Abstract

Increased tolerance to antimicrobial agents is thought to be an important feature of microbes growing in biofilms. We address the question of how biofilm organization affects antibiotic susceptibility. We established Escherichia coli biofilms with differential structural organization due to the presence of IncF plasmids expressing altered forms of the transfer pili in two different biofilm model systems. The mature biofilms were subsequently treated with two antibiotics with different molecular targets, the peptide antibiotic colistin and the fluoroquinolone ciprofloxacin. The dynamics of microbial killing were monitored by viable count determination, and confocal laser microscopy. Strains forming structurally organized biofilms show an increased bacterial survival when challenged with colistin, compared to strains forming unstructured biofilms. The increased survival is due to genetically regulated tolerant subpopulation formation and not caused by a general biofilm property. No significant difference in survival was detected when the strains were challenged with ciprofloxacin. Our data show that biofilm formation confers increased colistin tolerance to cells within the biofilm structure, but the protection is conditional being dependent on the structural organization of the biofilm, and the induction of specific tolerance mechanisms.

Introduction

Natural conjugative plasmids express factors that can stimulate planktonic bacteria to form biofilm communities [1]. Biofilm formation also favors the infectious transfer of plasmids [2,3], and many medically relevant microbial pathogens are often associated with a variety of conjugative plasmids indicating an increased tendency for biofilm formation [1,4–6]. Microbial biofilms are notoriously hard to treat with antimicrobial compounds. The molecular nature of this apparent resistance is not well elucidated and a number of mechanisms have been proposed to explain the reduced susceptibility [7]. The architecture of mature biofilms varies considerably from flat homogenous layers of cells to highly organized cell clusters characterized by mushroom shaped structures interspersed by water-filled channels [8]. The organization of the biofilm affects the distribution of chemical and physiological gradients within the microbial assembly, which in turn affects the metabolic state of the bacteria, resulting in a heterogeneous congregation of cells in different physiological states [8–10]. The spatial and physiological heterogeneity of the biofilm architecture has the potential to affect the antimicrobial tolerance profiles of the organisms in the biofilm by forming microhabitats with diverse properties [11].

Escherichia coli (E.coli) K12 strains usually form unstructured flat biofilms in comparison with the highly organized and substantial biofilms formed by bacteria such as Pseudomonas aeruginosa (P.aeruginosa) [6,12]. Introduction of transfer constitutive IncF plasmids dramatically changes the organization of E.coli biofilms to a structure resembling those reported for P.aeruginosa [1,6]. Biofilm organization is determined by the configuration of the transfer pili since various mutants affected in the processing and activity of the pili exhibit differentially structured biofilms [6].

The ability to survive exposure to cationic antimicrobial peptides is an essential virulence property for many microbial pathogens [13]. Antimicrobial peptides are an integral part of the host defense system and believed to kill microorganisms by affecting their membrane integrity. Colistin is an amphipathic polypeptide antibiotic utilized in the treatment of multi-resistant P.aeruginosa causing chronic pulmonary infections among cystic fibrosis patients. The peptide antibiotic is also extensively used both as feed additive and in the treatment of severe colibacillosis caused by pathogenic E.coli in livestock [14,15]. Resistance towards antimicrobial peptides such as colistin is seldom identified in the clinical setting [16]. Gram-negative bacteria have evolved mechanisms that remodel the composition of the outer membrane through modification of the lipopolysaccharide (LPS) molecules [13]. The modifications lead to an increased tolerance to antimicrobial peptide exposure, and is controlled by a two-component regulatory system encoded by homologs to the Salmonella typhimurium pmkA/B genes in many bacterial species [17,18]. LPS alterations are thought to play a significant role during infection by conferring increased bacterial tolerance to host antimicrobial factors and avoidance of immune system recognition [13].

In contrast to colistin, the primary E.coli target for fluoroquinolone antibiotics such as ciprofloxacin is the topoisomerase II...
enzyme, DNA gyrase, which is essential for DNA synthesis [19]. Resistance to fluoroquinolones in *E. coli* occurs by chromosomal mutation and requires alterations in either of the genes that encode the topoisomerases (gyrA and gyrB, encoding gyrase, and parC and parE, encoding topoisomerase IV) or in genes that influence cell permeability or drug export [20,21].

In this report, we challenge *E.coli* biofilms with differential structural organization with colistin or ciprofloxacin, two clinically relevant antibiotics with different molecular targets and mechanisms of resistance. We show that biofilm formation increases the survival of the microbes after challenge by the peptide antibiotic but not by the fluoroquinolone. The increased survival is dependent on the *traR/traS* two-component system, since disruption of their encoding genes abolishes the observed protective effect while not affecting the organization of the biofilm. We demonstrate that the increased survival is due to development of a tolerant subpopulation within the biofilm and that the formation of the subpopulation is influenced by the spatial organization of the biofilm indicating that intra-biofilm physiochemical gradients promote antibiotic tolerance within spatially structured environments.

**Results**

**E.coli** cells in biofilms differing in organization display differential survival after colistin treatment

The presence of transfer constitutive IncF plasmids fundamentally influences the architecture and organization of *E.coli* biofilms [6]. The final spatial arrangement of the biofilm is determined by the configuration of the transfer plasmid, as various mutants in the processing and activity of the organelle display differentially organized biofilms [6]. F pilus synthesis requires 13 essential genes and 4 auxiliary genes of plasmid origin. TraQ is required for insertion of propilin (TraA) in the inner membrane. Absence of the TraQ chaperone results in rapid degradation of TraA in the cytoplasm [22]. TraX is responsible for acetylation of the N-terminus of mature pilin, but the modification is non-essential for pilus elaboration and transfer proficiency [23,24]. TraD is not involved in pilus assembly but instead has an essential role in DNA transfer at a stage following cell/cell contact [25]. To study the effects of differential biofilm organization in relation to antibiotic tolerance, we established a static biofilm and survival assay of *wildtype*, *traQ*, *traX*, and *traD* mutant derivatives of pOX38 and plasmid-free *SAR18* biofilm cultures. The strains were inoculated in Luria broth using standard polystyrene culture tubes and incubated under shaking (rpm 220) conditions for 24 h. The cultures were then challenged with 10 μg/ml colistin for an additional 24 h and surviving colony-forming units (CFU) were determined by serial dilution and plating on culture media. For all strains approximately 3×10^8 CFU had survived the colistin challenge, while the untreated control cultures had reached a CFU of 1×10^9. There was no significant difference in survival between wildtype, *traQ*, *traX*, and *traD* mutant derivatives of pOX38 and plasmid-free *SAR18* biofilm cultures (Fig. 1D). If the shaken over night cultures instead were challenge with 0.3 μg/ml ciprofloxacin for 24 h, 2×10^6 CFU were detected corresponding to a 3 log difference of survival between the different growth model systems (Fig. 1D). These data indicate that the presence of the F plasmid and its mutant derivatives does not affect antibiotic tolerance during planktonic growth.

**Mutations in the transfer operons do not confer protection in planktonic cultures**

Mutations in the transfer operon may have important pleiotropic consequences independent of the biofilm context, we therefore investigated the survival after colistin and ciprofloxacin treatment of planktonic cultures to the of wildtype, *traQ*, *traX*, and *traD* mutant derivatives of pOX38 and plasmid-free *SAR18* biofilm cultures. The strains were inoculated in Luria broth using standard polystyrene culture tubes and incubated under shaking (rpm 220) conditions for 24 h. The cultures were then challenged with 10 μg/ml colistin for an additional 24 h and surviving colony-forming units (CFU) were determined by serial dilution and plating on culture media. For all strains approximately 3×10^8 CFU had survived the colistin challenge, while the untreated control cultures had reached a CFU of 1×10^9. There was no significant difference in survival between wildtype, *traQ*, *traX*, and *traD* mutant derivatives of pOX38 and plasmid-free *SAR18* biofilm cultures (Fig. 1D). If the shaken over night cultures instead were challenge with 0.3 μg/ml ciprofloxacin for 24 h, 2×10^6 CFU were detected corresponding to a 3 log difference of survival between the different growth model systems (Fig. 1D). These data indicate that the presence of the F plasmid and its mutant derivatives does not affect antibiotic tolerance during planktonic growth.

**The increased survival of cells in colistin treated biofilms is dependent on a functional *basS* gene**

The *E.coli* K12 strain MG1655 is better characterized genetically than the *SAR18* strain. Moreover, *SAR18* harbors a number of antibiotic resistance markers that make genetic manipulation difficult (Table 1). We included the strain AF504, a nalidixic acid resistant derivative of MG1655, and a curli over-producing *ompR234* mutant of MG1655 in the investigation. Curli overproduction results in increased biofilm formation in the biofilm flow chamber system in an F-plasmid independent manner [6]. To investigate if the observed increase in colistin tolerance of biofilm cells is genetically determined or due to a generic difference in biofilm permeability, we introduced insertion deletions into the *basS* gene of AF504, using one-step allelic replacement [29]. We established biofilms of these mutant strains and compared the survival of the bacteria with or without the F-plasmid and its mutant *traD* variant after colistin treatment. As expected, introduction of the F-plasmid or its *traD* mutant derivative into AF504 drastically increased biofilm formation as well as colistin tolerance of the resulting strains (Fig. 2A and B). The protective effect of biofilm formation with F*+* and F*traD* was completely abolished in the *basS* mutants, and the survival reached a level comparable to the survival of plasmid free AF504 (Fig. 2B). If the
*basR* and *basS* genes were reintroduced on a multicopy plasmid into the *basS traD* mutant biofilm protection was restored (Fig. 2B), while if the same plasmid was introduced into AF504 or AF504 *F**(traX)* no effect on survival could be detected (Fig. 2B). If the vector alone was introduced to the strains, no effect on biofilm formation or survival could be detected (data not shown). The nalidixic acid resistance of AF504 does not affect the outcome of the biofilm survival assay since there is no detectable difference in survival between MG1655 and AF504 after colistin challenge (data not shown). These results indicate that a specific genetic mechanism is underlying the observed increased tolerance in biofilm forming *E. coli*. Over-expression of the *curli* fimbriae drastically influences the biofilm development in *E. coli* and such strains form biofilms comparable to the ones formed by *F**(traD)* and *F**(traX)* mutants (Fig. 2A [6,30]). No increase in survival after colistin treatment compared to the plasmid free AF504 was detected, demonstrating that biofilm formation and survival after antibiotic treatment are not directly correlated (Fig. 2B). If the same *basS* mutation was introduced into Sar18 *F*, pil mediated protection was abolished, showing that *basS* dependence is not exclusive for AF504 (Fig. 1B). Increased concentrations of colistin decreased the number of viable cells within the microtiter wells in a concentration dependent manner. Colistin concentrations above 60 μg/ml reduced the number of viable cells to below the detection level of 100 CFU/ml (Data not shown). The MIC for colistin for all of the AF504 derived strains were 1 μg/ml in microtiter assay.

**E. coli** flow-chamber biofilms are sensitive to the cationic antimicrobial peptide colistin

To be able to study biofilm formation *in vitro* in the hydrodynamic continuous-flow biofilm model system [31] we tagged AF504 *F* with the green fluorescent protein (GFP) under the control of a ribosomal promoter forming the strain AF504 *gfp*.

---

**Figure 1.** *E. coli* cells in biofilms differing in organization display differential survival after colistin treatment. A. Microtitre plate biofilm assay with the parental *E. coli* SAR18 in the presence and absence of the de-repressed IncF plasmid and mutants in the IncF plasmid (traO, traX, and traQ) stained with crystal violet. Values are normalized with respect to the mean of SAR18 *F**(traD)* set as 100% B. A microtitre plate biofilm antibiotic tolerance assay, in which the indicated strains were challenged with 10 μg/ml colistin for 24h and bacterial survival determined by viable count as indicated by white bars. Untreated control is indicated by black bars. C. Microtitre plate biofilm antibiotic tolerance assay, in which the indicated strains were challenged with 0.3 μg/ml ciprofloxacin for 24h and bacterial survival determined by viable count as indicated by white bars. Untreated control is indicated by black bars. D. The survival of planktonically cultured stationary phase *E. coli* SAR18 in the presence and absence of the de-repressed IncF plasmid and mutants in the IncF plasmid (traO, traX, and traQ) after a 24 h 10 μg/ml colistin or 0.3 μg/ml ciprofloxacin challenge. Black bars indicate CFU in untreated culture while white bars indicate treated cells as specified in the figure.

doi:10.1371/journal.pone.0001891.g001
A colistin tolerant subpopulation is formed in mature E. coli flow-chamber biofilms

E. coli mature biofilm cells are killed efficiently by colistin through gradual penetration into the centre of the microcolonies (Fig 3 A, B and C). However, in large microcolonies we observed a surviving population gradually increasing in biomass during the first 48 h after addition of colistin. This phenomenon was observed using 10 μg/ml and 25 μg/ml colistin (Fig. 3D and E).

Colistin tolerance is not due to resistance mutations

Based on these findings we wanted to investigate if the developing colistin tolerant subpopulations were the results of genetically mutated resistant cells, or if they represented phenotypically tolerant variants. We isolated the biofilm material from 2-day-old flow channel biofilms, grown in glucose minimal media, which had subsequently been exposed to 10 μg/ml or 25 μg/ml colistin, for 24–48 h. The isolated biofilm samples were homogenized and stained with PI after which individual cells were
treatment and reached 10^2 CFU/OD_{450} after 48 hours of drug-exposure. The number of viable cells in the control channel remained constant during the time of the experiment (10^6 CFU/OD_{450}). If 50 μg/ml colistin was used, an exposure for 24 h was enough for complete eradication of the biofilm and indeed no growth was observable after plating. If plasmid free AF504 gfp cells were inoculated in the flow channel system a few scattered loosely attached cells could be detected through the channel after 24 h and after a 24 h, 25 μg/ml colistin challenge very few bacteria could be detected (data not shown).

Table 1. Bacterial strains used in this study.

| Strains  | Description | Reference |
|----------|-------------|-----------|
| SAR18    | CSH26 attB:bla-PAT1/04/03gfpmut37Cm' | [6]        |
| AF504    | MG1655 Las' ilvG, rfb-50, rph-1 | This work. |
| AF504 gfp| AF504 attB:bla-mmbP1::gfpmut3b* | This work |
| AF518    | AF504 basS:kan | This work |
| AF574 gfp| AF504 basS::cm attB:bla-mmbP1::gfpmut3b* | This work |
| PHL 628  | MG1655 ompR234 | [63]      |
| PHL 628 gfp| PHL628 ompR234 attB:bla-mmbP1::gfpmut3b* | This work |
| XL10-Gold| TetA (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacIqZAM15 Tn10 (Tet)] | Stratagene |

F'. The flow chambers were seeded with the strain and incubated for 48h at 30°C in glucose minimal medium. The mature biofilms were then exposed to 25 μg/ml colistin for 0h (untreated control), 6.5h and 24h and stained with propidium iodide (PI) to distinguish living GFP expressing cells and dead PI stained cells (Fig. 3 A, B and C). The untreated control biofilm exhibited a few dead (red) cells spread out through the biofilm (Fig. 3A) while the majority of the cells in the treated biofilm were clearly dead after 24 h (Fig. 3B and C). The killing activity of the colistin moved from the edge of the microcolonies to the inner part as shown in time series image capturing (Movie S1). The time series also confirmed that detachment was absent or low under these conditions. To determine the proportion of viable cells in the biofilm channels, the biofilm was dislodged from the flow-channel and the CFU/ml were determined and normalized by the OD450 value obtained for each channel. The number of viable cells recovered from the channel decreased from 10^9 to 10^3 CFU/OD_{450} in 24 hours of

Figure 2. The increased survival of cells in biofilms is dependent on a functional basS gene. A) Microtitre plate biofilm assay with the parental E. coli AF504 in the presence and absence of the de-repressed IncF plasmid and mutants in the IncF plasmid (traD), insertion deletions of the basS and basR genes, and the curli overproducing ompR strain stained with crystal violet. Values are normalized with respect to the mean of AF504 F' (traD) set as 100%. B. A microtitre plate biofilm antibiotic tolerance assay in which the indicated strains were challenged with 10 μg/ml colistin for 24 h and bacterial survival determined. The fraction untreated/treated is indicated for each strain.

doi:10.1371/journal.pone.0001891.g002
sorted with a FACS cell sorter in red and green subpopulations. The red population was not viable as shown by plating on LB plates whereas the green population was growing on LB plates, but not on plates containing colistin (10 μg/ml or 25 μg/ml). Development of a tolerant subpopulation in the biofilm after treatment with colistin thus seems to be linked to adaptation or activation of a surviving phenotype suggesting that a transient colistin tolerant subpopulation is formed in the biofilm. When the cells were isolated from the flow chamber and re-grown without colistin they lost the tolerance to the peptide and became sensitive to the antibiotic, showing that the surviving tolerant cells were not genetically resistant mutants selected in the flow chamber.

Colistin tolerance is correlated to biofilm structure and architecture

To investigate the effect of biofilm architecture on antibiotic susceptibility we established flow chamber biofilms of E. coli in presence and absence of the de-repressed IncF plasmids (wildtype, traD, traQ and traX). It has been previously shown that the tra mutations lead to formation of biofilms with strikingly different spatial distribution [6]. The different biofilms were treated with colistin and the distribution of living and dead cells monitored. Interestingly, strains with the de-repressed IncF WT plasmid and the traD mutation forming biofilms with elaborate, rounded tower structures rising from the confluent cell layer, displayed regions of tolerant cells in the center of the biofilm micro-colonies after 24 h challenge with 10 μg/ml colistin. Moreover, this subpopulation formation seemed to be more extensive in the traD mutant biofilm compared to the wild type F’ (Fig. 4A). The strains lacking the IncF plasmid or carrying the traQ and traX mutant plasmids formed biofilms with a less structured organization, and without any recognizable mushroom shaped microcolony structures. In these biofilms no observable tolerant subpopulation was detected (Fig. 4A, data not shown). To evaluate the differences in bacterial survival in the biofilms quantitatively, we analyzed the CLSM data with the computer program COMSTAT [30]. The analysis showed that approximately 35% of the cells survived in the biofilm formed by SAR18 F’(traD), while survival was significantly lower (P<0.0030) for SAR18 F’ biofilms, in which around 15% of the cells were still green after 24h colistin treatment. In the biofilm formed by SAR18 F’(traX) less than 5% of the cells were viable after colistin challenge which is significantly less than SAR18F’ (P<0.0061). If the AF504 F’(traD) were challenged with colistin in the flow chamber system an extensive tolerant subpopulation was observed while when the AF504 F’(traX) strain no subpopulation could be detected (data not shown).

Induction of colistin tolerance is basS dependent

The development of the tolerant subpopulation can be due to a phenotypic change of the bacteria in the center of the large micro colonies or to a generic difference in biofilm permeability. We established flow chamber biofilms of E. coli AF504 F’ with additional chromosomal mutations in the basR/basS two-component regulatory system. There was no measurable effect of the basS mutation on biofilm structure, surface coverage and biomass as determined by COMSTAT analysis (data not shown). No tolerant subpopulation could be detected in the AF504 basS F’ biofilms after 24 h colistin treatment (Fig. 4B). COMSTAT quantification of the acquired images clearly showed a significant difference in the ratio between PI stained cells and GFP expressing cells in the AF504 F’ and the AF504 basS F’ (data not shown) (P<0.0095). These results suggest that subpopulation formation is genetically regulated.

The yfbE gene is induced within structured biofilms prior to colistin challenge

The yfb operon consists of seven genes (yfbE, F, G, H, yfbT, yfbW and yfb) that are implicated in the process of addition of the 4-amino-4-deoxy-L-arabinose (L-Ara4N) moiety to the phosphate groups of lipid A leading to increased polymyxin tolerance [32].
The induction of the yfb genes in vitro by low Mg\(^{2+}\) and high Fe\(^{3+}\) is under the control of the basR/basS two-component system [33,34]. To determine if tolerant subpopulation formation is correlated with increased yfbE operon expression, we constructed a plasmid harbouring the AF504 yfbE promoter in front of a promoterless gfp gene and introduced it into our collection of AF504 strains with differential biofilm formation ability. Expression of the yfbE::gfp reporter was induced when the strains were grown in N-minimal media with 10 \(\mu\)M Mg\(^{2+}\) and 100 \(\mu\)M Fe\(^{3+}\) relative to the expression levels when the strains were grown in media with 10 mM Mg\(^{2+}\)(Fig. 5A). No increase in fluorescence could be detected in strains, with a mutated basS gene, even when they were cultivated under inducing conditions (Fig. 5A), and no induction of the yfbE reporter could be detected by the addition of colistin to the N-minimal media under low or high Mg\(^{2+}\) conditions (data not shown). We then investigated GFP fluorescence of the different strains in microtiter biofilm assays. The bacterial population in the microtiter wells is highly heterogeneous and a direct measurement of the total fluorescence in the wells can be misleading. Instead, we determined the proportion of cells in the wells with elevated GFP fluorescence by using a strain with constitutive GFP expression to determine maximum range fluorescence and a strain with a promoterless vector control to determine minimum fluorescence. The proportion of induced fluorescent cells was significantly elevated in the biofilms formed by the AF504 \(F^+\) and AF504 \(F^+\)\(\Delta\)tauD strains compared to the biofilms formed by the wild type AF504 strain and the \(F^+\)\(\Delta\)tauX mutant (Fig. 5B). Interestingly, no increased fluorescence was detected in strains harboring a basS mutation indicating that observed increased fluorescence in the \(F^+\) and \(F^+\)\(\Delta\)tauD strains is due to elevated yfbE expression, and that the induction occurs prior to colistin challenge (Fig. 5B).

The yfbE::gfp reporter expression was also investigated in the hydrodynamic flow chamber system. AF504 \(F^+\) and AF504 \(basS\) \(F^+\) strains were inoculated in the system and GFP expression monitored after 24h our incubation without colistin treatment. Green fluorescence could be detected in the centre of large micro colonies in AF504 \(F^+\) biofilm while no fluorescence could be detected in \(basS\) mutant biofilm indicating that there is a correlation of yfbE expression and sub population formation in flow chamber system (Fig. 5C).

**Discussion**

The formation of bacterial biofilms is often associated with a reduced susceptibility to antimicrobial challenge [35,36]. The mechanisms underlying the observed increased tolerance are poorly elucidated and a direct causal relationship between antibiotic tolerance development and biofilm formation is inadequately supported [37,38]. Recently, Mah et. al. discovered that periplasmic glucans interact with the aminoglycoside tobramycin in *P. aeruginosa* biofilms and were proposed to delay by sequestration the effect of the antibiotic [39]. *P. aeruginosa* also has the ability to convert to an antibiotic-resistant phenotype after antibiotic exposure with enhanced biofilm forming ability [40]. A large proportion of the studies investigating biofilm tolerance are comparisons between cultures grown under shaking conditions (planktonic growth) and a particular biofilm model system [36,41–49]. The result of this kind of comparisons can be misleading because there are many other factors affecting antibiotic susceptibility other than biofilm formation in these systems. Cell number, growth phase, and media flow are factors that are varying in the different growth systems and can potentially affect antibiotic tolerance. There is a risk that results obtained under these conditions more reflect differences between the particular growth model systems rather than actually describing particular aspects of biofilm physiology compared to planktonically grown cells. In studies where tolerance has been investigated under physiologically similar conditions the differences in susceptibility has been less dramatic [43,50]. To better understand the influence of biofilm formation on the development of antibiotic tolerance, we exploited the recent discovery of Reisner et al. [6] that biofilm structure and organization can be manipulated by varying the IncF transfer plasmid configuration (Fig. 1A). Using this set of isogenic *E. coli* strains enable the investigation of antibiotic tolerance and resistance under nearly identical physiological conditions and the direct study of the influence of biofilm formation on antibiotic tolerance rather than comparing different growth model systems. We demonstrate that biofilm formation can
fundamentally influence bacterial survival upon antibiotic challenge, but the increased survival did not correlate directly with biofilm forming ability as such, but rather depended on the organization of the biofilm and on the antibiotic used (Fig. 1 and 2). When the biofilm forming strains were challenged with the surface-active peptide antibiotic colistin, there was a dramatic difference in survival between strains harboring the de-repressed incF transfer pilus and the plasmid free strains (Figs. 1 and 2). In contrast, when the biofilms were challenged with the fluoroquinolone ciprofloxacin, an antibiotic with an intracellular target, no significant differences in survival were observed between the investigated strains. Interestingly, resistance to ciprofloxacin is only known to occur through chromosomal mutations in *E. coli*, while antimicrobial peptide resistance and tolerance can arise through both physiological and mutational mechanisms [20,21,34,51]. In fact, our data suggest that physiological tolerance mechanism may be essential for the increased colistin tolerance of bacteria in biofilms. When flow chamber biofilms with complex spatial organization were challenged with colistin, subpopulations of tolerant cells were observed, while no such populations were detected in biofilms with flat, undifferentiated structure (Figs. 3 and 4). The localization of the subpopulation in the center of the micro-colonies indicates that the local environment within the biofilm induces subpopulation formation in our experimental system. Tolerance development is thus structurally dependent, influenced by the conditions created within the biofilm. However, we do not believe our data directly support a correlation of the difference in colistin tolerance to a distinct spatial organization and it is important to make distinction between a phenotype being structurally dependent and being correlated with a specific structure. When mutations in *basS* were introduced into the strains with wildtype F as well as the *traD* mutant, survival was comparable to the strain without F (Fig. 2A). Since *basS* is regulating the LPS modifications leading to augmented antimicrobial peptide tolerance, these data show that the increased survival is directly coupled to a genetically regulated event. We show that the expression of the *basR/S* regulated *yfbE* operon is up-regulated in the strains with increased survival and subpopulation formation, when the strains are grown as biofilms.

Figure 5. The *yfbE* gene is induced within structured biofilms prior to colistin challenge. A). Fluorescence of *yfbE*-gfp transcriptional fusion expressed in bacteria grown in N-minimal media, pH 7.7, containing 38 mM glycerol and either 10 mM Mg<sup>2+</sup> (black bars), or 10 μM Mg<sup>2+</sup> and 100 μM Fe<sup>2+</sup> (white bars). Data shown are the average of three experiments. Mean fluorescence units were detected by using a Becton Dickinson FACS Vantage SE cell sorter. Strains harboring the attB::bla-rrnB1::gfpmut3b or the pJBA113 vector alone demonstrated constitutive fluorescence and no fluorescence in all growth conditions, respectively. B). Fraction of fluorescent cells in the microtitre plate biofilm assay with the parental *E. coli* AF504 in the presence and absence of the de-repressed IncF plasmid and mutants in the IncF plasmid (traD), and the reciprocal insertion deletions of the *basS* and *basR* genes, harboring the *yfbE*-gfp transcriptional fusion. The threshold for GFP fluorescence was cells with a value above 600 arbitrary units. 99% of the constitutively expressing cells were above this threshold while 96% of the negative control was below. C). CLSM micrographs showing the expression of the *yfbE*-gfp transcriptional fusion in flow chamber biofilms of AF504/F<sup>+</sup> (left panel) and AF574 basS/F<sup>+</sup> (right panel). Green indicates GFP expressing cells all other cells appear red from treatment with SYTO62. doi:10.1371/journal.pone.0001891.g005
(Fig. 5B). Because the enzymes encoded by the operon are involved in the process of LPS modification, it is likely that it is through this process that the tolerant subpopulation is formed [32]. The increased expression of the operon occurs prior to colistin addition, indicating that the subpopulation formation process is not directly coupled to the addition of colistin or an adaptation to the presence of antimicrobial peptide (Fig. 5B). The main rational behind including the \( \text{basRS} \) mutant was to be able to differentiate between the development of a generic biofilm induced tolerance and specific genetic regulation to explain the observed increased survival in the \( F^+ \) and \( F^\text{traD} \) biofilms. Our data cannot rule out that there are other \( \text{basRS} \) dependent resistance mechanisms than LPS modification involved in the observed phenomena. This possibility does not change the fact that colistin tolerance is dependent on a specific genetic mechanisms rather than a generic biofilm property.

When \( P. \text{aeruginosa} \) was challenged with colistin in the flow chamber system, a tolerant subpopulation was also found [52]. In contrast to \( E. \text{coli} \), the surviving population was not observed in the center of large micro colonies but rather on the surface of the biofilm, and it was associated with a migrating cell population that eventually colonized the top of the microcolonies, resulting in the development of mushroom-shaped multicellular structures characterizing \( P. \text{aeruginosa} \) flow chamber biofilms [52]. The \( P. \text{aeruginosa} \) \( \text{pmr} \) system is known to be activated by the presence of cationic antimicrobial peptides [53,54]. The \( yfbE \) homolog, \( pmrH \), was indeed induced in the migrating tolerant subpopulation when the peptide was added to the system while cells in the stalks of the mushroom-shaped micro colonies seemed unable to induce or sense the presence of the peptide and were subsequently killed [52]. It is also interesting to note that even though the cells in the deeper layers of the flow chamber biofilm were coated by a thick layer of tolerant cells, this was not able to protect these cells from the action of the antibiotic [52]. No colistin-mediated induction has been shown for \( E.\text{coli} \), indicating a dissimilar regulation of homologous genes responding to disparate environmental stimuli since a migrating population is not present in the IncF mediated biofilm. Even though the tolerant subpopulations form at different locations within the \( E.\text{coli} \) and \( P.\text{aeruginosa} \) biofilms, there are notable common features. Both populations require the presence of genetically encoded tolerance mechanisms and seem to be influenced by specific environmental cues that are independent of general biofilm properties (Figs. 3 and 4) [52].

Physiochemical gradients have previously been demonstrated in microbial biofilms [9,10]. However, little is known about how these gradients affect the behavior of the cells integrated in the biofilm structure. The specific signals regulating the formation of the colistin tolerant subpopulation in \( E.\text{coli} \) are currently unknown, but since the development is under control of the \( \text{basR/basS} \) two-component system, it is probable that local environmental stimuli affect the cells in the biofilms. It is known that media with low \( \text{Mg}^{2+} \) in combination with high \( \text{Fe}^{3+} \) ion concentrations activate this regulatory network \textit{in vitro} [34]. We find it unlikely that such conditions prevail inside the large micro-colonies under the conditions tested. Luria broth is a medium with relatively low \( \text{Mg}^{2+} \) levels, but no extra iron is added in the experiments making it difficult to imagine a local accumulation of this ion in the micro colonies in sufficient amounts to induce \( \text{basS} \) activation. The magnesium ion concentration in the minimal medium used in flow chamber experiments is sufficiently high to prevent \( \text{basS} \) dependent induction, even in high iron conditions in a non biofilm setting and it is therefore unlikely that differential magnesium ion concentration is responsible for the observed induction [33]. The close proximity of the cells in the larger micro colonies in biofilm communities may create conditions mimicking the \textit{in vitro} effect of high iron levels. The bacteria may have to undergo complex physiological adaptations in response to the consequences of the high cell density within the micro-colonies. It is interesting to note, that the homologous operon to the \( \text{yfbE} \) gene in \( S. \text{typhimurium} \) is induced during swarm-cell differentiation, and an increased tolerance of swarmer cells to peptide antibiotics have been found to be due to an altered phenotypic state of the cells, in the absence of genetic changes [55,56]. It is possible that the conditions within the organized microcolonies mimic those that induce swarm-cell development. In addition, it cannot be ruled out that the \( \text{basR/basS} \) are components of a general stress response to toxic compounds produced by the metabolism of the cells in the micro-colonies.

The data presented here suggest that biofilm mediated antibiotic tolerance may often be a matter of subpopulation differentiation within the biofilm. The basis for tolerance development is not the biofilm formation \textit{per se}, but that the spatial and environmental heterogeneity provided by structurally organized biofilms have the capacity to promote tolerant subpopulation formation. In our experimental system, the increased antibiotic tolerance exhibited by biofilm formation is antibiotic specific and conditional, dependent on the actual biofilm structure and on the presence of a specific genetically encoded tolerance mechanism. Our data even suggest that specific modes of biofilm organization may induce antibiotic tolerance, and that the factors determining the properties of heterogeneous aggregated microbial communities may act on the individual cells within the structures rather than on the community. No heritable genetic changes are involved in the phenomenon; we suggest that many other cases of biofilm-induced tolerance to antibiotics could be the result of similar mechanisms. The biofilm mode of growth does not directly predict antibiotic resistance and we suggest that a complete elucidation of bacterial biofilm antibiotic tolerance phenomena merits investigations that are not limited by preconceived assumptions of the consequence of biofilm formation.

**Materials and Methods**

**Bacterial strains, plasmids and growth conditions**

The bacterial strains relevant to this study are listed in Table 1. Plasmids and PCR primers used in the study are listed in Table 2. Except where indicated, the strains used are derivatives of \( E. \text{coli} \) K-12 strain MG1655 or CSH26. Luria–Bertani (LB) broth or agar [54] was used for standard cultivation supplemented with 0.2% glucose. Appropriate antibiotics were added at the following final concentrations 100 \( \mu \text{g ml}^{-1} \) ampicillin \( (\text{Amp}) \), 10 \( \mu \text{g ml}^{-1} \) chloramphenicol \( \text{(Cm)} \), 100 \( \mu \text{g ml}^{-1} \) nalidixic acid \( \text{(Nal)} \) and 50 \( \mu \text{g ml}^{-1} \) kanamycin \( \text{(Km)} \) when required.

**Genetic techniques and mutant construction**

Unless otherwise stated all genetic methods followed standard procedures as described by Ausubel, F.M., and others [53]. \( E. \text{coli} \) phage P1 transductions were performed by the method of Miller [57]. To enable conjugative plasmid transfer a spontaneous nalidixic acid resistant mutant of MG1655 was isolated on LB agar plates containing 10 \( \mu \text{g ml}^{-1} \) nalidixic acid at 37°C and designated AF504. Conjugations of pAR108 pOX38-traD411 and pOX38-traX492 into AF504 from were performed by plate mating [58] with SAR18/pAR108, SAR18/pOX38-traD411 pSK410 and SAR18/pOX38-traX492 as respective donors. The defined insertion mutations in \( \text{basS} \) and \( \text{basR} \) were produced by using one-step allelic replacement utilizing the \( \lambda \) Red recombinase.
Table 2. Plasmids and primers used in this study.

| Plasmids                  | Description                        | Reference |
|---------------------------|------------------------------------|-----------|
| pAR108                    | pOX38Km aph::cat-PA1/04/03-cfp*     | [6]       |
| pOX38-traD411             | pOX38 traD::kan                     | [64]      |
| pOX38-traQ238             | pOX38 traQ::kan                     | [65]      |
| pOX38-traX482             | pOX38 traX::kan                     | [24]      |
| pSK410                    | F traD in pMS470.F8                 | [66]      |
| pSM1690                   | pLO2W Not(+)-rpB::gfpmut3b*         | [31]      |
| pKD3                      | Chloramphenicol insertion template. | [29]      |
| pKD4                      | Kanamycin insertion template.       | [29]      |
| pKD46                     | Red recombinase encoding plasmid    | [29]      |
| pLDR8                     | int gene expression helper vector   | [59]      |
| pLDRII                    | Cloning vector for attB integration | [59]      |
| pJBA113                   | Apr,PrUC18Not(+)-PA1/04/03-RBSII-gfpAV::-T0-T1a | [67] |
| pYFBe32                   | Derivative of pJBA113 carrying PybE PCR fragment | This work |
| p8ARs2                    | Derivative of pJBA113 carrying basRS fulllength PCR fragment | This work |

| Primers                  | Description                        | Reference |
|--------------------------|------------------------------------|-----------|
| basSmutf                 | TCCCCCGATTATCTTCTCATACATAATCCTGGTT- CTTCAATGTTAGGCTGAGCTGTCCT | This work |
| basSmutr                 | TCTGCGCGCCGAAATTCGTCGCCAACAGGCG- TGATAGGGAATATTGGCAGTGTTCC | This work |
| ecoBasRI                 | ATATAAGATCTTTCTTGGAATGACAAAGATTG | This work |
| xbaBasS1                 | AATTAATCTGAAAAATTTCCTTGTTGCTTTGC | This work |
| yfbEeco                  | AATTAAGATCTTGCAGGCGCAAAAAAGCTACAC | This work |
| yfbExba                  | ATATAATTCTGAGGAAAGCCGAATTCCTTCA | This work |

doi:10.1371/journal.pone.0001891.t002

Microtitre plate biofilm and antibiotic tolerance assays

Biofilm formation and antibiotic tolerance was quantified using the static microtitre plate biofilm assay. Bacterial cultures of the investigated strains were grown in 5ml LB in 37°C to turbidity. The cultures were diluted 1:300 and inoculated in triplicate in a 96-well microtitre plate (V-bottom; BioSterilin). Test plates were transferred to plastic bags to avoid evaporation and statically incubated for 24 h at 37°C. Biofilm formation was assayed by staining of attached cells with crystal violet as described [60]. In short, the growth medium was carefully removed from the biofilm microwells, washed twice with 0.9% NaCl solution, and covered with 0.1% CV for 15 min. The wells were again washed twice with 0.9% NaCl where after surface bound CV was extracted by addition of 96% ethanol. Absorbance of stained wells was determined at 590 nm with a VICTOR™ multilabel counter (Perkin Elmer, Inc.). For the antibiotic tolerance microtitre assay, biofilms were grown in microtitre plates without antibiotics as for biofilm quantification. After the 24 h incubation, colistin (H. Lundbeck, Copenhagen) or ciprofloxacin (Bayer) was carefully added to the wells at the indicated concentrations. After 24 h static additional incubation cells were vigorously re-suspended and the total CFUs were determined by serial dilution and plating on LB plates without prior washing. Consistent removal of the bacterial cells was confirmed by crystal-violet staining of the wells. For microtitre biofilm assays and tolerance assay the data is presented as mean±S.E.M. of 3 experiments performed in triplicate. In parallel with each biofilm experiment, a microtitre plate MIC was determined for the planktonic cells in the culture used as the inoculum. That the biofilm-associated cells truly were dispersed to single cells by vigorous aspiration was ascertained by observing the disrupted biofilm under a microscope prior to plating.

GFP Expression Assay and yfbE expression visualization

Strains harboring the plasmids pYFBe32 were grown in N-minimal media, pH 7.7, containing 38 mM glycerol with either 10 μM MgCl₂, or 10 μM MgCl₂ and 100 μM FeSO₄ and supplemented with 50 μg/ml ampicillin. GFP expression was analyzed after 6h of growth at 37°C by using a fluorescence-activated cell sorter (Becton Dickinson FACS Vantage SE). Assays were performed in triplicate. For yfbE expression in biofilms, strains were inoculated in 96 well microtitre plates as described for the antibiotic tolerance assay. After incubation for 24 h at 37°C
cells were vigorously re-suspended washed twice in 0.9% NaCl and GFP fluorescent monitored in the FACS. Strains harboring the attB::bla-mBP1::gfp-pmrA3b (GFP constitutive) and the pJBA113 vector alone (WT no reporter) demonstrated constitutive fluorescence and no fluorescence in all growth conditions, respectively. The threshold for GFP fluorescence was set as cells with a value above 600 arbitrary units, 95% of the constitutively expressing cells were above this threshold while 96% of the negative control was below. Experiments were done in triplicate. For visualization of yfbE expression in the hydrodynamic flow chamber systems the non-GFP fluorescent cells were counterstained with SYTO62 (Invitrogen) according to the manufacturers instructions.

Bacterial viability assay and fluorescence-activated cell sorting

The LIVE/DEAD BacLight bacterial viability kit (Invitrogen) was used to distinguish live and dead bacteria after antibiotic treatment according to the manufacturers instructions. For the experiments fluorescence-tagged bacteria (GFP, and red-fluorescent propidium iodide (PI) staining were used. Red fluorescing and green fluorescing cells from a colistin-treated biofilm were sorted by fluorescence-activated cell sorting (Becton Dickinson FACS Vantage SE) and plated on Luria Bertani plates to verify that red fluorescing cells were not viable and green fluorescing cells were viable.

Flow chamber biofilms

Cultivation of biofilms in laminar flow was done at 30°C as previously described [6]. Flow channels irrigated with AB minimal medium [61] supplemented with 0.01 mM Fe-EDTA, 1.0 mg/ml thiamine, or 1.0 mg/ml proline and uracil, where appropriate. The channels were inoculated with 250 μl of normalized dilutions (optical density at 600 nm of 0.05) of saturated bacterial cultures. After 24h incubation, biofilms were challenge with 50 μg/ml or 10 μg/ml colistin for an additional 24h where after the bacterial red fluorescent viability stain propidium iodide was injected into the flow channels and incubated for 15 min.

Images acquisition and analysis

All microscopic observations were performed by a Zeiss LSM510 Confocal Laser Scanning Microscope, CLSM (Carl Zeiss, Jena, Germany) equipped with an argon/krypton laser and detectors and filter sets for simultaneous monitoring of GFP (excitation 488 nm, emission 517 nm) and red-fluorescents emitted from the PI (excitation 543nm, emission 565 nm). For the quantification of biofilms and analysis of living and dead cells in treated biofilms the software COMSTAT was used [62]. Images were obtained using a 40×1.3 Plan-Neofluar oil objective. Multichannel simulated fluorescence projection (SFP, a shadow projection) images and vertical cross sections through the biofilm were generated by using the IMARIS software package (Bitplane AG, Zurich, Switzerland) running on a PC. Images were further processed for display by using Photoshop software (Adobe, Mountain View, Calif.). Time series experiments were performed on the Zeiss LSM510 microscope and movie sequences were produced using the Jase software, Animation Shop 3. For the quantification of E. coli F+ wild type and the different mutants three independent biofilm experiments were performed acquiring at least 9 image stacks for each strain and situation/time point randomly down through the flow channel using a 40×/1.3 Plan-Neofluar oil objective. For the quantification of living and dead cells, each strain was grown in three channels in three independent experiments, and from each experiment six image stacks were acquired randomly in the channel. Images were further treated using COMSTAT [62].

Statistical analysis

Statistical significance was determined using unpaired Student’s t-test, values of P<0.05 were considered significant.

Supporting Information

Movie S1 E.coli flow-chamber biofilms are sensitive to the cationic antimicrobial peptide colistin. Time series showing killing dynamic of a mature AF504 gfp/F" biofilm. Staining of the biofilm with PI (red) before/during image acquisition for monitoring of dead cells. Images were acquired every 15 min for 42 h. Found at: doi:10.1371/journal.pone.0001891.s001 (7.65 MB MPG)

Author Contributions

Conceived and designed the experiments: SM AF JH. Performed the experiments: AF JH CZ. Analyzed the data: SM AF JH CZ. Contributed reagents/materials/analysis tools: AF JH CS. Wrote the paper: SM AF JH.

References

1. Ghigo JM (2001) Natural conjugative plasmids induce bacterial biofilm development. Nature 412: 442–445.
2. Christensen BB, Stenberg C, Andersen JB, Ebel I, Möller S, et al. (1998) Establishment of new genetic traits in a microbial biofilm community. Appl Environ Microbiol 64: 2247–2255.
3. Molin S, Tolker-Nielsen T (2003) Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilaification of the biofilm structure. Curro Opin Biotechnol 14: 253–261.
4. Dudley EG, Abe C, Ghigo JM, Latour-Lambert P, Hormazabal JC, et al. (2006) An IncI1 plasmid contributes to the adherence of the atypical enteroaggregative Escherichia coli strain C1096 to cultured cells and abiotic surfaces. Infect Immun 74: 2102–2114.
5. Reiner A, Holler BM, Molin S, Zechner EL (2006) Synergistic effects in mixed Escherichia coli biofilms: conjugal transfer potentialed drives biofilm expansion. J Bacteriol 188: 4327–4336.
6. Reiner A, Haagensen JA, Schenbri MA, Zechner EL, Molin S (2003) Development and maturation of Escherichia coli K-12 biofilms. Mol Microbiol 48: 933–946.
7. Drenkard E (2003) Antimicrobial resistance of Pseudomonas aeruginosa biofilm. J Bacteriol 185: 3582–3588.
8. Miller SI, Ernst RK, Bader MW (2005) LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol 5: 35–46.
9. Münchian J, Kuch C, Lambert PA, Hoiby N, Elborn JS, et al. (2000) A ten year review of colomicin. Respir Med 94: 632–640.
10. Xu KD, McFeters GA, Stewart PS (2000) Biofilm resistance to antimicrobial agents. Microbiology 146: 547–549.
11. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Annu Rev Microbiol 49: 711–745.
12. Kuehn M, Hauner M, Bungartz HJ, Wagner M, Wilderer PA, et al. (1998) Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms. Appl Environ Microbiol 64: 4115–4127.
13. Miller SI, Ernst RK, Bader MW (2005) LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol 5: 35–46.
14. Lodgewood JM, Kuch C, Lambert PA, Hoiby N, Elborn JS, et al. (2000) A ten year review of colomicin. Respir Med 94: 632–640.
15. Harada K, Asai T, Kojima A, Oda C, Ishihara K, et al. (2005) Antimicrobial susceptibility of pathogenic Escherichia coli isolated from sick cattle and pigs in Japan. J Vet Med Sci 67: 999–1003.
16. La J, Nation RL, Mihoe RW, Turnidge JD, Goudhardt K (2005) Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. Int J Antimicrob Agents 25: 11–25.
17. Conrad RS, Galanos C (1989) Fatty acid alterations and polymyxin B binding by Pseudomonas aeruginosa adapted to polymyxin B resistance. Antimicrob Agents Chemother 33: 1724–1728.
18. Drenkard E (2003) Antimicrobial resistance of Pseudomonas aeruginosa biofilm. J Bacteriol 188: 3582–3588.
44. Amorena B, Gracia E, Monzon M, Leiva J, Oteiza C, et al. (1999) Antibiotic
43. Williams I, Venables WA, Lloyd D, Paul F, Critchley I (1997) The effects of
41. Larsen T, Fiehn NE (1996) Resistance of Streptococcus sanguis biofilms to
40. Drenkard E, Ausubel FM (2002) Pseudomonas biofilm formation and antibiotic
39. Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, et al. (2003) A genetic basis
37. Lewis K (2001) Riddle of biofilm resistance. Antimicrob Agents Chemother 45:
35. Nickel JC, Ruseska I, Wright JB, Costerton JW (1985) Tobramycin resistance of
34. Winfield MD, Groisman EA (2004) Phenotypic differences between Salmonella
33. Hagiwara D, Yamashino T, Mizuno T (2004) A Genome-Wide View of the
32. Trent MS, Ribeiro AA, Doerrler WT, Lin S, Cotter RJ, et al. (2001)
31. Sternberg C, Christensen BB, Johansen T, Toftgaard Nielsen A, Andersen JB, et
30. Heydorn A, Ersboll B, Kato J, Hentzer M, Parsek MR, et al. (2002) Statistical
29. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes
27. Storm DR, Rosenthal KS, Swanson PE (1977) Polymyxin and related peptide
24. Maneewannakul K, Maneewannakul S, Ippen-Ihler K (1995) Characterization
22. Maneewannakul K, Maneewannakul S, Ippen-Ihler K (1993) Characterization of
20. Drlica K, Zhao X (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones.
19. Gellert M, Mizuuchi K, O’Dea MH, Itoh T, Tomizawa JI (1977) Nalidixic acid
18. Storm DR, Rosenthal KS, Swanson PE (1977) Polymyxin and related peptide
17. Capra M, Hahn A, Givskov M, Parsek MR, et al. (2002) Quorum sensing and the
16. Halsey J (2000) Micromass Drug Susceptibility Assay for Staphylococcus aureus in biofilms developed in vitro. J Antimicrob Chemother 48: 584–590.
15. Anwar H, Strap JL, Chen K, Costerton JW (1992) Dynamic interactions of biofilms of mucoid Pseudomonas aeruginosa with tobramycin and piperacillin. Antimicrob Agents Chemother 36: 1208–1214.
14. Desai M, Buhler T, Weller PH, Brown MR (1998) Increasing resistance of planktonic and biofilm cultures of Burkholderia cepacia to ciprofloxacin and cefazidime during exponential growth. J Antimicrob Chemother 42: 153–160.
13. Kristina AG, Jennings RA, Naylor PT, Myvick QN, Webb LX (1989) Comparative in vitro antibiotic resistance of surface-colonizing coagulase-negative staphylococci. Antimicrob Agents Chemother 33: 813–816.
12. Hill D, Rose B, Pajkos A, Robinson M, Bye P, et al. (2005) Antibiotic susceptibilities of Pseudomonas aeruginosa isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions. J Clin Microbiol 43: 5085–5090.
11. Kuman T, Ono N, Isda M, Nickel JC (1995) Combination effect of fosfomycin and oxolinic acid against Pseudomonas aeruginosa growing in a biofilm. Antimicrob Agents Chemother 39: 1038–1044.
10. Sopering AL, Lewis K (2001) Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol 183: 6746–6751.
9. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes. Proc Natl Acad Sci U S A 97: 6640–6645.
8. Heydorn A, Ersholl B, Kato J, Hentzer M, Parsek MR, et al. (2002) Statistical analysis of Pseudomonas aeruginosa biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. Appl Environ Microbiol 68: 2008–2017.
7. Sternberg C, Christensen BR, Johansen T, Toftgaard Nielsen A, Andersen JB, et al. (1999) Distribution of bacterial growth activity in flow-chamber biofilms. Appl Environ Microbiol 65: 4108–4117.
6. Tsuru M, Welzel AA, Doerzer WT, Lin S, Cotter RJ, et al. (2001) Accumulation of a polysoprene-linked amino sugar in polymyxin-resistant Salmonella typhimurium and Escherichia coli: structural characterization and transfer to lipid A in the periplasm. J Biol Chem 276: 43132–43144. Epub 42001 Sep 19.
5. Hagiwara D, Yamashino T, Mizuno T (2004) A Genome-Wide View of the Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 91: 6649–6653.
4. Maneewannakul K, Maneewannakul S, Ippen-Ihler K (1993) Characterization of F19N, the F plasmid locus required for acetylation of F-plasmid subunits. J Bacteriol 175: 2957–2964.
3. Panicker MM, Mundley EG Jr (1985) DNA transfer occurs during a cell surface contact stage of F sex factor-mediated bacterial conjugation. J Bacteriol 162: 814–819.
2. Chandler M, Galas DJ (1983) Intergenerate formation mediated by TraT II. Activity of IS1 is modulated by external DNA sequences. J Mol Biol 170: 61–91.
1. Lewis K (2001) Riddle of biofilm resistance. Antimicrob Agents Chemother 45: 723–763.