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Invasion of *E. coli* biofilms by antibiotic resistance plasmids

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ABSTRACT

In spite of the contribution of plasmids to the spread of antibiotic resistance in human pathogens, little is known about the transferability of various drug resistance plasmids in bacterial biofilms. The goal of this study was to compare the efficiency of transfer of 19 multidrug resistance plasmids into *Escherichia coli* recipient biofilms and determine the effects of biofilm age, biofilm-donor exposure time, and donor-to-biofilm attachment on this process. An *E. coli* recipient biofilm was exposed separately to 19 *E. coli* donors, each with a different plasmid, and transconjugants were determined by plate counting. With few exceptions, plasmids that transferred well in a liquid environment also showed the highest transferability in biofilms. The difference in transfer frequency between the most and least transferable plasmid was almost a million-fold. The 'invasibility' of the biofilm by plasmids, or the proportion of biofilm cells that acquired plasmids within a few hours, depended not only on the type of plasmid, but also on the time of biofilm exposure to the donor and on the ability of the plasmid donor to attach to the biofilm, yet not on biofilm age. The efficiency of donor strain attachment to the biofilm was not affected by the presence of plasmids. The most invasive plasmid was pHH2-227, which based on genome sequence analysis is a hybrid between IncU-like and IncW plasmids. The wide range in transferability in an *E. coli* biofilm among plasmids needs to be taken into account in our fight against the spread of drug resistance.

1. Introduction

Biofilms are often defined as associations of microorganisms embedded in a polymeric matrix attached to a solid substrate or floating on liquid surfaces. They represent the most common form of microbial life in natural, industrial and hospital environments (Davey and O'Toole, 2000; Hall-Stoodley et al., 2004). Biofilms provide unique growth conditions, in which bacteria are less sensitive to harsh environmental conditions than their planktonic counterparts. A few mechanisms of biofilm resistance to antimicrobial compounds that are based on physical and biological properties have been proposed (Fux et al., 2005; Hoiby et al., 2010; Mah and O'Toole, 2001). However, the fastest mechanism of heritable acquisition of resistance against multiple antimicrobial agents is horizontal gene transfer (HGT) (Barlow, 2009). One of the major mechanisms of HGT is plasmid-mediated bacterial

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conjugation (O'Brien, 2002). Many plasmids from different incompatibility groups (Inc) that carry multiple drug resistance genes can conjugatively transfer between plasmid-bearing cells (donors, D) and plasmid-free cells (recipients, R) (Carattoli, 2009; Datta and Hedges, 1972; Strahilevitz et al., 2009; Venturini et al., 2010). Conjugation requires close contact between cells as well as minimal shear forces. Since both these features are characteristic for biofilms it has been taken for granted that conjugative gene transfer in microbial biofilms occurs with high efficiency (Molin and Tolker-Nielsen, 2003). However, the actual transfer rate in biofilms remains unclear, mostly because of problems with quantitative detection and good model systems (Król et al., 2011; Sørensen et al., 2005). Thus in spite of the important role that biofilms play in infectious diseases, we still have a poor understanding of the factors that affect plasmid transfer in biofilms.

The goal of this study was to compare the ability of natural multidrug resistance (MDR) plasmids to transfer into an existing biofilm of plasmid-free *Escherichia coli* cells in a short time period, and to determine what parameters affect the transfer efficiency of the plasmids. *E. coli* is a well-known model organism with widely available tools and well-characterized plasmid transfer mechanisms. While most laboratory strains of *E. coli* have lost the ability to form thick biofilms, many natural isolates form biofilms efficiently, causing threats to human health by colonizing not only abiotic surfaces but also food and medical devices (Lynch and Robertson, 2008; Marouani-Gadri et al., 2009; Rivas et al., 2007). We used a convenient and efficient system to grow biofilms on microscope slides (Król et al., 2011), and *E. coli* K12 MG1655 *csrA*, a mutant known to efficiently form biofilms (Jackson et al., 2002). Most plasmids used here were studied previously and were found to induce biofilm formation by *E. coli* directly or after co-inoculation with a recipient strain (Ghigo, 2001). Additional plasmids known to encode resistance to antibiotics and to transfer well in liquids or on agar plates were also included (see Table 1). We observed a wide diversity in the ability of these plasmids to invade an established *E. coli* biofilm, with frequencies varying almost a million-fold.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work and their relevant characteristics are listed in Table 1. The strains were grown in LB Miller broth (Sambrook and Russell, 2006), and when required, antibiotics were added at final concentrations of 10 μg ml^{-1} for tetracycline and gentamicin, 12.5 μg ml^{-1} for chloramphenicol, 100 μg ml^{-1} for ampicillin and rifampicin, and 75 μg ml^{-1} nalidixic acid.

2.2. Biofilm growth conditions and quantitative analysis

Biofilms were grown in a batch culture biofilm reactor as described previously (Król et al., 2011). Briefly, a sterile microscope slide was submerged in 25 ml of LB medium in a 50 ml conical tube. This bioreactor was then inoculated with 50 μl of an overnight grown culture (approx. 10^9 CFU) of strain *E. coli* MG1655 *csrA* Nal (Table 1), and incubated at 37 °C on an orbital shaker at 50 rpm. The slides were transferred daily to tubes with fresh medium. For biofilm quantitative analysis, slides with established (24 h, 48 h or 72 h) recipient biofilm were washed by submerging them in 50 ml saline to remove non-attached cells, and then transferred into 25 ml of 1 μM solution of SYTO®63 dye in saline buffer for 2 h, washed again and observed with the Olympus Fluoview FV1000 Confocal microscope (Olympus, Center Valley, PA) using 40× (LUMPlanFI/IR) lens. Biofilm images were processed using Olympus FV10-ASW 1.6 and analyzed by COMSTAT2 software (Heydorn et al., 2000) for biomass, average thickness, roughness, maximal thickness and surface to biovolume ratio.

2.3. Conjugation in liquids

As a reference point for plasmid transferability in liquid medium, so-called 'liquid matings' were performed. The following protocol was used to mimic the physiological state of the recipient cells used in the biofilm invasion assays. First, a recipient biofilm of *E. coli* MG1655 *csrA* Nal was grown on slides for 24 h, 48 h or 72 h, washed to remove the non-attached cells as described above and moved into 25 ml of an overnight culture of the *E. coli* K12Rif plasmid donor strain with one of the 19 plasmids (approx. 10^8 CFU/ml). *E. coli* MG1655 *csrA* Rif was used as donor in one experiment. To reduce the number of donor cells in some of the experiments, the overnight cultures were diluted 100× in fresh medium. Cells were immediately detached from the slide by a combination of scraping and vortexing (2 min), as described previously (Król et al., 2011), and the slide was removed. Mating mixtures were incubated on an orbital shaker at 37 °C at 50 rpm for 4 h (or 8, 12, 16, 20 or 24 h in the time course experiment). Conjugation was stopped by vortexing and serial dilutions were plated on selective media. The transfer efficiency after the specified incubation period is presented as the ratio of transconjugant cells to the product of the number of donor and recipient cells (T/R×D). The matings were done at least in triplicate using the same donor and recipient precultures for each of the 19 plasmid donors.

2.4. Biofilm invasion experiments

To measure plasmid transfer into *E. coli* biofilms, slides with established recipient biofilms (grown for 24, 48 or 72 h) were washed by submerging them in 50 ml saline to remove non-attached cells, transferred into 25 ml of an overnight grown culture of plasmid donor strain (approx. 10^8 CFU/ml), and incubated at 37 °C and 50 rpm for 4 h (or 8, 12, 16, 20 or 24 h in the time course experiment). As in liquid control experiment the donor was *E. coli* K12Rif with one of the 19 plasmids or *E. coli* MG1655 *csrA* Rif in one experiment. After incubation with the donor strain (from hereon called the infection time), slides were washed to remove the non-attached cells. Attached cells

Table 1
Bacterial strains and plasmids.

Bacterial strains or plasmids	Relevant phenotypes and genotypes	References or source
Strains		
E. coli K12 MG1655	Wild type	ATCC 700926
E. coli K12Rif	Rif^R [a]	Fox et al. (2008)
E. coli MG1655*csrA*	*csrA*:Tn*5*, Km^R	Jackson et al. (2002)
E. coli MG1655 *csrA* Nal	Nal resistant variant of MG1655 *csrA*	This work
E. coli MG1655 *csrA* Rif	Rif resistant variant of MG1655 *csrA*	This work
Plasmids		
pAR060302	IncA/C **Tc^R** Km^R Amp^R	Call et al. (2010)
F′	IncFI, **Tc^R**	Ghigo (2001)
pIP162	IncFI, **Tc^R** Sm^R **Amp^R** Sul^R Cm^R	Ghigo (2001)
R1	IncFII, Cm^R Km^R **Amp^R**	Ghigo (2001)
pSal8719	IncFII, Cm^R Km^R **Tc^R**	Call et al. (unpublished)
R64	IncI1, **Tc^R** Sm^R	Ghigo (2001)
pIP175	IncI2, **Amp^R**	Ghigo (2001)
pIP69	Inc7/L/M, **Tc^R** Km^R Amp^R	Ghigo (2001)
pIP135	Inc7/L/M, **Tc^R**	Ghigo (2001)
N3 (=RN3)	IncN, **Tc^R**	Glocker and Rasched (1990)
pIP113	IncN, **Tc^R**	Ghigo (2001)
pB10	IncP-1β, **Tc^R** Sm^R Amx^R Sul^R Hg^R	Schlüter et al. (2003)
RP4	IncP-1α **Tc^R** Amp^R Km^R	Datta et al. (1971)
pRGM1	IncU, **Gm^R** Sm^R Amp^R Sul^R Ery^R Sp^R Cef^R $Cipro^R$	Brown & Top (unpublished)
pHH2-227	IncW/IncU, **Tc^R** Sm^R Sul^R Pur^R	This work
RSa	IncW, **Cm^R** Km^R	Ghigo (2001)
pIP1100	IncX, Sm^R **Gm^R** Amp^R Ery^R	Ghigo (2001)
R6K	IncX, **Amp^R**	Ghigo (2001)
RIP71a	Inc9, **Tc^R** Cm^R Amp^R	Ghigo (2001)

[a] Abbreviations refer to resistances to the following antimicrobials: Rif^R, rifampicin; Km^R, kanamycin; Cm^R, chloramphenicol; Amp^R, ampicillin; Sm^R, streptomycin; Gm^R, gentamicin; Sul^R, sulfonamide; Ery^R, erythromycin; Cef^R, ceftazidime; Sp^R, spectinomycin; $Cipro^R$, ciprofloxacin; Pur^R, puromycin; Hg^R, mercury; Nal^R, nalidixic acid. Antibiotics used for selection are in bold.

were immediately detached from the slide into 25 ml of saline as described above and serial dilutions were plated on selective media. Transfer efficiency was presented as described above. Each experiment was performed with at least three replicates.

2.5. Statistical analysis

A standard unpaired *t*-test was used in the case of simple two-group comparisons. Biofilm parameters were analyzed using tests for comparing multiple treatments. In the case of analysis of transfer efficiency measured by T/(R×D) nonparametric methods were used instead of the standard analysis of variance (ANOVA), because one of the assumptions of ANOVA was violated for both raw and transformed data. In order to test if there were significant differences in T/(R×D) between the plasmids the Kruskal–Wallis test was applied (Kruskal and Wallis, 1952). After rejecting the null hypothesis of no differences between plasmids, the rank-based HSD (Honest Significant Difference) multiple comparisons procedure was used to determine for which plasmids T/(R×D) differed significantly. To test whether T/(R×D) increased or decreased as incubation time increased, the Jonckheere–Terpstra (J–T) test was applied (Jonckheere, 1954). To determine in which time intervals significant changes in T/R×D occurred over a specific time course, the Bonferroni multiple comparisons procedure was applied. Additionally, ANOVA followed by the HSD multiple comparisons procedure was used to test if the number of donors (D) changed significantly as the incubation time increased. Prior to the analysis of variance, the data were transformed using either log or Box–Cox transformation (Box and Cox, 1964). These analyses were performed using R and SAS software.

2.6. DNA sequence analysis of the plasmid pHH2-227

The genome sequence of plasmid pHH2-227 was determined by shotgun sequencing at the DOE Joint Genome Institute (Walnut Creek, CA), with 10× coverage. Regions of poor sequence quality were resequenced at the University of Idaho by PCR amplification followed by sequencing using a BigDye Terminator v.3.1 cycle sequencing kit and a 3730 DNA analyzer (Applied Biosystems, Carlsbad, CA). The sequence was automatically annotated by the J. Craig Venter Institute Annotation Service (http://www.jcvi.org/annotation/service/) and further annotated manually using Manatee (manatee.sourceforge.net). The sequence data of plasmid pHH2-227 have been submitted to the DDBJ/EMBL/GenBank databases under accession no. JN581942.

3. Results and discussion

3.1. Ability of 19 plasmids to transfer between E. coli hosts

First, we tested if all 19 drug resistance plasmids from more than 10 incompatibility groups were able to transfer in a liquid environment from *E. coli* MG1655Rif to MG1655 *csrA* Nal. To ensure that the numbers and physiological state of the recipient cells were similar to those of cells

used in the biofilm experiments, the recipient was first grown as a biofilm for 48 h. These biofilm cells were then resuspended in cultures of the donor strains, which were grown overnight without selection for the plasmid, and the mixtures were incubated for four hours. All plasmids transferred into the recipient during that short time period (4 h) (Fig. 1A). However, the transfer efficiencies differed greatly between plasmids, even within plasmids of the same incompatibility group, up to 5–6 orders of magnitude. The number of recipient cells was relatively similar in all matings, and all plasmids except pIP69 were maintained in *E. coli* without selection. Based on the transfer efficiency (T/R×D), the highest rank was assigned to the (Inc7/L/M) plasmid pIP69, but this was due to a lower number of plasmid-bearing donors in the mating mixture because of poor plasmid maintenance (Fig. 1A). High transfer efficiency was also observed for pHH2-227, a hybrid between IncU and IncW plasmids (see pHH2-227 DNA sequence analysis, Section 3.6), and for plasmids F′ and pIP162 (IncFI), which are known to transfer very efficiently in liquids. The IncP (IncP-1) plasmids pB10 and RP4 ranked above average, even though they are known to transfer better on solid surfaces than in liquids (Bradley et al., 1980).

The transfer efficiency was calculated as T/(R×D) as this method has been shown to work best in the case where the local density of donor and recipient cells is different than the total numbers (Angles et al., 1993; Jones et al., 1991; Sengelov and Sørensen, 1998; Sørensen and Jensen, 1998; Zhong et al., 2012).

3.2. Plasmid invasion of an E. coli biofilm

To assess the ability of the 19 plasmids to invade an *E. coli* biofilm, their efficiencies of transfer into a 48 h old recipient biofilm were compared after 4 h of incubation with the donors. The number of recipient cells recovered after that period was highly consistent across the 19 donors (Fig. 1B). The number of donors used was high ($3.81 \pm 1.69 \times 10^8$ CFU/ml), but the number actually attached to the biofilms after 4 h was at least 100 times lower (Fig. 1B). This number was surprisingly similar among the donors, and also similar to that of the same *E. coli* strain without plasmid (data not shown). Except for pIP69 (due to stability issues described above), none of the other 18 attached donor densities significantly differed from each other. Although sex pili involved in conjugation in Gram-negative bacteria have been shown to promote biofilm formation in several studies (Ghigo, 2001; Reisner et al., 2006), some reports have challenged their role in attachment to the biofilm (Burmølle et al., 2008; Ong et al., 2009). Our results showed that the type of plasmid pili did not affect *E. coli* biofilm attachment in a 4-h period.

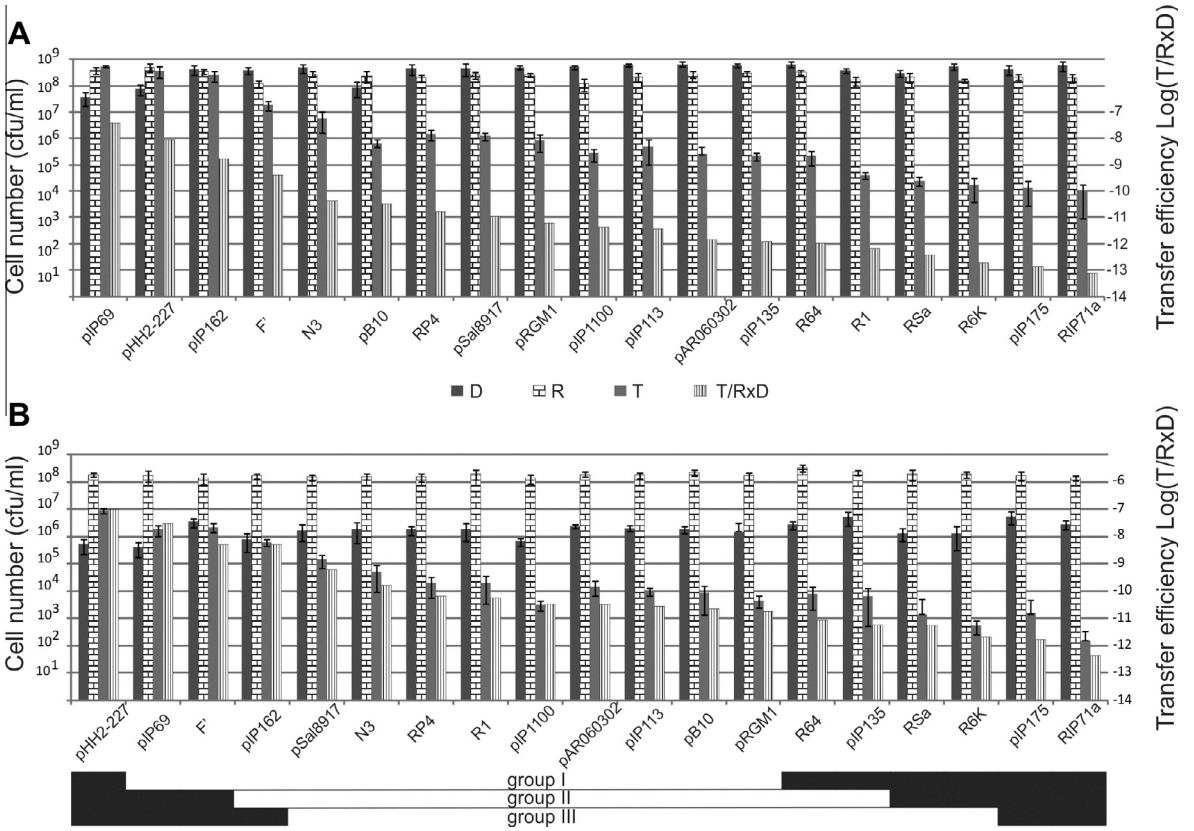

Fig. 1. Plasmid rankings based on efficiency of plasmid transfer (T/R×D) between *E. coli* cells in liquids (A) and biofilms (B). Data represent the number of donors (D), recipients (R) and transconjugants (T) (CFU/ml), and Log_{10} (T/R×D) after 4 h. Statistically significant groups in the biofilm experiment are marked as black squares.

A ranking of transfer efficiencies in biofilms (Fig. 1B) showed that in general, plasmids that transferred well in a liquid environment also showed the highest transferability in biofilms. Moreover, plasmids that transferred poorly in liquids were also ranked low in the biofilm assay. Based on a HSD multiple comparisons procedure we distinguished three sets of plasmid groups that showed significant differences in transfer frequencies (Fig. 1B). For example, the top-ranked plasmid pHH2-227 was significantly more transferable than the six lowest ranked plasmids, with transfer efficiencies differing between 10^4- and 10^6-fold (see group I in Fig. 1B).

Some interesting changes in ranking were observed between liquid and biofilm environments (Fig. 1A and B). The only two IncFII plasmids tested, R1 and pSal8719, ranked higher in the biofilm than in the liquid matings, while plasmids like pB10 (IncP-1) dropped down in the biofilm ranking. The latter is consistent with our previous observations that pB10 transferred poorly in submerged biofilms and more efficiently on agar surfaces and at the air–liquid interface (Fox et al., 2008; Król et al., 2011). Increased transfer of the two IncFII plasmids in biofilms compared to liquids may be caused by a temporal derepression in conjugation after the first round of transfer to the recipient cells (Lundquist and Levin, 1986; Willetts, 1974). Because of the mixing in liquids, an immediate second round is less likely, while in biofilms recipient cells are close to each other, allowing immediate subsequent rounds of transfer. Thus even plasmids like R1 that are repressed for transfer and previously shown not to invade in liquids or on agar surfaces (Lundquist and Levin, 1986; Simonsen, 1990; Simonsen, 1991) may transfer efficiently enough in biofilms to be maintained in *E. coli* populations as parasitic mobile elements.

The plasmid transfer efficiencies (T/R×D) were 7–700 times higher in biofilms than in liquids, suggesting that the biofilm mode of growth promotes plasmid spread from an invading donor population. That difference was mostly due to the low number of donor cells attached to the biofilm at the end of the 4 h period compared to the total number in liquid (see Fig. 1B), and not to higher absolute numbers of transconjugant (Fig. 1A and B). Therefore the mating experiment in liquid was repeated for plasmids pB10, RP4, F′ and pHH2-227 with a lower number of donor cells in the inoculum ($\sim 10^6$ instead of $\sim 4 \times 10^8$ CFU/ml). The efficiency of conjugation was still significantly lower than in biofilms, yet the difference was smaller for three of the four plasmids (by a factor 5 for pB10, 43 for RP4, 80 for F′, yet 10,300 for pHH2-227; $P < 0.001$). Thus for several plasmids the transfer efficiency which is a measure of plasmid invasion, was consistently higher in biofilms than in well-mixed liquids. No matter how the plasmid transfer efficiencies are compared between liquid and biofilm setups, the comparison is compromised by our inability to determine the true number of donors and recipients involved in plasmid exchange in the biofilms. The actual number of donors that transferred plasmids into the biofilms was probably higher than the population attached to the recipient biofilm after four hours since cells in the liquid phase may have attached just long enough to transfer their plasmid before they detached. Indeed, conjugative plasmid transfer takes only a few minutes (Achtman, 1975; Andrup, 1998; Babic et al., 2008; Lagido et al., 2003; Lawley et al., 2002; Seoane et al., 2011). Therefore comparisons of plasmid transfer efficiencies between totally mixed liquid system and biofilms should always be interpreted with caution.

3.3. Effect of biofilm age on plasmid transfer efficiency

A few reports have shown that plasmids can transfer only to the peripheral cells in the biofilm microcolonies and that deeper biofilm layers cannot be invaded (Christensen et al., 1998; Haagensen et al., 2002; Hausner and Wuertz, 1999; Nancharaiah et al., 2003). We tested the hypothesis that more structured and thicker biofilms result in a lower proportion of recipient cells transformed into plasmid-bearing cells. This was done by evaluating the effect of the recipient biofilm age on efficiency of plasmid invasion after incubating recipient biofilms formed for 24 h, 48 h and 72 h separately with eight plasmid donors (pB10, RP4, F′, pHH2-227, pRGM1, N3, pIP135 and pIP113). The differences in biofilm properties were observed at both macro- (Fig. 2A) and micro-scale (data not shown). Three biofilm areas were described, as we have shown previously that the biofilm structure varies with location along the slide (Król et al., 2011). The physical properties differed both with age and location on the slides (Fig. 2B). The numbers of recipient cells were consistent between matings and increased slightly every 24 h (Fig. 3). All analyzed biofilm parameters, except maximal thickness, showed statistically significant ($P < 0.0001$) differences between 24 and 48 h but not between 48 and 72 h (Fig. 2B). The number of donors attached to the biofilm did not depend on the biofilm age (Fig. 3). The efficiency of transfer T/(DxR) analyzed using a Jonckheere–Terpstra (J–T) test showed a statistically significant decrease with recipient biofilm age in the cases of pB10, pHH2-227, pIP113, and RP4 (data not shown). Detailed analysis using a Bonferroni multiple comparisons procedure showed that for the first three plasmids these differences were only significant between 24 h and 48 h or between 24 h and 72 h (pIP113). This correlated with significant changes in the biofilm structures. In the case of RP4 the differences were significant between all three time points. For the remaining plasmids (pRGM1, F′, pN3 and pIP135) the differences in transfer efficiency between biofilm ages were not statistically significant. These findings indicate that under our experimental conditions, plasmid transfer to older and thicker recipient biofilms never resulted in higher proportions of plasmid-bearing cells than transfer into thin young biofilms. This confirms earlier studies that plasmids do not easily invade into the deeper layers (Molin and Tolker-Nielsen, 2003; Seoane et al., 2011). Future studies should determine the effects of biofilm physical properties on plasmid invasion.

3.4. Prolonged invasion time affects transfer efficiency

The short incubation time of four hours in the experiments described above may not have allowed for deep plasmid invasion. We postulated that a longer ‘infection

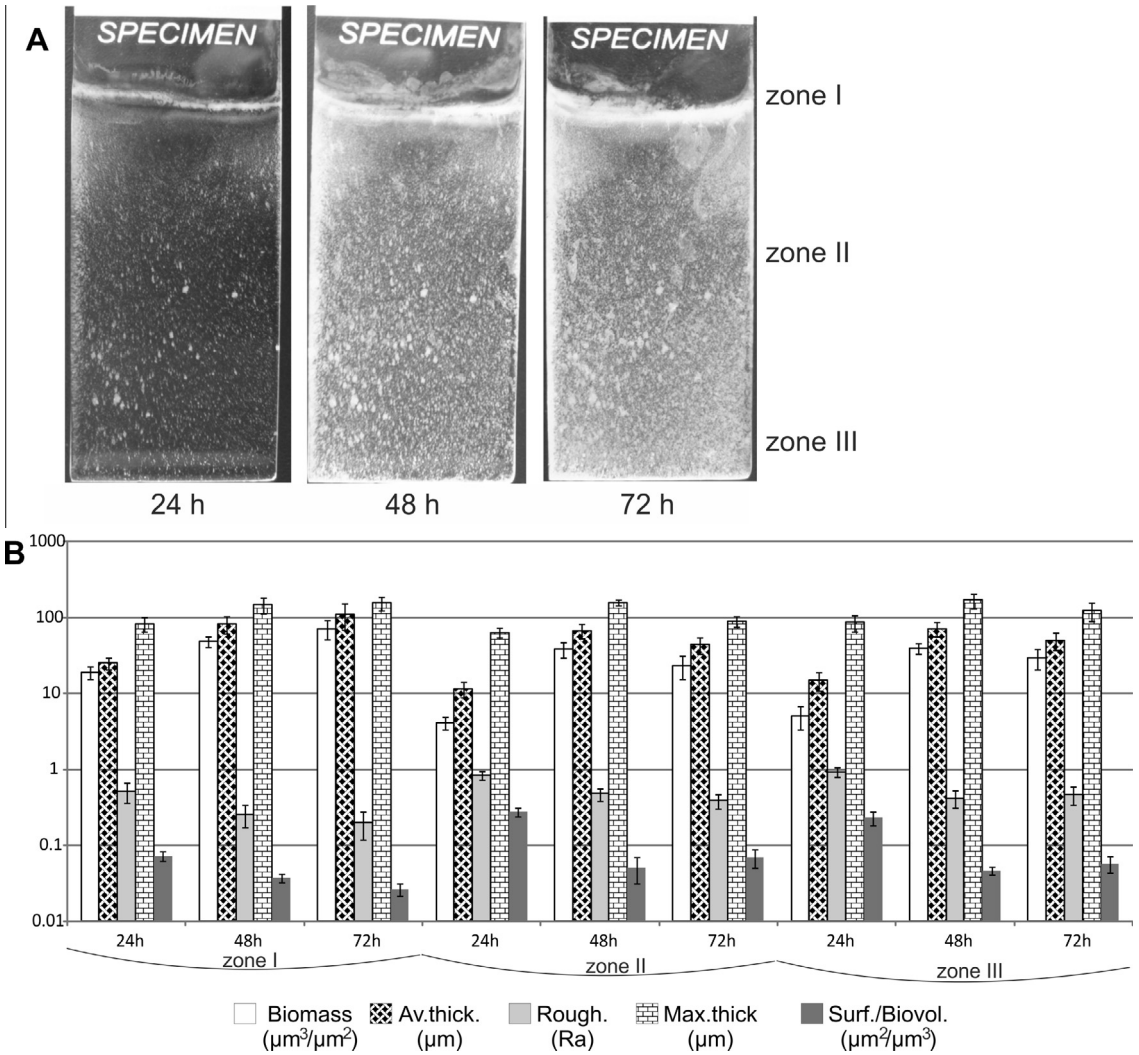

Fig. 2. Batch culture biofilms of *E. coli* MG1655 *csrA* Nal. (A) Microscope slides with 24 h-, 48 h-, and 72 h-old biofilms. (B) Quantitative analysis of biofilm features over a 72 h time period. CLSM images from three areas of the 24, 48 and 72 h-old biofilms were analyzed using the COMSTAT2 image analysis program (Heydorn et al., 2000) as described in the Methods. At least ten independent stacks were analyzed per area per day. Compared parameters are: biomass, average thickness, roughness, maximal thickness and surface to biovolume ratio in zone I (air–liquid interface), zone II (central part), and zone III (bottom part of the slide).

time' allows (i) more donors to attach to the biofilm, and (ii) plasmids to penetrate into deeper biofilm layers, resulting in higher number of transconjugants. As the first 24 h have been described to be important in plasmid biofilm infection (Licht et al., 1999), we increased the infection time in 4-h increments, up to 24 h using two IncP-1 plasmids, pB10 and RP4. In both cases we observed an increasing number of donors attached to the 48 h-old recipient biofilm, but the HSD multiple comparisons procedure showed that differences were only statistically significant between some time points (Fig. 4). The conjugation efficiency significantly increased over time in both experiments, based on the results of the J–T test. Thus even with increasing numbers of attached donors, the ratio T/R×D still increased over time due to a drastic increase in transconjugants.

These data suggest that for a similar initial number of donor cells, longer incubation times allow increased donor attachment and more efficient plasmid invasion. Monitoring this invasion microscopically in future studies will be essential to determine whether the 'wave' of plasmid spread is indeed able to penetrate deep into biofilms when donors are in contact with these biofilms for multiple hours or even days. We also noticed that longer incubation time without a new supply of nutrients led to cell detachment from the biofilm and affected the results (Fig. 4, 24 h). Such an exchange of attached cells between the biofilm and the aqueous phase under no flow conditions was found previously (Angles et al., 1993; Hermansson and Marshall, 1985).

The time-dependent increase of transconjugant numbers in laboratory liquid or filter matings is a well-known

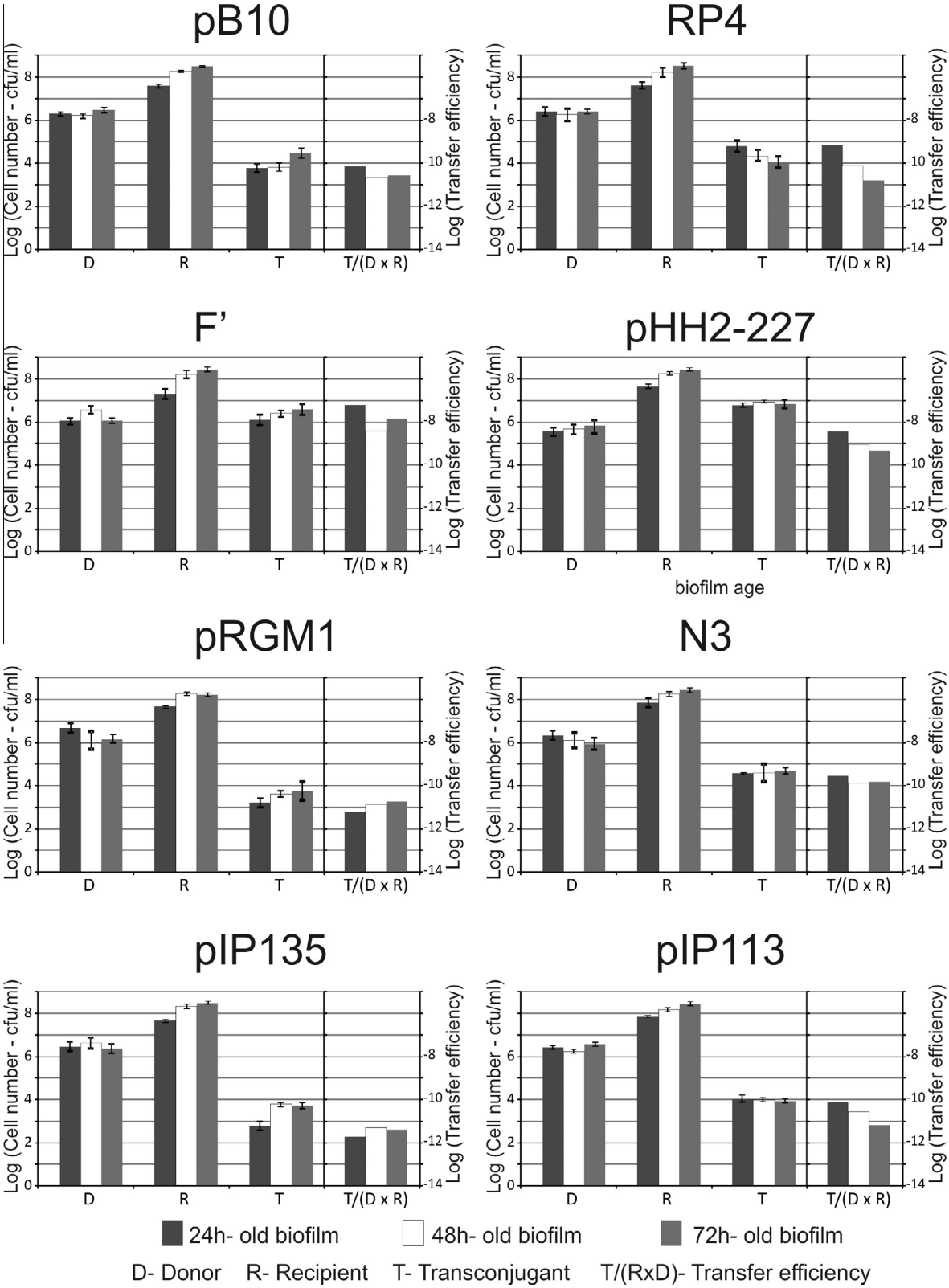

Fig. 3. Effect of biofilm age on plasmid transfer in *E. coli* biofilms. Number of donors (D), recipients (R) and transconjugants (T) as Log_{10} (CFU/ml), and the efficiency of plasmid transfer Log_{10} (T/DxR) in 24 h-, 48 h- and 72 h-old biofilms.

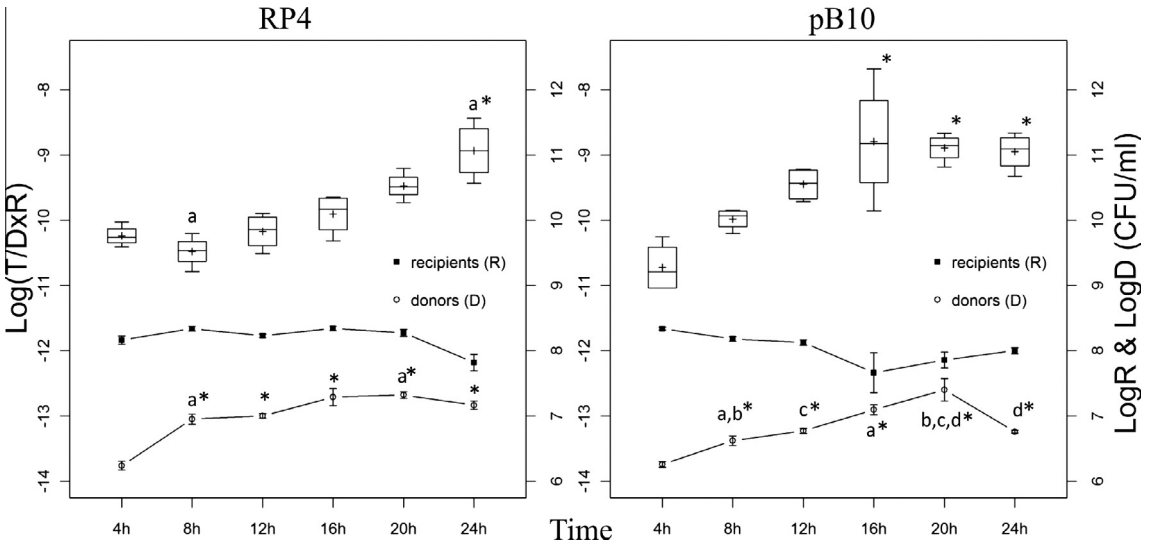

Fig. 4. Effect of infection time on invasion of plasmids RP4 and pB10 in *E. coli* biofilms. Efficiency of plasmid transfer is presented as a box plot of Log_{10} (T/(DxR)) values. Error bars for D and R show standard errors. Statistical differences ($P \leqslant 0.05$) between transfer efficiencies and numbers of attached donors at different time points are as follows: *all values that differ from the one at 4 h; letters a–d, data points with the same letter are statistically different from each other.

fact (Andrup and Andersen, 1999; Cullum et al., 1978; Levin et al., 1979; Snowsill et al., 1981; Willard et al., 1982; Williams et al., 1977). Previous observations of plasmid transfer in biofilms also showed that co-inoculation time affects transfer efficiency (Hausner and Wuertz, 1999; Licht et al., 1999). Here we showed that prolonged infection time results in increased donor attachment and transfer efficiency. Limiting the contact time between a drug resistance plasmid donor and a biofilm should thus help minimize antibiotic resistance transfer.

3.5. Increased plasmid host strain attachment enhances plasmid transfer

We hypothesized that donor strains that attach more efficiently to a biofilm would facilitate more efficient plasmid transfer. To test this hypothesis we compared the transfer efficiency of plasmids pB10 and RP4 in liquid and biofilm experiments between two donors: the efficient biofilm former *E. coli* MG1655*csrA*Rif, and the strain used above, MG1655Rif. For both plasmids, the number of MG1655 *csrA* cells attached to the biofilm was ~3-fold higher ($P = 0.03$) than that of wild-type MG1655, and this resulted in an almost 20- ($P = 0.007$) and 5-fold ($P = 0.0002$) increase in conjugation efficiency (T/DxR) for pB10 and RP4, respectively (data not shown). However, also in liquid matings a 6- and 5-fold increase ($P < 0.005$) in transfer efficiencies of pB10 and RP4 was observed for the *E. coli csrA* donor, suggesting that the efficiency of plasmid transfer was increased by the ability of the donor strain to attach to recipient cells in both environments. Since the *csrA* mutation affects other cellular processes, further work is needed to determine if donor attachment significantly affects plasmid invasion in established biofilms.

3.6. Sequence analysis of the most invasive plasmid pHH2-227

Since plasmid pHH-227 transferred at the highest efficiency into the *E. coli* biofilm, we determined the plasmid's genome sequence. This plasmid was exogenously isolated from an arable soil bacterial community in matings with an *E. coli* recipient and confers resistance to sulfadiazine, tetracycline and streptomycin (Heuer et al., 2009). The complete 38,963 bp DNA sequence of plasmid pHH2-227 showed a hybrid structure with elements from IncW and IncU/IncP-6 plasmid backbones and accessory elements (Fig. S1). The replication module with the *oriV*-region and the replication initiation gene *repA* is similar to that of IncU plasmids, and appears to have replaced the original IncW replication module (Fig. S1). The most similar *repA* gene found in GenBank is that of the IncU plasmid Rms149 (Haines et al., 2005) (71% nucleotide identity). The 27 kb segment of the plasmid backbone coding for plasmid stability, mating pair formation and DNA transfer resembles that of IncW plasmids pMAK3 (98.2%), R388 (98.0%) and R7K (98.0%) (Revilla et al., 2008). For the IncW plasmid RSa (also called pSa), which transferred much less efficiently in this study (Fig. 1), only 6 kbp of the backbone showed similarity (92%) to that of pHH2-227. Notably, the *oriT* regions of the two plasmids strongly differ in protein binding sites and the promoter sequence of *trwA*, which might affect transfer rates (Revilla et al., 2008). The *oriT* of pHH2-227 is identical to that of R7K. Plasmids carrying multiple *oriV* and *rep* genes from different plasmid groups have been described earlier (Pagotto and Dillon, 2001; Schlüter et al., 2007). Such plasmids may evolve to a hybrid plasmid like pHH2-227 by deletion of one replication module. The recently discovered group of Low GC-type plasmids also carries an acquired replication module, putatively replacing the ancestral *oriV-repA* (Heuer et al.,

2009). Accessory elements of pHH2-227 are a class 1 integron, the gene cassette *aadA13*, and the transposon Tn*1721*. The class 1 integron is most similar to that on plasmid pPA25 (EU930362). The gene cassette *aadA13* with recombination site *attC* is integrated at the same position as in plasmid R7K at an IntI1 secondary site (Revilla et al., 2008). The transposon Tn*1721* with intact inverted repeats and flanked by 5 bp direct repeats is inserted upstream of *kfrA* and separated from the acquired replication module by a small fragment (<150 bp) that contains signatures from the IncW backbone (Fig. S1). Detailed plasmid annotation is given in Table S1.

4. Conclusions

Although the role of bacterial plasmids in biofilm formation has been studied previously (Ghigo, 2001; May and Okabe, 2008; Reisner et al., 2006), the actual efficiency of invasion of antibiotic resistance plasmids into biofilm-embedded cells has not been studied in detail. Several authors have described plasmid transfer in bacterial biofilms (Angles et al., 1993; Christensen et al., 1996, 1998; Ehlers and Bouwer, 1999; Hausner and Wuertz, 1999; Król et al., 2011; Licht et al., 1999; Lilley and Bailey, 2002; Mølbak et al., 2007; Molin and Tolker-Nielsen, 2003; Nancharaiah et al., 2003; Normander et al., 1998; Ong et al., 2009), but differences in experimental settings such as bacterial strains and plasmids used, biofilm growth conditions and transfer detection methods, make it hard to compare plasmid transfer efficiencies. Here we report for the first time the ability of many drug resistance plasmids to invade *E. coli* biofilms under the same, reproducible conditions. We show that all plasmids could rapidly transfer into an existing biofilm and that the efficiency of this transfer differed widely between plasmids. A surprising observation is that donor attachment only marginally depended on the type of plasmid carried by the host. This finding depreciates the role of plasmid-encoded pili in short-term attachment to biofilm embedded cells. We also point out that the actual efficiency of plasmid transfer into biofilms and the comparison with transfer on plates or liquids has to be carefully interpreted given the differences in densities of parental strains. We hope that this work will aid future studies aimed at determining factors that can limit the spread of MDR plasmids in bacterial biofilms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plasmid.2013.03.003.

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