RESEARCH ARTICLE

Limited diffusive fluxes of substrate facilitate coexistence of two competing bacterial strains

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Abstract

Soils are known to support a great bacterial diversity down to the millimeter scale, but the mechanisms by which such a large diversity is sustained are largely unknown. A feature of unsaturated soils is that water usually forms thin, poorly-connected films, which limit solute diffusive fluxes. It has been proposed, but never unambiguously experimentally tested, that a low substrate diffusive flux would impact bacterial diversity, by promoting the coexistence between slow-growing bacteria and their potentially faster-growing competitors. We used a simple experimental system, based on a Petri dish and a perforated Teflon® membrane to control diffusive fluxes of substrate (benzoate) whilst permitting direct observation of bacterial colonies. The system was inoculated with prescribed strains of *Pseudomonas*, whose growth was quantified by microscopic monitoring of the fluorescent proteins they produced. We observed that substrate diffusion limitation reduced the growth rate of the otherwise fast-growing *Pseudomonas putida* KT2440 strain. This strain out-competed *Pseudomonas fluorescens* F113 in liquid culture, but its competitive advantage was less marked on solid media, and even disappeared under conditions of low substrate diffusion. Low diffusive fluxes of substrate, characteristic of many unsaturated media (e.g. soils, food products), can thus promote bacterial coexistence in a competitive situation between two strains. This mechanism might therefore contribute to maintaining the noncompetitive diversity pattern observed in unsaturated soils.

Introduction

The use of molecular tools in environmental microbiology has revealed a surprising extent of bacterial diversity in soils, such that soils are now considered as the main reservoir of prokaryotic diversity on Earth (Whitman *et al.*, 1998). Not only is the global bacterial diversity in soil very great, but the local diversity is also remarkably high. For example, recent estimates of the abundance of bacterial species exceed $10^5\,g^{-1}$ of soil (Gans *et al.*, 2005). Although our ability to investigate bacterial diversity in soil remains hindered by technical limitations, the presence of hundreds of thousands of operational taxonomic units (OTUs) within a few grams of soil appears to be the rule rather than the exception.

The ability for so many different bacteria to share such limited soil volumes is intriguing. According to classical competition theory, whenever two populations compete for a substrate or a resource, the weaker competitor declines and eventually disappears (Tilman, 1982). The potential for strong local competitive interactions in soil is high because it is unlikely that a soil sample of a few grams would contain as many types of nutrients as the number of different OTUs it harbours. The validity of the competitive exclusion principle has been demonstrated for bacteria competing for a limiting substrate in chemostats (Gottschal, 1993). Nevertheless, many aspects of soil make it a habitat very different from chemostats and may promote the coexistence of competitors which would have been impossible in such controlled systems. Some of the mechanisms that act to alleviate or counterbalance bacterial competition in complex environments have been identified (Gottschal, 1993; Horner-Devine *et al.*, 2004), among which some are

considered of special relevance to soil, such as niche diversity and spatial isolation, owing to the complex nature and structure of below-ground habitats (Young & Crawford, 2004).

The observations that, compared with saturated soils, unsaturated soils support higher bacterial diversity and are characterized by noncompetitive diversity patterns (i.e. absence of dominant OTUs) prompted the hypothesis of aquatic limitation imposed on competitive interactions in unsaturated soils (Zhou *et al.*, 2002, 2004). This hypothesis has been experimentally tested: a slow-growing bacterial strain was rapidly out-competed by a fast-growing competitor in saturated conditions, yet the two strains coexisted under low matric potential (Treves *et al.*, 2003). Based on the microhydrology of unsaturated porous media, Treves and collaborators suggested two explanations for the strains' coexistence in unsaturated conditions. Owing to the lack of aqueous connectivity, the fast-growing strain was unable to invade the zones occupied by the slow-grower; and the diffusion of substrate through water phases was slow and limited the growth of the otherwise stronger competitor.

A simple model, featuring two soil particles, their nutrient and biomass pools and the fluxes between them supports the hypothesis that bacterial competition is alleviated by low substrate fluxes (Zhou *et al.*, 2002). Using a more realistic spatially-explicit model, which integrates substrate diffusion and bacterial motility, Long & Or (2005) also concluded that low substrate diffusivity caused by low matric potential promotes the coexistence of two competing species in soil.

These investigations clearly suggest a role for aquatic fragmentation and associated low diffusive fluxes of substrate as a mechanism limiting bacterial competition. However, no unambiguous experimental evidence of this role has been provided. Such evidence is challenging to obtain using soil or other natural porous media due to the coupling between biological, physical and chemical parameters in such habitats. Specifically, when the soil matric potential decreases, the consequences are not only limited to reduced diffusive fluxes of solute: bacteria may also be affected by desiccation (Potts, 1994), increased osmotic potential (Hills *et al.*, 1997), and/or reduced motility due to the reduction of connectivity in hydrated pathways (Griffin & Quail, 1968). Consequently, microbiological observations made following a decrease in soil matric potential may not be unambiguously attributable to substrate flux limitations only. To circumvent such unavoidable couplings in porous media, we developed a simple experimental system, which, unlike soil or other porous media, allows for an independent control of diffusive flux of substrate whilst stabilising other ambient conditions and facilitating direct observation of growing bacterial colonies. In this system the bacteria grow on an agar layer, separated from the nutrient source by a perforated Teflon® membrane. The efficiency of the proposed system was established first using a tracer molecule in the absence of bacteria, and then by observing the colony growth kinetics of a model strain. The experimental system was then used to test the effect of diffusive fluxes of a substrate, benzoate, on competition between pairs of model bacterial strains of *Pseudomonas*.

Materials and methods

Bacteria

To test the influence of diffusive flux of substrate on bacterial competition, two strain pairs were used. To allow for their microscopic detection, the strains constitutively produced fluorescent proteins. The first pair was used as a control, and comprised two derivatives of *Pseudomonas putida* KT2240, a well-studied soil bacterium, originally isolated from the rhizosphere (Nelson *et al.*, 2002). The two KT2440 derivatives were identical, except that one, hereafter denoted KT2440 GFP, produces the green fluorescent protein (GFP), whereas the second, hereafter denoted KT2440 DsRed, constitutively produces the DsRed Express protein. The two strains were obtained by inserting the appropriate miniTn7 cassette into KT2440 chromosome, according to Lambertsen *et al.* (2004), and were expected to have similar growth rates.

The second pair comprised KT2440 GFP and *Pseudomonas fluorescens* F113 producing DsRed.T3_S4T, an improved DsRed variant (Dandie *et al.*, 2005). The latter is denoted F113 DsRed hereafter. All strains were routinely grown on AB10 medium (Nielsen *et al.*, 2000) supplemented with 5 mM benzoic acid. To selectively enumerate F113 DsRed, this medium supplemented with 50 $\mu g\,mL^{-1}$ kanamycin was used.

Control of diffusive flux of substrate

Benzoate was used as a model substrate. To manipulate the diffusive flux of this substrate to bacterial colonies, two types of Petri dishes (9 cm diameter) were prepared. High diffusive flux (HDF) plates, where bacteria grew directly on the top of a 17-mL (*c.* 0.3 cm thick) layer of solid AB10 (1.5% agar) supplemented with 5 mM benzoic acid, were compared with Low diffusive flux (LDF) plates. The LDF plates were prepared by pouring, in 2-cm high Petri dishes, two layers of AB10 agar separated by a perforated Teflon® membrane (85 mm in diameter and 0.5-mm thick, Brønnum Plast, Rødovre, Denmark). The bottom layer (17 mL) contained benzoic acid (5 mM), while the top layer (70 mL, corresponding to a thickness of 1.2 cm), on which the bacteria were inoculated, was devoid of a usable carbon source (Fig. 1). The Teflon® membrane was perforated with 14 regularly spaced 5-mm diameter holes, so that the substrate could diffuse from the bottom agar layer to the top layer through the holes. The diffusive path through the top agar layer (1.2 cm) was sufficient to suppress radial

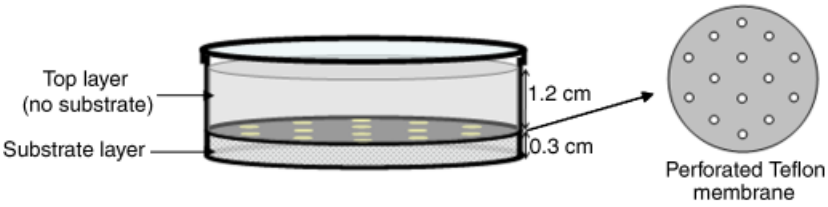

Fig. 1. Schematic representation of the LDF Petri dish system used to control substrate flux to colonies developing at the surface of the top agar layer.

heterogeneity in substrate flux caused by the holes in the membrane.

Validation of the diffusion control

To verify and quantify the ability of the Teflon® membrane to limit diffusive flux of benzoate, an experiment was conducted without bacteria and using nitrite, whose concentration can readily be measured, as a model solute instead of benzoic acid. The LDF plates were made of a 17-mL agar (1.5%) layer containing nitrite, 5 mM, topped by a 80-mL agar layer, the two layers being separated by the perforated Teflon® membrane. The temporal evolution of the nitrite concentration at the surface of the plates was monitored by placing and sampling 4.2-cm^2 pieces of filter paper on the top of the agar layer. The nitrite concentration in the soaked filter paper was determined spectrophotometrically in 1.5-mL Eppendorf tubes after formation of reddish-purple azo dye produced by coupling diazotized sulfanilamide with *N*-(1-naphthyl)-ethylenediamine dihydrochloride (Fox, 1985). The temporal evolution of the nitrite concentration at the surface of triplicate LDF plates was compared with that obtained with similar plates from which the Teflon® membrane had been omitted. The diffusion coefficients (D) of nitrite (1) through agar, and (2) through the agar-membrane stack were estimated by fitting the data to Fick's first law. To account for the finite size of both compartments (initially with and without substrate), the bottom and the top of the plate were considered as reflective boundaries (Crank, 1975).

Colony detection and size quantification

In experiments involving bacteria, the size of colonies growing on the surface of the plates was determined by epifluorescence microscopy. This was realized using a Leica MZ16 FA stereomicroscope with a $\times 1$ objective and a magnification of 15 or 20, depending on the colony sizes. Fields of view were examined to detect GFP- (excitation wavelength, 480 nm; emission, 525 nm) and DsRed- (excitation, 565 nm; emission, 620 nm) producing colonies. Images were acquired with a digital camera and analyzed with the IMAGE PRO software (version 5, Media Cybernetics, Silver Spring, MD) to automatically detect and measure the area and diameter of colonies. The automatic detection was visually checked and corrected if necessary. At least 30 (and typically, more than 60) colonies were measured to estimate average colony sizes for each treatment.

Single strain colony growth kinetics

Prior to inoculation of plates, KT2440 GFP was cultured in liquid AB10 supplemented with benzoic acid (5 mM). The $OD_{600\,nm}$ of the exponentially-growing culture was measured and the culture diluted in sterile NaCl (9‰) solution so as to obtain four dilutions containing between 700 and 30 CFU 100 μL^{-1}. The four dilutions were then inoculated on triplicate LDF and HDF plates (100 $\mu L\,plate^{-1}$), using sterile glass beads to ensure a random spatial distribution of the colonies. The plates were inoculated within 1 h following their preparation, so that as little benzoate as possible had diffused into the top agar layer of LDF plates. The inoculated plates were incubated at 30 °C in a closed container containing 1 L of KH_2SO_4-saturated solution, which ensured that the relative humidity of the headspace was maintained approximately at 97% (Winston & Bates, 1960). The sizes of the colonies on the plates were then recorded through time.

Competition experiments

Competition experiments between similar competitors (KT2440 GFP and KT2440 DsRed) and dissimilar competitors (KT2440 GFP and F113 DsRed) were performed in three systems: shaken flasks containing 17 mL of AB10 with 5 mM benzoate, HDF plates, and LDF plates. The strains were first individually grown overnight in liquid medium. The cultures were then diluted and combined by pairs to obtain a range of 100-µL inocula with various total cell densities and strain ratios (two different inocula for the competition between KT2440 GFP vs. KT2440 DsRed, four different inocula for the competition between KT2440 GFP vs. F113 DsRed). Each inoculum was used to initiate competition on triplicate flasks, HDF and LDF plates, following the protocol described in the previous section for plate inoculation and incubation.

The outcome of competition was quantified after the colonies had reached their final size. For the competitions on HDF and LDF plates, the quantification was done by measuring the colony sizes as explained above. For the competitions in flasks and for the competitions on plates between KT2440 GFP and F113 DsRed, the competitors' final abundance (cell number) was also determined. For the

flasks, this was realized by plating serial dilutions of 1-mL aliquots from the competition flasks. For the competition plates, 7.5-cm^2 zones of the surface of the plates were scraped using a sterile scalpel, the cells were suspended in sterile NaCl (9‰) and enumerated by plating serial dilutions.

Data analysis

Kruskal and Wallis tests were used to test for the existence of differences in mean colony area. The colony area data were also expressed as the ratio of the mean DsRed colony area relative to the mean GFP colony area. The 95% confidence intervals of such ratios were computed according to Cox (1985). When final bacterial abundances were determined, the relative fitness of the strains was calculated. The fitness (W) of Strain x relative to that of Strain y is:

$$W(x,y) = \frac{\ln\left(\frac{x_F}{x_0}\right)}{\ln\left(\frac{y_F}{y_0}\right)}$$

with x_0, denoting the abundance of Strain x at inoculation, and x_F denoting the final abundance of Strain x (Kerr *et al.*, 2002). Differences in relative fitness were tested using Student's t-tests.

Results and discussion

Validation of the low diffusive flux plates

The effect of the perforated Teflon® membrane on the diffusive flux of solute was first evaluated by assessing the evolution of nitrite concentration at the plate surface (Fig. 2). Whereas without the Teflon® membrane the surface nitrite concentration reached its maximum value within 1×10^3 min, in the presence of the membrane, it took 4×10^3 min to reach maximum concentration. Fick's first law was fitted to nitrite concentration profiles, resulting in an apparent diffusion coefficient in the absence of membrane of 1.6×10^{-5} cm^2 s^{-1}, in line with published values for somewhat larger molecules such as glucose, for which D has been estimated to 6.5×10^{-6} cm^2 s^{-1} (Stecchini *et al.*, 1998). The fit was less satisfactory for the plates with the membrane (Fig. 2). This is not surprising because of the heterogeneity of the compartment in which the nitrite diffused (membrane+top layer agar). Nevertheless, fitted diffusion values show that the insertion of the Teflon® membrane resulted in a fourfold decrease in apparent diffusion coefficient (4.2×10^{-6} cm^2 s^{-1}).

Because the molecular weight of benzoic acid is 2.7 times higher than the one for nitrite, it is expected that its rate of diffusion would be reduced by a factor of about 0.6, the ratio of diffusion coefficients being equal to the square root of the inverse of the ratio of the molecular weights (Schwarzenbach *et al.*, 1993). The rate of diffusion obtained with the LDF plate system is relevant to unsaturated soil conditions because diffusion coefficients in the range of 10^{-5}–10^{-6} cm^2 s^{-1} are expected for most soils with volumetric water content in the 25–60% range (Hu & Wang, 2003).

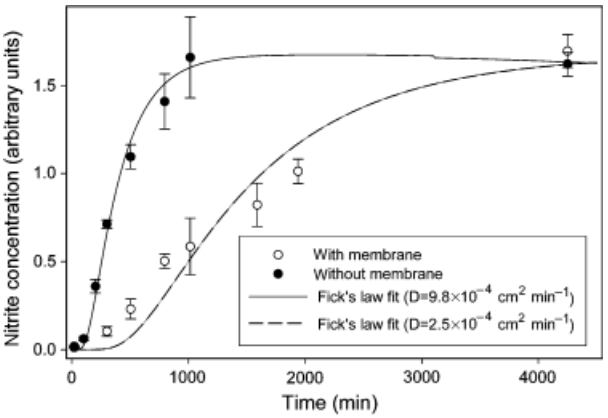

Fig. 2. Evolution of nitrite concentration at the surface of two types of plates. In one set of plates a Teflon® membrane, punched with holes, separated a nitrite-containing bottom agar layer from a 80-mL nitrite-devoid top agar layer, whereas in the second set of plates the membrane has been omitted.

Single strain colony growth kinetics on HDF and LDF plates

When KT2440 GFP was inoculated alone on HDF and LDF plates over a range of inoculum densities and its colony diameter was followed through time, the resultant kinetic curves can be divided into three phases (Fig. 3). First, the colony diameter increased linearly; then, the colony growth rate decreased before a plateau in the diameter was reached. The length of the linear phase was inversely related to the inoculation density, because intercolony competition for substrate developed earlier when many colonies were present on the plate (Cooper *et al.*, 1968). The slope of the linear phase is known to be proportional to the strain's maximal specific growth rate, μ_{max} (Pirt, 1967; Salvesen & Vadstein, 2000). Here, the slope was significantly smaller on LDF plates (23.9, SE 0.51) than on HDF plates (54.6, SE 0.83), suggesting that KT2440 GFP did not reach its μ_{max} on LDF plates, which we attribute to low diffusive fluxes of benzoate.

KT2440 GFP was also inoculated alone on a plate made by pouring 87 mL AB10 agar containing 0.98 mM benzoate. This corresponded to the benzoate concentration that would exist in LDF plates (17 mL 5 mM benzoate +70 mL devoid of benzoate) after equilibration of benzoate concentration in the two layers, in absence of bacterial consumption. Results show that the initial growth phase was very similar to that observed on HDF plates with 5 mM benzoate (the slopes of the linear phase are not statistically different; data not

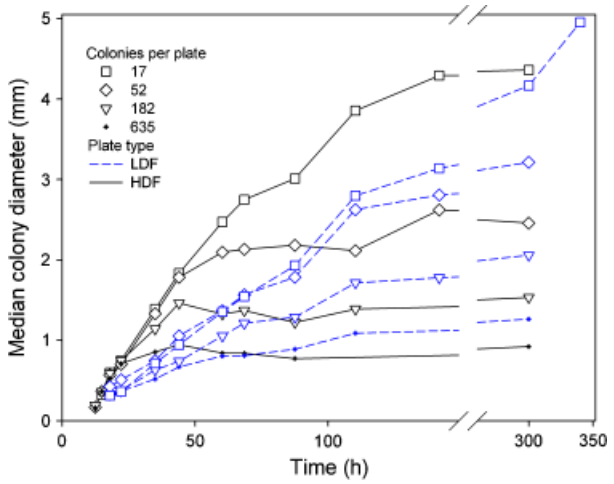

Fig. 3. Growth kinetics of KT2440 GFP colonies at the surface of HDF and LDF plates, respectively, as a function of inoculum density.

shown). Therefore, the low initial growth rate observed with LDF plates was not attributable to an overall lower concentration of benzoate in LDF plates than in HDF plates but was caused by smaller diffusive flux of benzoate. The diffusion-limited growth was maintained throughout the incubation because, even with as few as 60 colonies on the plates, no acceleration of growth was observed: the colony diameter increased linearly before a decrease in growth rate due to substrate exhaustion (Fig. 3).

Figure 3 also indicates higher final median colony sizes on LDF plates than on HDF plates although the total mass of carbon source was identical in both plates. This significant difference ($P < 0.05$ for all inoculation densities), coupled with the reduced fluorescent intensities of the colonies observed on LDF plates (data not shown) suggest that diffusion-limited growth resulted in wider and flatter colonies than the ones developing on HDF plates. This is in agreement with Pirt's model of colonial growth (Pirt, 1967), according to which colony height is limited by nutrient diffusion through the cell layers, and hence by nutrient concentration at the base of the colony. On LDF plates, the low local substrate concentration, sustained by slow substrate diffusion from the source and by bacterial metabolism, probably limited the vertical growth of the colonies.

Competition between similar competitors

The similarity in growth rate on benzoic acid of KT2440 GFP and KT2440 DsRed was first verified with competition experiments in stirred liquid medium (AB10+5 mM benzoic acid). After four successive subcultures, the abundance ratio of the two strains was not statistically different from the inoculation ratio ($P > 0.05$; data not shown). Therefore, the strains' relative fitness was not statistically different from unity, demonstrating the similarity of their growth rate.

Table 1. Influence of substrate flux provisioned by High or Low diffusive flux plates (HDF and LDF, respectively), and inoculum density on the competitive interaction between the same *Pseudomonas* strain tagged with GFP or DsRed, as measured by the ratio of their mean colony area

Approx. inoculation density (CFU)		95% confidence interval of mean colony area ratio (DsRed/GFP)	
KT2440 DsRed	KT2440 GFP	HDF plates	LDF plates
200	200	[0.876; 1.12]	[0.903; 1.015]
15	55	[0.856; 1.33]	[0.745; 1.049]

The strains were then inoculated on HDF and LDF plates and the final colony sizes recorded. In accordance with their similar fitness in liquid culture, the two strains formed colonies of very similar size: irrespective of the number of colonies per plate, the final mean colony size ratio was not statistically different from 1 on both LD and HD plates ($P > 0.05$; Table 1). This confirmed that substrate diffusivity did not impact upon competition between two similar competitors, and that no bias in colony size determination was caused by the type of fluorescent protein.

Competition between dissimilar competitors

It was verified that F113 DsRed and KT2440 GFP had different specific growth rates on benzoic acid. The two strains were coinoculated in shaken flasks (medium volume and concentration identical to the substrate source in the plate experiments). The relative abundance of F113 DsRed decreased sharply and was close to the detection limit at the end of two successive subcultures (data not shown). The fitness of F113 DsRed relative to KT2440 GFP was estimated to be 0.33 (SE 0.013). This value, significantly different from 1 ($P < 0.05$), signifies that F113 DsRed is a weak competitor vs. KT2440 GFP when substrate diffusion to cells is unimpeded.

The results obtained for the competitions on plates at several inoculation densities are shown in Table 2. The relative competitive inferiority of F113 DsRed observed in liquid cultures was also manifest on HDF plates: F113 DsRed colonies were significantly smaller than KT2440 GFP colonies as shown by the low values of the ratio of their mean colony sizes (DsRed/GFP). This ratio decreased as KT2440 GFP inoculation density increased because competition was more intense. Indeed, the mean distance between a F113 DsRed colony and a KT2440 GFP colony was smaller when more KT2440 GFP colonies were present per plate. The observations made on colony areas were consistent with cell counts, which served as the basis to compute the relative fitness values (Table 2): the relative fitness of F113 DsRed on

Table 2. Influence of substrate flux provisioned by High or Low diffusive flux plates (HDF and LDF, respectively) and inoculum density on the competitive interaction between F113 DsRed and KT2440 GFP, as measured by the ratio of their mean colony area and F113 DsRed fitness relative to KT2240 GFP

Approx. inoculation density (CFU)		95% confidence interval of mean colony area ratio (DsRed/GFP)		Mean relative fitness (DsRed/GFP) and SE	
F113 DsRed	KT2440 GFP	HDF plates	LDF plates	HDF plates	LDF plates
400	600	[0.019; 0.027]	[0.363; 0.440]	ND	ND
600	400	[0.037; 0.063]	[0.399; 0.502]	0.81 (0.023)	1.02 (0.010)
400	200	[0.056; 0.081]	[0.352; 0.483]	0.88 (0.009)	1.01 (0.015)
110	40	[0.080; 0.135]	[0.324; 0.426]	0.99 (0.015)	1.05 (0.036)

ND, not determined.

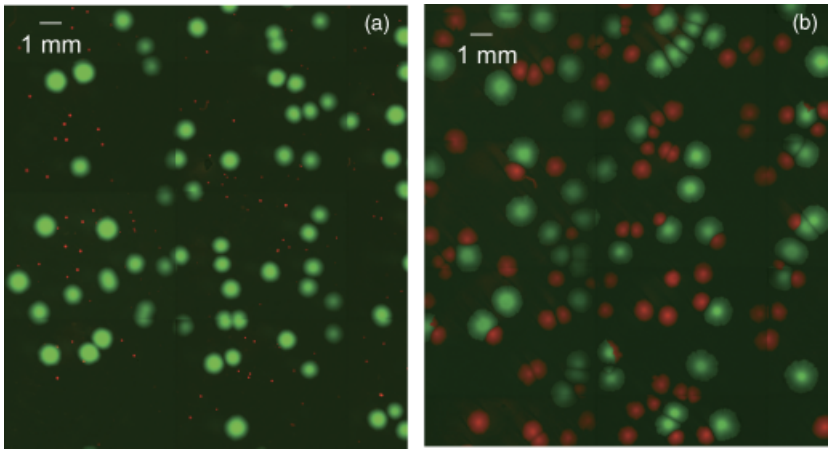

Fig. 4. Composite image (tiled from several fields-of-view) of the final stage of colony growth at the surface of High (a) and Low (b) diffusive flux plates (HDF and LDF, respectively) after an inoculation of *c*. 900 F113 DsRed and 400 KT2440 GFP CFUs per plate. The red colonies are F113 DsRed colonies (barely detectable on the HDF plates) and the green are KT2240 GFP. The image contrast has been digitally enhanced using IMAGE PRO PLUS.

HDF plates, albeit higher than observed in liquid medium, remained lower than unity. F113 DsRed's inferiority was more pronounced at high KT2440 GFP inoculation density. Very clear differences in colony area ratio were observed when comparing LDF and HDF plates (Fig. 4). Irrespective of the inoculation density of KT2240 GFP, the colony area ratio on LDF plates was in the 0.3–0.5 range and the relative fitness of F113 DsRed was not statistically different from 1 (i.e. the two strains had the same fitness, $P > 0.05$; Table 2).

Although pointing to the same conclusions, the results obtained by microscopy and those obtained by enumeration of cells were not identical. Based on cell counts, the prevalence of F113 DsRed on both types of plates was higher than suggested by colony size ratios (Table 2). Such discrepancy would arise if F113 cells were smaller than KT2440 cells. A colony of a given surface would then contain more cells if this colony is composed of F113 cells than if it is made of KT2440 cells. Nevertheless, the microscopic method detected the influence of low diffusive flux on competition more consistently than the cell spreading method. For example, on the plates where 40 KT2440 GFP cells were inoculated, LDF plates were associated with a significantly higher colony area ratio than HDF plates, whereas no significant difference in relative fitness was detected ($P > 0.05$; Table 2). This is probably because cell abundance values obtained by plate spreading were more variable and less precise than the colony size measurements, which were based on dozens of individual colony size determinations.

Relations between substrate diffusive flux and fitness of competitors

The fitness of F113 relative to KT2440 was estimated at 0.33 in liquid culture. F113 DsRed is quickly out-competed under such conditions of unimpeded substrate diffusion to the cells. In contrast, the surface of a solid medium is a spatially structured environment where bacteria are essentially stationary and where colonies rely on diffusion to get their nutrients (Korona *et al*., 1994). The spatial separation between freshly inoculated cells ensured that F113 colonies did not suffer from the effects of competition with KT440 colonies for the initial phases of colony growth. This would explain the relatively high fitness of F113 on HDF plates (> 0.8) compared with the stirred liquid medium situation (viz. 0.33). On LDF plates, KT2440 GFP lost its entire competitive advantage: even when as many as 400 KT2440 GFP colonies were inoculated on LDF plates, the relative fitness of F113 DsRed was not statistically different from 1 ($P > 0.05$; Table 2). Two nonexclusive mechanisms can explain this observation. The first is related to spatial isolation and the second to differences in the growth kinetic properties of the strains. As mentioned above, upon

inoculation, F113 does not suffer from competition by KT2440 when separation distances between colonies are sufficiently large. As a result of colony development, a zone of nutrient-depleted agar surrounding KT2240 colonies may reach the vicinity of F113 colonies, ending this spatial isolation phase and reducing the relative fitness of F113. LDF conditions were shown to decrease KT2440 colony development rate (Fig. 2). As a consequence, F113 colonies on theses plates could grow for a longer time without suffering from the competition of neighbouring KT2440 colonies. The spatial isolation phase was thus maintained for longer periods on LDF plates.

The second potential mechanism is directly linked to the intrinsic kinetic properties of the strains. In a chemostat, the outcome of competition between two species A and B is controlled primarily by the shape of their respective specific growth rate vs. substrate concentration relationships (Gottschal, 1993). If the curves exhibit a cross-over because, using Monod's formula, $Ks_{(A)} < Ks_{(B)}$ and $\mu_{max(A)} < \mu_{max(B)}$, B will out-compete A at high substrate concentration, but the opposite will occur at low substrate concentration (Gottschal, 1993). F113 has a lower μ_{max} than KT2440 but their relative *Ks* values are unknown. If $Ks_{(F113)}$ is smaller than $Ks_{(KT2440)}$ it is possible that on LDF plates F113 could have a growth rate similar or even superior to that of KT2440 due to the low substrate concentration sustained at the base of the colonies growing on these plates. The contribution of this second mechanism to the increased fitness of F113 on LDF plates is difficult to estimate in our experiments. In contrast to a chemostat, where substrate concentration is stable, our plates do not reach a steady state and the substrate concentration varies both in time and space. Therefore, knowing the intrinsic growth parameters of the two strains does not permit an easy prediction of their overall fitness on the plates. To distinguish the individual roles of intrinsic growth kinetic parameters and the spatial isolation mechanism, F113 DsRed and KT2440 GFP were coinoculated on HDF and LDF plates at a very high cell density (total 1.6×10^6 CFU plate^{-1}, 1 : 1 ratio) in order to reduce the spatial isolation factor. In this situation, an increase in the relative fitness of F113 s on LDF plates compared with HDF plates would mainly be attributable to the strains' intrinsic kinetic parameters. The fitness values observed on HDF and LDF plates were not significantly different under these inoculation densities (0.39 SE 0.049 vs. 0.45 SE 0.008, respectively, $P > 0.05$) and hence indicate that, for our pair of strains, this mechanism plays at most a modest role. Therefore, it can be concluded that the fitness gain realized by F113 DsRed on LDF plates as presented in Table 2 is primarily due to the spatial isolation between F113 and KT2440 colonies which is maintained longer on LDF than on HDF plates.

Conclusion

The work presented here demonstrates in controlled experimental circumstances that a lower diffusive flux of substrate increases the relative fitness of a slow-growing bacterial strain competing with a relatively faster-growing strain within a resource domain. For the competing strains prescribed for this work, this effect was attributed primarily to the longer spatial isolation phase promoted by low diffusive flux of substrate. This mechanism, which has been proposed in the context of plant competition (Huston & DeAngelis, 1994), acts irrespective of the intrinsic kinetic parameters of the strains but only if the competitors are spatially separated (i.e. occupy different soil microhabitats). In addition to this diffusive spatial isolation mechanism, low diffusive fluxes of substrate may increase the fitness of the strain that has the smallest half-saturation coefficient, possibly even resulting in an inversion of the dominance relationship. The use of a simplified experimental system allowed us to rule out the confounding effects associated with the use of soil microcosms, such as the multiplicity of potential nutrients sources or the hydrological couplings. Moreover, the interaction between the strains can be deemed to be solely competitive, thanks to the use of two well-studied strains belonging to closely related genera, growing on benzoate as the sole usable carbon source, which prevented metabolic crosstalk. The strain coexistence under low diffusive flux of substrate demonstrated here constitutes a potential explanation for the noncompetitive diversity pattern observed in unsaturated soils by Zhou *et al.* (2002, 2004). The effect of slower diffusion of solute is likely to extend to other forms of bacterial interactions, and impacting upon bacterial diversity. For example, Allison (2005) showed, using a modeling approach, that the benefit of producing extracellular enzymes to acquire carbon or nutrients when competing with 'cheaters' that do not produce exo-enzymes was higher under slow diffusion conditions. Slow diffusion of solute will also have an impact on interactions involving antibiotic-producing strains, because the spatial range of action of these molecules and the range of bacterial dispersion define the relative fitness of resistant, sensitive, and antibiotic-producing strains (Koch, 1999; Wiener, 2000; Kerr *et al.*, 2002). Further exploration of the role of slow diffusion of solutes on the shaping of microbial diversity patterns is of great importance because conditions conducive to low diffusive fluxes prevail not only in unsaturated soils but also in other environments such as food products (Stecchini *et al.*, 1998; Dens & Van Impe, 2000) or the permafrost (Rivkina *et al.*, 2000).

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