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ORIGINAL ARTICLE

Environmental modification and niche construction: developing O_2 gradients drive the evolution of the Wrinkly Spreader

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The evolutionary success of the novel Wrinkly Spreader (WS) genotypes in diversifying *Pseudomonas fluorescens* SBW25 populations in static liquid microcosms has been attributed to the greater availability of O_2 at the air–liquid (A–L) interface where the WS produces a physically cohesive-class biofilm. However, the importance of O_2 gradients in SBW25 adaptation has never been examined. We have explicitly tested the role of O_2 in evolving populations using microsensor profiling and experiments conducted under high and low O_2 conditions. Initial colonists of static microcosms were found to establish O_2 gradients before significant population growth had occurred, converting a previously homogenous environment into one containing a resource continuum with high and low O_2 regions. These gradients were found to persist for long periods by which time significant numbers of WS had appeared colonising the high O_2 niches. Growth was O_2 limited in static microcosms, but high O_2 conditions like those found near the A–L interface supported greater growth and favoured the emergence of WS-like genotypes. A fitness advantage to biofilm formation was seen under high but not low O_2 conditions, suggesting that the cost of biofilm production could only be offset when O_2 levels above the A–L interface were high. Profiling of mature WS biofilms showed that they also contained high and low O_2 regions. Niches within these may support further diversification and succession of the developing biofilm population. O_2 availability has been found to be a major factor underlying the evolutionary success of the WS genotype in static microcosms and illustrates the importance of this resource continuum in microbial diversification and adaptation.
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Introduction

Adaptive radiation is believed to be a driver of genetic and ecological diversification of bacterial lineages, driven by resource competition in environments containing ecological opportunity (i.e. multiple ecological niches). The mechanisms underlying radiation are readily investigated using artificial microcosms, where rapid growth and large populations are achievable within short time frames; comparative analysis of ancestor and evolved genotypes is possible and physical conditions can be manipulated (reviewed by Elena and Lenski, 2003; Maclean, 2005 and references therein). One such example of this approach has paired the soil and rhizosphere bacterium *Pseudomonas fluorescens* SBW25 with small liquid cultures incubated statically or with shaking (Rainey and Travisano, 1998, and subsequent publications). In microcosms such as these, O_2 availability is expected to be a key environmental factor influencing or driving the radiation of bacterial populations and the emergence of novel, adaptive genotypes. However, this expectation has never been explicitly examined, nor the assumption that static microcosms *per se* provide multiple niches.

Relatively short-term experiments using SBW25 result in adaptive radiation within 2–7 days and leads to the emergence of a novel genotype, the Wrinkly Spreader (WS), which produces a biofilm to colonise the air–liquid (A–L) interface in static microcosms (Figure 1). WS-like genotypes appear within 2 days and can account for ~50% of the population in 4 days (Rainey and Travisano, 1998), with competitive fitness (W) values of 1.5–1.7 compared with non-biofilm-forming strains including the wild-type or ancestral SBW25 ($W=1$ indicates no fitness advantage; $W>1$ indicates a

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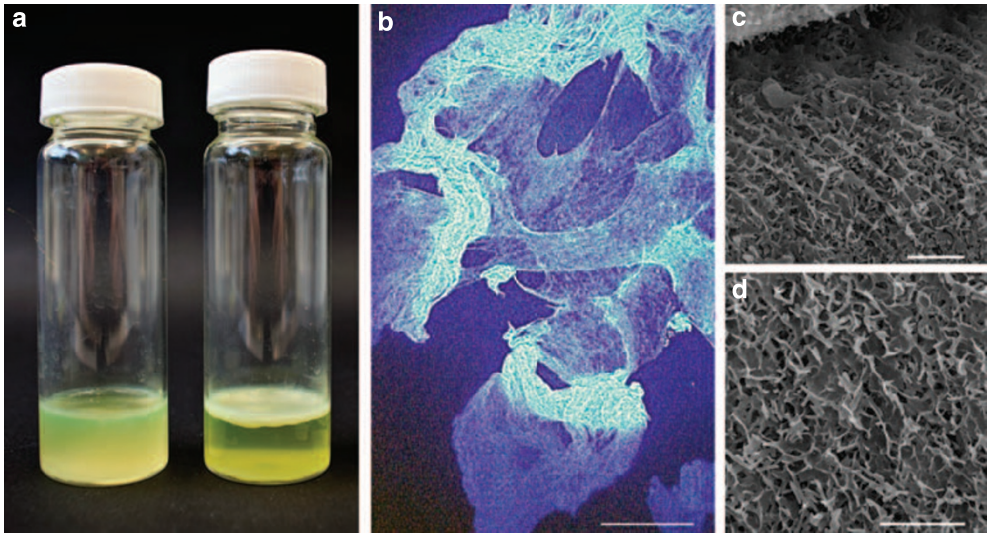

Figure 1 The WS produces a robust, cellulose matrix-based biofilm at the A–L interface of static liquid microcosms. Shown are images of the WS biofilm at different scales. (**a**) A photograph of two static microcosms with wild-type SBW25 (left) in which bacteria growth throughout the liquid column and the WS (right) in which most bacteria are found within the robust biofilm (the microcosms are 30 ml universal tubes containing 6 ml KB growth medium). (**b**) A low magnification image of WS biofilm material stained with calcofluor to reveal cellulose fibres and imaged using an epifluorescent microscope (source: AK & AS, unpublished) (scale bar: 200 μm). (**c**, **d**) Scanning electron microscopy images of fragments of biofilm after freeze-drying and shadowing with gold (scale bars: 10 μm).

fitness advantage of one strain over the other) (Rainey and Travisano, 1998; Spiers *et al.*, 2002). Such advantage explains the ability of the WS to dominate the non-biofilm-forming genotypes and the almost-certain appearance of a WS biofilm within 5–7 days in static microcosms inoculated with wild-type SBW25. The WS phenotype, a wrinkled colony morphology on agar plates and a physically cohesive-class A–L biofilm in static microcosms (Ude *et al.*, 2006), depends on the production of cellulose, lipopolysaccharide and an as yet unidentified pili-like attachment factor (Spiers *et al.*, 2002, 2003; Spiers and Rainey, 2005). Mutations increasing c-*di*-GMP (3′, 5′-cyclic diguanylate) levels in SBW25 appear to be the primary cause of the emergence of WS-like genotypes in static microcosms (Bantinaki *et al.*, 2007; McDonald *et al.*, 2009). In contrast, wild-type SBW25 will produce a very fragile, cellulose-based viscous mass class biofilm when non-specifically induced with iron (Koza *et al.*, 2009).

Despite our understanding of the ecological advantage and molecular biology of the WS, the selective pressure driving the emergence of WS-like genotypes in SBW25 populations in static microcosms has not been explicitly examined. O_2 availability was posited to be a significant factor in the fitness advantage of the WS (Rainey and Travisano, 1998) and preliminary experiments using mineral oil to reduce O_2 diffusion to the A–L interface suggested that it was a limiting factor in biofilm formation (Spiers *et al.*, 2003). O_2 is limiting in the A–L biofilm produced by *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*); like the WS biofilm, it also utilises a cellulose-based matrix though the *G. xylinus* biofilm can reach depths of 12–22 mm after 15 days (Verschuren *et al.*, 2000 and references therein), some 10 × deeper than WS biofilms (Spiers *et al.*, 2003). O_2 availability is also known to limit growth in flow-cell (submerged/solid-liquid interface) biofilms under conditions where nutrients are not limiting (Costerton *et al.*, 1995; De Beer and Kühl, 2001). The apparent half-saturation constant for O_2-uptake by aerobic bacteria such as SBW25 and *G. xylinus* is about 0.05% of normal levels of O_2 found in solution and their demand for and ability to remove O_2 from the local environment leads to very steep gradients at biofilm surfaces, sediments and soil aggregates (Sexstone *et al.*, 1985; Costerton *et al.*, 1995; De Beer and Kühl, 2001; Fenchel and Finlay, 2008; Stewart and Franklin, 2008). Such gradients provide a very strong selective pressure for adaptation to suboptimal O_2 levels or migration to more highly oxygenated regions and can result in highly differentiated communities on either side of the O_2 transition zone (Lüdemann *et al.*, 2000; Noll *et al.*, 2005).

In this work, we report a series of experiments investigating important changes occurring in static microcosms inoculated with a small number of SBW25 colonists, which grow and evolve over a period of 3–5 days to produce a population dominated by biofilm-forming WS-like genotypes (Figure 2). Specifically, we have examined (i) the establishment of O_2 gradients by SBW25 in static microcosms within several hours of inoculation and the persistence of these over a period of several days; (ii) whether the establishment of these gradients could be attributed to the respiration of the first colonists or the evolving population;

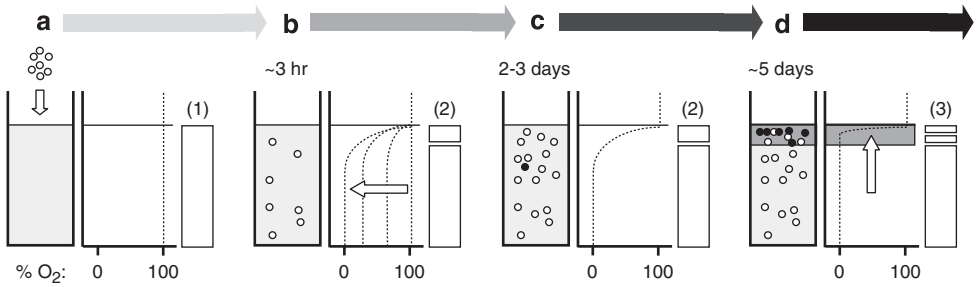

Figure 2 The colonists of static microcosms modify the original environment and generate new niches for their descendents. Shown are static microcosms with growing and diversifying bacterial populations (circles), corresponding O_2 gradients (dashed line) and niches (rectangular boxes). (**a**) Colonists of a single genotype are added to a homogenous environment with no O_2 gradient (i.e. a single niche). (**b**) Within 3 h and without significant population growth, the colonists have established a significant O_2 gradient, thus modifying the original environment to produce a resource continuum and establishing two new niches: a high O_2 zone at the A–L interface and a low O_2 region lower down the liquid column. (**c**) Over the next 2–3 days, significant population expansion and diversification occurs, during which WS-like genotypes appear (black circle). (**d**) In the following days, WS-like genotypes begin to dominate the A–L interface by producing a biofilm (grey rectangle). As the biofilm grows in depth, the transition zone between high and low O_2 environments will become fixed near the A–L interface, establishing physically structured high and low O_2 niches within the biofilm (a third unstructured low O_2 niche is below the biofilm), in which further adaptation and succession can occur.

(iii) whether O_2 availability limits SBW25 growth in static microcosms; (iv) what impact O_2 levels had on the emergence of WS-like genotypes; (v) the fitness advantage to WS biofilm formation in static microcosms and (vi) whether similar O_2 gradients develop in mature WS biofilms and its association with biofilm microstructure.

Materials and methods

The methodologies of key experiments are listed after the general sections detailing bacteria and culturing conditions and statistical analyses.

Bacteria and culturing conditions

Wild-type *P. fluorescens* SBW25 was provided by Rainey and Bailey (1996). The biofilm-forming WS (SBW25 *wspF* S301R) and the cellulose mutant SM-13 (SBW25 *wssB*::mini-Tn*5*-km; resistant to 50 μg ml^{-1} kanamycin) incapable of producing a biofilm were from Spiers *et al.* (2002). The construction of the WS-GFP mutant (WS::miniTn7 $(Gm)_{PAI/04/03}$ *gfp.ASV*-a) (Lambertsen *et al.*, 2004) is described in the Supplementary Information available at the ISME journal's website. Microcosms were 6 ml King's B (KB) liquid medium (King *et al.*, 1954) in 30 ml lidded universal glass vials which were incubated statically or with shaking at 20 °C. High O_2 conditions were produced under normal atmospheric conditions, whilst low O_2 conditions (micro-aerobic, $<0.05\%$ normal O_2 levels) were produced using AnaeroGen Compact kits (Fisher Scientific, Loughborough, UK) and monitored with Anaerobic Indicator strips (Fisher Scientific). Bacterial growth was determined by measuring optial density $(OD)_{600}$ using a Spectronic Helios Epsilon spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with 10 mm optical-path cuvettes and enumeration on KB plates (15 g l^{-1} agar). Plates were also used to assess colony morphology after 2–3 days incubation.

Statistical analyses

All data were found to be normally distributed after examination of the residuals using the Shapiro–Wilk W test. Differences between means were examined by *t*-test assuming unequal variances and by Tukey–Kramer HSD (T-K HSD) using JMP 7.0 (SAS Institute Inc., Cary, NC, USA). Means and s.e. are reported where appropriate.

Establishment and measurement of O_2 gradients in static microcosms

The establishment of O_2 gradients by SBW25 and WS colonists was investigated in static microcosms using a profiling system with a 100 μm O_2 microprobe (Unisense, Aarhus, Denmark). Microcosms were inoculated with 1, 10 and 100 μl of overnight stationary phase KB cultures ($\sim 10^3$–10^5 cells), providing cell densities equivalent to those used previously in adaptation and biofilm experiments (Rainey and Travisano, 1998; Spiers *et al.*, 2003). Replicate microcosms were profiled until five data sets were obtained for each inoculum without incident (microcosms disturbed by accident were discarded). Sterile microcosms were allowed to equilibrate at 20 °C with loosened lids for 12 h before use and the O_2 profile was measured to confirm that no gradient was present. The inoculum was added to the microcosm and mixed briefly before O_2 profile measurements were made every 10 min. The profiling system was also used to investigate O_2 gradients in long-term microcosms (up to 5 days) and through mature WS biofilms. In the latter case, microcosms were moved several millimetres horizontally after each profile measurement so that the next profile penetrated a new section of biofilm. The instrument set-up and

calibration was as follows: the profiling system ran SensorTrace PRO software v1.9 (Unisense) and was used in an air-conditioned environment (18–22 °C). The instrument was calibrated in air and a solution of 2% (w/v) sodium ascorbate in 0.1 M NaOH, to provide 100% and 0% normal levels of O_2, respectively, before each set of measurements (i.e. each replicate microcosm). The 100% value corresponds to the equilibrium O_2 concentration (284 μmol O_2 l^{-1}) at 21.3 kPa and 20 °C in water (or a low-salinity solution such as KB). O_2 levels are reported as the percentage of normal O_2 levels in solution (% O_2). Measurements were taken by integrating 3 s of signal made after a pause of 3 s following each 100–200 μm movement of the microprobe down the profile. Temperature variations affect the microprobe recordings, so all profile data were re-zeroed and normalised to allow comparison within and between experiments.

SBW25 respiration rates
O_2 uptake by SBW25 was determined by measurement of respiration rates. Stationary and log phase KB cultures were compared with stationary phase KB cultures, which had been treated with sodium azide (NaN_3) to partially inhibit respiration. Respiration rates were determined using O_2 microprobe measurements and long-necked 10 ml volumetric flasks each containing 10 ml KB and mixed constantly with a magnetic flea. Replicate flasks ($n = 5$ for each culture) were inoculated with 100 μl of SBW25 and the neck of the flask immediately filled with mineral oil to minimise O_2 diffusion into the flask. O_2 measurements were taken every minute for up to 10 min. Data were plotted to determine a time during which O_2 levels dropped linearly. The respiration rate per minute per cell (μmol O_2 min^{-1} $cell^{-1}$) was calculated for this period after cell numbers had been determined by serial dilution and plating of a sample of the inoculum. NaN_3 was used at a final concentration of 0.2% (w/v). Inocula were pretreated with NaN_3 for 30 min before transfer to flasks containing KB with NaN_3 and measurement.

Measurement of growth rates under different O_2 conditions
Growth rates of wild-type SBW25 and WS were determined from static and shaken microcosms incubated under high and low O_2 conditions. Replicate microcosms were inoculated with 10 μl of overnight KB cultures and were destructively sampled to measure OD_{600} after 4, 8, 12 and 24 h ($n = 5$ for each incubation period). Data were plotted and the maximum growth rate recorded (ΔOD_{600} h^{-1}).

Emergence of WS genotypes in evolving populations under different O_2 conditions
The emergence of WS genotypes in diversifying SBW25 populations was assessed in static microcosms incubated under high and low O_2 conditions. Replicate microcosms were inoculated with 10 μl of SBW25 from an overnight KB culture and incubated for up to 4 days. Each day, individual microcosms ($n = 5$ for each condition) were destructively sampled by thorough vortexing to disrupt biofilms, serial dilution and plating to determine the percentage of WS-like genotypes that had appeared in the population (wild-type and WS-like colonies were differentiable by colony morphology after 48 h incubation).

Competitive fitness of WS versus SM-13 in different O_2 conditions
The competitive fitness (W) of WS relative to the non-biofilm-forming mutant SM-13 was determined in static microcosms incubated under high and low O_2 conditions. Replicate microcosms ($n = 8$ for each condition) were inoculated with 60 μl of a 1:1 WS:SM-13 mixture made from overnight KB cultures. This mixture was also serially diluted and plated onto KB plates to determine initial cell numbers (WS_i and $SM\text{-}13_i$) (WS and SM-13 colonies were differentiable by colony morphology after 48 h incubation). The microcosms were incubated for 48 h before each was destructively sampled by thorough vortexing to disrupt biofilms, serial dilution and plating to determine final cell numbers (WS_f and $SM\text{-}13_f$). WS competitive fitness was determined by $W = \ln(WS_f/WS_i)/\ln(SM\text{-}13_f/SM\text{-}13_i)$ after Lenski *et al.* (1991).

Biofilm microstructure
WS-GFP biofilm samples were imaged by confocal laser scanning microscopy after staining with thiazine red R and calcofluor. Scanning electron microscopy was used to image WS-GFP biofilm samples. Further details are provided in the Supplementary Information available at the ISME journal's website.

Results

We have provided in Figure 2 a schematic detailing changes occurring in static microcosms inoculated with a small number of SBW25 colonists. In the following sections we demonstrate how the colonists modify their environment and construct new niches (i.e. new micro-habitats which induce different behaviour or require adaptation by the colonists), how O_2 levels impact on growth rate, the emergence of WS-like genotypes and fitness and how WS biofilms are modified themselves to produce new niches available for further colonisation and adaptation.

O_2 gradients are rapidly established by the first colonisers
We first investigated the establishment of O_2 gradients by wild-type SBW25 colonists in static

microcosms inoculated with $\sim 10^3$, 10^4 and 10^5 cells. Rapid changes in O_2 levels were observed after the introduction of SBW25 and the O_2 profiles from a representative experiment inoculated with $\sim 10^4$ cells are shown in Figure 3 in which measurements were made over 5 h. During the first 2 h, O_2 levels fell to $\sim$90% at 1 mm depth, in the following 2 h fell to $\sim$25% and by 5 h it had fallen to $\sim$5% with effectively all O_2 removed ($\leqslant$1%) from the microcosm at depths below 1.2 mm. After 5 h, more than 90% of the 1.6 ml-deep liquid contained $\leqslant$1% O_2, indicating that the aerobic respiration (O_2-uptake) by the colonists had a dramatic effect on the microcosm environment within a reasonably short period of time.

The establishment of O_2 gradients was clearly dependant on the size of the colonising population: static microcosms inoculated with 10^5 SBW25 cells lowered O_2 levels to 50% within 40 min at depth of 600 μm, whereas microcosms inoculated with 10^3 cells took over 300 min to reach 50% at the same depth. Although O_2 gradients were established within hours of the arrival of the colonists, they persist for at least 5 days with the transition between high and low O_2 habitats (arbitrarily set at 1% O_2) occurring at a depth of 100–200 μm (see profiles in Supplementary Figure 1 available at the ISME journal's website). It was assumed that static microcosms provide multiple niches for colonisation, but these observations show that it is the early colonisers, which modify the homogeneous environment from one with no O_2 gradient into one containing a resource continuum with novel high and low O_2 regions (Figures 2a and b).

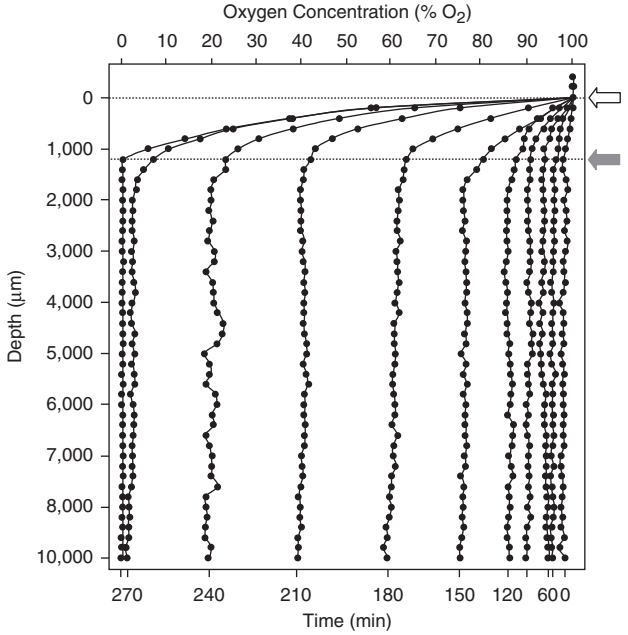

Figure 3 SBW25 colonists in the static microcosm rapidly establish an oxygen gradient. Shown is a representative experiment recording the development of the oxygen gradient over time in a static microcosm inoculated with 10^4 cells ml^{-1}. O_2 profiles were measured every 10 m for 300 min, but for clarity only profiles 30 min apart are shown. Measurements were taken every 200 μm down a profile of 10 mm, with the A–L interface at 0 μm and indicated by the clear arrow (the microcosms were $\sim$16 mm deep and the final 6 mm were not profiled). O_2 concentration is indicated as the percentage of normal O_2 levels in solution (% O_2) on the top *x* axis. Total elapsed time since inoculation is indicated on the bottom *x* axis. The grey arrow indicates $\leqslant$1% O_2 reached after 300 min at a depth of 1.2 mm.

The development of the O_2 gradient does not require population growth

The aerobic respiration of the SBW25 colonists and subsequent generations would be expected to generate the O_2 gradients observed in static microcosms. In confirmation of this, O_2 gradients were found to develop more slowly in the presence of the metabolic inhibitor sodium azide (NaN_3). However, it was not clear whether the respiration of the colonists alone was sufficient to establish these gradients, or whether significant population growth was also required. We investigated this by monitoring the colonist growth (OD_{600}) over 6 h after the inoculation of static microcosms with $\sim 10^5$ cells. No significant change in OD_{600} was observed during the first 3 h, during which O_2 gradients are established by this sized inoculum (*t*-test: $t_{2.696} = -1.9153$, $P = 0.1616$). After 3 h, the stationary phase colonists had entered log phase and rapid growth occurred. In contrast, SBW25 in shaken microcosms enters log phase within 60–90 min. O_2 uptake by SBW25 was found to vary with growth phase, with log phase cultures consuming O_2 7.9 × faster than stationary phase cultures (34.0 ± 7.7 and $4.3 \pm 0.9 \times 10^{-12}$ μmol O_2 min^{-1} cell^{-1}, respectively) (*cf.* NaN_3-inhibited stationary phase cultures showed a 0.01 × reduction in O_2 consumption compared with untreated cultures). These data show that the stationary phase colonists remove O_2 from static liquid microcosms faster than it can diffuse into the liquid column. This imbalance generates the O_2 gradients observed in static microcosms, modifying the original homogeneous environment into one containing a resource continuum with novel high and low O_2 regions (Figures 2a and b).

Growth rates in static microcosms are limited by O_2 availability

In order to determine whether SBW25 growth was limited by O_2 in static microcosms, we examined growth rates at high and low O_2 levels and under conditions of high and low availability. Microcosms were incubated normally or in AnaeroGen Compact bags to produce high and low O_2 conditions, respectively. Static and shaking incubation was used to manipulate O_2 availability (i.e. diffusion into the liquid), with shaking providing greater O_2 availability compared with static incubation. SBW25 growth was faster at high O_2 levels compared with low O_2 levels and faster under

Table 1 Growth rates of SBW25 under different O_2 conditions

	Growth rate in static microcosms with poor O_2 availability		*Growth rate in shaken microcosms with greater O_2 availability*	
	High O_2 levels	*Low O_2 levels*	*High O_2 levels*	*Low O_2 levels*
SBW25	0.047 ± 0.004^{c}	0.005 ± 0.001^{d}	0.119 ± 0.002^{a}	0.011 ± 0.000^{d}
WS	0.044 ± 0.000^{c}	0.006 ± 0.001^{d}	0.100 ± 0.001^{b}	0.010 ± 0.001^{d}

Low O_2 levels were produced using AnaeroGen Compact bags. High O_2 levels were provided by normal atmospheric conditions. Growth rate is $\Delta OD_{600}\ h^{-1}$. Comparison of means was by Tukey–Kramer HSD, $q^* = 3.150$, $\alpha = 0.05$; means not connected by the same letter are significantly different.

conditions of greater availability compared with poor availability (Table 1), clearly demonstrating that SBW25 growth is limited by O_2 levels and availability in KB microcosms. We interpret this to mean that the growth of SBW25 close to the A–L interface in static microcosms, where there are higher levels of O_2 and greater availability due to a short diffusion distance, is faster than for cells located further down in the liquid column where O_2 levels are lower and availability more limited.

The WS was also found to be similarly limited by O_2 (Table 1). Under most conditions, SBW25 and WS growth rates were not significantly different, except in shaken microcosms with high O_2 levels where SBW25 grew $1.19\times$ faster than the WS. This difference may reflect the extra cost incurred by the WS owing to the unproductive expression of cellulose and attachment factor.

The emergence of WS-like genotypes in evolving populations requires higher O_2 levels at the A–L interface

If higher O_2 levels at the A–L interface was the selective pressure driving the emergence of the WS in evolving populations of SBW25 in static microcosms, lower O_2 levels at the A–L interface would be predicted to result in a delayed or reduced emergence of WS-like genotypes. This was explicitly tested using populations incubated in static microcosms under normal and low O_2 levels over 4 days (Figure 4). WS-like genotypes were detectable after 24 h for both conditions, but while the percentage of WS-like genotypes increased to 20–30% under high O_2 levels after 4 days, it did not increase beyond 2% in the low O_2 microcosms. These findings suggest that it is the higher O_2 levels at the A–L interface which drives the emergence of WS-like genotypes in evolving SBW25 populations in static microcosms (Figure 2c).

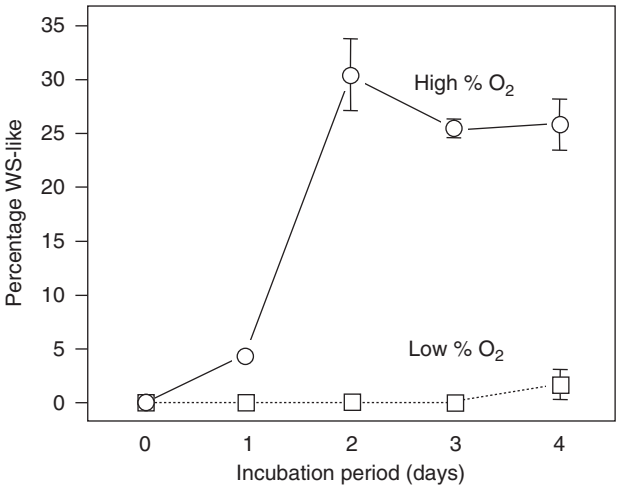

Figure 4 The emergence of WS-like genotypes is affected by O_2 conditions. Shown is the emergence of WS-like genotypes in static microcosms incubated under high (circles) and low O_2 (squares) conditions. Microcosms were inoculated with 10^4 wild-type SBW25 cells (i.e. 0% WS-like). Low O_2 levels were produced using AnaeroGen Compact bags. High O_2 levels were provided by normal atmospheric conditions. Replicate microcosms were destructively harvested every day and the percentage of WS-like genotypes determined by spreading onto KB plates and examining colony morphologies. Means ± s.e. are shown ($n = 5$). WS-like genotypes were not observed in the negative control (shaken microcosms under normal and low O_2 conditions) (data not shown).

Fitness advantage of biofilm formation is reduced in low O_2 microcosms

We predict that the fitness advantage of the biofilm-forming WS over non-biofilm-forming competitors in static microcosms is dependant on O_2 levels at the A–L interface. This was explicitly tested by determining the competitive fitness (W) of WS with respect to the non-biofilm-forming mutant SM-13 over a period of 2 days. In static microcosms under high O_2 conditions, the WS was fitter than SM-13 ($W = 1.23 \pm 0.15$), but when incubated in low O_2 conditions, WS was significantly less fit than SM-13 ($W = 0.12 \pm 0.07$) (t-test: $t_{9.9545} = 6.6955$, $P < 0.0001$). We interpret these results to suggest that the benefit in colonising the A–L interface in static microcosms is reduced with lowered O_2 levels at the surface, although the cost to producing the WS biofilm remains the same.

O_2 is depleted in the top layer of WS biofilms where the majority of active cells appear to be localised

Although we have focussed on the development of O_2 gradients by wild-type SBW25 during the early

period of colonisation of static microcosms, we can confirm that the WS produces similar gradients during this period (data not shown). We have also investigated O_2 gradients through 5-day-old WS biofilms having an average depth of 2.7 mm and estimated to contain $\sim 1.5 \times 10^{10}$ cells at a density of $\sim 2.9 \times 10^6$ cells μl^{-1} (see profiles in Supplementary Figure 2 available at the ISME journal's website). O_2 was clearly depleted from these biofilms, with the top 80 μm containing $\geqslant 50\%$ of normal O_2 levels and regions below 300 μm through to the bottom of the biofilm and into the liquid column below with $<0.5\%$. Preliminary investigation of WS-GFP biofilms by CSLM suggest that no cells are present in the top 4–5 μm of the biofilm and that bacteria are not evenly distributed vertically with fewer cells found in the lower region of the biofilm (see Supplementary Figure 3 available at the ISME journal's website), suggesting that cell activities or distributions change during the development of these structures.

Discussion

A significant body of research investigating *P. fluorescens* SBW25 adaptation in static microcosms has been undertaken over the past decade. Central to this work has been the assumption that better O_2 access at the top of the liquid column, compared with lower down, provides the ecological benefit to colonisation of the A–L interface by adapted genotypes such as the WS. By examining the development and persistence of O_2 gradients established by SBW25 in static microcosms, we provide a view of how the microcosm environment changes and the impact of this on the adaptation of SBW25 (Figure 2). The first bacterial colonisers rapidly establish a steep O_2 gradient (a resource continuum) generating two habitats within the previously homogeneous microcosm: a shallow high O_2 zone at the top of the liquid column and a deeper low O_2 region below. The transition zone between the two regions sees a change from 100–50% of normal O_2 levels to $<1\%$ in 1200 μm and is established within 5 h by a relatively small number of colonists. Similar rapid changes in O_2 gradients have been observed in flow-cell biofilms (Costerton *et al.*, 1995; De Beer and Kühl, 2001; Stewart and Franklin, 2008), *G. xylinus* A–L interface biofilms (Verschuren *et al.*, 2000) and in natural systems including marine sediments, paddy fields and soil aggregates (Sexstone *et al.*, 1985; Lüdemann *et al.*, 2000; Noll *et al.*, 2005). It appears that many microbial habitats are characterised by very steep O_2 gradients and the infiltration of O_2 into these usually dominates the spatial structure of microbial communities (Fenchel and Finlay, 2008). *Pseudomonas* such as *P. fluorescens* CHA0 respond to anaerobic conditions below $\sim$1–2% of normal O_2 levels with changes in gene expression patterns (Højberg *et al.*, 1999), including the de-repression of ANR-regulated genes involved in anaerobic respiration (Zimmermann *et al.*, 1991). It is likely that SBW25 is similarly sensitive and initiates anaerobic respiration at such low O_2 levels.

The differentiation between the high and low O_2 regions impacts directly on SBW25 adaptation as growth is limited by O_2 availability in static KB microcosms. The lack of O_2 and decreasing culture pH have been shown to limit the growth of another *P. fluorescens* strain before nutrients are exhausted (Sinclair and Stokes, 1962), although KB is sufficiently nutritious to support the production of two serial WS biofilms (Spiers *et al.*, 2003). In static microcosms, SBW25 in the high O_2 zone grows faster than those in the lower region, and as a result, WS-like genotypes emerge more quickly from the rapidly expanding and diversifying high O_2 population. WS-like genotypes have a fitness advantage over wild-type SBW25 in static microcosms and a simplistic explanation might be that they have a faster growth rate in KB. However, we have found that growth rates do not significantly differ, and suggest therefore, that the WS advantage is owing to the rapid colonisation of the A–L interface although the growth of the non-biofilm-forming population is dissipated throughout the liquid column. The apparent disparity between growth and competitive fitness assays emphasises that the latter are likely to be more accurate measurements of fitness than independent measurements of growth rate, especially when growth rates are low. The consequence of the rapid colonisation of the A–L interface is the early interception of O_2 diffusing into the liquid column allowing faster growth of bacteria in this zone and the gradual depletion of O_2 in the lower region. WS biofilms are thought to develop from bacteria attached to the microcosm vial walls at the meniscus and extend out across the A–L interface and rapid surface expansion is also seen with WS colonies on agar plates (Spiers *et al.*, 2003). The value of such rapid expansion and domination of surfaces is better O_2 and nutrient access, and has been demonstrated for the WS using mixed-colony-based competitive fitness assays (Spiers, 2007).

In the *G. xylinus* biofilm, cellulose expression and probably growth is restricted to a zone 50–100 μm below the A–L interface defined by downward-diffusing O_2 and upward-diffusing nutrient (Verschuren *et al.*, 2000). Motile bacteria are generally guided by chemotaxis in search of nutrients, but when cellular energy levels are stressed, aerotaxis and other energy-taxis behaviours dominate even strong chemotaxis responses (Taylor, 2004). However, many aquatic and soil bacteria, including some *Pseudomonas*, demonstrate a low-substrate-regulated microaerophilic behaviour and will move into less optimal low O_2 regions to maximise access to nutrients (Mazumder *et al.*, 2000). Bacterial taxis, metabolism, O_2 and nutrient diffusion will produce multiple resource continua and help define the optimal region for growth in both natural and

artificial environments. It is not clear what the fate is of bacteria outside the optimal region in biofilms, but the presence of large numbers of dead cells and remnants including extra-cellular DNA suggest that many do not survive. Those that do may adapt and successfully colonise these suboptimal niches.

G. xylinus biofilms are thought to develop by growth at the top surface which displaces older layers further into the liquid column (Schramm and Hestrin, 1954). SBW25 O_2 uptake rates are 170 × lower than that measured for *G. xylinus* (though comparable with *P. aeruginosa*) (Verschuren *et al.*, 2000; Geckil *et al.*, 2001). This difference may explain why the transition zone is twice the depth in WS biofilms and suggests that WS metabolic activity may extends to a greater depth than in *G. xylinus* biofilms. Xavier and Foster (2007) argue that EPS production in a biofilm is a selfish trait: it is altruistic to the later generations of cells above which are pushed into better O_2 conditions closer to the top surface of the biofilm, but detrimental to older cells below as O_2 access is steadily reduced. The O_2 gradients determined through WS biofilms here are consistent with this hypothesis and a layered-growth mechanism for the WS biofilm is suggested by the array of spaces seen in cross-sectional scanning electron microscope images (Figure 1). Sample preparation may have condensed the biofilm structure, as epifluorescent microscopy and density measurements (Spiers *et al.*, 2003) suggest that it is a very hydrated, open structure containing largely unattached bacteria with little evidence of highly packed cells characteristic of the archetypal flow-cell-type biofilm models (Costerton *et al.*, 1995; Stewart and Franklin, 2008). Nonetheless, the WS biofilm clearly has a complex physical structure which transverses a significant resource continuum. In mature biofilms, it is divided by the transition zone at ~100–200 μm, with high and low O_2 niches distinct from those established by the first colonisers of the static microcosms, which lacked physical structure. These new niches provide room for further bacterial adaptation and succession which may in part explain why WS-like genotypes with substantially different fitness can be isolated from biofilms (Bantinaki *et al.*, 2007). The diversification of WS into different ecological niches within the biofilm may help maintain the cooperation required for biofilm formation (Brockhurst *et al.*, 2006). This diversity may be further maintained by the trade-off between competitiveness for limiting resources and the need to maintain a structure capable of resisting physical disturbance (Engelmoer and Rozen, 2009).

The development of O_2 gradients and the impact they have in creating new microbial niches are important beyond the experimental system described here. Paddy field soil pore networks that are recently flooded show a rapid decrease in O_2 levels and distinctly different microbial communities are found in well-oxygenated versus anoxic regions (e.g. Lüdemann *et al.*, 2000). The periodic flooding of soil pore networks also occurs in terrestrial systems as the result of precipitation pulses, impacting on plant physiology, soils and ecosystems (Schwinning *et al.*, 2004 and references therein). Such flooding mobilises nutrients and microbial colonists, transporting them to different regions of the pore network. The ability of SBW25 colonists to generate O_2 gradients in static microcosms and the subsequent impact this has on SBW25 adaptation suggests that nutrient-rich networks flooded for 3 h–3 days have the potential to create novel niches for bacteria and sufficient time to select for adaptive mutants. Perhaps the propensity of soil-associated pseudomonads to produce A–L biofilms (Ude *et al.*, 2006), to grow in aerobic and microaerobic environments, are adaptations to the constantly changing water distribution in soils. The advantage of this is the ability to rapidly colonise the A–L interface where O_2 availability is maximised and nutrients diffusing through the saturated pore network are still accessible. Once O_2 gradients are established, interspecific competition for nutrients at the A–L interface and possibly within the biofilms may have a greater role in succession and further adaptation to this optimal environment (similarly, gradients established within pathogenic biofilms would also be expected to impact on bacterial diversification and adaptation).

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