Increased transfer of a multi-drug resistance plasmid in *E. coli* biofilms at the air-liquid interface.

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ABSTRACT

Although biofilms represent a common bacterial life style in clinically and environmentally important habitats, there is scant information on the extent of gene transfer in these spatially structured populations. The objective of this study was to gain insight into factors that affect transfer of the promiscuous multi-drug resistance plasmid pB10 in \textit{E. coli} biofilms. Biofilms were grown in different experimental settings and plasmid transfer was monitored using laser scanning confocal microscopy and plate counting. In closed flow cells, plasmid transfer in surface-attached submerged biofilms was negligible. In contrast, high plasmid transfer efficiency was observed in a biofilm floating at the air-liquid interface in an open flow cell under low flow rates. A vertical flow cell and a batch culture biofilm reactor were then used to detect plasmid transfer at different depths away from the air-liquid interface. Extensive plasmid transfer only occurred in a narrow zone near that interface. The much lower transfer frequency in the lower zones coincided with rapidly decreasing oxygen concentrations. However, when an \textit{E. coli} csrA mutant was used as recipient, a thick biofilm was obtained at all depths, and plasmid transfer occurred at similar frequencies throughout. These results and data from separate aerobic and anaerobic matings suggest that oxygen can affect IncP-1 plasmid transfer efficiency, not only directly but also indirectly through influencing population densities and therefore co-localization of donors and recipients. In conclusion, the air-liquid interface can be a hot-spot for plasmid-mediated gene transfer due to high densities of juxtaposed donor and recipients cells.
INTRODUCTION

Plasmid-mediated horizontal gene transfer is one of the key mechanisms of adaptive evolution in bacteria. Since many self-transmissible plasmids encode resistance to antibiotics and heavy metals, catabolic pathways or virulence and colonization factors (37, 68), horizontal gene transfer by plasmid-mediated conjugation plays an important role in adaptation of bacteria to variable conditions in natural and clinical environments (34). Despite the importance of these mobile elements in the rapid rise of multi-drug resistant bacterial pathogens (36), we still have a limited understanding of the environmental factors that affect rates of plasmid spread.

In many natural environments bacteria colonize different surfaces where they form microcolonies, often embedded in a polymeric matrix. These bacterial populations are spatially structured, thereby creating environmental gradients and exhibiting behaviors that are different from those in well-mixed liquid environments (16, 67). Such structured bacterial communities attached to a surface or to each other are called biofilms (16). The presence of transferrable plasmids and conjugation events has been shown to positively affect biofilm formation (10, 19, 29, 50, 54, 57, 69). Since bacterial cells in biofilms stay in close contact, it is believed that gene exchange by conjugation is favored by this “biofilm mode” of growth. However, several studies on the efficiency of plasmid transfer in laboratory-type bacterial biofilms have shown mixed results, which seem to be dependent on experimental conditions and transfer detection methods. For example, an early study by Angles and coworkers showed an increased transfer of plasmid RP1 in biofilms formed by Vibrio sp. on glass beads in a bioreactor compared to cells in the aqueous phase using a standard plating method (3). The application of fluorescent protein reporters in combination with confocal laser scanning microscopy (CLSM) has facilitated the in situ tracking of plasmid transfer in bacterial biofilms, up to the individual cell level. Hausner and
Wüertz found that the plasmid transfer rate quantified by *in situ* image analysis was 1000-fold higher than that determined by classical plating techniques (31), and similar results were obtained in other studies (39, 65). A combination of standard plating and fluorescent microscopy has been used to monitor transfer of IncP-9 plasmid pWW0 (12, 14, 30, 53, 62), IncP-1 plasmids pRK415 (31), pJP4 (5), IncX1 plasmid pMAS2027 (54) and the novel unclassified plasmids pQBR11 (47) and pBF1 (20) in biofilms grown under different experimental conditions.

Interestingly, plasmid transfer has often been shown to be limited to the surface of colonies or biofilms, and complete plasmid invasion was not observed (12, 14, 30). Possible inhibition of plasmid transfer by unfavourable cell contact mechanics (50, 62) or lack of nutrient availability in those deeper cell layers (27) have been suggested as potential explanations. Nutrients have been shown to positively affect plasmid transfer efficiency in some cases, but not in others (27, 31). In summary, most studies showed that plasmid transfer in bacterial biofilms is not easy to detect and depends on many different factors like plasmid type, host strain, media and biofilm growth conditions.

The IncP-1 plasmids are among the most promiscuous self-transmissible plasmids in Proteobacteria and often code for resistance to multiple antibiotics and mercury, or for the degradation of xenobiotics (1). They code for short rigid sex pili and have been observed to transfer at higher rates between cells growing on solid surfaces than in liquids (9), in part due to shear forces in liquids that hinder the formation and stability of mating pairs (70). Few studies have monitored the spread of IncP-1 plasmids in biofilms (3, 5, 31, 48), and to our best knowledge virtually nothing is known about the factors that affect their transfer efficiency in this important environment.
To improve our insights into the efficiency of IncP-1 plasmid transfer in biofilms, and to
determine what parameters affect the transfer frequency, we monitored transfer of plasmid pB10
in *E. coli* biofilms. This multi-drug resistance broad-host-range IncP-1β plasmid was isolated
from a waste water treatment plant (25) and its genome sequence is known (61). Several studies
have determined its ability to transfer to different hosts in a variety of experimental conditions
(8, 24, 27, 70). *E. coli* was chosen as the host because it is a well known model organism with
widely available tools and well-characterized plasmid transfer mechanisms. Also, many *E. coli*
isolates are known to form biofilms, and several threaten human health by colonizing medical
devices and causing recurrent urogenital infections (6, 40). Here we show that plasmid pB10
transfer between *E. coli* K12 cells occurs at a much higher efficiency in biofilms formed at the
air-liquid interface (also called pellicles) than in submerged, substrate-attached biofilms, and that
this is at least in part due to differences in oxygen concentration and spatial location of donor and
recipient cells.

MATERIALS AND METHODS.

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this work and
their relevant characteristics are listed in Table 1. The strains were grown in Luria-Bertani broth
(LB) (LB-Miller, Fisher Scientific, Pittsburgh, PA) or M9 mineral salts medium with 0.2 g/L
glucose (hereafter called M9) (60), and when required, antibiotics were added at final
concentrations of 10 µg ml⁻¹ for tetracycline and gentamicin, 25 µg ml⁻¹ for chloramphenicol,
100 µg ml⁻¹ for ampicillin and 50 µg ml⁻¹ for rifampicin, nalidixic acid, and kanamycin.

**Plasmid marking and localization of MaT7cat transposon insertion sites.** As an
alternative plasmid detection system to be used in batch culture biofilms besides the DsRed-
based plasmid pB10::rfp, pB10 was also marked with a T7 polymerase/cat gene cassette (MaT7cat) using a previously described approach (42). The donor of this plasmid was used in combination with the recipient strains E. coli MG1655 and MG1655csrA that carried one of three constructed reporter plasmids (pB2a-Evoglow®, pG-GFP, pG-RFP) (Table 1). The vectors were constructed as previously described (42). To determine the MaT7cat transposon insertion sites in five selected clones of pB10::T7cat, DNA was isolated by a standard alkaline lysis-phenol/chloroform extraction method, digested with EcoRI or SalI and cloned into pGem3Zf+ vector (Promega, Madison, WI). Recombinant clones were selected on LBA with ampicillin and gentamicin, or ampicillin and chloramphenicol, as the EcoRI and SalI sites are located between the gentamicin and chloramphenicol resistance genes in the MaT7cat transposon. This allowed us to determine the flanking sequences from both transposon ends, with primers BT20out2 and MaT7seq1 (42) for the gentamicin and chloramphenicol resistant clones respectively, using the Big Dye Terminator v.3.1 Cycle Sequencing Kit and a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, California). Sequence similarity searches were done using Blast (2). The five marked pB10::T7cat plasmids, varying only in the insertion site of the transposon tag, were transferred back into rifampicin-resistant E. coli K12 (K12Rif), and the resulting transconjugants were used as donors in conjugation experiments.

Conjugation experiments. A filter mating protocol was performed as described elsewhere (27). The 'transfer frequency' of plasmid pB10 after the specified incubation period is presented as the ratio of transconjugant cells (T) to the number of donor (D) or recipient (R) cells at the end of the matings. For conjugation in anaerobic conditions, media and saline supplemented with resazurine (0.0001%) after sterilization were purged with nitrogen (20 min.) and stored in an anaerobic (5.21% CO\textsubscript{2}, 5.18% H\textsubscript{2}, 89.61% N\textsubscript{2}) chamber [AS-580 Gloveless...
Cells from aerobically or anaerobically grown precultures (1ml) were spun down (4 min, 2,000xg), resuspended in 50µl of LB medium, mixed together and incubated in Eppendorf tubes, or on cellulose nitrate 0.45µm filters (Whatman Inc., Piscataway, NJ) in aerobic or anaerobic conditions for 18h at 37°C. Student’s t-test on log\(_{10}\) transformed data was used to compare transfer efficiencies in different conditions, as previously reported (66).

**Biofilm Growth Conditions.** Four different biofilm growth systems were used to monitor the pB10 plasmid transfer. The first one used a ‘closed horizontal flow cell’, either a Three Channel Flow Cell® (4 x 40 x 1 mm channels) or a Convertible Flow Cell® (24 x 40 x 8 mm channel) (Stovall Life Science Inc., Greensboro, NC; http://www.slscience.com/flow-cell). The medium used was M9 or 0.1 x LB broth; separate experiments were done with both systems unless otherwise stated. Flow cells were inoculated for 1h with a mixture of donor and recipient cells (1:1 ratio; 10\(^5\) cfu/ml; 200µl/ channel or 2 ml/ cell) static, and then medium flow was applied (0.1ml/min for the Three Channel Flow Cell® and 0.5ml/min for the Convertible Flow Cell®). Flow cells were placed in the typical horizontal position and incubated at 37°C. The flow rates ensured any planktonic cells were flushed out. Biofilms were analyzed as described below at various time points up to 6 days.

The second system was an ‘open horizontal flow cell’, which consisted of only the bottom part of the Convertible Flow Cell® (the top was removed). The cell was placed horizontally on a heating plate to maintain a temperature of 37°C and inoculated with 1ml of a donor:recipient mixture (1:1 ratio; 10\(^5\) cfu/ml); after 1h of static incubation, flow was applied at 0.11 ml/min. The biofilm that was floating at the air-liquid interface was collected on a microscope cover slip and analyzed as described below.
The third system consisted of a ‘vertical flow cell’ (Fig. 2A). The Convertible Flow Cell® was inoculated with 1 ml of donor:recipient mixture (1:1 ratio; $10^5$ cfu/ml) and kept in horizontal position for 1 h with no medium flow. Subsequently, the inoculum was removed, the flow cell was raised to a vertical position, and then medium flow was applied from the bottom at 0.330 ml/min. The flow cell was incubated vertically at 37°C. The liquid-air level was maintained at ~2 cm from the top of the flow cell by removing the liquid medium from the top using a blunted needle connected to a peristaltic pump (1.1 ml/min) (Fig. 2A). After 3 days, the needle was removed, the flow cell was filled with medium and the biofilm was analyzed as described below.

Finally, the fourth system was a simple batch culture biofilm reactor (Fig. 4A). A sterile microscope slide was submerged in 25 ml of medium (M9 or 0.1 x LB) in a 50 ml conical tube, and incubated at 37°C in an orbital shaker at 50 rpm. To grow the biofilm, the bioreactor was inoculated with 50 µl of overnight culture; the slides were transferred daily to tubes with fresh medium. For plasmid infection studies, slides with established (24-48 h) recipient biofilm were washed by dipping them in 50 ml saline to remove non-attached cells, and then transferred into 25 ml of an overnight culture of the plasmid donor (approx. $10^5$ cfu/ml) and incubated for 4 h at 37°C (50 rpm). The non-attached cells were washed off by submersion in saline and the slide was transferred into fresh medium. After 24 or 48 h, the slide biofilms were washed, submerged in saline in a Petri plate and analyzed by confocal microscopy.

A previously described *E. coli* K12Nal::*gfp* and plasmid pB10::*rfp* (27) marked with constitutively expressed *gfp* and *rfp* reporter genes (13) were used in the first three biofilm set-ups, while in the batch culture biofilm plasmid pB10::*T7cat* was used in combination with recipient strains with one of three reporter plasmids (pB2a-Evoglow®, pG-GFP, pG-RFP).
Microscopy techniques of biofilm analysis. Biofilms were observed at different time points with a standard bright field Olympus BX41 microscope with 5x lens (Plan-NEOFLUAR) or with the Olympus Fluoview FV1000 Confocal microscope (Olympus, Center Valley, PA) using 10x (UPLanFLN) or 60x (LUMPlanFI/IR) lenses, and settings for GFP, RFP or CFP (Evoglow) detection. The images were processed using FV10-ASW 1.6 (Olympus, Center Valley, PA) and Canon PhotoStitch software. For quantitative biofilm analysis, such as areal porosity measurements, the ISA2 software was used and at least 10 images from each zone were analyzed using standard settings (7).

Quantification of plasmid transfer in biofilm. To quantify pB10 plasmid transfer in biofilm by means of plate counting, the batch culture biofilm protocol with the E. coli MG1655 as plasmid donor and MG1655csrA recipient strain was used. After 4h incubation with the donor strain, the slides were washed with saline; the bacteria were resuspended in 25ml of saline by a combination of scraping and vortexing (2 min.), and serial dilutions were plated on selective media. The efficiency of transfer at different depths from the air-liquid interface was analyzed as follows. Biofilm samples (~1cm²) were collected with sterile swabs immediately after the 4h inoculation period, cells were resuspended in 1ml of saline and serial dilutions were plated onto selective media. The 'transfer frequency' was calculated as described above (Table 2). For statistical analysis, we first took log(T/R) or log(T/D) and then performed a t-test: for each replicate, we took log(T/R) or log(T/D) from zone 1 as x, and log(T/R) or log(T/D) from zone 2 as y, and performed a t-test for (x,y).

Oxygen measurements. The oxygen concentration was measured in the liquid column of the vertical flow cell and along the microscope slide for batch culture biofilms. Measurements were done immediately or within minutes after stopping the medium flow or shaking. An
amperometric dissolved oxygen microelectrode (DOM) was used for oxygen profile measurements as described previously (45). Briefly, the tip of a tapered Pt wire was plated with gold and used as the cathode. The outer case was made of a tapered Pasteur pipette (Fisher catalog #: 22-183-632). Then a homemade silver/silver chloride reference electrode was inserted and the DOM was filled with the electrolyte (0.3 M K$_2$CO$_3$, 0.2 M KHCO$_3$ and 1 M KCl). For this specific application, the tip of the microelectrode was around 50 µm and the length of the tip was 15 cm. The tip was covered with a silicone rubber membrane that allows diffusion of gases. The DOM was calibrated at zero oxygen concentration (in saturated Na$_2$SO$_3$ solution) and air-saturated water. The response time was around 1–3 s. A HP 4140B pA meter/DC voltage source device was used to polarize the gold cathode and to measure the current. The DOM was moved into the solution in 10 µm steps, using a Mercury-step stepper motor controller (Physik Instrumente, Auburn, MA, PI M-230.10S Part No. M23010SX). The microelectrode movement and data collection were controlled by custom-made software called Microprofiler®.

RESULTS

Low frequency of plasmid transfer in submerged biofilms. Several experiments wherein transfer of pB10::rfp in E. coli MG1655 biofilms was monitored in closed horizontal flow cells showed very low numbers of transconjugant cells in submerged biofilms. This was observed under many conditions, such as in two different closed flow cells with different media and flow rates, as well as different combinations of parental cells (premixed donor and recipients, pre-grown donor biofilm inoculated by recipients and vice versa). If any transconjugants were observed, they were represented by single cells and did not form multi-cell
clusters (data not shown). These results suggested that only a first round of conjugation occurred, with no secondary transfer events.

To examine this low occurrence of plasmid transfer, we monitored submerged biofilms formed on the glass bottom of an open horizontal flow cell using the upright confocal laser scanning microscope (CLSM) with water immersion lenses. In these experiments, we observed spatial separation of donor and recipient cells. When the cells were first mixed and then allowed to form a biofilm, the donor cells formed a biofilm attached to the glass, while the recipient cells formed a discrete layer above the donor biofilm. The spatial separation was even more striking when an already established donor biofilm was inoculated with recipients (Fig. 1A), or when an established recipient biofilm was infected by donor cells. In the latter case, the donor cells seemed to have migrated through the recipient biofilm and settled down onto the slide surface, somehow pushing up the layer of the recipient cells (Fig. 1B). The same phenomenon was observed when the experiment was repeated, and it persisted over a prolonged incubation time (data not shown). At the interface between donors and recipients, few transconjugant cells were observed and never any clusters indicative of multiple rounds of transfer. These results suggest that the spatial separation of plasmid-bearing and plasmid-free cells was largely responsible for the inefficient spread of an otherwise highly transferable IncP-1 plasmid.

**Increased plasmid transfer at the air-liquid interface.** In the open horizontal flow cell under conditions of low or no liquid flow, an additional pellicle floating at the air-liquid interface was observed. After carefully transferring this floating layer of cells from the interface to a microscope slide, we observed a high number of transconjugants (Fig. 1C). Moreover, it appeared that more transconjugant cells were located in the upper layer (facing the air) than in the lower part that faced the liquid (data not shown). The finding suggests that plasmid pB10
transfers much more efficiently at the air-liquid interface than in a submerged biofilm attached to a glass substrate.

To further study plasmid transfer in a biofilm at the air-liquid interface in more detail and under different conditions, we designed a ‘vertical flow cell’, as shown in Fig. 2A. In this set-up, nutrients and oxygen were continuously supplied from the bottom of the cell through the incoming growth medium flow, and additional oxygen was available at the top through gas exchange at the air-medium interface. That interface was kept at the same level during the course of the experiment. The biofilm formed by wild-type E. coli K12 in this vertical flow cell showed high substrate coverage and biovolume along the glass surface (data not shown). We observed structural differences between the three zones. In zone 1 (submerged, close to the air-liquid interface), bacteria were closely packed and only a few small isolated microcolonies were observed (Fig. 2B, C, D), while the biofilm further away from the interface tended to consist of more isolated microcolonies of increasing size. The degree of coverage of the glass surface by the biofilm was quantified by the areal porosity parameter, defined as the ratio of the combined areas of the voids to the total area (7, 45). Areal porosity increased with distance from the air-liquid interface (zones 2 and 3), from 0.57±0.03 for zone 1 to 0.74±0.03 for zone 2 and 0.75±0.05 for zone 3 (P<0.003). As the flow cell was initially inoculated with approximately $10^5$ cfu of donor E. coli K12Rif (pB10::rfp) and recipient K12Nal::gfp (1:1 ratio), we assumed that, as in submerged biofilms, plasmid bearing cells would attach to the glass surface more efficiently. However, we observed that green recipient cells formed the main part of the biofilm. Only in zone 1 a large number of red donor cells as well as yellow transconjugants was observed (Fig. 2B, D). This zone extended down about 0.7 mm from the air-medium interface. In the lower zones, we observed several microcolonies formed by donor cells surrounded by the
recipients. In these cases, even if the cells were in close contact along the colony border, only a limited number of transconjugants, located mostly on the top of the microcolony, were detected (Fig. 2D). These results are consistent with those from the open horizontal flow cell, i.e. that there is more invasion of the IncP-1 plasmid pB10 in *E. coli* K12 biofilms at the air-liquid interface than in submerged biofilms.

**Oxygen concentration at different depths in the vertical flow cell.** The simplest explanation for the intensive plasmid transfer at the air-medium interface could be the high oxygen level. It is well known that oxygen concentrations drop rapidly both inside biofilms as well as in the liquid column (23). To test the oxygen level at different depths in the vertical flow cell, a 15cm-long oxygen microelectrode was used. A rapid drop in oxygen concentration was observed immediately away from the surface (Fig. 3); at 1.5-2 mm from the surface the oxygen concentration had dropped from 8 mg/L to the lowest level. The minimal O$_2$ level depended on the medium used. In the M9 medium where the total number of cells reached ~10$^6$-10$^7$ cfu/ml, the lowest oxygen concentration was 3 mg/L. In the rich LB medium, where the number of bacteria was at least 10 times higher, the oxygen concentration reached 1mg/L.

**Effect of oxygen on transfer of plasmid pB10.** To determine, if the presence of oxygen directly affects conjugation efficiency of plasmid pB10, four combinations of filter matings were performed. Using aerobically or anaerobically grown donor and recipient pre-cultures matings were performed under both aerobic and anaerobic conditions (for 18h at 37ºC), followed by enumeration of donor, recipient and transconjugant cells on selective media. The average ratio of transconjugants/recipients (T/R) in aerobic and anaerobic conditions using aerobically grown pre-cultures was 0.40 (±0.13) and 0.32 (±0.14) respectively, and not significantly different (P=0.2, Standard *t*-test). However, when anaerobically grown donor and recipient pre-cultures were
used, significant differences in transfer frequencies were observed between aerobic and anaerobic matings (P<0.001): 0.60 (±0.25) and 0.022 (±0.006), respectively. When results from aerobic pre-cultivation and mating conditions were compared with those from fully anaerobic conditions, the difference was also highly significant (P<0.001).

As spatial separation of donor and recipient cells could affect conjugation efficiency in filter matings, we performed additional liquid matings under the same four conditions of aerobic and anaerobic precultivation and matings. Here we observed an even more striking difference in T/R frequency for anaerobically grown precultures: 1.79 x 10^{-2} and 4.68 x 10^{-6} for aerobic and anaerobic matings, respectively (P<0.001). However, just like in the filter matings, the difference was not significant when donor and recipient cells were pre-grown aerobically, suggesting that even residual amounts of oxygen are sufficient for efficient transfer of plasmid pB10. The low frequencies in completely anaerobic conditions were not due to plasmid loss in the donor strain, based on counts of plasmid-bearing donor cells (data not shown). In conclusion, anaerobic conditions negatively affected the conjugation efficiency of the IncP-1 plasmid pB10, but trace amounts of oxygen, as expected in biofilm flow cells, should not.

**Effect of cell density on plasmid transfer in a batch culture biofilm.** To confirm that there is a higher degree of plasmid invasion in *E. coli* populations at the air-liquid interface than in biofilms submerged by liquid, the transfer of pB10 was monitored in a fourth system, i.e., biofilms grown on vertically placed microscope slides in batch culture reactors (Fig. 4A). Here a different marker system was used to visualize transconjugants. An *E. coli* donor carrying pB10::T7cat was used in combination with recipients carrying different reporter plasmids (pG-GFP, pG-RFP, and pB2a-Evoglow®, encoding the oxygen-independent fluorescence protein Evoglow®) (see Table 1). Since each of these recipients required the presence of T7 polymerase
to express the fluorescent reporter protein, the only fluorescent cells were recipients that acquired the plasmid. The observed plasmid transfer patterns were similar to those observed in the vertical flow cell, i.e. efficient plasmid transfer in the SI zone, and lower numbers of transconjugants in the lower zones. This coincided again with the very uneven distribution patterns of bacteria on the slide, and with an oxygen profile similar to that in the vertical flow cell (data not shown).

Juxtaposition of donor and recipients was only observed in the zone 1 at the air-liquid interface, where most of the biofilm was formed (Fig. 4B). Only a thin layer of single cells or small microcolonies were formed by plasmid-bearing donor cells in the deeper zones (data not shown). These findings strongly suggest that here too, spatial separation of donor and recipient cells can explain the lack of gene transfer in the lower submerged zones.

To test the hypothesis that the low frequency of plasmid transfer in submerged biofilms was due to spatial separation of donors and recipients, we monitored plasmid transfer into an *E. coli* MG1655*csrA* mutant that has increased biofilm formation ability (35). This strain not only formed a substantial biofilm at the air-medium interface in the batch culture reactor, but also efficiently covered the microscope slide in deeper zones (Fig. 4C). Using this strain, fluorescent transconjugants were observed all the way down along the slide in experiments with pG-GFP (Fig. 4D) as well as pG-RFP and pB2a-Evoglow® as reporter plasmids (data not shown). This strongly suggests that the very low frequency of plasmid transfer in wild-type *E. coli* MG1655 submerged biofilms was largely due to the poor biofilm formation ability of this strain, causing spatial separation of donor and recipient cells.

To quantify the efficiency of plasmid transfer in the three different zones of the batch reactor biofilm of *E. coli* MG1655*csrA* away from the surface, parental and transconjugant cells were enumerated by the classical dilution/plating method (Table 2). We found a higher absolute
number of transconjugants in zone 1, but the differences in T/R ratios in all zones were not statistically significant (P=0.23 between zone 1 and 2). This was because lower transconjugant densities in the lower zones were correlated with lower recipient densities. On the other hand, the T/D ratios in all zones were statistically different (P<0.01 between zone 1 and 2) because the donor densities did not decrease as drastically with depth. Thus, for a given number of invading donor cells, transconjugant densities were highest at the air-liquid interface because of the higher recipient densities at that location. Again, the air-liquid interface seemed to promote biofilm growth of the csrA recipient strain, and therefore allowed more extensive plasmid invasion.

DISCUSSION

Due to the close cell-to-cell contact and minimal shear force in biofilms, the hypothesis has been raised that exchange of genetic information mediated by conjugative plasmid transfer should be highly efficient in this ubiquitous form of bacterial growth. To test this postulate, different experimental settings for growing biofilms, different bacterial hosts and plasmids, as well as different plasmid transfer detection methods, have been used in previous studies (3, 12, 14, 26, 31, 46, 47, 49-51, 53, 54). As the detection of plasmid transfer in situ in spatially structured bacterial populations has many technical challenges (65), direct evidence of extensive conjugative gene transfer in biofilms is not convincing so far. In this study we developed and used several biofilm flow cell and reporter systems to determine some of the basic parameters that govern transfer of the IncP-1 plasmid pB10 in E. coli biofilms. Our results indicate that plasmid transfer occurs at very low frequencies in the typical thick biofilms submerged by liquid, due to the intricate spatial architecture of biofilms, sometimes causing physical separation between clonal populations. However, the broad definition of biofilms also includes pellicles.
floating at the air-liquid interface (16). We found that plasmid pB10 could efficiently invade floating pellicles, as well as surface-attached biofilms close to this interface. Recently, Nguyen et al. showed that transfer of chromosomal markers among Mycobacterium smegmatis strains also occurred predominantly at the air-liquid interface (52).

Our findings strongly suggest that many bacterial communities that are naturally floating at the air-liquid interface could be ‘hot-spots’ for plasmid-mediated gene transfer. Examples of such natural hot-spots are the scum layers on standing bodies of water and the surface microlayers (SML) in lakes and oceans (17, 28). Our postulate is corroborated by various studies that observed plasmid transfer or detected plasmids in such natural ecosystems (4, 20, 21, 32, 38, 44). There are multiple possible explanations for the observed increased plasmid transfer at the air-liquid interface compared to submerged biofilms, and they likely involve a combination of the physical properties of the environment as well as the physiological state of the cells. Here we discuss only a few possible scenarios, which can be separated as the ‘co-localization hypothesis’ and the ‘oxygen effect on conjugation hypothesis’.

First, the co-localization hypothesis states that higher densities of well-mixed donor and recipient populations at the air-liquid interface, as shown in Fig. 2 and Fig. 4, promote gene transfer by conjugation. It is known that in spatially structured populations initial cell densities of both the donor and recipient, as well as their relative position in the matrix, strongly determine the success of plasmid invasion, a phenomenon not observed in mixed liquids (27, 43, 64). Juxtaposition of donor and recipient cells is absolutely required for cells to exchange DNA by conjugation. In our horizontal flow cells, the formation of a surface-attached E. coli K12 biofilm required the presence of plasmid pB10, and therefore donor and recipient populations were separated out rather than being well-mixed. This is in agreement with previously published
observations that *E. coli* K12 strains barely formed biofilms in various flow cells (56, 57) and that their ability to adhere to surfaces was increased by the presence of conjugative plasmids (10, 29, 50, 54, 56, 57, 69). In the batch culture system *E. coli* MG1655 without plasmid formed a biofilm mostly at the air-liquid interface (Fig. 4B). Previous studies have also shown an increased ability of *E. coli* strains to form biofilms at the air–liquid interface, even in the absence of conjugative plasmids (11, 15, 22). Thus the low transfer frequencies in submerged biofilms of *E. coli* K12 must be in part due to the poor ability of the plasmid-free strain to adhere and form a biofilm.

To test our hypothesis that the absence of dense mixed populations of donor and recipient cells in the lower zones of the vertical biofilms is the basis of poor plasmid transfer, we used an *E. coli csrA* mutant, known to form thick biofilms, as the recipient strain (35). This recipient strain also formed the densest biofilm at the air-liquid interface, but unlike *E. coli* MG1655, it also covered deeper zones on the microscope slide in the batch culture biofilm reactor (Fig. 4C). Because of the gradient in recipient density along the slide, there were more transconjugants at the air-liquid interface again per donor cell attached (Table 2). However, similar transconjugant/recipient ratios were found at different depths, suggesting that the recipient density was the limiting factor and not the conjugation efficiency itself. While the *csrA* mutation may have unknown effects on plasmid transferability, comparison of the results of *E. coli* wt and *csrA* suggests that the air-liquid interface is a hot spot for plasmid invasion because it facilitates the formation of dense well-mixed biofilms of both plasmid donors and recipients.

One of the factors causing the higher densities of donor and recipient cells at the air-liquid interface is likely the higher oxygen concentrations in these zones. Steep oxygen gradients were observed in the vertical flow cell (Fig. 3) and batch culture reactor (data not shown), and
correlated with decreasing cell densities in the biofilms along the glass surface. While the
physicochemical conditions of the air-liquid interface may also directly promote adherence of *E. coli* to each other and to the glass surface, the mere presence of more cells in that zone due to the
aerobic conditions may well explain the higher proportion of adhering cells, and therefore
increased cell-to-cell contact. However, previous studies have shown that *E. coli* did not form
biofilms under anaerobic or anoxic conditions, suggesting that there may be a specific biofilm
inhibition mechanism in the absence of oxygen (11, 15). Future studies will have to clarify the
role of aerobiosis in *E. coli* biofilm formation, and determine if characteristics of the air-liquid
interface other than oxygen levels are responsible for enhanced plasmid transfer.

The observation that single donor and recipient cells grew out into separate donor and
recipient layers (Fig. 1) or microcolonies (Fig. 2C) in submerged biofilms, thus limiting gene
exchange, is consistent with a recent observation made in *Pseudomonas aeruginosa* biofilms.
Klayman *et al.* showed that a biofilm initiated with isogenic strains of *P. aeruginosa* carrying
reporter plasmids (expressing CFP or YFP) was composed of separate clusters of cells
containing a single fluorescent protein rather than clusters containing mixed populations (41).
Tolker-Nielsen *et al.* (67) also observed that *Pseudomonas putida* biofilms were formed as
separate clusters of cells but a few single cells were able to move between these microcolonies.
The movement of recipient cells into a microcolony of donors, or the rare mating in the donor-
recipient contact zone, could result in conjugation, and probably explains the presence of single
fluorescent transconjugant cells in our submerged horizontal and vertical flow cell biofilms.

The second hypothesis is that conjugation itself is affected by the oxygen concentration
gradient in the biofilms, either directly through an oxygen-regulated mechanism, or indirectly
through its effect on the cell physiology. Conjugation is known to require energy needed for
DNA replication and protein biosynthesis in both donor and recipient cells (18, 58). Christensen et al. showed that the presence of transconjugants at the surface of biofilm microcolonies coincided with a greater metabolic activity of those cells (12). In addition, Salyers and coworkers concluded previously that efficient transfer of IncP-1 plasmids requires oxygen (59, 63). Our observations support these conclusions since plasmid transfer frequencies in both filter and liquid mating experiments were significantly lower under anaerobic conditions than under aerobic conditions. Yet traces of oxygen left in the mating from aerobic pre-cultivation were sufficient to obliterate this difference. The cells in our submerged biofilms were likely exposed to traces of oxygen (Fig. 3). Moreover, the presence of transconjugants in the deeply submerged zones of the batch culture E. coli csrA biofilms at similar T/R ratios as at the air-liquid interface (Fig. 4D) indicates oxygen was not the limiting factor. We therefore conclude that our biofilm results cannot be explained by a direct effect of oxygen on plasmid transfer frequencies.

While it is known that DsRed and GFP require oxygen for proper folding and fluorescence (55), we do not think that lack of fluorescence was the cause of the low densities of transconjugants observed in submerged biofilms. First, single or small clusters of fluorescent transconjugants were observed in those locations (e.g. Fig. 2B; 2D; 4D), indicating that the fluorescent proteins were expressed and folded correctly. Second, the last experiments in batch culture reactors with the wild-type and csrA mutant showed similar transconjugant/recipient ratios along the slide for GFP (Fig. 4), DsRed as well as the oxygen-independent Evoglow® protein.

Based on previous reports and our own observations, we conclude that conjugative transfer of IncP-1 plasmids was largely hindered in the submerged E. coli K12 biofilms by spatial separation of donor and recipient cells, and promoted at the air-liquid interface by the...
high densities of juxtaposed recipient and donor cells. However, plasmid transfer was not limited
to the air-liquid interface: when the plasmid-receiving *E. coli* strain was a good biofilm former,
as is the case for many pathogenic *E. coli* strains (6, 40), conjugation also occurred extensively
in submerged biofilms. Given the high medical, environmental and industrial importance of
bacterial biofilms and of the promiscuous multi-drug resistance and catabolic plasmids of the
IncP-1 group, greater insight is needed into the conditions and plasmid/host combinations that
affect the rates of gene spread through these ubiquitous bacterial ecosystems. The experimental
systems described here can be further used to study the effect of various parameters on plasmid
transfer in biofilms.

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J.E.K. designed the dual reporter system, constructed all strains and vectors, designed vertical
flow cell and batch culture biofilms experimental settings, performed the plasmid transfer
experiments, interpreted data and wrote the manuscript draft. H.D.N. and H.B measured and
analyzed oxygen profiles. L.M.R. provided technical assistance and helped with manuscript
writing. E.M.T. and S.M.K. oversaw the project, provided help with the experimental design and
data interpretation, and assisted in writing the manuscript.

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   exposed to 2,4-D: bacterial community development and establishment of conjugative


Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Relevant phenotypes and genotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> S17-1 λpir</td>
<td><strong>Tmp</strong>&lt;sup&gt;R&lt;/sup&gt; <strong>Sm</strong>&lt;sup&gt;R&lt;/sup&gt; <strong>recA</strong> <strong>thi pro hsdR</strong>&lt;sup&gt;+&lt;/sup&gt; <strong>M</strong>&lt;sup&gt;+&lt;/sup&gt; RP4:2-Tc:Mu:Km::Tn7 λpir.</td>
<td>(33)</td>
</tr>
<tr>
<td><em>E. coli</em> K12 MG1655</td>
<td>Wild type</td>
<td>ATCC700926</td>
</tr>
<tr>
<td><em>E. coli</em> K12Rif</td>
<td>MG1655, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(27)</td>
</tr>
<tr>
<td><em>E. coli</em> K12Nal</td>
<td>MG1655, Nal&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(27)</td>
</tr>
<tr>
<td><em>E. coli</em> K12Nal::gfp</td>
<td>K12Nal containing mini-Tn5-*P&lt;sub&gt;Al-&lt;/sub&gt;&lt;sup&gt;0403&lt;/sup&gt;::gfpmut3-cassette, Nal&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(27)</td>
</tr>
<tr>
<td><em>E. coli</em> MG1655csrA</td>
<td>csrA:: mini-Tn5, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(35)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pB10</td>
<td>IncP-β, Te&lt;sup&gt;R&lt;/sup&gt; Sm&lt;sup&gt;R&lt;/sup&gt; Amx&lt;sup&gt;R&lt;/sup&gt; Sul&lt;sup&gt;R&lt;/sup&gt; Hg&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(61)</td>
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<tr>
<td>pB10::rfp</td>
<td>pB10 with mini-Tn5-Km-*P&lt;sub&gt;Al&lt;/sub&gt;&lt;sup&gt;0403&lt;/sup&gt;::rfp</td>
<td>(27)</td>
</tr>
<tr>
<td>pB10::T7cat</td>
<td>pB10 with a mini-MaT7cat</td>
<td>This work</td>
</tr>
<tr>
<td>pMaT7cat</td>
<td>Mini-marinier transposon containing T7 RNA polymerase; Cm&lt;sup&gt;R&lt;/sup&gt; Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(42)</td>
</tr>
<tr>
<td>pB2a-Evoglow®</td>
<td>pGem3Zf+ with *pT7-Evoglow placer&lt;sub&gt;dSRedcat&lt;/sub&gt; cassette in PvuII site - reporter plasmid; Amp&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pG-RFP</td>
<td>pGem3Zf+ with <em>pT7-dsRed in SmaI</em></td>
<td>This work</td>
</tr>
</tbody>
</table>
pG-GFP pGem3Zf+ with \textit{pT7-gfpmut3} in SmaI This work

|---|---|---|

site-reporter plasmid; Amp$^R$ Cm$^R$
Table 2. Efficiency of pB10 transfer in batch culture biofilms at different depths from the air-liquid interface.

<table>
<thead>
<tr>
<th>Environment</th>
<th>Number of donors (D) (cfu/ml)</th>
<th>Number of recipients (R) (cfu/ml)</th>
<th>Number of transconjugants (T) (cfu/ml)</th>
<th>T/D</th>
<th>T/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm (total)*</td>
<td>2.29 x 10^6</td>
<td>4.73 x 10^7</td>
<td>7.37 x 10^3</td>
<td>3.21 x 10^3</td>
<td>1.56 x 10^-4</td>
</tr>
<tr>
<td>Zone 1#</td>
<td>4.43 x 10^6</td>
<td>2.84 x 10^8</td>
<td>2.86 x 10^4</td>
<td>6.46 x 10^3</td>
<td>1.01 x 10^-4</td>
</tr>
<tr>
<td>Zone 2#</td>
<td>3.35 x 10^6</td>
<td>4.34 x 10^7</td>
<td>8.11 x 10^3</td>
<td>2.42 x 10^3</td>
<td>1.87 x 10^-4</td>
</tr>
<tr>
<td>Zone 3#</td>
<td>2.61 x 10^6</td>
<td>2.62 x 10^7</td>
<td>2.67 x 10^3</td>
<td>1.02 x 10^3</td>
<td>1.02 x 10^-4</td>
</tr>
</tbody>
</table>

* Mean values of 6 independent experiments; cell counts represent cells attached to the glass after rinsing (see Materials and Methods).

* Mean values of 5 independent experiments;
**Fig. 1.** Biofilm formed in the closed (A, B) and open (C) horizontal flow cell showing donors (red), recipients (green) and transconjugants (yellow). A) Side view of a submerged 68.5h-old recipient MG1655::gfp biofilm observed 2.5h after the donor MG1655 (pB10::rfp) was inoculated (1h static and 1.5h with media flow) B) Side view of a submerged 75.5h-old donor MG1655 (pB10::rfp) biofilm observed 4.5h after the recipient MG1655::gfp was inoculated (1h static and 3.5h media flow). Similar results were obtained when the donor and recipient cells were inoculated together and incubated for 90h (data not shown). C) Air-liquid interface biofilm formed 90h after inoculation of the same donor and recipient applied together, and transferred onto a microscope cover slip; GFP channel (left); RFP channel (central); both channels (right panel).

**Fig. 2.** Vertical flow cell biofilm 72h after inoculation of donor MG1655 (pB10::rfp) and recipient MG1655::gfp. Red arrows indicate the air-liquid interface. From the left: Photograph of the vertical flow cell (A), the red bar extending out of the top is the needle used to maintain the liquid level; Composite of biofilm images along the flow cell, indicating high levels of transconjugants as represented by the yellow cells (GFP, RFP and bright light channels combined; magnification [mag.] 10x, white bars – 0.5mm) (B); Bright field microscope images of three zones (mag. 4x) (C); Microphotographs of the biofilm sampled at zone 1 (upper panels), and donor colonies surrounded by recipient cells and very few transconjugants in lower zones 2 and 3; (GFP, RFP channels; mag. 60x, bars - 10µm)(D).

**Fig. 3.** Oxygen profiles along the liquid column in the vertical flow cell (see Fig. 2A) 24h after
inoculation with donor and recipient in 0.1xLB or M9 medium. Oxygen concentrations were measured using a custom-made microelectrode. Similar profiles were observed at 48h and 72h after inoculation. The picture above the graph shows part of the vertical flow cell from Fig. 2B, indicating zone 1 (zones 2 and 3 are beyond the 5 mm shown in this figure).

Fig. 4. The batch culture biofilm reactor (A) and the glass slide biofilm (24h) of *E. coli* MG1655 (B) and *E. coli* MG1655*csrA* (C), 24h after infection with *E. coli* MG1655 (pB10::*MaT7cat*). D) Microphotographs of the MG1655*csrA* (pG-GFP) biofilm infected with *E. coli* MG1655 (pB10::*MaT7cat*), sampled from three zones: the zone 1 at the air-liquid interface (marked with the red arrows); zone 2, and zone 3 (GFP, bright field channels; mag. 60x, red bars - 20µm).
Oxygen concentration (mg/L) vs Depth (mm) for LB E. coli (black dots) and M9 E. coli (grey circles). The graph shows a decrease in oxygen concentration as depth increases, with a distinct zone labeled as zone 1. The transition from air to liquid is indicated by a vertical dashed line at depth 0.