RESEARCH ARTICLE

An individual-based approach to explain plasmid invasion in bacterial populations

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Abstract

We present an individual-based experimental framework to identify and estimate the main parameters governing bacterial conjugation at the individual cell scale. From this analysis, we have established that transient periods of unregulated plasmid transfer within newly formed transconjugant cells, together with contact mechanics arising from cellular growth and division, are the two main processes determining the emergent inability of the pWW0 TOL plasmid to fully invade spatially structured *Pseudomonas putida* populations. We have also shown that pWW0 conjugation occurs mainly at advanced stages of the growth cycle and that nongrowing cells, even when exposed to high nutrient concentrations, do not display conjugal activity. These results do not support previous hypotheses relating conjugation decay in the deeper cell layers of bacterial biofilms to nutrient depletion and low physiological activity. We observe, however, that transient periods of elevated plasmid transfer in newly formed transconjugant cells are offset by unfavorable cell-to-cell contact mechanics, which ultimately precludes the pWWO TOL plasmid from fully invading tightly packed multicellular *P. putida* populations such as microcolonies and biofilms.

Introduction

Vertical gene transfer among bacteria involves the inheritance of a copy of the parental cell's genetic material by the offspring after cell division. Horizontal gene transfer (HGT) processes, by contrast, move genetic material between cells, independent of cell division. Mobile genetic elements play an important role in the evolution and rapid adaptation of prokaryotes by conferring adaptive traits to their hosts. Among the different existing HGT mechanisms, conjugation (the transfer of DNA between bacterial cells after cell-to-cell contact) is considered to be a major pathway of lateral gene transfer between bacteria. The process and rates of conjugation affect potential beneficial applications such as the conveyance of xenobiotic degradation abilities in the bioremediation of organic compounds or metal tolerance in engineered reductive precipitation. Understanding the kinetics of conjugation is crucial to quantify these impacts, to estimate the related risks and to provide new insights into the evolution of microorganisms (Birge, 1994; Maloy *et al.*, 1994; Davison, 1999).

During the last two decades, much experimental and theoretical effort has gone into obtaining better knowledge about the processes and factors that govern conjugation. Plasmid conjugation rates are reported as being positively affected by many biotic and abiotic factors such as bacterial cell density (Normander *et al.*, 1998), cell metabolic activity (Smets *et al.*, 1993), plasmid donor and recipient relatedness (Majewski, 2001), nutrient availability (Fox *et al.*, 2008) or the spatial architecture of the bacterial community (Molin & Tolker-Nielsen, 2003). However, the underlying regulatory mechanisms explaining these effects have remained elusive to date.

One well-studied example that illustrates the degree of complexity often attained by plasmid transfer regulatory systems is the TOL plasmid pWW0, a 116 580 bp plasmid belonging to the *Pseudomonas* incompatibility group IncP-9 (White & Dunn, 1978), which confers the ability to biodegrade simple monocyclic aromatic compounds (Williams & Murray, 1974; Mosqueda *et al.*, 1999; Greated *et al.*, 2004). The observed continuous expression of the transfer genes

led researchers to consider pWW0 constitutively derepressed for transfer (Bradley & Williams, 1982; Park *et al.*, 2003). Later, Lambertsen *et al.* (2004) obtained similar results for conjugal gene expression in pWW0, but they demonstrated the existence of an autogeneous regulatory mechanism controlling pWW0 transfer by the inactivation of the *tra* and *mpfR* genes, which are involved in plasmid transfer and mating pair formation, respectively. The authors propose that the *mpfr* gene product, Mpfr, controls the expression of the transfer operons (*mpf*) via a negative control on transcription from the promoter regions *mpfRp1* and *mpfRp2*. Under steady-state conditions, low levels of transfer operon expression would then be maintained. However, it can be surmised that the expression of transfer genes and conjugal pili synthesis could be drastically enhanced immediately after plasmid entry into a recipient cell. However, once the plasmid establishes, the presence of MpfR would again regulate both transfer gene expression and conjugal pilus synthesis. This regulation mechanism would presumably aim to reduce the metabolic burden and the risks of male-type specific phage infection that could be caused by plasmid gene expression and conjugal pilus synthesis at high levels (Thomas, 2006). The apparent tight control of transfer gene expression achieved in this system does not cause low plasmid invasiveness: pWW0 has been observed to attain transfer frequencies as high as one transconjugant per donor under optimal conditions (Ramos *et al.*, 1997). Hence, pWW0 could be considered as a very transfer-proficient conjugative plasmid. Surprisingly, previous studies have observed that pWW0 is unable to fully invade colonies (Christensen *et al.*, 1996) and biofilms (Christensen *et al.*, 1998) via conjugation. This observed conjugation decay in the deeper cell layers of tightly packed bacterial structures has been proposed to be related to nutrient and oxygen depletion, low metabolic activity and low transfer gene expression levels. However, it has also been shown that even starved cells may perform plasmid transfer and that above a certain threshold activity, the level of conjugation is independent of metabolic activity (Normander *et al.*, 1998; Hausner & Wuertz, 1999). Therefore, the reasons why a conjugal plasmid cannot fully invade a receptive bacterial population remain unclear.

In a first attempt to overcome experimental limitations, Stewart & Levin (1977) applied a mathematical approach to describe plasmid fate in a bacterial population under different conditions. This model correctly describes observed plasmid transfer in active planktonic cells, and it has been used widely to compute conjugation rates in various other settings such as in rhizosphere and phyllosphere environments (Knudsen *et al.*, 1988), in liquid cultures (Simonsen, 1991) and in biofilms (Smets *et al.*, 1994; Beaudoin *et al.*, 1998). However, in spatially structured environments, perfect cell mixing cannot be assumed. Therefore, mass-action models fail when applied to surface-associated populations (Arango Pinedo & Smets, 2005; Krone *et al.*, 2007). Furthermore, plasmids such as those belonging to the IncP-9 incompatibility group are reported to transfer more efficiently on solid surfaces than in liquid, due to the nature of the rigid pili they encode (Bradley, 1983). In addition, the Stewart & Levin model does not include any delays or lags, which are often observed experimentally (Massoudieh *et al.*, 2007). This oversimplification has a drastically negative impact on the ability of a model to predict the plasmid fate within a bacterial population. For these reasons, it is not possible to simply extrapolate the conclusions obtained through mass-action models to plasmid spread in spatially structured populations.

As suggested previously (Gilmour *et al.*, 2001; Sorensen *et al.*, 2005; Hellweger & Bucci, 2009), individual-based modeling (IbM) (Kreft, 2004; Picioreanu *et al.*, 2004) appears to be the most appropriate framework for modeling gene transfer by conjugation, as this is a discrete event between individual cells that form a mating pair. In addition, this methodology presents multiple advantages: it allows to capture the intrapopulation variability, to track the changes that may occur during the mating process (e.g. the relative position of transconjugants with respect to recipients), to link the mechanisms involved at the individual cell level to the emerging population behavior and to capture the effect of spatially structured habitats (such as biofilms and microcolonies). The most relevant parameters to describe a conjugation event at the individual cell level in a structured environment are: (1) the conjugation rate (Stewart & Levin, 1977; Simonsen, 1990; Smets *et al.*, 1994); (2) the donor–recipient distance (Gregory *et al.*, 2008); and (3) the lag times between plasmid receipt and plasmid transfer (Massoudieh *et al.*, 2007). However, the parameter estimates available to date derive from population-averaged instead of individual-based observations, which makes it very difficult in practice to apply IbM to the study of bacterial conjugation. The fast development of individual-based observation technology (Brehm-Stecher & Johnson, 2004) now provides the opportunity to create a specifically designed experimental framework focusing on the study of HGT at the individual cell scale.

The main objective of this study is to provide an experimental framework specifically designed to obtain accurate estimates of the core parameters describing conjugal plasmid transfer at the individual cell level. In addition, using the proposed methodology, we explore from an individual-based perspective the process behind the apparent inability of plasmids to completely invade surface-associated bacterial populations (biofilms). To this end, we have used pWW0 (an archetypical TOL plasmid) as a model plasmid (Williams & Murray, 1974; Bradley & Williams, 1982). Our results suggest that transient periods of increased transfer

rates by newly formed transconjugant cells, and cell-to-cell contact mechanics that arise from cellular growth and division are the main processes supporting pWW0 invasion in a bacterial population.

Materials and methods

Bacterial strains and conjugal plasmid

The plasmid donor strain was kindly provided by Lars Molbak (Molbak *et al.*, 2003). Briefly, *Pseudomonas putida* KT2442 (Bagdasarian *et al.*, 1981) was chromosomally tagged with *lacIq* and a constitutively expressed *dsRed* gene fused to an *rrnB*P1 promoter on a mini Tn5 cassette (Tolker-Nielsen *et al.*, 2000). The plasmid donor strain is resistant to Rifampicillin and Kanamycin. *Pseudomonas putida* KT2440 (Bagdasarian *et al.*, 1981) chromosomally tagged with *Plac*::yfp on a miniTn7 cassette (a gift from Fatima Yousef) served as the recipient strain.

Our model plasmid, pWW0, had been tagged with a mini-Tn5 insertion of a *gfpmut3b* gene downstream of a synthetic LacI-repressible $P_{A1/O4/O3}$ promoter and a kanamycin resistance *npt* gene following established procedures without significantly modifying its transfer rate (Christensen *et al.*, 1998; Normander *et al.*, 1998) probably because the mini-Tn5 was inserted outside of any region regulating pWW0 transfer (Lambertsen *et al.*, 2004). The expression of the *gfp* gene was repressed in plasmid donor cells, but restored upon transfer to a recipient cell.

Growth conditions

Both donor and recipient strains were tested for transfer in minimal AB medium (Clark & Maaløe, 1967) either without an added carbon source, with *m*-toluic acid (AB*m*-Tol, 5 mM) or with citrate (ABC, 1 or 10 mM) and supplemented with agar (15 g L^{-1}) (Table 1).

Conjugation experiments on filters

Donor and recipient cells were harvested from 1 mL overnight cultures in AB*m*-tol and ABC media, respectively. The OD of the cell suspensions was adjusted to the desired values, and the suspensions were combined to yield the desired initial donor–recipient ratio and total density. To estimate the mean intercellular distance (d_{cc}, μm) as a function of cell density, we have deduced the following relation between cell–cell distance and the cell area density (ρ, μm^2 per cell) (see Supporting Information for further details):

$$d_{cc} = \sqrt{\frac{2}{\sqrt{3}}\frac{1}{\rho}} \cong 1.075\sqrt{\frac{1}{\rho}}$$

Table 1. Experimental framework proposed for the quantification of bacterial conjugation parameters at the individual cell level

Method	Parameter targeted	Unit	Growth conditions*
Filter mating	Conjugation rate†	% h^{-1}‡	AB (no growth) AB*m*-toluic acid (5 mM) ABC (0.5;1;10 mM)
	Cell-to-cell distance	μm	AB (no growth)
Perfusion chamber	Conjugation time lag	h	ABC (1 mM)
	Orientation	–	ABC (1 mM)
	Donor–recipient contact	μm	ABC (1 mM)
	Recipient cells elongation	%	ABC (1 mM)

*ABC and AB *m*-toluic media composition are described in detail in Materials and methods.

†Conjugation rate is measured as the ratio of donor and transconjugants' cell contacts over donor and recipient cell contacts per unit time, hence unbiased by cell growth and retransfer.

‡During filter matings, cells were immobile.

For each donor : recipient initial ratio, 100 μL of the cell suspension was mixed with 4.9 mL of phosphate-buffered saline (PBS) [for 1 L: 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate dibasic (Na_2HPO_4) and 2.4 g potassium phosphate monobasic (KH_2PO_4) to 1 L dH_2O, pH adjusted to 7.4]. After 2 min of vortexing at the maximal speed, the suspensions were filtered onto 0.2 μm pore-size black filters (Pall Corporation), which were then placed on plates containing the desired media for incubation at 30 °C. The mating occurred on the filter surface. At each time point, three replicate filters for each donor–recipient ratio were stored at 4 °C for 2 days before observation to allow for the full maturation of fluorescent proteins. Images were obtained over six randomly chosen positions for every filter using a confocal laser scanning microscope (CLSM) (TC-SP5, Leica, Germany).

Real-time visualization of HGT

Cells were grown in a perfusion chamber as described earlier (Reinhard & Van der Meer, 2009). Briefly, after cells were grown overnight, 1-mL aliquots were collected, pelleted and resuspended in PBS. A 10-μL sample was pipetted onto a 100-μL agarose slab (ABC 0.5 mM medium, 1.5% agarose), which was placed inside the perfusion chamber with the surface containing the cells directly against the glass cover of the chamber. Once the chamber closed, positions along the agarose slab were randomly selected for automatic image capture as described below. For substrate addition, the perfusion chamber was opened and a 10 μL droplet of a 10 mM carbon substrate solution was added on the top of the agarose slab. This operation did not disturb the spatial organization of the microcolonies.

In order to determine the contribution of cell elongation to conjugation, perfusion chamber experiments were run under a moderate nutrient concentration (ABC 1 mM) and in the presence or absence of the β-lactam mecillinam (200 μg mL^{-1}, Sigma-Aldrich). Successful mating pair frequencies were determined after 4 h of incubation at 30 °C.

Microscopy and image analysis

Image acquisition was performed using CLSM (TC-SP5 Leica) to visualize cells expressing green, yellow and red fluorescent proteins. A × 63 oil immersion lens with a numerical aperture of 1.4 was used for obtaining all images. The fluorescence emitted from recipient cells [i.e. yellow fluorescent protein (YFP)], transconjugant cells [i.e. green fluorescent protein (GFP)] and donor cells (i.e. DsRed) was detected in fluorescent channels PMT1 (408–450 nm), PMT2 (519–543 nm.) and PMT3 (585–677 nm), respectively.

The fraction of successful mating pairs in the filter experiments was determined by image analysis using custom-built macros in IMAGE PRO PLUS, version 5.1 (Media-Cibernetics). To derive this fraction, the surface occupied by successful donors (defined as donor cells situated within < 1.1 μm distance of a transconjugant cell) was divided by the surface occupied by donors potentially engaged in a mating pair (defined as donors situated at < 1.1 μm of a recipient). In this way, donor–recipient cell growth and retransfer from transconjugant cells were automatically removed from the image analysis, allowing us to obtain unbiased estimates of the conjugation frequency of the original donors.

In vivo HGT visualization was achieved by combining the multiple position marker function with the autofocus and time-lapse functions of the LAS AF 1.7.0 software (Leica). Images obtained in this way were analyzed using IMAGEJ FREEWARE (NIH) and LAS AF LITE (Leica) as follows: time series obtained from the automatic image capture were scanned visually for the appearance of a first transconjugant at the donor–recipient interface. Once detected, both the donor and the recipient cells involved were backtracked from their appearance until the occurrence of conjugation by manually outlining their cell wall on every image. Objects obtained in this way were analyzed for the length, orientation and distance between cells using the IMAGEJ plug-in ROI manager (NIH).

Statistical analysis

We compared the frequencies obtained in different analysis using Pearson's χ^2 test with Yates' continuity correction. Acceptance or rejection of the null hypothesis (i.e. that the successful mating frequencies are independent of the cells orientation) was based on a significance level (α) of 0.05.

Results and discussion

Direct visualization of HGT at the individual cell level

Matings were carried out in a perfusion chamber (Reinhard & Van der Meer, 2009) containing an agarose slab inoculated with cells on its surface facing the glass cover of the chamber. Both donor and recipient cells were easily detected, and transconjugants were easily identifiable (Fig. 1). A successful mating pair was defined as a single transconjugant cell (which expresses simultaneously GFP from the plasmid and YFP from the chromosome) and its closest donor (expressing DsRed from the chromosome). When the donor and recipient microcolonies entered in contact, highly transfer-efficient transconjugants were formed at the interface. Consequently, the original donors were no longer in contact with recipient cells, but these transconjugants transferred the plasmid to the adjacent recipients, leading to a rapidly spreading front of plasmid transfer through the recipient population (Video S1).

Estimating the effects of donor–recipient distance and relative orientation on conjugation

Images of successful mating pairs were collected on filter-grown populations inoculated with different initial donor–recipient ratios as a way of controlling the initial donor–recipient distance (see Supporting Information for further details). We visually confirmed that the cells remained immobile on filters before analysis. Images of 855 successful mating pairs found on filters in the absence of an added carbon source were collected. In 91.1% of the cases, successful mating pairs occurred through direct cell-to-cell contact (0–1 μm range). Only 8.6% were in a range of 1–5 μm and 0.2% were beyond this range (5–10 μm). Therefore, our results support the notion that direct cell-to-cell contact is not essential to conjugation and that pWW0 transfer may occur between two distant cells by conjugational type IV pili (Bradley, 1983), although at a comparatively low frequency. This is in accordance with studies in *Escherichia coli* that have shown that gene transfer may occur between cells up to 12 μm apart (Harrington & Rogerson, 1990; Babic *et al.*, 2008; Shu *et al.*, 2008). However, it is not well established whether the pilus is used to transfer genetic material (Harrington & Rogerson, 1990; Babic *et al.*, 2008) or just to pull recipients into the proximity of the donor cells before a separate conjugative junction is formed by the fusion of a portion of the cell membranes (Panicker & Minkley, 1985).

In vivo visualization of 34 mating pair formation events also allowed us to clarify the role of conjugative pili in the case of pWW0 transfer. In all cases, cell-to-cell contact arose randomly from microcolony morphogenesis and no occurrence of pulling action (by conjugal pili) was observed,

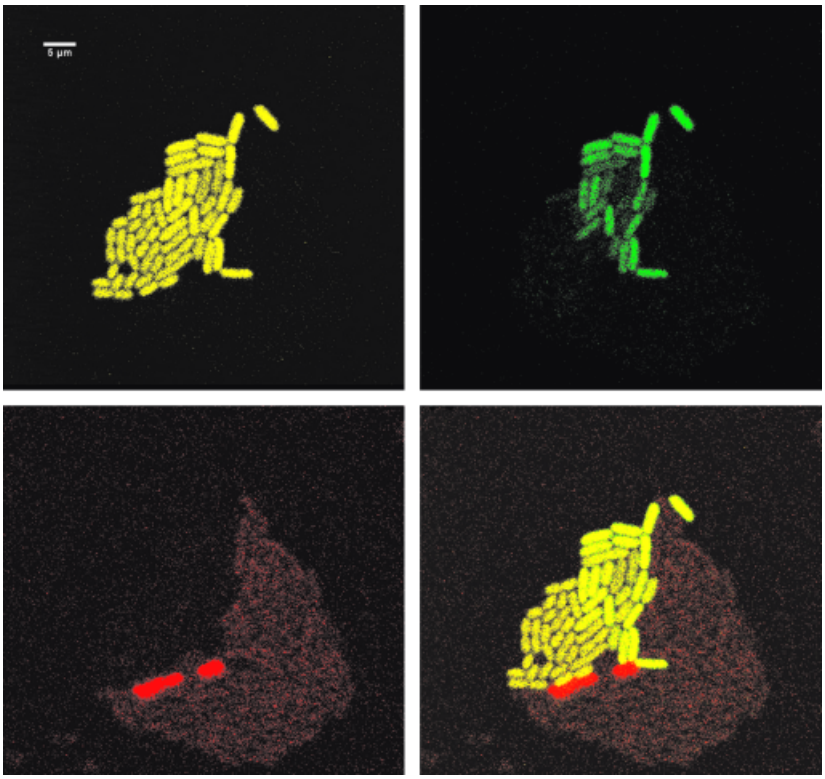

Fig. 1. *In situ* visualization of conjugation within a microcolony where donor *Pseudomonas putida* KT2440 cells expressing DsRed and LacIq (bottom, left channel, red) transfer pWW0 TOL::GFP (top right channel, green) to recipient *P. putida* KT2440 cells expressing YFP (top left channel, yellow). The lower-right image displays the overlay of all these fluorescence images. pWW0 was tagged with the *gfp* gene controlled by a lac promoter, which is downregulated in the donor cell by a chromosomal repressor (*lacIq*). Nondividing inoculated donors present higher red intensities due to previous DsRed maturation. See Video S1 for time-lapse visualization of plasmid spreading within the microcolony.

Table 2. Surface of contact and relative donor–recipient orientation frequencies observed at the moment of transfer

Case	Orientation*	Surface contact (µm)†	Successful pairs (%)†	Regular pairs (%)†
1		1.1 ± 0.27	24	13
2		1.7 ± 0.28	61.6	41
3		–	13.6	32
4		1.1 ± 0.12	0.79	13

*The relative orientation ($n = 380$) of donor (black cell) and recipient (white cell) and the surface of contact ($n = 36$) at the moment of transfer were analyzed. Regular nonconjugative pairs were also analyzed for relative orientation ($n = 107$).

†Means (± SE) and orientation frequencies (%) are given.

suggesting that cell-to-cell contact mechanics arising during colony morphogenesis affects plasmid invasion between and within microcolonies.

In addition, we have also checked the effect of the relative orientation of donor cells vis-a-vis recipient cells during plasmid transfer on filters. We have determined the frequency of occurrence of four orientation classes (Lawley *et al.*, 2002) for pairs that have been engaged in a successful mating (Table 2). Because all four classes were represented, our results suggest that pWW0 plasmid junctions may appear at any point of contact on the surface of donors and that DNA can be transferred to any available location along the recipient membrane. Similar observations were made for plasmids R751 (Lawley *et al.*, 2002) and RP4 (Samuels *et al.*, 2000) in *E. coli*. In addition, they support previous observations showing that proteins involved in DNA transfer are localized nonspecifically throughout the perimeter of the cell membrane (Gilmour *et al.*, 2001). However, orientation effects on plasmid transfer are highly significant (P-value < 0.0001), some orientations being more favorable than others. Indeed, while orientations 1 and 4 (recipient pole vs. donor lateral wall and vice versa) should have the same probability to occur, orientation 1 is over-represented within the successful mating pairs (Table 2). This indicates that conjugation is more likely to occur through the lateral wall of the donor than through the poles, which is consistent with previous observations showing that plasmids are situated preferentially at the characteristic center or quarter cell position in the cell and not in the poles (Lawley *et al.*, 2002). However, the differences obtained when the donor cell's pole is attached to the recipient (orientation 3 vs. 4) were unexpected and will need further research to be explained. These studies become particularly interesting when related to bacterial colony morphogenesis: previous biophysics work (Cho *et al.*, 2007; Volfson *et al.*, 2008) applied to two-dimensional microcolonies growing in chambers have suggested that cell growth and colony

expansion trigger the formation of the orientational (nematic) order in *E. coli* microcolonies after a few generations, maximizing the surface of contact between the lateral wall of cells and hence between donors and recipients at the transfer interface region. Therefore, the 'cell-to-cell contact mechanics' arising from cellular growth can be interpreted as a process maximizing the probability of conjugation during the early stages of microcolony formation.

Conjugation rate measurements at the individual cell level and its dependence on nutrient availability

Given the previous results, we assume that direct contact is the preferred condition for conjugal pWW0 transfer. Therefore, the results we present next refer only to the number of donors in contact with recipients that transfer at a certain moment of time over the total number of donors in direct contact with recipient cells (Fig. 2). In other words, donor–recipient cell growth and retransfer from transconjugant cells are automatically removed from the image analysis as described in Materials and methods, which allows us to obtain unbiased estimates of the conjugation frequency of the original donors. The effect of nutrient availability was analyzed by comparing the mating frequencies on media containing different concentrations of citrate or *m*-toluic acid. As shown in Fig. 2, conjugation can occur even in the absence of an added carbon source, but at a very low rate (maximum 10% of the donors have transferred the plasmid after 24 h). The conjugation rate in situations where nutrients were available for both donors and recipients (ABC) was higher than that observed when a carbon source specific to the donors was used (AB*m*-tol). Similarly, pWW0 transfer occurred at a higher rate when the initial concentration of nutrients was increased 10-fold. These results suggest that a high concentration of nutrients available for both donors and recipients enhances the conjugation rates significantly. However, under all tested conditions, plasmid transfer from the original donors stopped 4–5 h after the start of the experiment because they became separated from the recipients by newly formed transconjugant cells, which, in turn, became the main active donors. These observations are in agreement with previous findings (Fox *et al.*, 2008), which, however, relied on transconjugant cultivation and, therefore, did not distinguish transfer events from post-transfer selection (clonal growth of newly formed transconjugant cells). Theoretically, the most accurate metrics to report plasmid transfer efficiency are given by the number of transfer events per donor–recipient encounters (Sorensen *et al.*, 2005). The main advantage of the method presented here relies in its ability to generate this metric as an output, and hence, to produce more accurate estimates of the conjugal ability of a given plasmid.

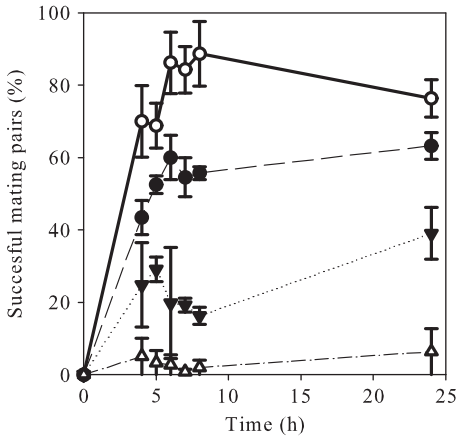

Fig. 2. Changes over time in the number of successful pWW0 TOL::GFP transfers in filter matings between *Pseudomonas putida* KT 2440 strains for various medium compositions: AB (△), AB *m*-tol 0.5 mM (▼), ABC 1 mM (●) and ABC 10 mM (○).

Elongation phase and conjugation

In order to investigate whether conjugation occurs at a specific phase of the recipient cell growth cycle, time-lapse observations were conducted on cells growing on a nutrient agarose slab. Thirty-five successful mating events were selected for image analysis: individual recipient/transconjugant cell length was measured during the entire growth cycle until division was attained and two transconjugant daughter cells were formed (Fig. 3). GFP expression was not observed

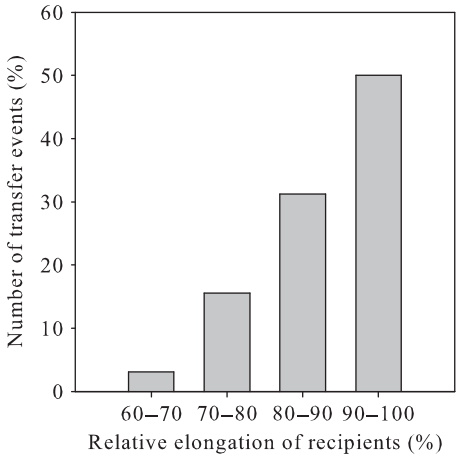

Fig. 3. Degree of elongation of *Pseudomonas putida* KT2440 recipient cells (calculated as the ratio of the cell length to its maximal length, attained immediately before septum appearance and division) at the moment when their GFP expression (from a newly received pWW0 TOL::GFP) was first detected (i.e. detection as transconjugants); 96.9% of the cells were over 70% of their relative division length at the moment of transfer detection. The cell duplication time was estimated to be around 100 min and the GFP minimal maturation time before signal detection was considered to be around 10 min. Time-lapse resolution = 20 min; transfer events considered = 34.

before the new transconjugant cells reached an elongation of 60–70% compared with their maximal length (length attained immediately before septum appearance and division), and in 75% of cases, the cells exceeded 80% elongation. No successful mating pair was detected that comprised recipient cells that did not divide shortly after transfer. These results suggest that recipients are more susceptible to receive the plasmid at advanced stages of cell growth cycle. Similar observations have been obtained previously with *Pseudomonas areuginosa* (Ghigo, 2001) and implemented successfully in theoretical studies (Gregory *et al.*, 2008). In addition, we have determined the contribution of cell elongation comparing successful mating pair frequencies obtained after 4 h in a perfusion chamber in the presence/absence of the β-lactam mecillinam (200 mg L^{-1}). This antibiotic was chosen because while the growth of *P. areuginosa* has been reported to be unaffected by concentrations as high as 400 mg L^{-1} (Noguchi *et al.*, 1979; Eng *et al.*, 1988), it inhibits lateral wall elongation, resulting in a morphological change from rod-shaped to coccoid cells (Supporting Information, Fig. S1). This allowed us to obtain a dividing, but not elongating population of spherical cells within our perfusion chambers. The antibiotic effect on the successful mating pair frequency was found to be highly significant (*P*-value < 0.0001), decreasing from 57% [control without antibiotic ($n = 578$)] to 29% in the presence of the antibiotic ($n = 221$). The surface of contact between coccoid mating partners was estimated to be 0.74 ± 0.14 μm ($n = 100$), a value not significantly different from the values found for 'normal' rod-shaped mating pairs oriented perpendicular with the pole of the recipient against the lateral wall of the donor (1.1 ± 0.27), which also showed very similar success rates (24%, Table 2). Therefore, these results suggest that the probability of the occurrence of a successful conjugation event is positively correlated with the surface of contact within the mating pair.

Quantifying conjugational lag times at the individual cell scale and its effects on plasmid invasion kinetics within a microcolony

Two different time-lapse experiments were carried out in a perfusion chamber (Fig. 4). First, plasmid transfer events between individual cells growing on AB-*m*-tol plus carbon traces were followed, and measurements were made of the time period between the acquisition of the plasmid by a recipient and its subsequent transfer to another recipient (t_{lag}). We consider three types of t_{lag} based on which a cell acted as a donor (Fig. 5): t_{lagD}, the time for an original donor to transfer after contact with a recipient; t_{lagT1}, the time for

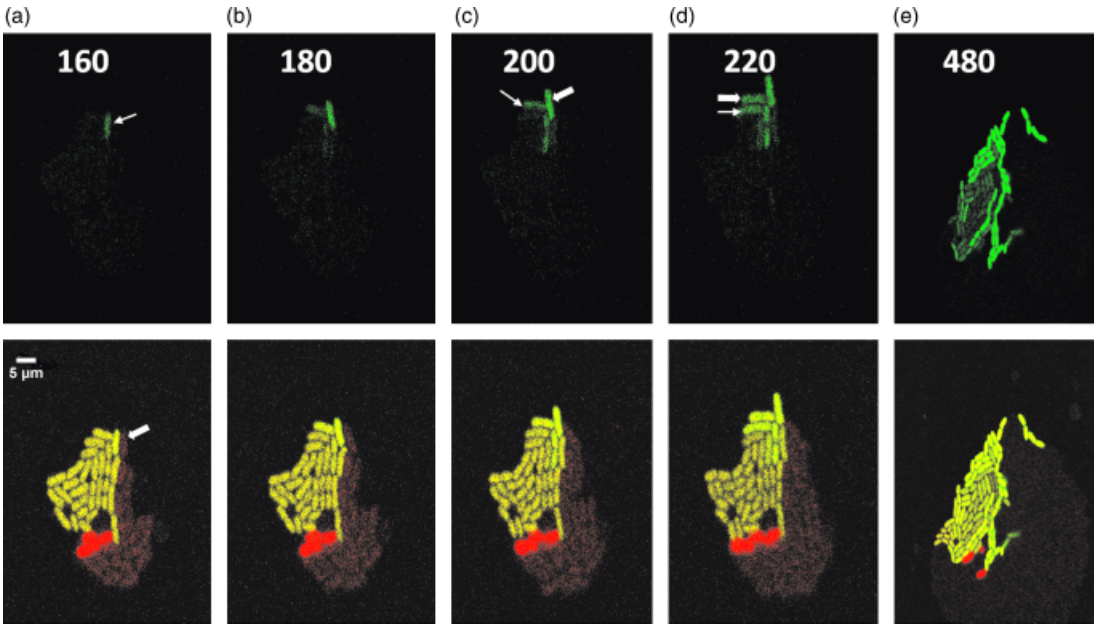

Fig. 4. Time-lapse CLSM images of a microcolony where donor *Pseudomonas putida* KT2440 cells expressing DsRed and LacIq are transferring pWW0 TOL : : GFP to recipient *P. putida* KT2440 cells expressing YFP. Transconjugants are simultaneously expressing GFP and YFP. The GFP signal (transconjugant cells) and the corresponding overlay of all fluorescence signals (all cell types) are displayed on the top and on the bottom channels, respectively. Nondividing inoculated donors have higher red intensities due to previous DsRed maturation. Thick arrows mark the individual cell transferring the plasmid while thin arrows indicate the resulting new transconjugant cell. The cells were inoculated on a nutrient agar slab, and images were taking every 20 min. After 160 min of donor–recipient contact, conjugative transfer was detected (a). It then took < 40 min for this transconjugant to retransfer twice (b, c) and < 20 min for the new transconjugants to retransfer again (d). After 480 min (approximately five division cycles), most of the recipients in the microcolony contained the plasmid (e, see also Video S1).

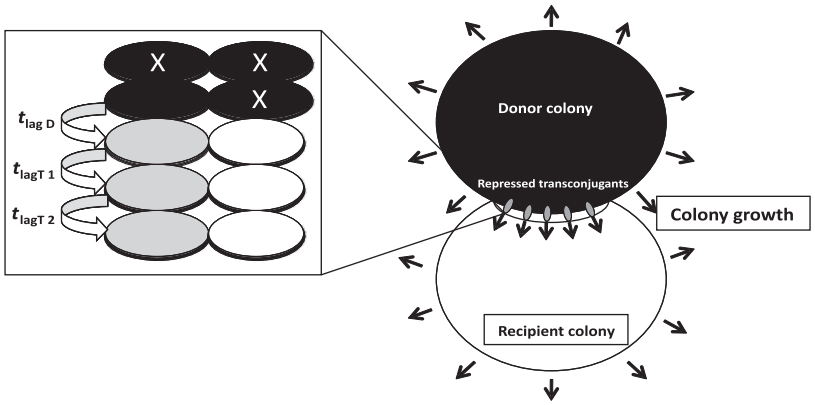

Fig. 5. Conceptual model for plasmid transfer on surfaces at the individual cell level. When a donor (black) and a recipient (white) colony meet, a transfer interface area is formed (modified after Simonsen, 1990). Incompatible cells for transfer are labeled with a cross (other donors). After a certain period of donor–recipient contact (t_{lagD}), a donor cell transfers the plasmid to a recipient cell. The new transconjugant cells formed (gray) can retransfer after a shorter transconjugant–recipient contact time (t_{lagT1}), leading to the appearance of a second transconjugant that can retransfer even faster (t_{lagT2}). Transfer stops when high cell density precludes cell growth and division.

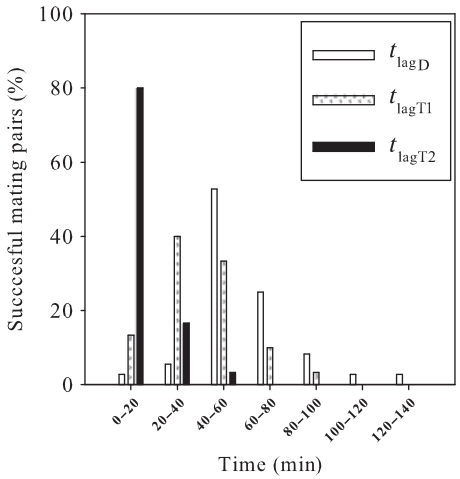

Fig. 6. Evolution of the conjugal lag times as an indirect method to evaluate plasmid transfer regulation impacts on plasmid invasion. $t_{lagD} > t_{lagT1} > t_{lagT2}$ with t_{lagD}, the time that a donor needs to make a transfer when in contact with a recipient; t_{lagT1}, the delay time between when the new transconjugant originated after t_{lagD} has first received a plasmid and when it is able to transfer the plasmid to other cells; and t_{lagT2}, the time that the transconjugant created after t_{lagT1} requires to make further transfers itself. $n = 30$ transfer events. All time lags include a GFP minimal maturation time before detection, which was considered to be around 10 min.

this newly formed transconjugant to transfer to an adjacent recipient.; and t_{lagT2}, the time for a subsequent transfer to the next row of recipient cells. Our results (Fig. 6) show that t_{lagT2} is significantly shorter than t_{lagT1}, which, in turn, is significantly shorter than t_{lagD}. These results indicate that t_{lag} decreases as the plasmid progresses towards the interior of the recipient microcolony: this phenomenon increases the plasmid spread significantly.

This observation is not consistent with a constant lag value for all newly formed transconjugants, as assumed typically in theoretical studies on plasmid conjugation that have included lag values (Massoudieh *et al.*, 2007; Gregory *et al.*, 2008). After only two retransfer events, these lag times decrease under the 20 min time step resolution of this study. Considering that the minimal maturation time for GFP detection is as low as 10 min (Reischer *et al.*, 2004), plasmid transfer from newly formed transconjugant cells appears to be very fast and occurring within the range of minutes. To the best of our knowledge, there is only one previous study estimating conjugation lag times on solid surfaces (Lagido *et al.*, 2003). In that study, using *Pseudomonas fluorescens* and RP4 as model bacterial strains and plasmid, the minimum time to detect a transfer event to occur and be detected was estimated at 3.5–4.0 min and the lag period between two rounds of transfer from the same donor was found to range from 5 to 30 min. In addition, we have shown experimentally that the lag times needed for newly formed transconjugant cells to transfer pWW0 are significantly shorter than in the case of initial donor cells. These results are consistent with the work by Lambertsen *et al.* (2004), who provided strong evidence that pWW0 transfer is autogenously regulated through a negative feedback mechanism. According to this work, most of the initial donors in the system are likely fertile, although conjugative transfer genes are expressed at minimal levels. As pWW0 is a low-copy plasmid (Duetz & Van Andel, 1991) and in view of the low expression of transfer genes levels, we would expect a single copy of pWW0 to be transferred to the transconjugant cells formed after t_{lagD}. However, the absence of transfer regulation gene products at the moment of pWW0 entry into the newly formed transconjugant cells would allow the full expression of the strong promoters regulating both *Tra* and *Mpf* operons for a short period. Therefore, multiple plasmid replications could be initiated, which would result in t_{lagT1} shorter than t_{lagD}. We speculate that after t_{lagT1} several plasmid copies could be transferred in a very short period to a single transconjugant cell via independent simultaneously formed mating pores. Then, the concentration of *Tra* and *Mpf* expression products needed to trigger transfer

could be attained even faster due to their increased gene copy number. Hence, t_{lagT2} would be reduced further compared with t_{lagT1}.

However, plasmid invasion stopped as soon as the recipient microcolony stopped dividing. This usually occurred when the recipient microcolony became a continuous monolayer of cells without free inner spaces (Video S2). In order to determine whether conjugation stopped due to nutrient depletion inside the agarose slab, 24 h time-lapse experiments were run. After this period, nutrients were replenished and the transconjugant cell clusters directly in contact with recipients were followed. Recipient microcolonies at the periphery of the inoculation zone rapidly resumed growth. In more central areas of the filter (but $< 50\,\mu m$ distance from the periphery), neither growth nor new conjugation events were detected, but reductions in cell size and changes in cell shape (which became more rounded) could be observed (Video S2). These observations suggest that cells within the densely populated central parts of the colony were under mechanical pressure from the actively growing peripheral cells, effectively preventing their elongation and division. Considering the almost complete absence of transfer under nongrowing conditions shown in Fig. 2, the conjugation dependence on the donor–recipient contact surface and orientation discussed previously and the advanced stages of the cell cycle at which transfer has been observed to occur (Fig. 3), we suggest that individual cell elongation during bacterial growth is the main process facilitating plasmid spreading through bacterial populations. Although the molecular mechanisms behind these observations are beyond the goal of our experiments, we hypothesize that the conjugation of pWW0 occurs through mating pores, such as those described previously for the RP4 plasmid in *E. coli* (Samuels *et al.*, 2000; Lawley *et al.*, 2002). It has been proposed that each donor cell contains several mating pores, each of which is potentially functional, but only become active when docked successfully on a recipient cell membrane (an essentially random event) (Lawley *et al.*, 2003). Based on this model, we suggest that cell elongation would facilitate conjugation not only by increasing the mating pair contact surface but also through the dynamic contact occurring between a mating pair during cell growth, which would highly increase the possibilities for at least one mating pore from the donor to dock successfully on the recipient cell and become viable. A complementary explanation could be that during growth, the loosening of the peptidoglycan matrix that forms the cell wall would facilitate the mating pore formation. In this sense, the mechanical interaction between neighboring cells [which has already been shown to affect the early stages of colony and biofilm formation (Cho *et al.*, 2007; Volfson *et al.*, 2008)], could also hinder cell elongation and division under high cell densities and therefore prevent successful contacts and mating pore formation between growing mating pairs. This could lead to a reduction or even the cessation of plasmid spread at advanced stages of multicellular population morphogenesis, as has been observed experimentally here and in previous studies (Christensen *et al.*, 1996, 1998).

In conclusion, we have provided a general experimental framework appropriate to identify and quantify the main parameters controlling bacterial conjugation at the individual cell level. We have used the proposed individual-based methodology to investigate the apparent inability of the pWW0 TOL plasmid to invade a *P. putida* population. Combining the different analyses presented in this work, we obtained accurate estimates of the targeted parameters (conjugation rate, donor–recipient distance and lag times between plasmid receipt and plasmid transfer) for the pWW0 plasmid. We have identified two key processes that control plasmid invasion in a bacterial population: transient periods of elevated plasmid transfer in newly formed transconjugant cells and cell-to-cell contact mechanics, which favor transfer in growing and incipient microcolonies, but limit transfer in spatially constrained populations.

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References

Arango Pinedo C & Smets BF (2005) Conjugal TOL transfer from *Pseudomonas putida* to *Pseudomonas aeruginosa*: effects of restriction proficiency, toxicant exposure, cell density ratios, and conjugation detection method on observed transfer efficiencies. *Appl Environ Microb* **71**: 51–57.

Babic A, Lindner AB, Vulic M, Stewart EJ & Radman M (2008) Direct visualization of horizontal gene transfer. *Science* **319**: 1533–1536.

Bagdasarian M, Lurz R, Ruckert B, Franklin FCH, Bagdasarian MM, Frey J & Timmis KN (1981) Specific-purpose plasmid cloning vectors. 2. Broad host range, high copy number, Rsf1010-derived vectors, and a host–vector system for gene cloning in *Pseudomonas sp. Gene* **16**: 237–247.

Beaudoin DL, Bryers JD, Cunningham AB & Peretti SW (1998) Mobilization of broad host range plasmid from *Pseudomonas putida* to established biofilm of *Bacillus azotoformans*. I. Experiments. *Biotechnol Bioeng* **57**: 272–279.

Birge EA (1994) *Bacterial and Bacteriophage Genetics*. Springer-Verlag, New York.

Bradley DE (1983) Specification of the conjugative pili and surface mating systems of *Pseudomonas* plasmids. *J Gen Microbiol* **129**: 2545–2556.

Bradley DE & Williams PA (1982) The TOL plasmid is naturally derepressed for transfer. *J Gen Microbiol* **128**: 3019–3024.

Brehm-Stecher BF & Johnson EA (2004) Single-cell microbiology: tools, technologies, and applications. *Microbiol Mol Biol R* **68**: 538–559.

Cho H, Jonsson H, Campbell K *et al.* (2007) Self-organization in high-density bacterial colonies: efficient crowd control. *PLoS Biol* **5**: e302.

Christensen BB, Sternberg C & Molin S (1996) Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (GFP) from *Aequorea victoria* as a marker. *Gene* **173**: 59–65.

Christensen BB, Sternberg C, Andersen JB, Eberl L, Moller S, Givskov M & Molin S (1998) Establishment of new genetic traits in a microbial biofilm community. *Appl Environ Microb* **64**: 2247–2255.

Clark DJ & Maaløe O (1967) DNA replication and division cycle in *Escherichia coli*. *J Mol Biol* **23**: 99–112.

Davison J (1999) Genetic exchange between bacteria in the environment. *Plasmid* **42**: 73–91.

Duetz WA & Van Andel JG (1991) Stability of tol plasmid Pww0 in *Pseudomonas putida* Mt-2 under nonselective conditions in continuous culture. *J Gen Microbiol* **137**: 1369–1374.

Elsas JD & Bailey MJ (2002) The ecology of transfer of mobile genetic elements. *FEMS Microbiol Ecol* **42**: 187–197.

Eng RHK, Liu R, Smith SM, Johnson ES & Cherubin CE (1988) Amdinocillin: interaction with other beta-lactam antibiotics for gram-negative bacteria. *Chemotherapy* **34**: 18–26.

Fox RE, Zhong X, Krone SM & Top EM (2008) Spatial structure and nutrients promote invasion of IncP-1 plasmids in bacterial populations. *ISME J* **2**: 1024–1039.

Ghigo J-M (2001) Natural conjugative plasmids induce bacterial biofilm development. *Nature* **412**: 442–445.

Gilmour MW, Lawley TD, Rooker MM, Newnham PJ & Taylor DE (2001) Cellular location and temperature-dependent assembly of IncHI1 plasmid R27-encoded TrhC-associated conjugative transfer protein complexes. *Mol Microbiol* **42**: 705–715.

Greated A, Lambertson L, Williams PA & Thomas CM (2004) Complete sequence analysis of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. *Environ Microbiol* **4**: 856–871.

Gregory R, Saunders JR & Saunders VA (2008) Rule-based modelling of conjugative plasmid transfer and incompatibility. *Biosystems* **91**: 201–215.

Harrington LC & Rogerson AC (1990) The F-pilus of *Escherichia coli* appears to support stable DNA transfer in the absence of wall-to-wall contact between cells. *J Bacteriol* **172**: 7263–7264.

Hausner M & Wuertz S (1999) High rates of conjugation in bacterial biofilms as determined by quantitative *in situ* analysis. *Appl Environ Microb* **65**: 3710–3713.

Hellweger FL & Bucci V (2009) A bunch of tiny individuals: individual-based modeling for microbes. *Ecol Model* **220**: 8–22.

Knudsen GR, Walter MV, Porteous LA, Prince VJ, Armstrong JL & Seidler RJ (1988) Predictive model of conjugative plasmid transfer in the rhizosphere and phyllosphere. *Appl Environ Microb* **54**: 343–347.

Kreft J-U (2004) Biofilms promote altruism. *Microbiology* **150**: 2751–2760.

Krone SM, Lu R, Fox R, Suzuki H & Top EM (2007) Modelling the spatial dynamics of plasmid transfer and persistence. *Microbiology* **153**: 2803–2816.

Lagido C, Wilson IJ, Glover LA & Prosser JI (2003) A model for bacterial conjugal gene transfer on solid surfaces. *FEMS Microbiol Ecol* **44**: 67–78.

Lambertsen LM, Molin S, Kroer N & Thomas CM (2004) Transcriptional regulation of pWW0 transfer genes in *Pseudomonas putida* KT2440. *Plasmid* **52**: 169–181.

Lawley TD, Gordon GS, Scott A, Wright D & Taylor DE (2002) Bacterial conjugative transfer: visualization of successful mating pairs and plasmid establishment in live *Escherichia coli*. *Mol Microbiol* **44**: 947–956.

Lawley TD, Gilmour MW, Gunton JE, Tracz DM & Taylor DE (2003) Functional and mutational analysis of conjugative transfer region 2 (Tra2) from the IncHI1 plasmid R27. *J Bacteriol* **185**: 581–591.

Majewski J (2001) Sexual isolation in bacteria. *FEMS Microbiol Lett* **199**: 161–169.

Maloy SR, Cronan JEJ & Freifelfer DL (1994) *Microbial Genetics*. Jones & Bartlett, Boston.

Massoudieh A, Mathew A, Lambertini E, Nelson KE & Ginn TR (2007) Horizontal gene transfer on surfaces in natural porous media: conjugation and kinetics. *Vadose Zone J* **6**: 306–315.

Molbak L, Licht TR, Kvist T, Kroer N & Andersen SR (2003) Plasmid transfer from *Pseudomonas putida* to the indigenous bacteria on alfalfa sprouts: characterization, direct quantification, and *in situ* location of transconjugant cells. *Appl Environ Microb* **69**: 5536–5542.

Molin S & Tolker-Nielsen T (2003) Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Opin Biotech* **14**: 255–261.

Mosqueda G, Ramos-González M-I & Ramos JL (1999) Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1 strain, and its role in solvent impermeabilization. *Gene* **232**: 69–76.

Noguchi H, Matsushasi M & Mitsusashi S (1979) Comparative studies of penicillin-binding proteins in *Pseudomonas aeruginosa* and *Escherichia coli*. *Eur J Biochem* **100**: 41–49.

Normander B, Christensen BB, Molin S & Kroer N (1998) Effect of bacterial distribution and activity on conjugal gene transfer on the phylloplane of the bush bean (*Phaseolus vulgaris*). *Appl Environ Microb* **64**: 1902–1909.

Panicker MM & Minkley EG (1985) DNA transfer occurs during a cell-surface contact stage of F-sex factor-mediated bacterial conjugation. *J Bacteriol* **162**: 584–590.

Park W, Jeon CO, Hohnstock-Ashe AM, Winans SC, Zylstra GJ & Madsen EL (2003) Identification and characterization of the conjugal transfer region of the pCg1 plasmid from naphthalene-degrading *Pseudomonas putida* Cg1. *Appl Environ Microb* **69**: 3263–3271.

Picioreanu C, Kreft J-U & van Loosdrecht MCM (2004) Particle-based multidimensional multispecies biofilm model. *Appl Environ Microb* **70**: 3024–3040.

Ramos JL, Marqués S & Timmis KN (1997) Transcriptional control of the *Pseudomonas* Tol plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. *Annu Rev Microbiol* **51**: 341–373.

Reinhard F & Van der Meer JR (2009) Microcolony growth procedures. *Handbook Hydrocarb Lipid Microbiol* **5**: 3562–3570.

Reischer H, Schotola I, Striedner G, Pötschacher F & Bayer K (2004) Evaluation of the GFP signal and its aptitude for novel on-line monitoring strategies of recombinant fermentation processes. *J Biotechnol* **108**: 115–125.

Samuels AL, Lanka E & Davies JE (2000) Conjugative junctions in RP4-mediated mating of *Escherichia coli*. *J Bacteriol* **182**: 2709–2715.

Shu AC, Wu CC, Chen YY, Peng HL, Chang HY & Yew TR (2008) Evidence of DNA transfer through F-pilus channels during *Escherichia coli* conjugation. *Langmuir* **24**: 6796–6802.

Simonsen L (1990) Dynamics of plasmid transfer on surfaces. *J Gen Microbiol* **136**: 1001–1007.

Simonsen L (1991) The existence conditions for bacterial plasmids: theory and reality. *Microbial Ecol* **22**: 187–205.

Smets BF, Rittmann BE & Stahl DA (1993) The specific growth rate of *Pseudomonas putida* PAW1 influences the conjugal transfer rate of the TOL plasmid. *Appl Environ Microb* **59**: 3430–3437.

Smets BF, Rittmann BE & Stahl DA (1994) Stability and conjugal transfer kinetics of a TOL plasmid in *Pseudomonas aeruginosa* PAO 1162. *FEMS Microbiol Ecol* **15**: 337–349.

Sorensen SJ, Bailey M, Hansen LH, Kroer N & Wuertz S (2005) Studying plasmid horizontal transfer *in situ*: a critical review. *Nat Rev Microbiol* **3**: 700–710.

Stewart FM & Levin BR (1977) The population biology of bacterial plasmids: a priori conditions for the existence of conjugationally transmitted factors. *Genetics* **87**: 209–228.

Thomas CM (2006) Transcription regulatory circuits in bacterial plasmids. *Biochem Soc T* **34**: 1072–1074.

Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS & Molin S (2000) Development and dynamics of *Pseudomonas sp*. biofilms. *J Bacteriol* **182**: 6482–6489.

Volfson D, Cookson S, Hasty J & Tsimring LS (2008) Biomechanical ordering of dense cell populations. *P Natl Acad Sci USA* **105**: 15346–15351.

White GP & Dunn NW (1978) Compatibility and sex specific phage plating characteristics of the TOL and NAH catabolic plasmids. *Genet Res* **32**: 207–213.

Williams PA & Murray K (1974) Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt-2: evidence for the existence of a TOL plasmid. *J Bacteriol* **120**: 416–423.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effect of mecillinam on the morphology of *Pseudomonas putida* KT2440.
Video S1. Real-time visualization of bacterial conjugation.
Video S2. 24 h time-lapse experiment following donor *Pseudomonas putida* KT 2440 (expressing DsRed from the chromosome) and recipient *P. putida* KT 2440 microcolonies (expressing YFP from the chromosome).

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