flocculation requires specific interactions between FS cells is supported by the observation that the ancestral (SM) genotype does not flocculate and remains in suspension when mixed 1:1 with FS in statically incubated microcosms (data not shown).

Although FS grows poorly in the broth phase of structured microcosms, growth sometimes occurs after several days incubation. As SBW25 is known to modify its growth environment with a resultant increase in pH over time (Table 4B), we speculated that the tendency for FS to form flocs
might be pH-dependent. To test the effect of pH on growth and flocculation, we recorded (again by
time-lapse photography) the growth of FS across a range of pHs from 6.5-8.5 (File S4). At pH 8
and above, growth occurred predominantly in the broth phase. At pH 7.5, growth occurred initially
at the AL-interface, with rafts of cells falling to the microcosm floor, but after 24 hours the broth
phase began to cloud with growth. Broth phase growth was less marked at pH 7 and did not occur
at pH 6.5. The pH-dependent differences in FS growth were not due to an effect of pH on growth
rate: populations were titered at 72 h and cell density (as cfu.ml$^{-1}$) did not differ between treatments
(Table 4A). Similarly, post-growth flocculation of dispersed cells occurred only at low pH (File
S5). From this we conclude that the failure of FS to grow freely in the broth phase of structured
microcosms is a consequence of flocculation following cell separation and that the particular cell
surface characteristics that promote flocculation are charge-mediated.

**fuzY mutation in WS results in a compromised ability to form mats:** The $fuzVWXYZ$ locus was
previously identified as a suppressor of the WS phenotype in the LSWS (large spreading wrinkly
spreader; $wspF_{A901C}$) and AWS (alternate wrinkly spreader; $awsX_{A100-138}$) mutants (MCDONALD et
al. 2009; Figure S1), suggesting a genetic link between the WS and FS phenotypes. To explore
this further, $fuzYT_{443G}$ and $ΔfuzY$ mutations were reconstructed in a $wspF_{236insGACCGTC}$ background
(WS; PBR366). The resulting mutants were compromised in their ability to form substantive mats
at the AL-interface: WS-$fuzYT_{443G}$ (PBR754) and WS$ΔfuzY$ (PBR755) mutant colonies show a
decreased wrinkled appearance on KB plates and produce feeble mats (fWS) that readily collapse to
the microcosm floor (Figure 5B). Comparison of mat growth over time revealed striking
differences between WS and WS-$fuzYT_{443G}$: WS mat formation initiates with the appearance of cell
aggregates at the AL-interface, which in time grow together to form a thin but robust film,
interspersed with denser aggregates of rope-like growth (File S6). In contrast WS-$fuzYT_{443G}$, while
also initiating growth at the AL-interface, forms ‘rafts’, many of which fall to the microcosm floor.
Over time these rafts expand to form a dense mat of surface growth, this, however, being unstable
with the periodic appearance of ‘holes’ as biomass drops to the microcosm floor. No networks of rope-like structures were observed. Mat strength was measured by the glass bead assay (RAINEY and RAINEY 2003). Whereas 3-day-old WS mats were able to support an average of 0.24 ± 0.07 g of two-millimeter glass beads (0.01 g) before collapsing, WS-ΔfuzY mats were unable to support even a single bead. Wild-type mat formation and wrinkled colony morphology was restored to WS-ΔfuzY by over-expression of fuzY<sub>wt</sub> from pSX (PBR756; Figure 5FG). As with SBW25, over-expression of fuzY<sub>wt</sub> in WS (PBR757) produced no discernible effect on either phenotype or growth or the ability to support beads (data not shown).

In contrast to FS, the genetic routes to WS are many and varied, with mutation within three distinct diguanylate cyclase-containing pathways (Wsp, Aws and Mws) conferring cellulose overproduction and a mat-forming phenotype (McDONALD et al. 2009; Figure S1). However, deletion of fuzY had the same phenotypic effect on each of three distinct WS genotypes (PBR755, PBR758, PBR759; data not shown). Thus, the putative LPS defect caused by loss of FuzY function has consequences for the downstream effect of the WS mutations, most likely the secretion and arrangement of acetylated cellulose polymers, rather than a specific interaction with the particular genes or protein components encoded by the wsp operon (SPIERS and RAINEY 2005).

**fuzY<sub>T443G</sub> confers resistance to SBW25Φ2:** A previously noted feature of ‘fuzzy spreader’ genotypes is their resistance to the lytic bacteriophage SBW25Φ2. Droplets of phage suspension produce clear zones on lawns of SBW25 but no clearing is observed on lawns of FS (Figure 6). All typical FS isolates, including the sFS subclass and SBW25ΔfuzY, are phage resistant (Table S2). Interestingly, the three broth-dwelling aFS isolates with mutations within fuzY are also phage resistant. We conclude that all (known) mutations in fuzY confer resistance to phage, regardless of any other effects of the mutations on niche preference. Single deletions of genes fuzVWX and fuzZ (but not fuzX) also conferred resistance to SBW25Φ2 (Table 3). ‘Rough’ fuzW mutants (PBR746-R) are phage resistant whereas ‘smooth’ fuzW mutants (PBR746-S) are phage sensitive.
Location of the FS causal mutation to a gene product predicted to modify LPS allows us to speculate upon the Φ2 receptor: LPS serves as a receptor for a broad range of bacteriophage in many bacterial species (for examples see MICHEL et al. 2010; FILIPPOV et al. 2011; GARBE et al. 2011; SHIN et al. 2012). It appears likely that phage resistance in FS is conferred by alterations in LPS structure. SBW25Φ2 proteins involved in phage attachment and entry are most closely related to P. aeruginosa-specific ΦKMV-like phages of the T7 supergroup, including ΦLKA1 (BLASTp, data not shown). Notably, ΦLKA1 infection requires expression of algC (CEYSSENS et al. 2011) — a gene encoding phosphoglucomutase, required for the synthesis of a complete lipopolysaccharide core (COYNE et al. 1994).

**An evolutionary arms race at the FS locus:** The fact that fuzY mutations confer resistance to bacteriophage SBW25Φ2 but have deleterious pleiotropic consequences for subsequent evolution of WS — the superior competitor at the AL-interface (RAINEY and TRAVISANO 1998) — raises the possibility that a tradeoff between phage resistance, and FS versus WS strategies to colonize the AL-interface might underlie — at least in part — the previously reported arms race between P. fluorescens SBW25 populations and SBW25Φ2 (BUCKLING and RAINEY 2002a). Such a possibility can be envisaged as follows: in the absence of phage the ancestral SM genotype rapidly diversifies into numerous niche specialist types, including WS and FS. Presence of phage selects for phage resistant genotypes, in particular, FS morphotypes, while at the same time selecting against phage sensitive genotypes such as WS. As FS types increase in frequency the phage titer — under a serial transfer regime — declines. Once at low density, opportunity exists for the competitively superior WS types to re-emerge — either from pre-existing sensitive types, or de novo, by mutation, from the dominant FS population. However, for WS to evolve from FS, a mutation that reverts (or compensates for) the fuzY mutation is required. Given that FS and WS share dependence on a functional fuz operon, it is possible that the bacterial response to the presence of phage may involve continual cycling between phage sensitive WS and phage resistant FS, effected by mutational
changes at the \textit{fuzY} locus.

We explored this possibility by asking whether WS genotypes can evolve from FS and, if they do, whether the FS-derived WS are phage sensitive. Fifty-six independent FS mutants representative of the range of \textit{fuzY} mutational types were propagated for three-day periods by serial dilution in structured microcosms until the emergence of colony morphologies resembling WS types (Table S3). All (bar three) FS genotypes gave rise to WS types within four serial transfers (with WS emergence most commonly requiring just one or two successive transfers to detect). However, the majority of the derived types produced feeble mats (fWS), indicative of the fact that the \textit{fuzY} mutation had neither been reversed, nor compensated for, by a change restoring functionality to the \textit{fuz} locus. Consistent with this thesis, most weak fWS types remained phage resistant (Table S3). However, strong mat-forming WS-derivatives (typical of regular WS mats) did arise from a number of FS isolates and repeatedly arose from two FS isolates in particular: FS108 (\textit{fuzY}_{432insCCTGGCGG}) and FS156a (\textit{fuzY}_{C1096T}) (Table S2). These derived WS types were phage-sensitive. A more extensive collection of FS-derived WS genotypes was selected from 12 replicate populations of each of FS108 and FS156a after five days growth in structured microcosms (data not shown). Sequencing of \textit{fuzY} from 22 phage-sensitive FS108-derived WS isolates revealed a reversion to the wild-type sequence (by retraction of an eight bp duplication of adjacent sequence at bp 432) in every case. Sequencing of \textit{fuzY} from 17 phage-sensitive FS156a-derived WS isolates revealed a reversion of C1096T to the wild-type T1096C in seven cases (restoring a Gln codon from an amber stop), and an additional SNP (A1097G) in ten cases (creating a Trp codon from an amber stop). Taken together, these data demonstrate that while fWS are more likely to evolve from FS (probably by a single mutation), true WS do emerge and are most likely the consequence of two mutations: the first reverting the \textit{fuzY} gene to wild type (and thus generating the SM morph) followed by a second mutation – in either the \textit{wsp}, \textit{aws}, or \textit{mws}-determined pathways (MCDONALD \textit{et al.} 2009) – to give WS. The extent to which this is possible is likely to depend on the nature of the FS-causing \textit{fuzY} mutation. Certain \textit{fuzY} mutations, by virtue of their genetic context (for
example, those involving expansion or contraction of repeated sequence) are more likely to revert than others. When reversion of \textit{fuzY} occurs, phage sensitive WS are inevitable and are likely competitively superior to phage resistant fWS: The relative fitness of fWS (WS-\textit{fuzY}_{1443G}) in three-day structured microcosms was just 0.80 ± 0.06 whereas the relative fitness of WS was 0.97 ± 0.05 \((t\) test: \(P < 0.0001, n=8\)). The strong mat-forming WS is substantially fitter than weak mat-forming WS-\textit{fuzY}_{1443G} and the major component of the differential fitness is the ability to exploit the AL-interface when competed in a structured environment. Thus, given sufficient generational time, it is predicted that phage-sensitive WS types will derive from FS progenitors in spatially structured environments once the dominance of FS genotypes has driven SBW25\text{\texttrademark}2 to near extinction.

An on-going arms race requires that phage sensitive WS can generate phage resistant FS by mutation. From knowledge of the underlying genetics this is highly probable, but is likely to require two mutations: one to revert the WS to SM and a second to generate FS. From twelve replicate populations of each of three distinct FS-derived WS genotypes propagated for two successive three-day periods and three successive seven-day periods by serial dilution in structured microcosms, 23 produced FS at detectable frequencies, with earlier emergence of ‘step one’ SM and/or fWS types as predicted (Table S4). All of the derived FS types were phage resistant. Thus, the plausibility of a continuing arms race with changes wrought at the \textit{fuzY} locus is demonstrated.
DISCUSSION

How mutational processes create the molecular variation upon which natural selection acts is a question readily addressed by experimental evolution (Beaumont et al. 2009; Blount et al. 2012). Ultimately, we seek to complete a comprehensive account of the common evolutionary trajectories during *P. fluorescens* SBW25 adaptive radiation, understand the underlying genetic architecture, mutational processes and targets, and the emergent ecological interactions that drive adaptive evolution. Here, we have described the genetic and ecological bases of FS fitness. Moreover, our genetic analyses have led to a reassessment of the niche preference of FS and the ecological processes underpinning the Pseudomonas radiation.

The FS mutational spectrum: Previous work has catalogued the underlying mutational bases of WS genotypes and identified the number of mutational routes to the WS phenotype (Spiers et al. 2002; Goymer et al. 2006; Bantinaki et al. 2007; McDonald et al. 2009). While a multitude of pathways lead to WS, the genetic underpinnings of FS are much simpler. Nevertheless, our catalogue of FS mutants provides useful information about the mutational processes that generate the diversity upon which natural selection acts. The spectrum of FS causal mutations is highly diverse — encompassing SNPs causing both missense and nonsense mutations, small INDELs causing frame-shifts, and both small and large deletions — with mutations spaced throughout the length of the gene. A number of studies, both experimental and comparative, have reported mutational spectra across genes and genomes in bacterial lineages experiencing hard or soft selection (Miller 1985; Leong et al. 1986; Mo et al. 1991; Garibyan et al. 2003; and reviewed in Ochman 2003; Wolff et al. 2004). More recently, experimental evolution studies, long-term experiments in particular, have provided the material for cataloguing mutational spectra — both neutral and those subject to positive selection — across whole genomes (Wielgoss et al. 2011; Lee et al. 2012). These studies reveal commonalities (such as a general two-fold bias for transitions...
over transversions, in particular the G:C>A:T substitution) but also idiosyncrasies. Indeed it can be difficult to compare mutational spectra between species, and even between genes within the same species, since mutation rates and classes are affected by gene-specific parameters such as distance of the gene from the replication origin, the direction of transcription relative to the passage of a replication fork, and the level of transcription (reviewed by OCHMAN 2003; JUURIK et al. 2012).

Various studies have reported mutational spectra for single genes encoding proteins constrained by functions essential for viability (GARIBYAN et al. 2003; WOLFF et al. 2004) making it difficult to draw conclusions about the relative occurrence SNPS and INDELS and the six classes of substitution. Though it may be difficult, perhaps impossible, to infer how the mutations sampled in this study might compare with the full spectrum of *fuzY* mutations occurring through time in each evolving population, the mutations we sampled (at least those of the ‘typical’ FS class) did not differ markedly in their relative fitness, indicating a phenotypic equivalence. Knowing that all confer a similar phenotype, it is possible to argue that the spectrum of sampled mutations is likely to be largely representative of the broader pool of those occurring in *fuzY* sequence space.

Considering unique SNPs only, the TsTv ratio in our study was 1:1.3. Considering the transitions, the distribution of AT>GC and GC>AT substitutions (after correcting for GC content) did not differ from that expected by chance ($\chi^2 = 1.517, P = 0.2180$, d.f. = 1, chi-square test). Similarly, the relative frequencies of the four possible transversions did not differ from that expected ($\chi^2 = 2.000, P = 0.5724$, d.f. = 3, chi-square test).

Curious, is the finding that a quarter of all the independently isolated fuzzy spreaders were caused by an identical SNP — the same T443G transversion carried by the resequenced ‘archetypal’ isolate — causing valine to be substituted for glycine at position 148 (V148G). Fitness assays performed under conditions similar to those in which our independent FS isolates evolved show that this mutation does not confer a fitness benefit substantially greater than any other FS mutation; indeed, mathematical models conclude that the differential fitness necessary to account for the preferential selection of T443 is beyond biological reality (E. LIBBY unpublished). The more
frequent occurrence of this mutation suggests that it has an increased probability of being generated, likely as a consequence of the local DNA sequence or secondary structure. A comparison of the DNA sequence surrounding the T443G ‘hotspot’ with nearby sequences failed to identify any imperfect palindromic matches likely to template a T > G substitution at position 443, thus replication template switching within imperfect repeat (quasipalindromic) sequences during passage of a replication fork is not obviously behind the generation of T443G (DE BOER and RIPLEY 1984; MO et al. 1991; DEMARINI et al. 1998; VISWANATHAN et al. 2000; LOVETT 2004).

Ecological interactions underpinning the Pseudomonas radiation: How genetic diversity translates into phenotypic diversity and how phenotypic diversity in turn drives the interactions between species and their environment is of considerable interest. The interplay of predominant emergent types during *P. fluorescens* SBW25 radiations has been extensively described (RAINEY and TRAVISANO 1998; RAINEY and RAINEY 2003; RAINEY 2005). However, closer inspection of FS growth within spatially structured microcosms – motivated initially by the overlap of the genetic architecture underpinning both FS and WS – has led to a significant revision of our understanding of the ecology of this particular morphotype: previously designated a bottom-dweller, FS innocula observed over time initially begin growth at the AL-interface, with flocs of cells attempting to spread as a biofilm but ultimately falling to the microcosm floor. This pattern of growth indicates that FS competes with WS for occupancy of the AL-interface, which fits with the fact that oxygen is the primary limiting resource driving the Pseudomonas radiation. FS performs poorly in competition with WS (RAINEY and TRAVISANO 1998; RAINEY 2005); however, it repeatedly arises and increases in frequency whenever spatially structured microcosms are seeded with the ancestral type. Moreover, that aFS and sFS types repeatedly arise in parallel adaptive radiations implies an ecological complexity within the simple structured broth microcosm that remains to be fully described. Our working model is as follows: growth of the ancestral SM genotype rapidly depletes the environment of oxygen, which establishes conditions that favor evolution of mat-forming WS
types. WS mats dominate for several days before collapsing (RAINEY and RAINEY 2003). Mat collapse opens a niche for FS types, which exploit this opportunity via the formation of transient rafts that exist for sufficient time to enable FS cells to access oxygen that is otherwise absent from the microcosm. Interestingly, SM types, being unable to adhere to FS types, cannot hitchhike with FS and are thus unable to take advantage of FS rafts in the same way that they can take advantage of the WS mats. This accounts for the previously noted inhibitory effect of FS on SM (RAINEY 2005): growth of FS takes oxygen from the uppermost layer of broth thus depriving SM of this limiting resource. SM growth is subsequently compromised (RAINEY 2005). Eventually WS types reinvade and perpetuate the cycle. Diversity is maintained by time-lagged frequency-dependent selection fueled by the fallibility of the surface-colonizing strategies of both WS and FS.

**The effect of fuzY mutation on WS phenotype:** The genetic link between FS and WS — the two key emergent types whose negative frequency-dependent interactions drive the dynamics within diversifying structured microcosms — is an unanticipated outcome of these studies. The fuzVWXYZ locus was previously identified as a suppressor of the LSWS (WspF_A901C) and AWS (awsX_A100-138) -based WS morphotypes (McDONALD et al. 2009), an association that was reconfirmed here. WS-fuzY_T443G and WSDfuzY mutant colonies showed a decreased wrinkled appearance on KB and produced mats that collapsed easily to the microcosm floor.

The role of LPS as a major component of WS biofilm strength and integrity, as a consequence of interactions between LPS and the cellulose matrix of the biofilm, was revealed in an earlier study (SPIERS and RAINEY 2005) and is well supported (LAU et al. 2009; NIELSEN et al. 2011; NILSSON et al. 2011). Alteration of the bacterial cell surface, with consequent changes to cell hydrophobicity, adhesive properties, and motility, likely perturbs the cell-cell interactions that maintain biofilm structure. The relationship between LPS defects and the adhesive, cohesive, and viscoelastic properties of biofilms and how these properties correlate with biofilm structure has been extensively characterized in *P. aeruginosa* PAO1 (LAU et al. 2009). Essentially, more
dramatic LPS defects (such as full truncation of O-antigen) result in stronger adhesion of cells to
glass and other abiotic surfaces and stronger cell-cell interactions (evident by flocculation).
Furthermore, mutants lacking O-antigen produce biofilms with weaker viscoelastic properties.
Recent studies with *P. putida* KT2440 EPS mutants support a model in which LPS mediates the
necessary cell-cell interactions that maintain biofilm matrix stability following attachment
(NILSSON *et al.* 2011), with EPS in a stabilizing role and cellulose polymers providing additional
structural support (NIELSEN *et al.* 2011; NILSSON *et al.* 2011).

**An evolutionary arms race due to tradeoffs in niche specialization:** Phages are important
determinants of microbial evolution and population structure (reviewed in STERN and SOREK 2010;
KOONIN and WOLF 2012). Their success in the face of asymmetry in the coevolutionary
potential of phages, relative to their more complex bacterial hosts, raises questions as to their
persistence (LENSKI 1984). Various explanations have been put forward: one is that phages and
their hosts participate in an on-going arms race mediated by endless cycles of bacterial mutations to
phage resistance and phage counter defenses (RODIN and RATNER 1983a; b). Evidence consistent
with this comes from experiments with SBW25 and SBW25Φ2 (BUCKLING and RAINEY 2002a; b;
BROCKHURST *et al.* 2004; 2006; BENMAYOR *et al.* 2008; PATERSON *et al.* 2010; GOMEZ and
BUCKLING 2011) and with other bacterial systems (for examples see KASHIWAGI and YOMO 2011;
DENNEHY 2012; SEED *et al.* 2013; LEVIN *et al.* 2013). An alternative explanation posits that phages
may persist if there exists a mixture of sensitive and resistant hosts where the resistant types are less
fit in competition for resources than the sensitive types (LEVIN *et al.* 1977). Such a scenario stands
to allow phage persistence if resistant bacteria revert to sensitivity and in so doing restore some
capacity that is lost by the resistance-generating mutation (LENSKI 1984). Such mutations would be
likely once phages are rare, thus allowing phage re-invasion. This scenario appears to be relevant
to our findings – at least in part – thus providing potential for persistent co-evolution. Phage-
sensitive WS are superior colonizers of the air-liquid interface, however, in the presence of phages
their success is compromised: FS instead dominate. However, as phage titers decline (with declining availability of WS hosts) there exists opportunity for mutants that revert to phage-sensitive, competitively superior, WS types. We show that this requires two mutations; nonetheless, such a possibility clearly exists.

The necessity of phage resistance in an environment where phages are abundant entails a trade-off against the optimal strategy for bacterial colonization: in the case of our simple experimental system, phage resistance is traded-off against the ability to generate stable biofilms enabling optimal access to O\textsubscript{2} for growth; in native environments, phage resistance is predicted to trade off against strategies for the establishment of infection and colonization of host organisms — strategies typically mediated by bacterial surface structures. Indeed, O-antigen is essential for the establishment of infection (or protection against host defenses) in a diverse array of plant and animal pathogens (Cava \textit{et al.} 1989; Dekkers \textit{et al.} 1998; Yang \textit{et al.} 2000; Kannenberg and Carlson 2001; Poon \textit{et al.} 2008; Kesarwat \textit{et al.} 2009); moreover, the O-antigen serves as a receptor for phages (see Michel \textit{et al.} 2010; Filippov \textit{et al.} 2011; Garbe \textit{et al.} 2011; Shin \textit{et al.} 2012). Thus it would appear that O-antigens are subject to two conflicting selective pressures: pressure to change to avoid phage predation and pressure to be maintained to enable colonization of host species during infection. Such a tradeoff may be widely relevant to persistence of phage and their bacterial hosts.

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TABLE 1.

Spectrum of *fuzY* mutations isolated

<table>
<thead>
<tr>
<th>mutational class</th>
<th>unique mutations (number of mutants)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNPs</strong></td>
<td></td>
</tr>
<tr>
<td>Transitions</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>AT &gt; GC</td>
<td>10 (14)</td>
</tr>
<tr>
<td>GC &gt; AT</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Transversions</td>
<td>13 (40)</td>
</tr>
<tr>
<td>AT &gt; CG</td>
<td>1 (28)</td>
</tr>
<tr>
<td>GC &gt; TA</td>
<td>6 (6)</td>
</tr>
<tr>
<td>AT &gt; TA</td>
<td>2 (2)</td>
</tr>
<tr>
<td>GC &gt; CG</td>
<td>4 (4)</td>
</tr>
<tr>
<td><strong>INDELs</strong></td>
<td></td>
</tr>
<tr>
<td>+1 insertions</td>
<td>5 (6)</td>
</tr>
<tr>
<td>+2 insertions</td>
<td>2 (2)</td>
</tr>
<tr>
<td>-1 deletions</td>
<td>10 (10)</td>
</tr>
<tr>
<td>-2 deletions</td>
<td>4 (4)</td>
</tr>
<tr>
<td>small insertions (5-8 bp)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>large insertions (951 bp)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>small deletions (3-6 bp)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>large deletions (24-3373 bp)</td>
<td>7 (8)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>59 (92)</td>
</tr>
</tbody>
</table>

Of the 112 independent FS and FS-like mutants isolated, 92 harbored mutations within *fuzY*. The mutations ranged from SNPs causing missense or nonsense mutations, to small and large deletions and insertions (INDELs) causing frame-shifts throughout the length of the gene. Unbracketed figures indicate the number of unique mutations isolated, while bracketed figures indicate the total number of mutants isolated for each class. 

T443G occurred 28 times (31% of all mutants); C565T occurred twice; C622T occurred three times; G857A occurred twice; Δ882-947 occurred twice. Of the seven large deletions, two extended into *fuzZ* and one deleted all of *fuzX* and *fuzY* and some of each of *fuzW* and *fuzZ*. Of the large insertions, one resulted...
from the duplication of adjacent sequence and the other from the insertion of a putative transposon encoded
elsewhere in the genome. Mutant genotypes are listed in full in Table S2.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>class</th>
<th>nucleotide change</th>
<th>amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>FS</td>
<td>Δ4 (-1 Fs)</td>
<td>M1Δ (37)</td>
</tr>
<tr>
<td>73</td>
<td>FS</td>
<td>Δ28-33</td>
<td>ΔLQ</td>
</tr>
<tr>
<td>104</td>
<td>sFS</td>
<td>268insT (+1 Fs)</td>
<td>F90Δ (4)</td>
</tr>
<tr>
<td>133</td>
<td>sFS</td>
<td>C288G (Tv)</td>
<td>N96K</td>
</tr>
<tr>
<td>156b</td>
<td>sFS</td>
<td>G352T (Tv)</td>
<td>G118W</td>
</tr>
<tr>
<td>145b</td>
<td>sFS</td>
<td>T376A (Tv)</td>
<td>Y126N</td>
</tr>
<tr>
<td>108</td>
<td>sFS</td>
<td>432insCCTGGCGG</td>
<td>R144Δ (14)</td>
</tr>
<tr>
<td>FS</td>
<td>FS</td>
<td>T443G (Tv)</td>
<td>V148G</td>
</tr>
<tr>
<td>152</td>
<td>FS</td>
<td>Δ568-569 (-2 Fs)</td>
<td>A190Δ (93)</td>
</tr>
<tr>
<td>143</td>
<td>FS</td>
<td>G620T (Tv)</td>
<td>G207V</td>
</tr>
<tr>
<td>56</td>
<td>aFS</td>
<td>A638G (Ts)</td>
<td>K213R</td>
</tr>
<tr>
<td>163</td>
<td>FS</td>
<td>Δ638-639 (-2 Fs)</td>
<td>I212Δ (71)</td>
</tr>
<tr>
<td>12</td>
<td>sFS</td>
<td>C662A (Tv)</td>
<td>A221E</td>
</tr>
<tr>
<td>117a</td>
<td>FS</td>
<td>fuzYΔ770-1149, fuzZΔ1-220</td>
<td>T256Δ (220), ΔFuzZ</td>
</tr>
<tr>
<td>145a</td>
<td>sFS</td>
<td>809ins126nt (duplication of 126 nt immediately upstream of insertion point)</td>
<td>Y271Δ (153)</td>
</tr>
<tr>
<td>44</td>
<td>FS</td>
<td>G857A (Ts)</td>
<td>G286D</td>
</tr>
<tr>
<td>191</td>
<td>FS</td>
<td>951ins Tn pflu2158, pflu4347, pflu5832, or pflu4873</td>
<td>G318Δ (10)</td>
</tr>
<tr>
<td>192b</td>
<td>aFS</td>
<td>C947A (Tv)</td>
<td>A316E</td>
</tr>
<tr>
<td>111b</td>
<td>aFS</td>
<td>G953A (Ts)</td>
<td>G318D</td>
</tr>
<tr>
<td>142</td>
<td>FS</td>
<td>1059insT (+1 Fs)</td>
<td>R353Δ (19)</td>
</tr>
</tbody>
</table>

*Isolates are ordered by position of mutation (5’ to 3’ direction); mutant genotypes are listed in full in Table S2.*

*Fs = frameshift; Tv = transversion; Ts = transition.

*Bracketed figures indicate the number of amino acid residues from the mutation site until STOP.*
TABLE 3.

Phenotypes of *fuzVWXYZ* single gene knock-out mutants

<table>
<thead>
<tr>
<th>mutant</th>
<th>colony morphology</th>
<th>niche occupancy (static microcosm)</th>
<th>SBW25Φ2 sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ <em>fuzV</em></td>
<td>small, smooth</td>
<td>broth phase (slow growth)</td>
<td>resistant</td>
</tr>
<tr>
<td>Δ <em>fuzW</em></td>
<td>smooth (like SM); rough</td>
<td>broth phase</td>
<td>resistant (rough)</td>
</tr>
<tr>
<td>Δ <em>fuzX</em></td>
<td>smooth (like SM)</td>
<td>broth phase</td>
<td>sensitive</td>
</tr>
<tr>
<td>Δ <em>fuzY</em></td>
<td>fuzzy spreader</td>
<td>microcosm floor</td>
<td>resistant</td>
</tr>
<tr>
<td>Δ <em>fuzZ</em></td>
<td>large, smooth</td>
<td>broth phase</td>
<td>resistant</td>
</tr>
<tr>
<td>Δ <em>fuzVWXYZ</em></td>
<td>small, smooth</td>
<td>microcosm floor (slow growth)</td>
<td>resistant</td>
</tr>
</tbody>
</table>

---

4 Colony morphologies were scored after 48h growth on KB plates.
5 Niche occupancy was scored after 72h growth in 6 ml KB microcosms at 28°C.
6 All mutants were tested for sensitivity to SBW25Φ2.
7 The *fuzZ* mutant appears as a fuzzy spreader when cultured on LBA plates.
TABLE 4.

The effect of pH on the growth of *P. fluorescens*.

<table>
<thead>
<tr>
<th></th>
<th>FS</th>
<th>SBW25 ΔfuzY</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>mean cfu.ml(^{-1}) ± SEM (P value)</td>
<td>mean cfu.ml(^{-1}) ± SEM (P value)</td>
</tr>
<tr>
<td>6.5</td>
<td>8.7 × 10(^8) ± 1.3 × 10(^8)((0.0733))</td>
<td>1.2 × 10(^8) ± 5.3 × 10(^8)((0.3213))</td>
</tr>
<tr>
<td>7.0</td>
<td>5.5 × 10(^8) ± 4.9 × 10(^7)</td>
<td>5.7 × 10(^8) ± 1.3 × 10(^8)</td>
</tr>
<tr>
<td>7.5</td>
<td>4.9 × 10(^8) ± 1.4 × 10(^8)((0.7372))</td>
<td>3.8 × 10(^8) ± 1.3 × 10(^8)((0.3578))</td>
</tr>
<tr>
<td>8.0</td>
<td>6.5 × 10(^8) ± 1.3 × 10(^7)((0.1084))</td>
<td>6.7 × 10(^8) ± 7.9 × 10(^7)((0.5265))</td>
</tr>
<tr>
<td>8.5</td>
<td>5.9 × 10(^8) ± 1.3 × 10(^8)((0.7403))</td>
<td>8.6 × 10(^8) ± 2.9 × 10(^8)((0.4047))</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>pH prior to growth</th>
<th>pH after growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>6.55 ± 0.01</td>
<td>8.21 ± 0.09</td>
</tr>
<tr>
<td>7.5</td>
<td>7.60 ± 0.03</td>
<td>8.61 ± 0.10</td>
</tr>
<tr>
<td>8.5</td>
<td>8.27 ± 0.03</td>
<td>8.86 ± 0.07</td>
</tr>
</tbody>
</table>

(A.) Rows 3-7 give the viable titer (as cfu.ml\(^{-1}\)) of FS and SBW25 ΔfuzY in 6 ml KB microcosms of differing pH following 72h static incubation at 28°.

(B.) The pH of KB was measured post autoclaving but prior to inoculation with FS. Inoculated microcosms were incubated statically at 28° for 72h. Spent media was sterilized by filtration through 0.22µm filters and the pH was again measured. Titers and pH measurements are a mean of three independent replicates and variance is given as the standard error of the mean. P values are derived from unpaired t-tests comparing titer at each pH with that at pH 7. No statistically significant differences were found for any of the contrasts.
**FIGURE 1.** Comparison of the arrangement of genes at the ‘fuzzy spreader locus’ in *Pseudomonas* species. The predicted ‘fuzzy spreader’ operon in *P. fluorescens* SBW25 comprises five genes with predicted functions relating to LPS modification: *fuzV* (537073-538830) encodes a predicted carbamoyltransferase (NodU family), *fuzW* (538831-539727) encodes a putative transcriptional regulator (Mig-14 family), *fuzX* (539697-541124) encodes a predicted deacetylase, *fuzY* (541184-542332) encodes a predicted β-glycosyltransferase (GT1 family), and *fuzZ* (542329-543210) encodes a predicted α-glycosyltransferase (GT2 family). Many of the genes within this hypothetical operon have highly conserved orthologues within a range of *Pseudomonas* species. Orthologous genes were identified by Reciprocal Best Blast Analysis/Orthologue performed upon data collated in the *Pseudomonas* Genome Database and are grouped by shade (or pattern). ‘*’ or ‘**’ indicate glycosyltransferases of different GT1 ortholog groups. Unfilled boxes indicate genes with no orthologues within the regions depicted. Overlapping boxes indicate ORFs that initiate within the coding sequence of the upstream ORF. Thin arrows indicate computationally predicted operons from the Database of prOkaryotic OpeRons (DOOR), where available.
**Figure 2.** Mutation of fuzY causes the ‘fuzzy spreader’ (FS) phenotype. fuzY\textsubscript{T443G} and ΔfuzY mutations reconstructed in SBW25 gave a phenotype indistinguishable from FS (B, C, D). Reconstruction of the wild-type fuzY sequence in FS gave a phenotype indistinguishable from SBW25 (A, E). Upper panels show 48h colonies on KB plates. Lower panels show growth after 48h incubation in structured KB microcosms.

Fitness of the reconstructed mutants was measured relative to lacZ-marked derivatives of FS (F) or SBW25 (G) (see ‘Methods’) with either shaken (light shaded bars) or static incubation (dark shaded bars). No statistically significant fitness differences were measured between native and reconstructed FS mutants under any conditions [One-way ANOVA: P=0.5048 (shaken), P=0.4406 (static)]. Similarly, fitness differences between native and reconstructed SBW25 were not statistically significant [Dunnett’s test: P=0.0745 (shaken), P=0.5727 (static)]. Fitness measures are a ratio of Malthusian parameters and are the mean of six independent replicates; error bars show standard error of the mean. fuzY\textsubscript{T443G} (carried by FS) was complemented by over-expression of the wild-type fuzY gene in trans from the vector pSX (H, I, J, K).
**Figure 3.** Relative fitness and diversification of independent FS isolates after three days incubation in spatially structured or unstructured environments. Fitness was measured relative to a lacZ-marked derivative of FS (PBR742). ‘Typical’ FS mutants (open bars) display the archetypal ‘fuzzy’ colony morphology and accumulate at the bottom of structured microcosms. ‘Subtle’ FS mutants (light grey bars) display the archetypal ‘fuzzy’ colony morphology and accumulate at the bottom of microcosms but also grow to a degree within the broth phase. ‘Atypical’ FS mutants (dark grey bars) display colony morphology intermediate between FS and SM and grow within the broth phase of structured microcosms. Fitness measures are a ratio of Malthusian parameters and are the mean of six independent replicates; error bars show standard error (A, B, D, E). Asterisks indicate statistically significant contrasts in comparison to the archetypal FS isolate PBR367 (multiple comparisons tests as stated below: * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001).

For competitions within spatially structured environments (A), differences in mean fitness for some pairs of mutants were statistically significant (Welch’s ANOVA, P = 0.0001), though only mutant 104 was significantly fitter than the archetypal FS (P = 0.0109, Dunnett’s multiple comparisons test). For competitions within unstructured environments (B), differences in mean fitness for some pairs of mutants were statistically significant (Welch’s ANOVA, P = 0.0001); mutants 111b, 12, 145b, 192b, and 56 were significantly fitter than the FS archetype (P <0.0001, 0.034, 0.0075, <0.0001, <0.0001, respectively, Dunnett’s multiple comparisons test).

Population diversification was measured as the proportion of colonies whose morphology deviated from the ancestral type after 72 hours static incubation; values represent a mean of six independent replicates and error bars show standard error (C). Differences in diversification for some pairs of mutants were statistically significant (Welch’s ANOVA, P ≤ 0.0281), though only mutant 145a diversified to a significantly greater extent than the archetypal FS (P = 0.0025, Dunn’s multiple comparisons test).

When the data for individual mutants was pooled by FS classification, the contrasts were strongly significant [Welch’s ANOVA: P < 0.0001 (D, E, F)]. For competitions in spatially structured environments (D), the sFS and aFS groups were of equal fitness but each was significantly more fit than the FS group (P < 0.0001, Dunnett’s multiple comparisons test). For competitions in unstructured environments (E), each group mean was significantly different to every other group mean, with aFS being the fittest group and FS the least fit (P < 0.0001, Tukey’s multiple comparisons test). Similarly, the sFS and aFS groups both diversified to a significantly greater extent than the FS group [P < 0.0001, Dunn’s multiple comparisons test (F)].
**FIGURE 4.** Colony morphologies of single *fuz* gene deletion mutants and a *fuzVWXYZ* deletion

*mutant.* Deletion of the *fuzVWXYZ* operon produced a small-colony variant, distinct in phenotype from both FS and SM, indicating that one or more of the genes surrounding *fuzY* also contribute to cell surface characteristics. Each single gene deletion (bar *fuzX*) produced a mutant with distinctly altered colony morphology, unlike either FS or SM (Table 3). Deletions were constructed in a SBW25 background via a two-step allelic exchange strategy as described previously (RAINEY 1999; BANTINAKI *et al.* 2007). Base numbers refer to the SBW25 genome sequence (NC_012660.1, SILBY *et al.* 2009). Images show 48h colonies on KB plates.
**Figure 5.** *fuzY* mutation in LSWS compromises mat integrity. *fuzY*<sub>T443G</sub> (not shown) and Δ*fuzY* mutations reconstructed in WS (PBR366) produced colonies with a decidedly less wrinkled appearance and, though to the eye capable of forming a thick mat at the AL-interface, produced weak mats incapable of supporting 2mm glass beads (A, B - upper panels show 48h colonies on KB plates; lower panels show growth after 48h static incubation in KB microcosms). Phage SBW25Φ2 can plaque on WS (C) but not on either WS-*fuzY*<sub>T443G</sub> (PBR754; D) or WS Δ*fuzY* (PBR755; E). *fuzY*<sub>T443G</sub> was complemented by over-expression of the wild-type *fuzY* gene in trans from the vector pSX (F, G - upper panels show 48h colonies on KB plates; middle and lower panels show growth after 48h static incubation in KB microcosms).
**FIGURE 6.** $fuzY_{T443G}$ confers resistance to SBW25$\Phi$2.

Phage SBW25$\Phi$2 forms plaques on *P. fluorescens* SBW25 (A) and the reconstructed wild-type FS-$fuzY_{G443T}$ (E) but is unable to form plaques on *fuzY* mutants (B, C, D).