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The evolution of biofilm-forming Wrinkly Spreaders in static microcosms and drip-fed columns selects for subtle differences in wrinkleality and fitness

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ABSTRACT

Experimental evolution studies are used to investigate bacterial adaptive radiation in simple microcosms. In the case of the Wrinkly Spreader, a class of biofilm–forming adaptive mutants of *Pseudomonas fluorescens* SBW25, the current paradigm is that they are only evolutionarily successful in static microcosms where they out-compete other lineages for O_2 at the air-liquid interface. However, we have isolated Wrinkly Spreaders from drip-fed glass bead columns as an example of parallel evolution. These mutants are adaptive, with competitive fitness advantages on columns of 1.28 – 1.78. This might be explained by the enhanced attachment characteristically shown by Wrinkly Spreaders, allowing them to resist liquid flow through the column pore network. A comparison of column and static microcosm–isolated Wrinkly Spreaders showed that many aspects of wrinkleality, including colony reversion, microcosm growth, biofilm strength and attachment, as well as fitness in static microcosms, were significantly different within and between the two groups of mutants. These findings indicate that the two environments had selected for Wrinkly Spreaders with subtly differing degrees of wrinkleality and fitnesses, suggesting that aspects of the Wrinkly Spreader phenotype may have different relative values in static microcosms and drip-fed columns.

INTRODUCTION

Divergent selection arising from environmental differences and competition for limited resources are drivers of ecological adaptive radiation and ultimately speciation (reviewed by Schluter, 2000). Adaptive radiation has been investigated in experimental evolution studies, often using bacterial populations in simple environments or microcosms in which ecological opportunity can be varied to alter selective pressures, to relax stabilising selection, or to create conditions that generate diversifying selection (reviewed by MacLean, 2005; Buckling *et al.*, 2009; Shapiro *et al.*, 2009; Spiers, 2013). A common feature of these studies is the consistent evolution of similar phenotypes across replicates and experiments, often referred to parallel evolution (Wood *et al.*, 2005; Arendt & Reznick, 2008).

One particularly successful example of the investigation of bacterial adaptive radiation has used glass vials containing liquid growth medium which where incubated with shaking to provide a single homogeneous environment, or statically to provide a heterogeneous, structured environment in which access to O_2 at the air-liquid (A-L) interface imposes a strong selection on bacterial populations (Spiers, 2013). When King's B (KB) growth medium-containing static

microcosms are inoculated with wild-type *Pseudomonas fluorescens* SBW25 (Rainey & Bailey, 1996), an O_2 gradient is rapidly established and differentiating the top O_2-rich layer from the deeper O_2-depleted zone (Koza *et al*., 2011). The gradients persist for long periods (up to 5 days) and select for biofilm-forming mutants (lineages) such as the Wrinkly and Fuzzy Spreaders which colonise the A-L interface where O_2 levels are the greatest (Rainey & Travisano, 1998; Spiers *et al*., 2002; Koza *et al*., 2011; Ferguson *et al.,* 2013) (A-L interface biofilms are often refereed to as 'pellicles' but should be considered a specialised biofilm in their own right, see Spiers, 2013; Spiers *et al*., 2013; Armitano *et al.,* 2014).

The underlying molecular biology of the archetypal Wrinkly Spreader (strain PR1200 in Spiers *et al*., 2002) is well understood, with WS-activating mutations identified in a range of diguanylate cyclase (DGC) or DGC–associated genes for a number of independently-isolated mutants (an example of parallel evolution; Bantinaki *et al*., 2007; McDonald *et al*., 2009; McDonald *et al*., 2011; recently reviewed by Spiers, 2014). These result in increased levels of c-*di*-GMP that lead to a common final pathway, including the over-expression of a pili-like attachment factor and cellulose (Spiers *et al*., 2002; Spiers *et al*., 2003; Spiers & Rainey, 2005), to produce the WS phenotype (in the archetypal Wrinkly Spreader a mutation in the chemosensory-like Wsp complex WspF subunit results in the over-phosphorylation of the DGC WspR; Bantinaki *et al*., 2007).

Here we define the WS phenotype *sensu stricto* as a wrinkled colony morphology, robust, well-attached biofilm formation at the A-L interface of static microcosms, and copious cellulose expression in both colonies and biofilms (see Figure 1 images of Wrinkly Spreader colonies and a biofilm *in situ*). Variations in WS phenotype, or wrinkleality (see Spiers, 2013; Spiers, 2014), can be quantified using a number of assays including colony expansion and reversion rates, and microcosm growth, biofilm strength and attachment levels (Spiers *et al.,* 2003; Ude *et al.,* 2006; Spiers, 2007; Robertson *et al.,* 2013). The archetypal Wrinkly Spreader mutation is also pleiotropic, having a significant effect on physiology (Huang *et al*., 2007) and the expression of several proteins found in metabolic pathways not required for the WS phenotype *per se* (Knight *et al.,* 2006) (other Wrinkly Spreaders have been reported to have similar metabolic deficiencies, e.g. MacLean *et al.,* 2004).

Wrinkly Spreaders are generally adaptive mutants, as shown by a competitive fitness (W) advantage compared to the ancestral strain or other non-biofilm–forming competitors in static microcosms of 1.5 – 2.5 (i.e. $W > 1$) (Spiers *et al.,* 2002; Spiers, 2007; Green *et al*., 2011), though this advantage is frequency-dependent and is reduced when Wrinkly Spreaders become common. Other measurements of fitness in static microcosms have been made elsewhere using different approaches, and Wrinkly Spreaders have also been reported to arise in soil microcosms where they are also adaptive (Gómez & Buckling, 2013). However, the Wrinkly Spreader is poorly

adapted to shaken microcosms (W = ~0.3 – 1.0) and agar plates (W = 0.15) demonstrating that there is a significant cost to the WS phenotype (Spiers *et al.,* 2002; Spiers, 2007; Green *et al*., 2011). As a result, the current paradigm is that Wrinkly Spreaders are only evolutionarily successful in static microcosms, though variation in fitnesses when compared to the archetypal Wrinkly Spreader (W = ~0.7 – 1.1) suggests that some may be more successful than others (Bantinaki et al., 2007; McDonald *et al*., 2009; McDonald *et al*., 2011). Wrinkly Spreaders may also be adapted to micro-niches within mature biofilms, allowing multiple Wrinkly Spreaders to stably coexist in static microcosms (Brockhurst *et al*., 2006).

However, experiments investigating biofilm-formation by wild-type *P. fluorescens* SBW25 in drip-fed glass bead columns resulted in the isolation of Wrinkly Spreaders, suggesting that the WS phenotype might provide a fitness advantage in this environment. In these columns (see Figure 1), we presume that the increased levels of attachment and cellulose production help retain Wrinkly Spreader cells and biofilms against the flow of KB medium, and that the pore network provides a very complex heterogeneous environment which will generate diversifying selection (see the visualisation and modelling of biofilms in porous media in Graf von der Schulenburg *et al*., 2009; Bottero *et al*., 2013). Here we investigate the rise of Wrinkly Spreaders in drip-fed columns. We use quantitative measurements of wrinkleality and fitness (based on assays from Spiers *et al.,* 2003; Ude *et al.,* 2006; Spiers, 2007; Robertson *et al.,* 2013) to determine whether the Wrinkly Spreaders isolated from drip-fed columns are different to those isolated from static microcosms, and whether the two environments that appear to be selecting for the same general phenotype are in fact selecting for subtly different variations. We demonstrate that they are adaptive mutants with significant fitness advantage over a non-biofilm–forming reference strain.

MATERIALS AND METHODS

Experimental system

A partially-saturated glass bead column irrigated by drip-fed growth medium was developed to investigate the evolution of *Pseudomonas fluorescens* SBW25 (Rainey & Bailey, 1996) populations, the rise of Wrinkly Spreader mutants, and to test the fitness of these in comparison to the non-biofilm–forming mutant SM-13 (*P. fluorescens* SBW25 *wssB*::mini-Tn*5*-km; maintained with 50 µg mL^{-1} kanamycin; Spiers *et al*., 2002). Plastic chromatography columns (15 mm inner diameter, 0-20 ml, Bio-Rad, UK) and plugged with glass wool, were filled with 1-2 mm diameter glass balls (SIGMA, UK) to produce a 5 cm bead bed (see Figure 1). The top of the columns were sealed with foil and then pieced by a P1000 pipette tip positioned 2 cm from the top of the bed. A

peristaltic pump was used to drip-feed King's B (KB) medium (10 g glycerol, 1.5 g K_2HO_4, 1.5 g $MgSO_4.7H_2O$ and 20 g Proteose peptone No. 3 (Becton, Dickinson and Company, UK) per L) into the tip at 1 mL min^{-1} and a short length of drainage tubing was fixed to the bottom of the column to maintain a constant matric potential of 1 kPa and to prevent flooding. Before inoculation, the drain was sealed and KB added to submerged the beads. Over-night KB culture (100 µL, wild-type *P. fluorescens* SBW25 or mixtures of different Wrinkly Spreaders and SM-13, see below) was then added and the bacteria allowed to attach for 1 h before the drain was opened. Replicate drip-fed columns were run at 18 – 20°C for up to 5 days before destructive sampling (columns were occasionally abandoned due to contamination or blockage). The beads were transferred to 50 mL tubes containing 10 mL sterile water and vortexed vigorously for 1 min. The bacterial suspension was then serially-diluted and aliquots spread onto KB plates which were incubated at 28°C for 48 – 72 h before inspection.

Evolution of Wrinkly Spreaders

A total of 24 Wrinkly Spreaders were randomly chosen from plates spread with samples from replicate 3 day-old static microcosms (6 mL KB in 30 mL lidded universal glass vials, incubated without shaking) (WS-1 to WS-12; one isolated from each of 12 replicate microcosms) and drip-fed columns operated for the same period (WS-13 to WS-24; three isolated from each of 4 replicate columns and listed in order), all of which were initially inoculated with wild-type *P. fluorescens* SBW25. The WS phenotype was confirmed for all isolates by testing for biofilm–formation in static microcosms and inspection of biofilm samples by fluorescent microscopy for cellulose after staining with Calcofluor (SIGMA-ALDRICH, UK) after Spiers *et al.* (2003). Wrinkly Spreader isolates were maintained at -80°C in KB containing 30% (v/v) glycerol.

Fitness of Wrinkly Spreaders on columns

The fitness of representative Wrinkly Spreaders relative to SM-13 was determined by competitive fitness (W) assays on drip-fed columns. A mixture of over-night KB cultures of WS and SM-13 (100 µL, 1:1 ratio) was used to inoculate replicate columns (n = 8). Initial numbers of attached WS and SM-13 bacteria were determined by destructively sampling four columns after washing with 30 mL sterile water, whilst the remaining columns were run for 3 days before sampling for final numbers. Bacteria were recovered from columns as above, and W was calculated as ln [WS_f / WS_i] / ln [$SM\text{-}13_f$ / $SM\text{-}13_i$], where i denotes the initial numbers of attached bacteria and f the final numbers after Lenski *et al.* (1991).

Fitness of Wrinkly Spreaders in static microcosms

Fitness relative to SM-13 was determined by competitive fitness (W) assays in static microcosms after Green *et al.* (2011). A mixture of over-night KB cultures of representative Wrinkly Spreaders and SM-13 (100 µL, 1:1 ratio) was used to inoculate replicate microcosms (*n* = 4). The mixture was also used to determine initial numbers after serial dilution and spreading onto KB plates. Microcosms were incubated for 3 days at 28°C before destructive sampling by vortexing for 1 min, serial dilution and spreading onto KB plates to determine final numbers. W was calculated as for the column assays.

Quantitative assessment of wrinkleality

Wrinkeality was determined by measuring colony expansion and reversion on KB plates, and microcosm growth, biofilm strength and attachment levels in static microcosms. Overnight KB cultures were used to dot-inoculate (5 µL*, n* = 4) KB plates and colony spreading was determined by measuring diameters (mm) after 3 days at 28°C (Spiers *et al.,* 2003). Reversion to a wild-type–like phenotype was determined using dot-inoculated (*n* = 4) KB plates incubated for 3 days at 28°C. Colony material was re-suspended in sterile water before serial dilution and spreading onto KB plates which were incubated at 28°C for 48 – 72 h to determine the percentage of Wrinkly Spreaders (% WS) (Spiers, 2007). Static microcosm growth, biofilm strength and attachment levels were determined using a combined biofilm assay after Robertson *et al.* (2013). Briefly, replicate static microcosms (*n* = 8) inoculated with 100 µL of overnight KB cultures were incubated for three days at 28°C before assay. Biofilm strength was determined by the maximum deformation mass (MDM) assay where glass beads (mean weight of 0.0115 g) were added until the biofilm was destroyed (Ude *et al.,* 2006). The contents of the microcosm were then transferred into a new tube and vortexed vigorously for 1 min before a sample was taken to determine microcosm growth by optical density (OD_{600}) measurement. Finally, the original microcosm tube was washed, stained with Crystal violet, and the absorbance (A_{570}) of the eluted stain measured to determine attachment levels after Spiers *et al*. (2003).

Impact of WspR9 on attachment

Plasmids pVSP61-WspR9 and pVSP61 (control) were used to assess the impact of the expression of WspR9 (Goymer *et al*., 2006) in representative Wrinkly Spreaders. Plasmid DNA was isolated using a GeneJet Plasmid Miniprep Kit (Thermo Scientific, UK) and dialysed against deionised water for 1 h using 0.025 µm VSWP membrane filters (Millipore, UK). Aliquots of cells (100 µL) were prepared from overnight KB cultures, washed twice and then re-suspended in an equal volume of ice-cold 10% (v/v) glycerol, 1 mM HEPES solution. Freshly-prepared cells were electroporated with 10 µL DNA in ice-cold 1 mm gap-width cuvettes using an Electroporator 2510 (Eppendorf, UK) set at 200 Ω, 1.75 kV and 25 µF. Cells were out-grown in 1 mL KB for 1 h at 28°C

before aliquots were spread on KB plates containing 50 µg mL^{-1} kanamycin and incubated for 48 h at 28°C. Transformant colonies were re-streaked onto fresh selective plates and stored at -80°C as for the Wrinkly Spreader isolates. Attachment levels were determined as for the wrinkleality assay in static microcosms containing 50 µg mL^{-1} kanamycin. Repression of attachment was calculated as A_{570} pVSP61 strain / A_{570} pVSP61-WspR strain.

Genome sequencing

Whole genome re-sequencing of WS-14 was undertaken by the Centre for Genomic Research, University of Liverpool. Briefly, a library was produced using purified genomic DNA and sequenced with other samples (not part of this work) on one flowcell of a MiSeq 2000 with 2 x 150 bp paired-end sequencing and v2 chemistry. The read library was aligned against the reference genome sequence (RefSeq ID: NC_012660.1) with 3,698,789 aligned reads after filtering, resulting in a breadth of coverage of 6,178,150 bp (91.9% of the whole genome) with a mean depth of 79.7x, and sufficient for variant calling using UnifiedGenotyper, snpEff v3.2a and BREAKDANCER (Chen *et al.,* 2009; McKenna *et al.*, 2010; DePristo *et al.*, 2011; Cingolani *et al.*, 2012).

Statistical analyses and modelling

Experiments and assays were conducted with replication as stated, and means ± SE provided where necessary. Data were examined using JMP Statistical Discovery Software (JMP 7.0, SAS Institute Inc., USA) and SPSS Statistics (IBM, USA). T-tests were used to determine whether competitive fitness was significantly different to one ($W \neq 1$). Wrinkleality and fitness were investigated using a nested ANOVA (standard least squares) approach with origin (drip-fed columns and static microcosms) and isolate [origin] as factors. Competitive fitness was examined using a general linear model (GLM) approach with fitness as a response variable and origin as the main factor, and colony expansion and reversion, microcosm growth, biofilm strength and attachment levels as covariates. Similarities between Wrinkly Spreaders were also explored by principal component analysis (PCA) on correlations of mean wrinkleality and fitness data.

RESULTS AND DISCUSSION

P. fluorescens SBW25 populations developing on columns undergo adaptive radiation

Populations established by wild-type *P. fluorescens* SBW25 on replicate drip-fed columns expanded rapidly, from the initial inoculum of ~10^5 un-attached bacteria to 7.1 ± 0.7 x 10^{10} cells in 3 days, and plateauing at 7.5 ± 1.1 x 10^{10} cells after 5 days (Figure 2). The accumulation of biomass in columns could be seen in the clumping of beads by adhering biomass when emptied

from the columns (see Figure 1) and in µX-ray CT imaging of biofilms *in situ* obtained with the help of W. Otten (SIMBIOS Centre) (Figure 3). Pore spaces between beads containing biofilms and those with only liquid were not differentiated by µX-ray CT as Wrinkly Spreader biofilms are highly hydrated with a density almost equivalent to KB (Spiers *et al*., 2003). However, the images suggest that not all spaces have developed biofilms which might reflect a high degree of environmental heterogeneity within the drip-fed column. In particular, flow dynamics and shear stress might effect initial colonization of the bead surfaces and impose a physical limit to biofilm-formation, and liquid depth and distance to the nearest A-L interface might impact on O_2 availability and growth rates. Note however, that the simple assumption that nutrient and O_2 supply in biofilms is restricted to diffusion has been challenged; when liquid flow is allowed within biofilms, computer simulations show more patchy and realistic distributions of biomass within pore networks (Thullner & Baveye, 2008). Such patchy distributions have been seen in *Shewanella* biofilms developing in a glass bead column after imaging with high-energy synchrotron-based µX-ray CT (Iltis *et al.,* 2011).

During the five day incubation, *P. fluorescens* SBW25 populations developing on drip-fed columns also underwent radiation; producing a number of mutants with altered colony characteristics including mucoidal and siderophore-deficient mutants, as well as Wrinkly Spreaders which represented 35 ± 8 % of the total cell numbers after 3 days (in comparison, wild-type *P. fluorescens* SBW25 populations developing in parallel static microcosms reached $1.6 \pm 0.2 \times 10^{13}$ cells after 3 days, producing 29 ± 7 % Wrinkly Spreaders) (a total of 24 Wrinkly Spreaders were isolated from static columns (WS-1 – WS-12) and columns (WS-13 – WS-24) after 3 days for further comparison). The large variation in Wrinkly Spreader numbers in both columns and microcosms seen here and elsewhere (e.g. Green *et al*., 2011) is in part explained by the randomness with which WS-inducing mutations occur in growing populations: if mutations occur early, the proportion of Wrinkly Spreaders will be high at the end compared with populations in which mutations occurred later on (notwithstanding the selective advantage these mutants might have that will further increase the proportion of Wrinkly Spreaders).

Testing of the 12 randomly-chosen column-isolated Wrinkly Spreaders (WS-13 – WS-24) confirmed that they had the classical WS phenotype, including a wrinkled colony morphology on KB plates, biofilm-formation in static microcosms, and the expression of large amounts of cellulose (Rainey & Travisano, 1998; Spiers *et al*., 2002; Spiers *et al*., 2003). The adaptive nature of these Wrinkly Spreaders was demonstrated by determining the competitive fitness (W) on drip-fed columns of four representative isolates (WS-14, WS-17, WS-20 and WS-23) relative to the reference strain SM-13 (Spiers *et al*., 2002). The biofilm-deficient strain SM-13 was used in these assays instead of wild-type *P. fluorescens* SBW25 because the latter forms a weak biofilm in KB when Fe^{3+} levels are high (Koza *et al*., 2009) which might confound our observations. The

competitive fitnesses of the four Wrinkly Spreaders on columns, calculated as the ratio of Malthusian parameters after Lenski *et al*. (1991), ranged from 1.28 – 1.78 (Figure 4), demonstrating that each were adaptive mutants with significant advantages over SM-13 (i.e. W > 1) in the environment from which they were isolated. The range of competitive fitness advantage seen here for the column-isolated Wrinkly Spreaders is similar to that reported for the archetypal Wrinkly Spreader in static microcosms (W = 1.5 – 2.5; Rainey and Travisano, 1998; Spiers *et al*., 2002; Green *et al*., 2011).

Column-isolated Wrinkly Spreaders are similar to the archetypal Wrinkly Spreader, but there is evidence for quantitative differences in phenotype and underlying genetics

Preliminary testing showed very low levels of attachment of SM-13, WS-14, WS-17, WS-20 and WS-23 cells to the glass beads of drip-fed columns (0.1% SM-13 and 0.09 – 4.8% for the Wrinkly Spreaders). Nonetheless, the highest level of attachment shown by a Wrinkly Spreader relative to SM-13 seen here (4.8*x*) is similar to that reported for the archetypal Wrinkly Spreader in static microcosms (~3 – 5*x* relative to wild-type *P. fluorescens* SBW25; Spiers *et al*., 2003; Spiers & Rainey, 2005), suggesting that these isolates are not unusual. This is further supported by the finding that the WS colony phenotype of all four column-isolated Wrinkly Spreaders, plus four static microcosm-isolated Wrinkly Spreaders chosen for comparison (WS-1 – WS-4), revert to wild-type–like colonies when the dominant-negative WspR mutant, WspR9 (G296R) (Goymer *et al*., 2006), was expressed *in trans* from the plasmid pVSP61-WspR9 (no change in colony morphology was observed for the control plasmid pVSP61). Previously, WspR9 has been shown to revert the WS phenotype of the archetypal Wrinkly Spreader (Goymer *et al*., 2006) and acts to inhibit the normal functioning of the chromosomal copy of WspR, preventing the normal production of c-*di*-GMP required for the WS phenotype. These observations suggest that the Wrinkly Spreaders investigated in this work are likely to have similar WS-activating pathways as has been reported for the archetypal Wrinkly Spreader and other Wrinkly Spreader isolates recovered from static microcosms (Bantinaki *et al*., 2007; McDonald *et al*., 2009; McDonald *et al*., 2011) and are the result of parallel evolution.

In order to investigate this further, we have determined a draft sequence of the WS-14 genome with a breadth and depth of coverage of 91.9% and 79.7x, respectively. Bioinformatics analysis identified a single base mutation in *wspF* (G823T resulting in a change in amino acids from glycine to cysteine) likely to be responsible for the WS phenotype, though this preliminary finding has not yet been confirmed by allele replacement experiments (as in Bantinaki *et al*., 2007). The same mutation has been seen previously, and two additional mutations found within two base pairs of this position in other Wrinkly Spreaders (WS_C, G823T; WS_F, C821T; and WS_U, Δ823-824;

Bantinaki *et al*., 2007), suggesting this region of the protein is particularly susceptible to adaptive mutation.

Although the static microcosm and column-isolated Wrinkly Spreaders showed similar qualitative responses to WspR9 expression *in trans*, quantitative measurements of the repression of attachment levels by WspR9 determined in static microcosms showed clear differences between WS-1 – 4, WS-14, WS-17, WS-20 and WS-23 (Table 1), suggesting that different WS-activating mutations might generate quantitatively-different phenotypes (particularly in the case of WS-23 which shows a low level of WspR9-mediated repression of attachment levels). However, an alternative explanation that cannot as yet be excluded is that Wrinkly Spreaders contain secondary mutations not directly associated with the WS pathway which might nonetheless affect some aspects of the WS phenotype. Previously, only qualitative comments on variations of colony morphology have been reported for independently-isolated Wrinkly Spreaders (Rainey & Travisano, 1998; Buckling *et al*., 2000; Kassen *et al*., 2000; Buckling *et al*., 2003).

Different environments select for subtly different wrinklealities

Quantitative measurements of wrinkleality were used to determine whether Wrinkly Spreaders could be differentiated on the basis of the environment in which they originated (i.e. origin). Phenotype means ranged from 14.5 – 34.8 mm for colony expansion and 5.5 – 100 % WS for colony reversion on KB agar plates, and 0.77 – 1.80 OD_{600} for microcosm growth, 0.6 – 0.75 g for biofilm strength, and 0.17 – 0.27 A_{570} for attachment levels in static microcosms (Figure 5). The phenotype data were analysed by ANOVA with origin and isolate[origin] (i.e. isolate nested within origin) as factors, as the origin of each of the Wrinkly Spreaders was presumed likely to have an impact on phenotypes. Analyses of the data demonstrated that isolate[origin] had a significant effect on each of the phenotypes measured ($P < 0.0001$), and in all but the colony expansion assay, origin also had a significant effect on phenotype ($P \leq 0.0496$) (Table 2). These findings provide the first quantitative demonstration that the WS phenotype *sensu stricto* varies between Wrinkly Spreader isolates, confirming our earlier impression based on the repression of attachment by WspR. Furthermore, the comparison of isolates from drip-fed columns and static microcosms suggests that the two environments had selected for Wrinkly Spreaders with differing degrees of wrinkleality.

Wrinkly Spreaders have different fitnesses in static microcosms

The competitive fitness (W) of each of the Wrinkly Spreaders was also determined in static microcosms in comparison with the reference strain SM13 and analysed as for the measurements of wrinkleality. Mean fitness ranged from 1.17 – 1.90 and 0.84 – 1.86 for drip-fed column and static

microcosm-isolated Wrinkly Spreaders, respectively (Figure 5), and both isolate [origin] and origin had a significant effect on fitness ($P < 0.0001$) (Table 2). This adds further support to the suggestion that the two environments had selected for differing Wrinkly Spreaders. Finally, no significant correlation was observed between the drip-fed column and static microcosm competitive fitnesses for WS-14, WS-17, WS-20 and WS-23 ($P = 0.9865$).

Modelling fitness with wrinkleality

In order to investigate in more detail the relationship between competitive fitness and wrinkleality, a GLM approach was used to model fitness as a response variable and origin as the main factor, with colony expansion and reversion, microcosm growth, biofilm strength and attachment levels as covariates (Table 3A). In this analyses, origin was found to be marginally insignificant ($P = 0.058$), supporting our earlier ANOVA findings that the environment from which the Wrinkly Spreaders had been isolated had a significant effect on fitness, colony reversion, microcosm growth, biofilm strength and attachment levels. Significant between-subject effects were also found between origin x colony reversion ($P = 0.044$), origin x microcosm growth ($P = 0.017$) and origin x attachment levels ($P = 0.007$). This suggests that there was an interaction between the environment in which Wrinkly Spreaders were selected and some aspects of wrinkleality, i.e. the drip-fed column and static microcosm-isolated Wrinkly Spreaders differed in terms of colony reversion, microcosm growth and attachment levels. An examination of parameter estimates showed further differences between effects when the column and static microcosm-isolated Wrinkly Spreaders were considered separately though the linear correlations were poor ($R^2 \leq 0.306$) (Table 3B). We interpret this to mean that the phenotypes quantified in these assays may not map directly onto the Wrinkly Spreader characteristics that are critical for competitive success in static microcosms, *i.e.* competitive fitness in static microcosms is more complex than that described by the wrinkleality data. Nonetheless, the environment in which Wrinkly Spreaders originated provides a legacy effect on wrinkleality and fitness, as shown by the PCA clustering of Wrinkly Spreaders by origin (Figure 6).

Explaining variability in wrinkleality and fitness

Although variation in WS phenotype have been noted (Rainey and Travisano, 1998; Buckling *et al*., 2000; Kassen *et al*., 2000; Buckling *et al*., 2003), this is the first report quantifying variation using a number of different phenotypic assays for wrinkleality as well as competitive fitness. It is clear that some phenotypic variation is the result of differential selection in the environments from which the Wrinkly Spreaders originated (Table 4). Furthermore, the variation in fitnesses also suggests that the Wrinkly Spreaders may not be optimally adapted to their original environment. Instead, they might be better adapted to micro-environments defined by physical heterogeneity,

nutrient and O_2 availability within maturing static microcosm or drip-fed column biofilms, or they carry secondary mutations which reduce their over-all fitness. The variation not explained by environment might reflect the nature of the WS phenotype–inducing mutation in each Wrinkly Spreader and the presence of additional secondary mutations.

Although the genotypes of the Wrinkly Spreaders examined here are unknown (with the exception of WS-14 in which preliminary analysis has identified a *wspF* mutation), we would predict that those having mutations in the chemosensory-like Wsp complex leading to the activation of WspR (as in the case of the archetypal Wrinkly Spreader and ~50% of all other independently-isolated Wrinkly Spreaders; Bantinaki *et al*., 2007; McDonald *et al*., 2009; McDonald *et al*., 2011) could be differentiated from those with mutations in other diguanylate cyclases or DGC-associated pathways (including phosphodiesterases which destroy c-*di*-GMP), as both sets of mutations might be expected to increase c-*di*-GMP to varying levels which may then differentially affect attachment factor and cellulose expression and DGC or c-*di*-GMP–associated pleiotropies. However, the presence of non-WS phenotype inducing mutations, regardless of whether they are compensatory, or add additional phenotypic burden, would be expected to increase wrinkleality variation even further.

CONCLUDING STATEMENT

As has been previously shown in static microcosms, populations of wild-type *P. fluorescens* SBW25 undergo adaptive radiation in drip-fed columns and give rise to Wrinkly Spreader mutants. Although these are adaptive on columns as well as in static microcosms, column and static microcosm-isolated Wrinkly Spreaders can be differentiated on the basis of wrinkleality and fitness, suggesting that the two environments select for subtly different mutants. This parallel evolution in columns and static microcosms suggests that the adaptive advantage provided by the WS phenotype is not restricted to static microcosms in which enhanced attachment levels and the over-expression of cellulose allows the colonisation of the A-L interface through the production of a robust biofilm. These characteristics also help retain colonising bacteria and developing biofilms in the pore network of the glass bead column, providing resistance to fluid flow and shear stress. Nonetheless, adaptation in drip-fed columns and static microcosms clearly selects for different Wrinkly Spreaders, suggesting that small differences in phenotype are also important in the colonisation of these opposite environments. Such heterogeneity within an adaptive phenotype also has wider implications for bacterial ecology and medicine, and may help explain the

colonisation of heterogeneous environments and the broadening of host ranges, as well as the variable efficacy of antibiotic intervention.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Table 1. Wrinkly Spreader attachment levels in static microcosms is repressed by WspR9 expressed *in trans*.

Origin	Isolate	Relative repression
Static microcosms	WS-1	2.1 ± 0.4
	WS-2	3.0 ± 0.5
	WS-3	2.8 ± 0.6
	WS-4	3.4 ± 0.5
Drip-fed columns	WS-14	1.8 ± 0.4
	WS-17	2.8 ± 0.6
	WS-20	1.9 ± 0.4
	WS-23	0.6 ± 0.1

Means and standard errors are shown. Relative repression of attachment was determined by measuring the levels of attachment in static microcosms (A_{570} after Crystal violet staining, $n = 8$) for Wrinkly Spreaders carrying pVSP61 and pVSP61-WspR9 and calculated as A_{570} (pVSP61) / A_{570} (pVSP61-WspR9).

Table 2. Analysis of Variance testing the effects of isolate and origin on wrinkleality and fitness

		Isolate [Origin]				Origin			
Assay	R^2	NP	DF	F	P	NP	DF	F	P
Attachment levels	0.32	22	22	3.41	<0.0001	1	1	3.91	0.0496
Biofilm strength	0.53	22	22	6.82	<0.0001	1	1	40.48	<0.0001
Colony expansion	0.77	22	22	10.56	<0.0001	1	1	0.0	1.0000
Colony reversion	0.97	22	22	41.61	<0.0001	1	1	1315.41	<0.0001
Microcosm growth	0.81	22	22	8.17	<0.0001	1	1	540.63	<0.0001
Fitness in static microcosms	0.86	22	22	10.98	<0.0001	1	1	23.32	<0.0001

Wrinkleality and competitive fitness in static microcosms data were analysed using an ANOVA (standard least squares) approach with isolate (WS-1 – 24) nested within origin (static microcosm and column) and origin as factors. Each assay was analysed independently. R^2, coefficient of determination; NP, number of parameters; DF, degrees of freedom; F, F statistic; P, P-value**.** The low R^2 values suggest that this was not particularly robust for attachment levels and biofilm strength.

Table 3. Test of between-subject effects, parameter estimates and linear correlations.

(A) Source	SS	DF	MS	F	Sig.
Origin	0.176	1	0.176	4.402	0.058
Origin x Attachment levels	0.606	2	0.303	7.578	0.007
Origin x Biofilm strength	0.187	2	0.094	2.338	0.139
Origin x Colony expansion	0.040	2	0.020	0.503	0.617
Origin x Colony reversion	0.328	2	0.164	4.097	0.044
Origin x Microcosm growth	0.470	2	0.235	5.868	0.017

(B) Parameters	B	SE	t	Sig.	Linear R^2
Origin (Column) x Attachment levels	-2.830	2.460	-1.151	0.272	-0.101
Origin (Static) x Attachment levels	11.309	3.041	3.719	0.003	0.076
Origin (Column) x Biofilm strength	-1.520	0.730	-2.161	0.052	-0.306
Origin (Static) x Biofilm strength	0.043	0.642	0.067	0.948	0.019
Origin (Column) x Colony expansion	0.002	0.016	0.107	0.917	-0.114
Origin (Static) x Colony expansion	-0.032	0.032	-0.997	0.338	0.236
Origin (Column) x Colony reversion	-0.005	0.006	-0.831	0.422	0.325
Origin (Static) x Colony reversion	-0.016	0.006	-2.739	0.018	-0.258
Origin (Column) x Microcosm growth	1.216	0.727	1.672	0.120	0.008
Origin (Static) x Microcosm growth	1.629	0.545	2.990	0.011	0.026

Competitive fitness was examined using a GLM approach with fitness as a response variable, origin as the main factor, attachment levels, biofilm strength, colony expansion, colony reversion and microcosm growth as covariates. $R^2 = 0.764$ (Adjusted $R^2 = 0.548$). Abbreviations: B, Unstandardized coefficient; DF, Degrees of freedom; F, F statistic; MS, Mean square; SE, Standard error; SS, Type III Sum of squares; t, t statistic; Sig., Significance; R^2, Linear correlation (sign indicates slope of the line). a, Set to zero because it is redundant.

Table 4. Selective pressures operating in drip-fed columns and static microcosms.

	Selection	Drip-fed columns	Static microcosms
Proximal / Immediate	Physical disturbance	Very important as the early colonising cells and developing biofilm needs to resist fluid flow and shear stress.	Less important as developing biofilms are not continuously subject to physical disturbance.
Medial / Intermediate	O_2 availability	Less important as the constant flow of fluid brings new O_2 to most regions of the column pore network.	Very important as static microcosms are rapidly divided into low and high–O_2 regions by the metabolic activity of the early colonists.
Distal / Longer term	Environmental heterogeneity	Very important in mature columns where cells and regions of biofilms may be protected from fluid shear, subject to different flow dynamics and O_2 availability.	Very important in mature biofilms where the low and high–O_2 division occurs within the biofilm, and biofilm structure adds additional heterogeneity.
	Competition	Very important in mature columns where biofilms support non-biofilm–forming mutants and high cell-densities enhance competition for diffusion-limited nutrients and O_2.	Very important in mature biofilms in static microcosms for similar reasons.

FIGURE LEGENDS

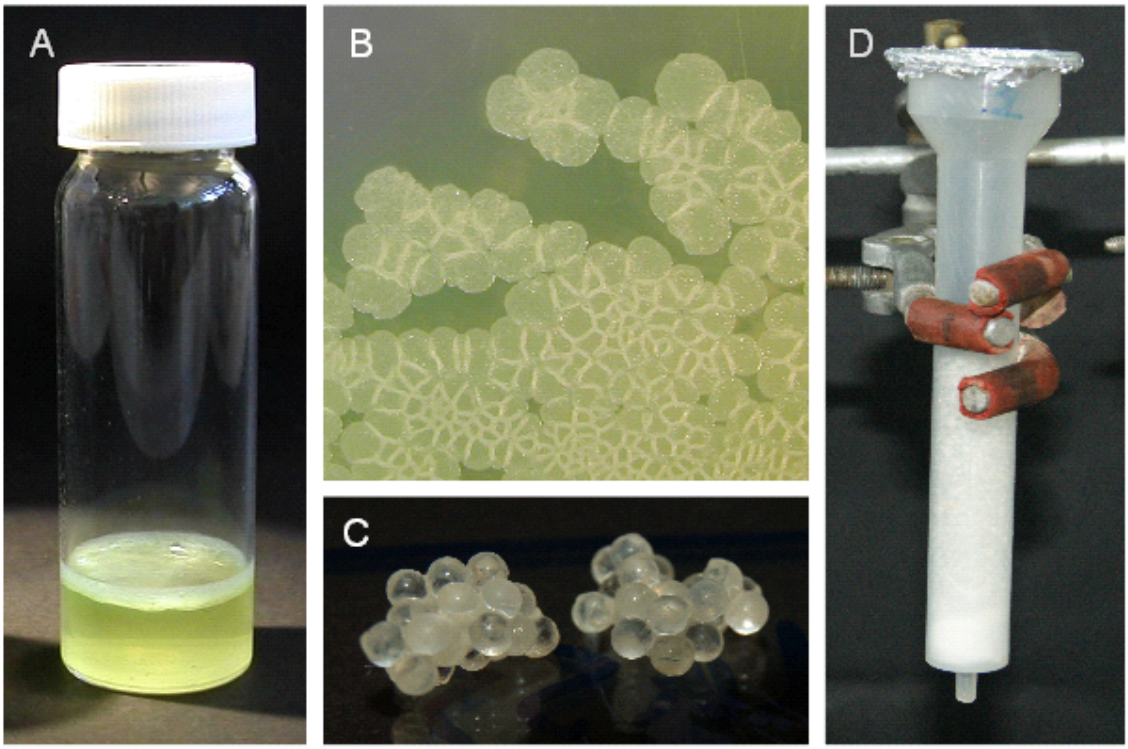

Figure 1 The Wrinkly Spreader adaptive mutant produces a robust biofilm in static microcosms and a wrinkled colony morphology. Wrinkly Spreaders arise in populations of wild-type *P. fluorescens* SBW25 radiating in static microcosms, producing a biofilm at the air-liquid (A-L) interface of static microcosms (left) and a wrinkled colony morphology on agar plates (top middle). Wrinkly Spreaders can also be isolated from the glass beads (bottom middle) recovered from drip-fed glass bead columns (right).

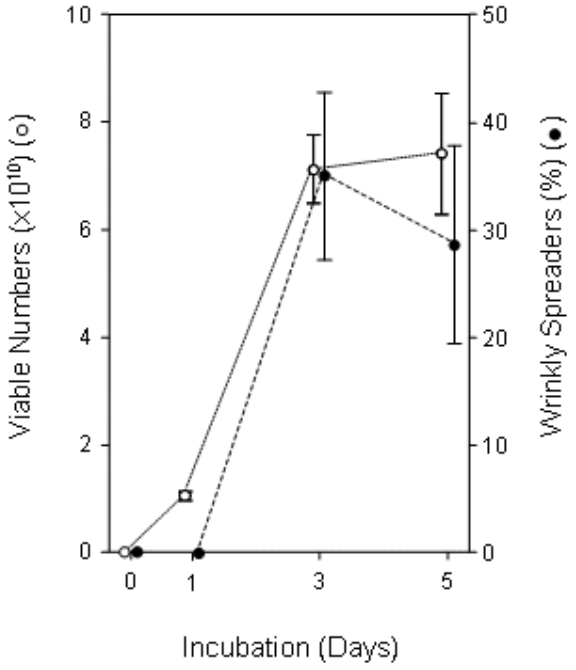

Figure 2 *P. fluorescens* SBW25 populations developing on columns giving rise to Wrinkly Spreader mutants. Wild-type *P. fluorescens* SBW25 populations develop quickly on columns as determined by measurements of viable numbers on KB plates. During this period the population radiates, as shown by the detection of significant numbers of Wrinkly Spreader mutants by day 3. Replicate columns were destructively sampled after 1, 3 and 5 days to determine viable numbers and the percentage of Wrinkly Spreaders (% WS). Means and standard errors are shown (n = 4).

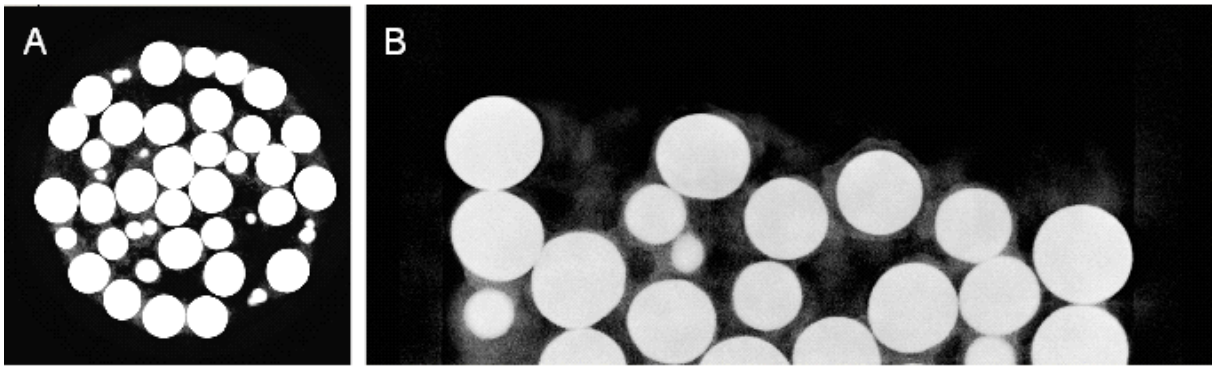

Figure 3 Biofilm formation in glass bead columns appears not to be uniform throughout the pore network. µX-ray CT was used to image biofilm formation in glass bead columns. Shown here are two representative images (axial, left; sagittal, left) of the top region of a drip-fed column inoculated with wild-type *P. fluorescens* SBW25 after 5 days in which glass balls (white), air spaces (black) and regions containing biofilms and liquid (grey) can be identified. The plastic column is not visible in these images. The glass bead bed is 15 mm wide.

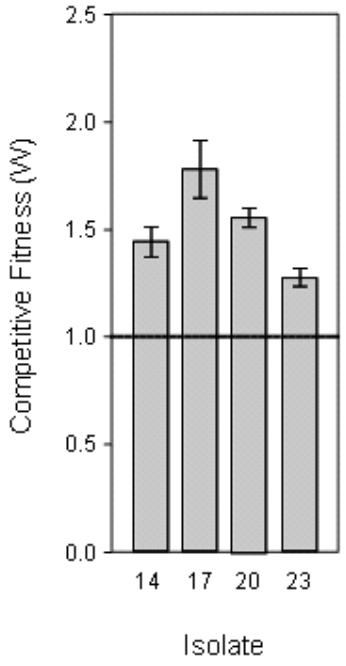

Figure 4 Column-isolated Wrinkly Spreaders are adaptive. Representative Wrinkly Spreaders recovered from columns show a competitive fitness (W) advantage on replicate drip-fed columns compared to the non-biofilm–forming reference strain SM-13. Fitness was calculated as the ratio of Malthusian parameters for both strains, using the number of attached bacteria at the beginning of the assay and final numbers after three days. Means and standard errors are shown (n = 8). For all four Wrinkly Spreaders, $W \neq 1$ (t test, $P < 0.05$).

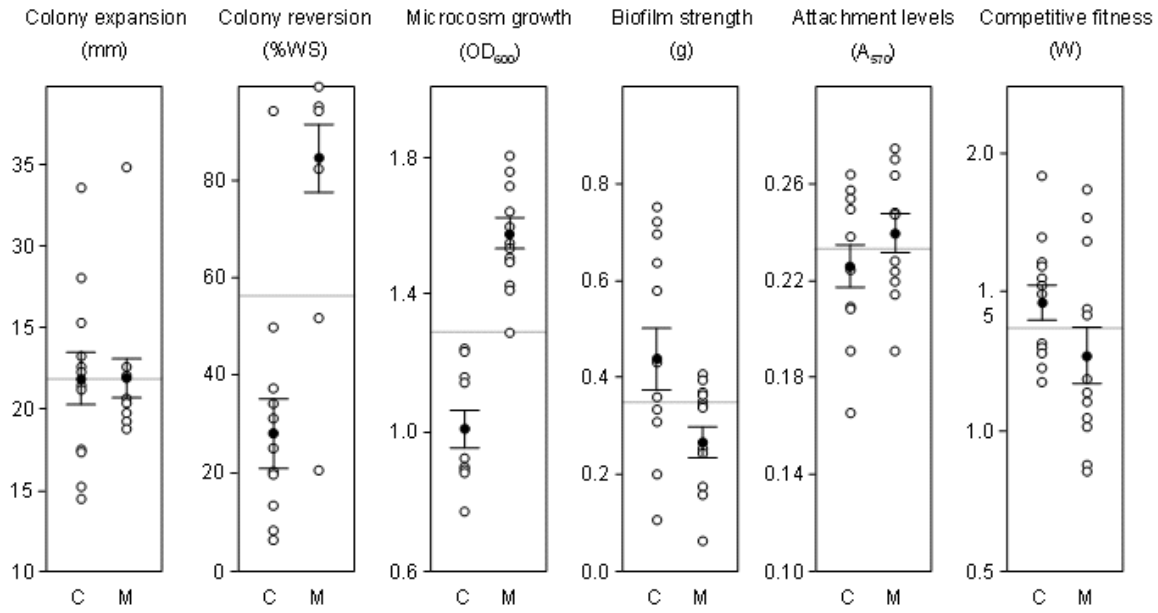

Figure 5 Quantitative measurements of wrinkleality and fitness show differences amongst Wrinkly Spreaders and between origins. Colony expansion, colony reversion, microcosm growth, biofilm strength, attachment levels, and competitive fitness in static microcosms were determined for each of the twenty-four Wrinkly Spreaders (left to right). Data are shown for each of the two environments from which the Wrinkly Spreaders originated (C, drip-fed columns; M, static microcosms). Means are shown for individual Wrinkly Spreaders (white circles) (*n* = 24, some may be obscured). Means (black circles) and standard errors are shown for each origin (*n* = 12). The grand means across origins are indicated by the grey horizontal lines. Statistical analyses of these data are presented in Tables 2 and 3.

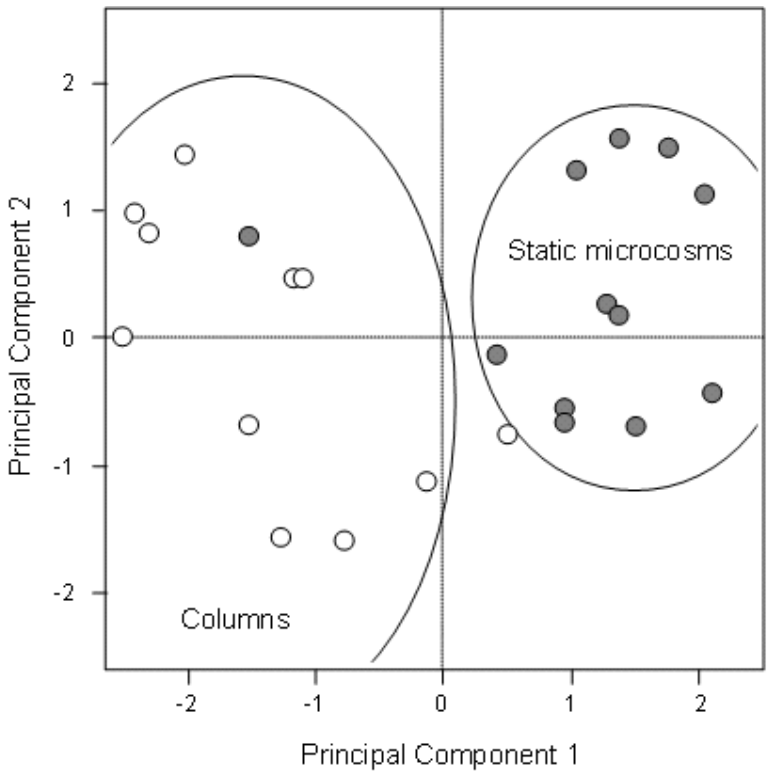

Figure 6 The origin of Wrinkly Spreaders can be distinguished by wrinkleality and fitnesses. A principal components analysis of the mean wrinkleality and fitness data demonstrates that individual Wrinkly Spreaders group according to origin (white circles, column isolates; grey circles, microcosm isolates). Six components (mean colony expansion, colony reversion, microcosm growth, biofilm strength, attachment levels, and competitive fitness in static microcosms) were used in this analysis. The score plot shown here uses principal components 1 and 2. The associated Eigenvalues are 2.3172 and 1.2173, respectively, and account for 58% of the total variation. The arcs delineating the two origins are descriptive only.