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Common β-lactamases inhibit bacterial biofilm formation

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Summary

β-Lactamases, which evolved from bacterial penicillin-binding proteins (PBPs) involved in peptidoglycan (PG) synthesis, confer resistance to β-lactam antibiotics. While investigating the genetic basis of biofilm development by *Pseudomonas aeruginosa*, we noted that plasmid vectors encoding the common β-lactamase marker TEM-1 caused defects in twitching motility (mediated by type IV pili), adherence and biofilm formation without affecting growth rates. Similarly, strains of *Escherichia coli* carrying TEM-1-encoding vectors grew normally but showed reduced adherence and biofilm formation, showing this effect was not species-specific. Introduction of otherwise identical plasmid vectors carrying tetracycline or gentamicin resistance markers had no effect on biofilm formation or twitching motility. The effect is restricted to class A and D enzymes, because expression of the class D Oxa-3 β-lactamase, but not class B or C β-lactamases, impaired biofilm formation by *E. coli* and *P. aeruginosa*. Site-directed mutagenesis of the catalytic Ser of TEM-1, but not Oxa-3, abolished the biofilm defect, while disruption of either TEM-1 or Oxa-3 expression restored wild-type levels of biofilm formation. We hypothesized that the A and D classes of β-lactamases, which are related to low molecular weight (LMW) PBPs, may sequester or alter the PG substrates of such enzymes and interfere with normal cell wall turnover. In support of this hypothesis, deletion of the *E. coli* LMW PBPs 4, 5 and 7 or combinations thereof, resulted in cumulative defects in biofilm formation, similar to those seen in β-lactamase-expressing transformants. Our results imply that horizontal acquisition of β-lactamase resistance enzymes can have a phenotypic cost to bacteria by reducing their ability to form biofilms. β-Lactamases likely affect PG remodelling, manifesting as perturbation of structures involved in bacterial adhesion that are required to initiate biofilm formation.

Introduction

β-Lactam antibiotics inhibit bacterial growth by inactivating penicillin-binding proteins (PBPs) which are involved in synthesis of the cell wall polymer, peptidoglycan (PG). To evade these drugs, bacteria have evolved several mechanisms of resistance including (i) variation of the target (PBPs) (ii) expression of efflux pumps to reduce drug permeation across the outer membrane (iii) modification of PG structure and (iv) production of β-lactamase enzymes (Hakenbeck and Coyette, 1998). β-Lactamases are widely distributed among both Gram-negative and Gram-positive bacteria and inactivate penicillins and cephalosporins through hydrolysis of the crucial 4-carbon β-lactam ring structure. PG

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transpeptidases and carboxy-peptidases normally bind to the terminal D-Ala-D-Ala residues of a donor strand pentapeptide, forming an acyl enzyme intermediate between the carbonyl group of the D-Ala residue and the active site serine residue of the PBP (Green, 2002). β-Lactam antibiotics, which are structural mimics of the D-Ala-D-Ala moiety of PG, can form an essentially irreversible acyl complex with the catalytic serine of the PBPs. Unlike PBPs, β-lactamases have evolved the ability to undergo deacylation of the acyl complex, completing the hydrolysis reaction that is responsible for the destruction of β-lactam antibiotics.

The genes encoding β-lactamase enzymes are found on the bacterial chromosome or on transmissible plasmids (Williams, 1999). Plasmid-encoded β-lactamases are often expressed in large amounts (Jacoby, 1994), while chromosomally encoded β-lactamases are typically expressed at low levels until induced by the presence of their substrates. In Gram-negative bacteria, β-lactamases are exported to the periplasm, whereas in Gram-positive bacteria they are secreted from the cell (Rice *et al*., 2001). β-Lactamase enzymes exhibit diversity in both structure and function, and are divided into four classes (A–D) based on their primary sequence and catalytic mechanisms. The class B β-lactamases are zinc-dependent whereas the remaining three classes are serine hydrolases.

Of these, class A is the most numerous and well studied. Historically, these enzymes were described as penicillinases because they were able to catalyse penicillin hydrolysis. The ubiquitous TEM-1 enzyme, often encoded on cloning vectors to confer ampicillin resistance, belongs to class A, and was one of the first β-lactamases for which the crystal structure was solved (Jelsch *et al*., 1992). The class A β-lactamases have a molecular mass of approximately 29 kDa and are comprised of 260–280 residues, of which nine appear to be highly conserved: four residues, Ser-70, Lys-73, Ser-130 and Glu-166 are critical for catalysis whereas the five remaining residues Gly-45, Pro-107, Asp-131, Ala-134 and Gly-236 likely play a role in structural integrity (Matagne *et al*., 1998). Furthermore, three additional residues: Asn (in most sequences)-132, Lys/Arg-234 and Ser/Thr-235 also play important roles in enzyme activity. Sequence analysis revealed similarity between class A β-lactamases and the low molecular weight (LMW) PBPs such as PBP4 of *Escherichia coli* (Massova and Mobashery, 1998). In addition, the crystal structure of class A β-lactamases revealed homology with the catalytic domain of PBP5 from *E. coli* (Nicholas *et al*., 2003). However, even though the PBPs and β-lactamases share strong structural homologies and have a common ancestry, β-lactamases are thought to have lost the ability to interact with PG while functioning efficiently as resistance enzymes (Frere, 1995).

The class B β-lactamases are monomeric proteins that require divalent cation(s), predominantly zinc, for activity and are consequently inhibited by metal chelators such as EDTA and EGTA. The class C β-lactamases are also monomeric proteins with a molecular mass of approximately 39 kDa. They are typically encoded on the chromosomes of Gram-negative bacteria and are expressed at low levels unless induced by the products of cell wall damage (Jacobs *et al*., 1994).

Class D β-lactamases, such as the Oxa enzymes, are monomeric proteins ranging from 27 to 31 kDa in molecular mass. The crystal structures of the Oxa-10 and Oxa-13 enzymes have been determined at resolutions of 2.0 Å and 1.8 Å respectively (Majiduddin *et al*., 2002; Maveyraud *et al*., 2000; Paetzel *et al*., 2000; Pernot *et al*., 2001). Their name is derived from the ability to efficiently hydrolyse oxacillin, amoxicillin and cloxacillin, unlike most class A and all of the class C enzymes (Matagne *et al*., 1999). This difference in substrate profile may be attributed to the larger binding cleft of the Oxa enzymes (Maveyraud *et al*., 2000; Paetzel *et al*., 2000; Majiduddin *et al*., 2002). Of the few studies that have focused on this class of β-lactamases, no site-directed mutagenesis experiments have been performed and so

only comparisons with the other serine β-lactamases can be made. As class D β-lactamases have only recently been identified in clinically relevant pathogens, there are few structural or mechanistic studies available (Fisher *et al*., 2005). Like the class A and class C enzymes, class D β-lactamases are characterized by four catalytic elements, Ser-72, Ser-115, Lys-205 and the Ω-loop (Majiduddin *et al*., 2002). Unlike classes A and C, however, the general base for class D enzymes is still unknown, although Lys-70 appears to play an important role in the nucleophilic attack of the active site serine (Maveyraud *et al*., 2000; Paetzel *et al*., 2000; Pernot *et al*., 2001; Majiduddin *et al*., 2002). Golemi *et al*. (Golemi *et al*., 2001) demonstrated that CO_2 activates the class D β-lactamases. Briefly, the active site lysine is N-carboxylated as a result of addition of CO_2 to the lysine side chain amine (Golemi *et al*., 2001). Finally, a carbamate functional group is formed via hydrogen bonding with the active site serine (Maveyraud *et al*., 2000; 2002). The lysine is predicted to be fully carboxylated *in vivo* and to act as the general base activator for both the acylation and deacylation steps of catalysis (Maveyraud *et al*., 2000; 2002).

Our laboratory studies the development of biofilms, which are surface-adherent communities of microorganisms able to withstand extremely high levels of antibiotics. While performing genetic complementation experiments, we noticed that plasmid vectors carrying β-lactamase genes as selectable markers had detrimental effects on biofilm formation and motility phenotypes previously shown to be important for normal biofilm development (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Chiang and Burrows, 2003; Klausen *et al*., 2003). We show here that this antibiofilm effect is specific to class A and D β-lactamases, because otherwise identical plasmids bearing class B or C β-lactamases, tetracycline or gentamicin resistance markers do not decrease biofilm formation. We discuss these results in the context of a proposed model in which β-lactamases may interfere with PG remodelling required for correct assembly of large macromolecular complexes participating in surface attachment and subsequent biofilm development.

Results

TEM-1 impairs biofilm formation by *Pseudomonas aeruginosa* and *E. coli*

Pseudomonas aeruginosa is a common model organism for investigations of bacterial biofilm formation. It is motile by polar flagella (swimming motility) and retractile type IV pili (twitching motility) (O'Toole and Kolter, 1998; Wall and Kaiser, 1999; Mattick, 2002). During investigations of the role of twitching motility in *P. aeruginosa* biofilm formation (Chiang and Burrows, 2003), we noted that transformation of the wild-type PAK strain with pUCP20, a shuttle vector encoding the ampicillin resistance marker TEM-1, reduced the size of twitching motility zones by approximately 50% compared with transformation with the essentially identical, but tetracycline-resistance-encoding vector, pUCP26 (Fig. 1A). To confirm that the negative effect on twitching motility was due to expression of TEM-1, the *bla* gene on pUCP20 was disrupted by insertion of a gentamicin-resistance cassette, forming pUCP20Gm (Chiang *et al*., 2005). Disruption of the *bla* gene restored wild-type twitching motility in pUCP20Gm transformants (Fig. 1A).

Motility has previously been shown to play an important role in biofilm formation by both *P. aeruginosa* and *E. coli* (O'Toole and Kolter, 1998; Pratt and Kolter, 1998). Therefore, we compared the ability of *P. aeruginosa* bearing pUCP20, pUCP20Gm and pUCP26 to form biofilms in a static assay in the absence of antibiotics. Figure 1B shows that there are no differences in planktonic growth among the strains, but that the strain carrying pUCP20 forms less biofilm than other transformants. The plasmids were stably maintained without antibiotic selection (data not shown). When the same set of plasmids was introduced into *E. coli* XL-1 Blue, we noticed defects in biofilm formation exclusively in the pUCP20 transformants (Fig. 1B). Therefore, biofilm impairment is not restricted to *P. aeruginosa*,

but appears to be a general effect on Gram-negative bacteria. We also compared the adhesion of *E. coli* XL-1 Blue bearing pUCP20, pUCP20Gm and pUCP26 to glass and saw a similar defect in biofilm formation in the strain harbouring pUCP20 (Fig. 1C), showing the effect was substratum-independent. Similar results (not shown) were obtained with two additional strains of *E. coli*, the K12-derivative W3110 and a clinical isolate, strain CVG, showing the effect is not strain-specific. *E. coli* W3110 was used for subsequent studies.

The active site Ser of TEM-1 is required to impair *E. coli* biofilm formation

To understand if the biofilm defect of these strains was related to the pUCP20 plasmid, *E. coli* W3110 harbouring the plasmid vector pBR322 (Balbas *et al*., 1986), which encodes resistance to both ampicillin (TEM-1) and tetracycline (TetA), was monitored for its ability to bind to silicone coupons. Figure 2A shows that adherence (the first stage of biofilm formation) of *E. coli* bearing pBR322 is reduced compared with the untransformed strain, showing that the presence of other markers or differences in plasmid backbone does not impede the biofilm defect conferred by TEM-1. Disruption of the *bla* gene on pBR322 with a gentamicin-resistance cassette (pBR322Gm) restored the binding to levels comparable to the untransformed wild type (Fig. 2A). These data support the notion that the biofilm impairment phenotype can be directly attributed to the presence of the TEM-1 β-lactamase, and reinforces the observation that the effect is independent of the substratum (silicone, in this instance). In addition, these data show that the defect in biofilm formation manifests as early as 2 h, during the initial attachment phase of biofilm formation.

To distinguish whether the TEM-1 β–lactamase protein per se, or rather its enzymatic activity, was required for the inhibition of biofilm formation, we used site-directed mutagenesis to alter the catalytic Ser residue within the active site. When the active-site Ser of TEM-1 was mutated to Ala (S70A) to generate pBR322T1, no defect in adherence was observed (Fig. 2A). Thus, loss of enzyme activity leads to restoration of the wild-type biofilm phenotype.

Both class A and class D β-lactamases reduce attachment to surfaces

β-Lactamases are classified into four molecular classes, designated A to D. Classes A, C, and D encompass evolutionarily distinct groups of serine hydrolases, and class B, the zinc-dependent (‘EDTA-inhibited’) enzymes (Ambler, 1980). To determine if the biofilm inhibition was restricted to class A β-lactamases such as TEM-1, we cloned genes encoding a representative of each of the four classes into the multiple cloning site of the pUCP20Gm vector, under control of the vector’s *lac* promoter. All of these recombinant plasmids conferred ampicillin resistance (data not shown), confirming that the cloned genes were expressed from the constitutive promoter. The ability of *E. coli* W3110 bearing either no plasmid, the gentamicin-resistance vector only, or the vector expressing each of the four classes of β-lactamase to adhere to silicone coupons was measured over time in the absence of antibiotics (Fig. 2B). The number of attached bacteria expressing class B (BlaS) or C (AmpC) enzymes was comparable to the untransformed wild type (Fig. 2B). In contrast, presence of a plasmid encoding β-lactamases TEM-1 (Class A) or Oxa-3 (Class D) impaired attachment of *E. coli* W3110 to the coupons (Fig. 2B), suggesting that the effect is restricted to enzymes of those classes. The morphology of early (24 h) biofilms formed on the coupons by *E. coli* W3110 expressing TEM-1 or Oxa-3 is shown in Fig. 3. *E. coli* W3110 carrying pUCP20Gm, in which the TEM-1-encoding gene is disrupted, exhibited uniform coverage of the substratum, while the same strain carrying pUCP20, pUCP20Gm (TEM-1) or pUCP20Gm (Oxa-3) showed substantial reductions in colonization of the substratum (Fig. 3).

To confirm the observation that both class A and D enzymes exerted negative effects on bacterial attachment and subsequent biofilm formation, the ability of both *E. coli* W3110 and *P. aeruginosa* PAO1 harbouring pUCP20Gm, pUCP20Gm (TEM-1), pUCP20Gm (BlaS), pUCP20Gm (AmpC) or pUCP20Gm (Oxa-3) to form biofilms in a static microtitre plate assay was determined. Regardless of the species tested, a decrease in biofilm formation was observed for strains expressing either TEM-1 or Oxa-3 (Fig. 4), showing the effect is not species-specific.

Role of Oxa-3 active site residues in the biofilm-deficient phenotype

Using site-directed mutagenesis, we demonstrated that the catalytic Ser residue of TEM-1 was critical for the biofilm inhibition phenotype (Fig. 2). Similarly, we sought to determine if the enzymatic activity of Oxa-3 was required for its effects on biofilm formation. Surprisingly, an S72A/T73A double mutant of Oxa-3 (designated Oxa-3*) continued to impair *E. coli* W3110 adherence to a similar extent as the unmodified enzyme (Fig. 5), even though it no longer conferred resistance to ampicillin (data not shown). However, when expression of Oxa-3 was abrogated by alteration of Phe59 to a premature stop codon (designated Oxa-3**), adherence by *E. coli* W3110 transformed with this construct was restored to wild-type levels (Fig. 5).

Both class A and class D β-lactamases impair twitching motility of *P. aeruginosa*

Based on our original observation of reduced twitching motility in *P. aeruginosa* strain PAK harbouring pUCP20, we tested whether this effect could also be caused by the class D β-lactamase. To show that the effect was not restricted to strain PAK, we used the common laboratory strain, PAO1, for these experiments. Figure 6 shows that twitching motility of PAO1 was reduced when it was transformed with pUCP20, pUCP20Gm (TEM-1) or pUCP20Gm (Oxa-3) compared with pUCP20Gm transformants. Interestingly, transformation of PAO1 with pUCP20Gm (Oxa-3*) or pUCP20Gm (Oxa-3**) partially impaired twitching motility, causing a 30% and 40% reduction, respectively, compared with the wild type (Fig. 6), but less effect than the construct encoding the unmodified Oxa-3 enzyme. The reduced twitching phenotype could not be attributed to a decrease in surface piliation (as determined by SDS-PAGE of sheared surface proteins; data not shown).

Deletion of LMW PBPs impairs *E. coli* biofilm formation

We hypothesized that the observed phenotypes of class A and D β-lactamase-expressing transformants could be due to the ability of these β-lactamases to bind to or modify PG, therefore interfering with the activity of related PBPs. As these β-lactamases are most closely related to the LMW PBPs of *E. coli*, we tested the ability of a strain lacking PBP5 (the LMW PBP most closely related to TEM-1) to form biofilms compared with the wild type. In support of our hypothesis, deletion of this PBP resulted in a decrease in biofilm formation (Fig. 7A) of a magnitude similar to that seen in the isogenic *E. coli* strain expressing TEM-1 on pBR322 (data not shown).

Taking advantage of a previously constructed, comprehensive set of *E. coli* PBP mutants (Denome *et al*., 1999), we then tested the biofilm formation capacity of strains lacking LMW PBPs 4 or 7, as well as strains lacking double and triple combinations of PBPs 4, 5 and 7. Biofilm formation was reduced in these strains in a cumulative manner, with the triple mutant (lacking all three PBPs) showing the largest reduction in biofilm formation (Fig. 7). These reductions in biofilm were not due to growth defects, as growth of all mutants was similar to that of the wild type (data not shown). The decrease in biofilm formation was evident when the strains were grown in either rich [Luria–Bertani (LB)] or minimal [Davis Minimal Media (DMM)] medium (Fig. 7A), showing the phenotype is highly reproducible and independent of nutrient conditions. Confocal microscopy analysis of 96 h *E. coli*

biofilms grown in flow cells under continuous irrigation showed that the PBP5 mutant had decreased coverage of the substratum compared with the wild type, and the PBP4,5,7 triple mutant demonstrated poor substratum coverage (Fig. 7B).

Discussion

There has been a concerted effort in the past decade to understand the molecular mechanisms that underlie the development of bacterial biofilms from single attached cells to complex, three-dimensional communities. In this work we investigated the effects of β–lactamase expression on biofilm formation after observing that the presence of the *bla* gene on the multicopy plasmid pUCP20 decreased the amount of biofilm formed by both *E. coli* and *P. aeruginosa* strains harbouring this vector. This phenomenon was specific to the resistance marker, because disruption of the *bla* gene with a gentamicin-resistance cassette restored the wild-type biofilm phenotype. In addition, strains carrying pUCP26, which is essentially identical to pUCP20 except for the presence of a tetracycline resistance cassette in place of the *bla* gene, did not impair biofilm formation. The observed adherence and biofilm defects in strains carrying a plasmid with a *bla* gene was independent of species (*P. aeruginosa* or *E. coli*), strain (at least two strains of each species tested), coexpression of other markers (Gm^R or Tet^R), substratum (polystyrene, silicone or glass), nutrients (DMM and LB) and growth conditions (static and flowing systems).

On the basis of their structure and catalytic mechanisms, β-lactamases have been divided into four major groups, A–D. In this work, we have demonstrated that only Class A (TEM-1) and class D β-lactamases (Oxa-3) caused defects in biofilm formation without affecting growth rates, and that these defects manifested at the point of initial attachment, because differences in numbers of attached cells were apparent as early as 2 h after incubation of bacteria with the substratum. Class A and D β-lactamases are both serine hydrolases but are evolutionarily distinct from class B and C β-lactamases. Enzymes of classes A and D are well-characterized penicillinases and in some cases have evolved the ability to hydrolyse cephalosporins (Hall and Barlow, 2004), while class C β-lactamases are able to hydrolyse a very broad spectrum of cephalosporins (Joris *et al*., 1991). Based on structure-based phylogeny analysis, the class C β-lactamases have been shown to predate the divergence of the related classes A and D from a common ancestor (Hall and Barlow, 2004). The evolutionary and functional relationship of class A and D enzymes suggests that the mechanism by which they modulate biofilm formation are similar, and that class B and C enzymes do not exert this effect at the levels of expression used in this study. Our working hypothesis is that β-lactamases of classes A and D, which have evolved from LMW PBPs, may bind to and sequester PG substrates from the LMW PBPs and thus interfere with normal cell wall remodelling. This hypothesis is supported by the phenotype of mutants lacking one or more LMW PBPs, which exhibit comparable decreases in biofilm formation.

Class A β-lactamases have previously been shown to be highly effective in binding and hydrolysis of β-lactam antibiotics at the expense of their DD-peptidase activity (Sun *et al*., 2003). Although the evidence suggests that β-lactamases have lost DD-carboxypeptidase activity, a small but detectable level of DD-peptide hydrolysis was observed when Chang *et al*. (Chang *et al*., 1990) tested a chimeric protein, created by replacing 28 amino acids of the TEM-1 enzyme with the homologous portion of PBP5 from *E. coli*. Furthermore, Rhazi *et al*. (Rhazi *et al*., 1999) have shown evidence that all classes of β-lactamases have the ability to hydrolyse various N-acylated dipeptides. Taken together, these studies indicate that although the peptidase activity of β-lactamases are significantly less than that of PBPs, these enzymes are still able to bind to and act upon a PG substrate. Computer modelling (not shown) using the published crystal structure of TEM-1 indicate that its active site can readily accommodate the pentapeptide region of PG. The binding of TEM-1 and Oxa-3,

which are expressed from multicopy plasmids, to this region of PG may impede LMW PBPs from accessing their substrate. Because the β-lactamases themselves are poor carboxypeptidases, the overall effect may be altered cross-linking of PG. This hypothesis is supported by the phenotype of mutants lacking LMW PBPs, which, like class A and D β-lactamase-expressing strains, exhibit impaired biofilm formation.

In addition to the conserved catalytic Ser residue, primary structure alignments reveal that Class D β-lactamases have a conserved Glu or Asp residue and Tyr-Gly-Asn motif, whose locations correspond to the critical Glu-166 and Tyr-150 residues in class A and C β-lactamases respectively (Joris *et al*., 1991; Ledent and Frere, 1993; Matagne *et al*., 1999). Interestingly, mutagenesis of the catalytic Ser in TEM-1 resulted in restoration of a wild-type biofilm phenotype, while a similar mutation in the Oxa-3 enzyme (Oxa-3*) did not relieve the biofilm deficit. Although the Ser residue in TEM-1 is crucial for catalysis, it may also participate in stabilization of substrate binding, as repeated attempts to cocrystallize an S70A mutant TEM-1 enzyme with its substrate were not successful even though the structure of the mutant protein appeared to be similar to the wild type (J.M. Frere, pers. comm.). In the case of the Oxa-3 enzyme, an inactivating mutation (S72A/T73A) did not prevent the enzyme from exerting its effect, while introduction of a premature stop caused the biofilm repression to be relieved. These results may reflect differences in the participation of active site residues in substrate binding by these enzymes, related to dissimilarities in the structure of their active sites.

A common strategy to identify genes involved in biofilm formation has been the screening of transposon libraries for mutants defective in biofilm formation (Loo *et al*., 2000; Di Martino *et al*., 2002). This useful approach has identified many interesting genes, including some implicated in bacterial cell wall biosynthesis. In a screen of *Streptococcus gordonii* transposon insertion mutants impaired in biofilm formation, Loo *et al*. (Loo *et al*., 2000) identified the PG biosynthetic genes encoding PBP2B and PBP5, as well as genes *glmM* and *bacA*, involved in the synthesis of PG precursors. In addition, the importance of normal PG turnover for biofilm formation was recently demonstrated by Mercier and colleagues (Mercier *et al*., 2002), who showed that a *Lactococcus lactis* mutant lacking a PG hydrolase were also deficient in the ability to form biofilms. These studies provide further evidence to support the notion that PG remodelling may play a role in biofilm formation.

How could alterations in PG remodelling result in adherence and biofilm defects? We speculate that this phenotype arises due to effects on the assembly or function of large macromolecular organelles such as pili/fimbriae, flagella or protein secretion systems that must pass through the PG layer of the cell wall to be correctly displayed on the surface of the cell. Because the size of such complexes typically exceeds the mesh size of PG (*ca*. 50 kDa cut-off), localized remodelling is necessary to permit their transit. In support of this idea, we have shown that biofilm defects caused by expression of either TEM-1 or Oxa-3 β-lactamases in *P. aeruginosa* are correlated with defects in twitching motility, a phenotype known to be important for biofilm formation (O'Toole and Kolter, 1998; Chiang and Burrows, 2003; Klausen *et al*., 2003). Similarly, the observed decreases in *E. coli* adherence and biofilm formation upon expression of TEM-1 or Oxa-3 could be due to effects on flagellation, expression of type I fimbriae or the antigen 43 (FluA) adhesin, all of which are required for normal biofilm development (Danese *et al*., 2000; Schembri and Klemm, 2001; Schembri *et al*., 2003). These possibilities are currently under investigation.

Previous studies of the effects of subminimal inhibitory concentration (MIC) concentrations of β-lactam antibiotics on bacterial adherence (Schifferli and Beachey, 1988), also support the concept that partial inhibition of PBP function can affect adhesion. For example, Stephens and colleagues (Stephens *et al*., 1984) determined that treatment of *Neisseria* spp.

with 1/2 MIC of penicillin inhibited assembly of surface pili without impairing pilin synthesis and impaired adherence of treated bacteria to human cells. Similarly, *P. aeruginosa* strains treated with sub-MIC concentrations of ceftazidime showed decreases in adherence to mucin (Vishwanath *et al*., 1987). Treatment of *E. coli* with sub-MIC concentrations of penicillin caused loss of fimbriation and adherence, as did loss of carboxypeptidase activity in a temperature-sensitive mutant grown at the non-permissive temperature (Ofek *et al*., 1979). Together these studies support the idea that normal PG biosynthesis is required for optimal expression and/or function of surface adhesins and consequent colonization of surfaces.

In conclusion, we have shown that expression of class A and D β-lactamases from plasmid vectors can negatively impact biofilm formation by Gram-negative bacteria. This finding is an interesting corollary to previous studies showing that biofilms enhance transmission of resistance plasmids (Li *et al*., 2001), and that conjugative resistance plasmids can enhance biofilm formation (Ghigo, 2001). Our study shows that bacteria may gain resistance to some antibiotics at the expense of other protective phenotypes such as the ability to form biofilms, and cautions that investigators should be aware of potential confounding effects of vector markers on bacterial phenotypes under investigation.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 1. Growth media included DMM (Difco), LB and agar (Difco) and *Pseudomonas* Isolation Agar (PIA; Difco). For some experiments, a 50:50 mixture of LB and DMM, or DMM supplemented with 1% LB was used. Antibiotics were used where indicated at the following concentrations: ampicillin, 50 μg ml^{-1} for *E. coli*, pipercillin, 50 μg ml^{-1} for *P. aeruginosa;* gentamicin, 15 μg ml^{-1} for *E. coli* and 200 μg ml^{-1} for *P. aeruginosa*, and tetracycline, 15 μg ml^{-1} for *E. coli* and 50 μg ml^{-1} for *P. aeruginosa*. Plasmids encoding class B, C and D β-lactamases were the kind gift of F. Sanschagrin, U. Laval.

DNA manipulations

Restriction digestions and ligations were performed using enzymes from Life Technologies as per the manufacturer's instructions. The construction of pUCP20Gm was described previously (Chiang and Burrows, 2003). pBR322Gm was generated by linearizing pBR322 at the unique ScaI site within *bla* and inserting a 0.8 kb gentamicin resistance cassette released from pUCGm using SmaI. The QuikChange™ Mutagenesis kit (Stratagene, Cedar Creek, TX) with the modifications described by Wang and Malcolm (Wang and Malcolm, 1999) was used to generate pBR322-T1 (S70A), *oxa*-3* (S72A, T73A) and *oxa*-3** (F59Stop). Briefly, complementary mutagenic oligonucleotide primers were designed with the desired sequence alterations, as described by Stratagene. These primers were used to PCR-amplify the entire pBR322 or pUCP20Gm (*oxa-3*) plasmid with the incorporated changes using the proof-reading *Pfu* DNA polymerase (Stratagene). Two separate PCR reactions, each containing only one of the primers, were performed for three cycles. The two reactions were pooled and allowed to proceed for a further 18 cycles. Methylated template DNA was removed by digestion with DpnI (Stratagene), and the digestion reaction was transformed into *E. coli* DH5α. The introduction of site-specific alterations; pBR322-T1 (S70A), *oxa*-3* (S72A, T73A) and *oxa*-3** (F59Stop) were confirmed by DNA sequencing.

Twitching motility assay

Pseudomonas aeruginosa strains were tested for twitching motility by stabbing a sterile toothpick inoculated with a small amount of cells to the bottom of a 3 mm thick 1% DMM :

LB (50:50) agar plate without antibiotics (Semmler *et al*., 1999). After incubating overnight at 37°C in a humidified chamber, the presence (or absence) of a hazy zone radiating from the point of inoculation at the agar–plastic interface, indicating twitching motility, was recorded. To improve visualization of twitching zones, agar was removed from the plate and the twitching zones were stained with crystal violet for 5 min then rinsed with tap water to remove unbound dye.

Static biofilm assays

Static assays of biofilm formation were performed as described previously, with modifications (O'Toole and Kolter, 1998). Overnight cultures of *E. coli* strains were standardized to an optical density at 600 nm of 0.2 (Pratt and Kolter, 1998), while *P. aeruginosa* strains were standardized to 0.056, corresponding to 10^7 cfu ml^{-1}. All standardized cultures were inoculated 1/20 in 200 μl of DMM in a 96-well polystyrene microtitre plate that was incubated overnight at room temperature. Eight wells per experiment were inoculated for each strain. After removal of planktonic cells, the wells were rinsed with tap water and biofilms stained with 200 μl of 1% crystal violet (CV). After 15 min the plate was rinsed with tap water and retained CV was solubilized with 200 μl of 95% ethanol. The OD_{600} of planktonic cells (growth control) and absorbance of CV at 600 nm were measured using an automated 96-well plate reader ($Versa_{MAX}$ Microplate Reader) and the data compiled with Softmax PRO software (Molecular Dynamics). For some experiments, standardized strains were aliquoted as above into 96-well polystyrene microtitre plates modified to accept a 96-peg lid (Nunc). Biofilms form on the pegs during overnight incubation at room temperature. The lid was then rinsed twice in PBS to remove planktonic cells and the biofilms stained with 200 μl of 1% CV. The amount of biofilm formed on each peg was determined spectrophotometrically after solubilizing retained CV with 200 μl of 95% ethanol.

Bacterial adhesion to silicone coupons

To analyse adherence of each *E. coli* strain to silicone coupons, equal amounts of each strain were used to inoculate 5 ml of DMM/LB (50:50) media containing a 10 mm × 10 mm × 2 mm sterile surgical-grade silicone coupon as previously described (Finelli *et al*., 2002). Briefly, each strain was then incubated at 37°C with 100 r.p.m. shaking for a period of 2, 4, 6 or 24 h with the coupon. After each incubation period, the coupon was rinsed in sterile saline to remove non-adherent bacteria and placed in 5 ml sterile phosphate buffered saline (PBS) containing five or six 3 mm diameter glass beads. The coupons were subjected to three 30 s rounds of sonication on ice followed by vortexing for 60 s to dislodge biofilm bacteria. The number of bacteria on the coupon was determined by tenfold serial dilution and plate counts.

Scanning electron microscopy (SEM)

For SEM analysis, *E. coli* strains were grown as individual biofilms on silicone coupons in DMM : LB (50:50) without antibiotics at 37°C with 100 r.p.m. shaking as above. After 24 h, the coupon was rinsed in sterile saline to remove non-adherent bacteria and placed in 5 ml of 2% gluteraldehyde. Samples were processed in the EM department at the Hospital for Sick Children, Toronto, ON. After fixation in OsO_4 for 1 h at room temperature, specimens were washed three times in PBS, followed by a rinse in ddH_2O. The samples were dehydrated in a graded ethanol series, critical-point dried and mounted on SEM stubs for viewing with a JEOL 820 SEM and Iridium image capturing software.

Confocal scanning laser microscopy (CSLM) of biofilms

Escherichia coli strains were grown as individual biofilms as described previously (Finelli *et al*., 2003) in a commercially available flow cell (Stovall) under continuous irrigation with DMM medium supplemented with 1% LB at room temperature. After 96 h, biofilms were stained with BacLight Live/Dead stain (Molecular Probes) and images were acquired with a Zeiss CSLM and processed with Zeiss LSM510 software.

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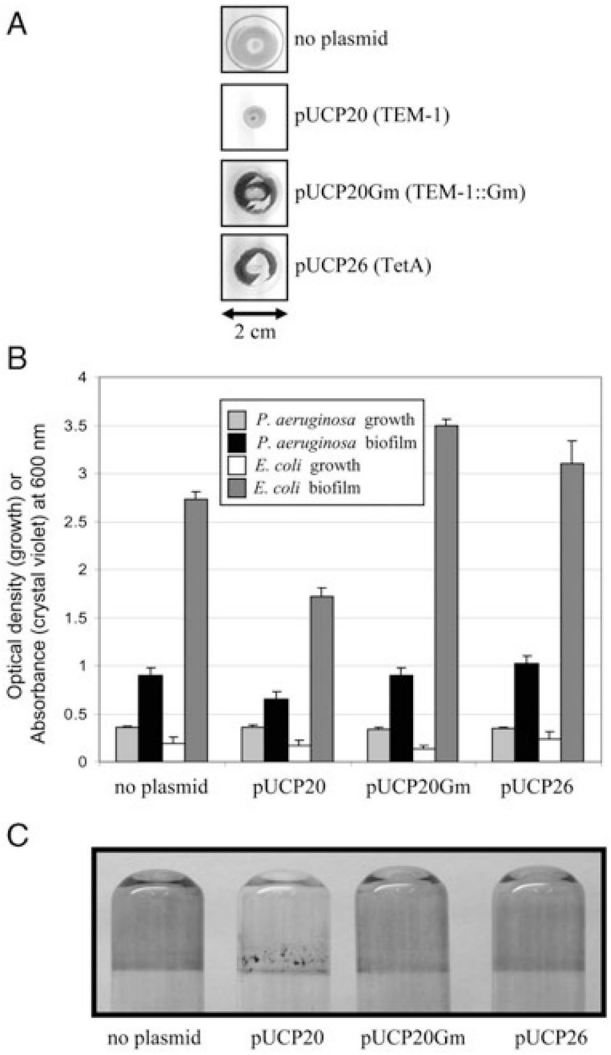

Fig. 1. Effect of TEM-1 on twitching motility and biofilm formation

A. Twitching motility on 1% agar of *P. aeruginosa* strain PAK and its transformants. Twitching zones on the Petri dish were stained with 1% crystal violet. Twitching motility is markedly reduced only in transformants expressing TEM-1.

B. Static biofilm formation on polystyrene by *P. aeruginosa* PAO1 and *E. coli* XL-1 Blue and transformants of each. Results represent the average of two independent experiments, each performed in triplicate, in the absence of antibiotics. While growth is not affected by the presence of plasmids, transformants expressing TEM-1 show a decrease in biofilm formation.

C. Biofilm formation of *E. coli* XL-1 Blue harbouring pUCP20, pUCP20Gm and pUCP26 on glass test tubes. After 24 h incubation and removal of non-adherent cells by washing, adherent cells were stained with crystal violet.

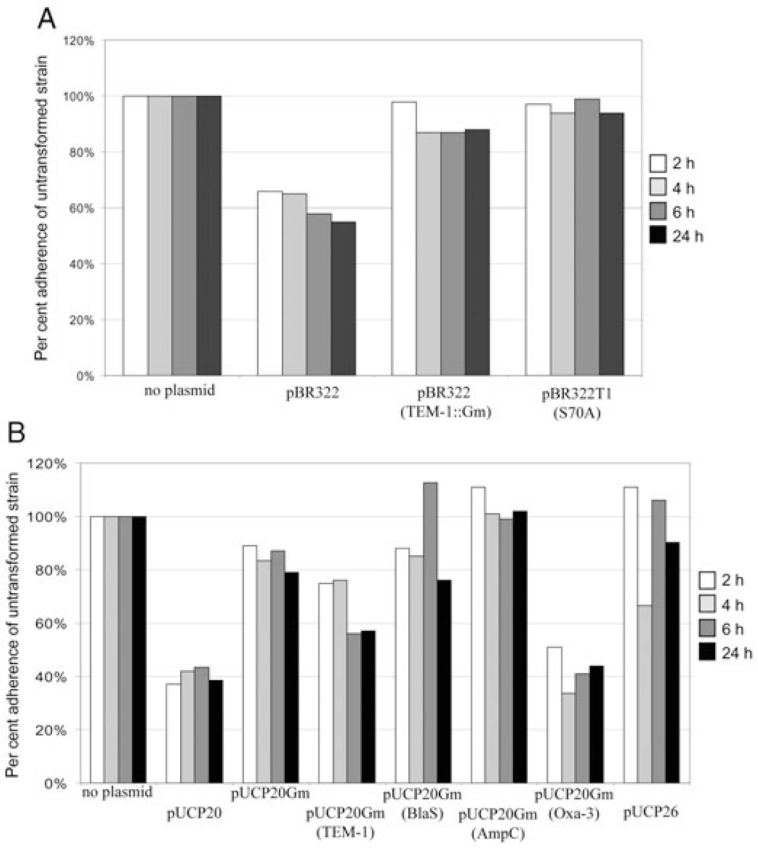

Fig. 2. Adherence of *E. coli* W3110 and transformants to silicone over time

A. Sterile silicone coupons (10 mm × 10 mm × 2 mm) were immersed in standarized suspensions of *E. coli* W3110 or its transformants and triplicate samples removed at the indicated time points for viable plate counts of attached bacteria as outlined in *Experimental procedures*. Each experiment was performed twice, the results averaged and expressed as percent adherence compared with the untransformed strain. Expression of TEM-1 from pBR322 impairs adherence of *E. coli* to silicone at all time points. Inactivation of TEM-1 on pBR322 either by disruption with a Gm^R cassette, or by site-directed mutagenesis of the catalytic Ser70 residue, restores adherence to wild-type levels.

B. *E. coli* W3110 transformants were tested for adherence to silicone as outlined above. Expression of TEM-1 (class A β-lactamase) from its native promoter on pUCP20, or from the *lac* promoter preceding the multiple cloning site on pUCP20Gm, impaired adherence. Expression of Oxa-3 (class D) but not BlaS (class B) or AmpC (class C) from the *lac* promoter of pUCP20Gm also reduced adherence of *E. coli* to silicone. Expression of markers conferring resistance to gentamicin (pUCP20Gm) or tetracycline (pUCP26) did not impair adherence to silicone.

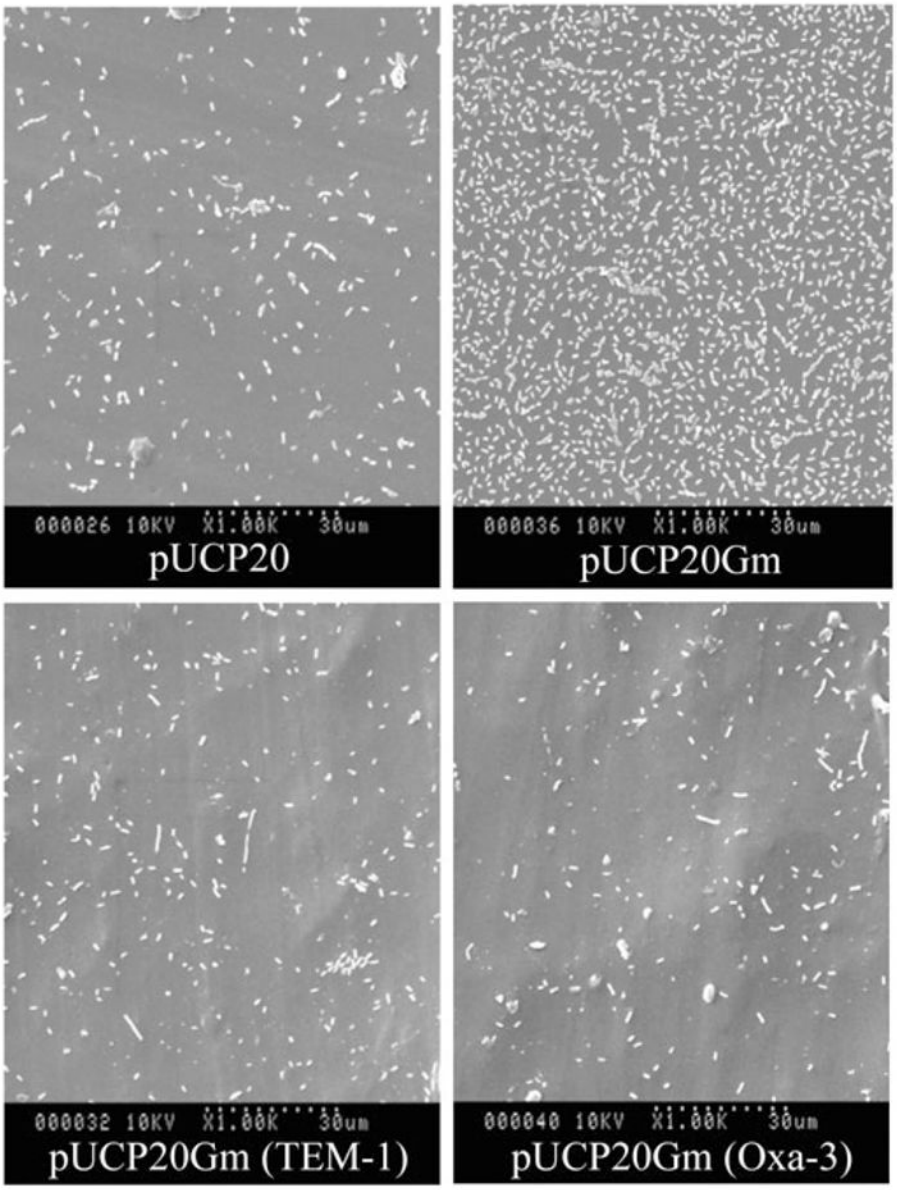

Fig. 3.

Morphology of *E. coli* W3110 transformants attached to silicone coupons after 24 h. Silicone coupons were exposed to standardized cultures of *E. coli* W3110 transformants for 24 h as described in *Experimental procedures*, fixed and imaged by SEM. Strains expressing either TEM-1 [pUCP20 and pUCP20Gm (TEM-1)] or Oxa-3 [pUCP20Gm (Oxa-3)] demonstrate reduced surface coverage compared with the strain carrying pUCP20Gm, in which the *bla* gene encoding TEM-1 has been disrupted. Magnification, 1000 ×; dotted scale bar, 30 μm.

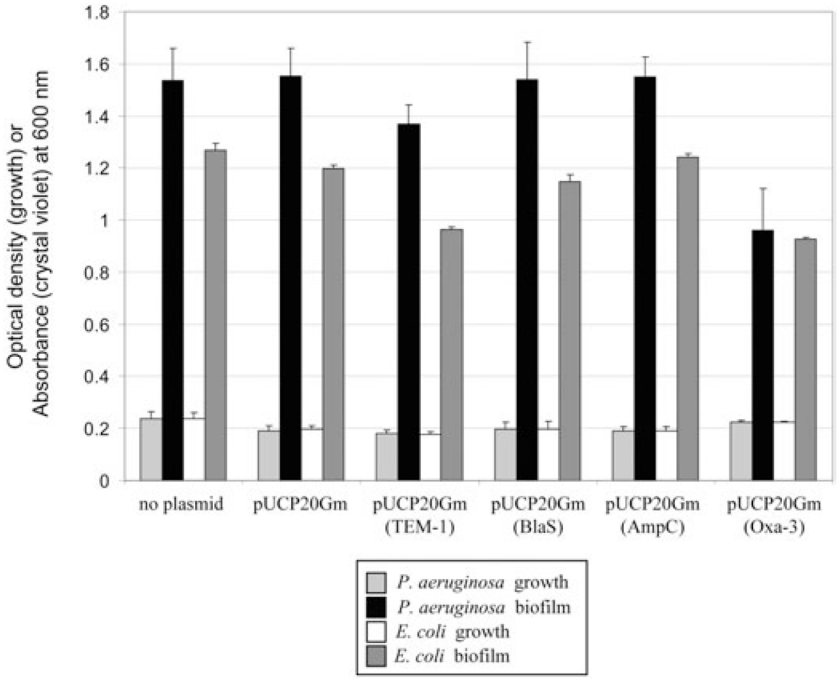

Fig. 4.

Static biofilm formation by *P. aeruginosa* PAO1 and *E. coli* W3110. Static biofilm formation on polystyrene by *P. aeruginosa* and *E. coli* and transformants of each species was measured as described in *Experimental procedures*. Results represent the average of two independent experiments, each performed in triplicate, in the absence of antibiotics. While growth is not affected by the presence of plasmids, transformants expressing TEM-1 and Oxa-3, but not BlaS nor AmpC, show a decrease in biofilm formation compared with the untransformed strain.

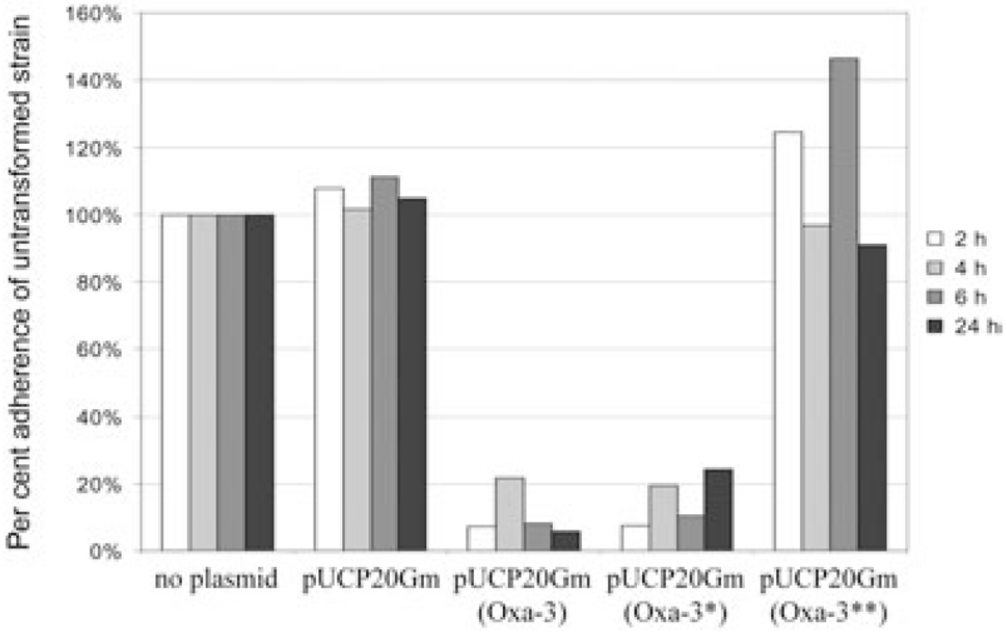

Fig. 5.

Effect of Oxa-3 mutations on adherence of *E. coli* W3110 transformants to silicone. *E. coli* W3110 transformants were tested for adherence to silicone as outlined in *Experimental procedures*. Simultaneous mutation of two potential catalytic residues in Oxa-3 to create Oxa-3* (S72A, T73A) resulted in loss of ampicillin resistance but did not restore biofilm formation to levels seen in the non-transformed strain. In contrast, introduction of a premature stop codon after the first 58 amino acids of Oxa-3, to create Oxa-3**, resulted in restoration of biofilm formation to levels seen in the untransformed strain.

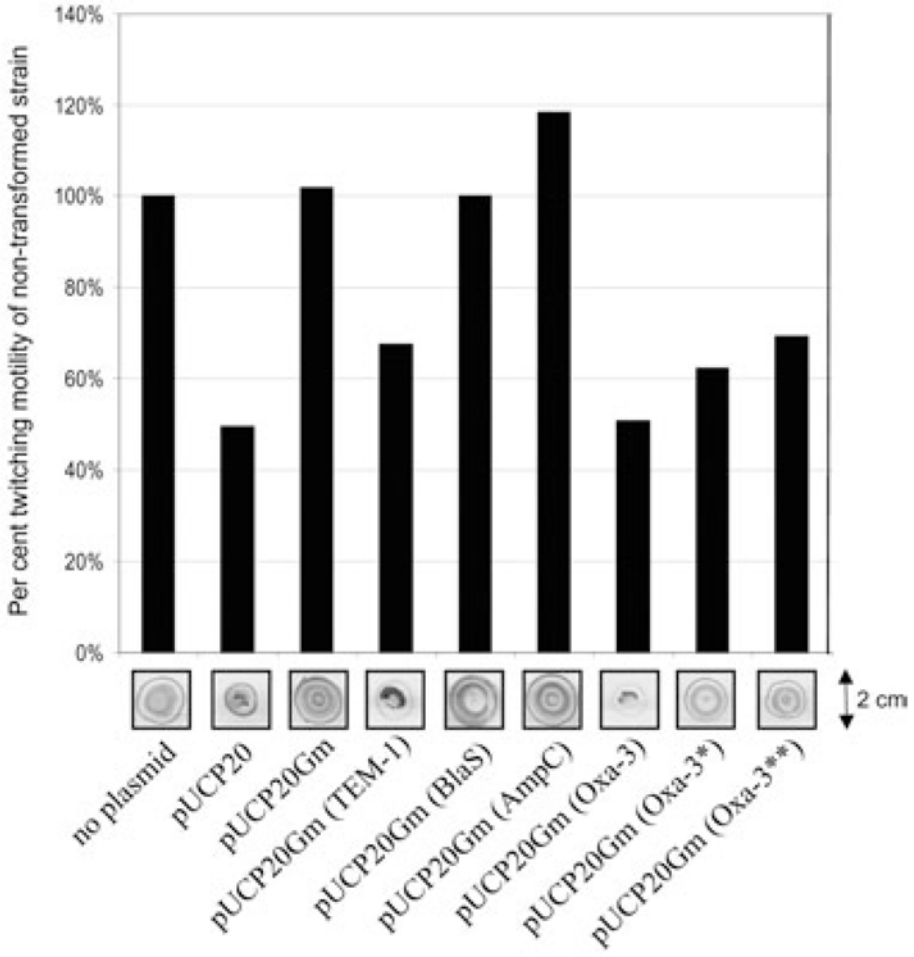

Fig. 6.

Twitching motility of *P. aeruginosa* strain PAO1 and its transformants. The twitching motility of each strain was evaluated on a 50:50 LB : DMM 1% agar plate as previously described by Semmler *et al*. (1999). The average diameter of six individual zones for each strain was measured using NIH Image software and is represented graphically as a percentage of the untransformed strain, with a representative twitching zone for each transformant shown. As observed for strain PAK (Fig. 1A), expression of TEM-1 or Oxa-3 reduces twitching motility. For *P. aeruginosa*, inactivation of Oxa-3 by either site-directed mutagenesis of the catalytic residues (Oxa-3*) or introduction of a premature stop (Oxa-3**) restores twitching motility to 60–70% of the levels of the untransformed strain.

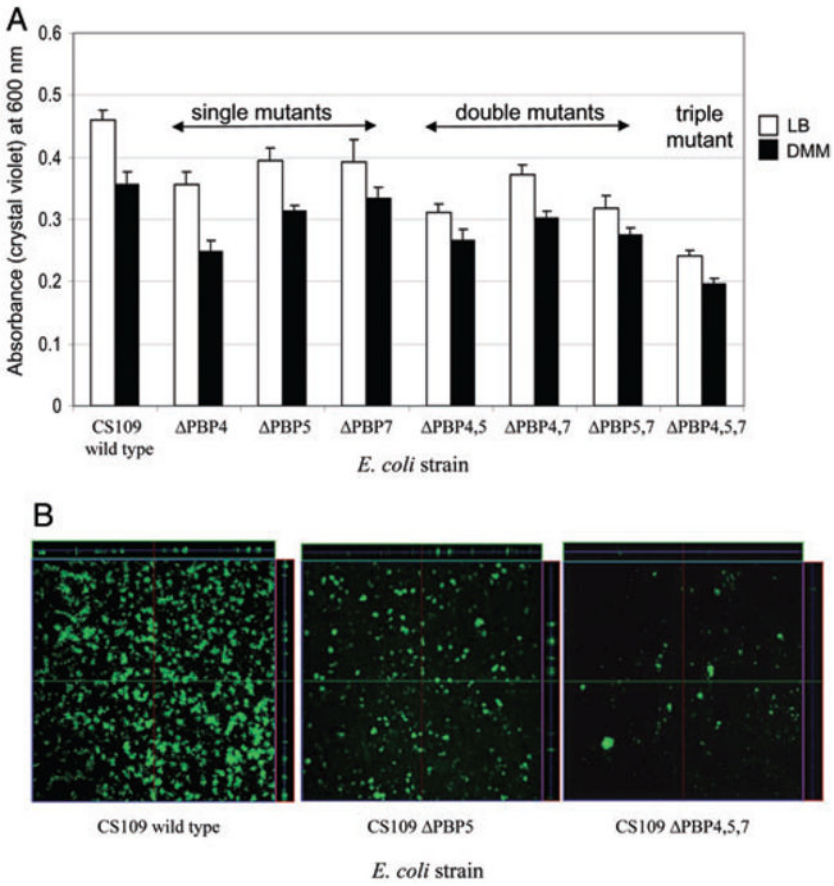

Fig. 7. Effect of deleting one or more low-molecular weight PBPs on *E. coli* biofilm formation

A. The ability of *E. coli* strain CS109 and its PBP deletion mutants to form biofilms on polystyrene pegs, in two media (LB, rich broth; DMM, minimal medium) was tested as outlined in *Experimental procedures*. No differences in growth among these strains were noted (not shown). Results are the average of duplicate experiments each performed in triplicate. Deletion of LMW PBPs has cumulative effects on biofilm formation, with a triple mutant showing the greatest decrease in biofilm levels.

B. Confocal Laser Scanning Micrographs (CLSM) of *E. coli* CS109, *E. coli* CS109 (ΔPBP5) and *E. coli* (ΔPBP4,5,7) biofilms, grown for 96 h in DMM media supplemented with 1% LB in a flow cell system (Stovall) stained with BacLite® Live/Dead dye. *E. coli* CS109 (ΔPBP5) and *E. coli* CS109 (ΔPBP4,5,7) show reduced biofilm formation compared with the wild type, with the triple mutant showing a more severe reduction.

Table 1

Bacterial strains and plasmids used in this study.

Species or plasmid	Strain	Relevant characteristics	Reference or source
Bacteria			
Escherichia coli	TOP10	F$^-$ *mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ(lac)X74 *rec*A1 *deo*R araD139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
	XL1-Blue	Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F′*pro*AB *lac*I^{q}ZΔM15 Tn5 (Kan^r)]	Stratagene
	W3110	F$^-$ λ$^-$ IN(*rrnD-rrnE*)1	Yao and Valvano (1994)
	CS109	F$^+$ λ$^-$ *glnV rpoS rph*	Denome *et al*. (1999)
	CS109 ΔPBP4 (CS11-2)	F$^+$ λ$^-$ *glnV rpoS rph ΔdacB*	Denome *et al*. (1999)
	CS109 ΔPBP5 (CS12-7)	F$^+$ λ$^-$ *glnV rpoS rph ΔdacA*	Denome *et al*. (1999)
	CS109 ΔPBP7 (CS9-19)	F$^+$ λ$^-$ *glnV rpoS rph ΔpbpG*	Denome *et al*. (1999)
	CS109 ΔPBP4,5 (CS219-1)	F$^+$ λ$^-$ *glnV rpoS rph ΔdacB ΔdacA*	Denome *et al*. (1999)
	CS109 ΔPBP4,7 (CS203–1B)	F$^+$ λ$^-$ *glnV rpoS rph ΔdacB ΔpbpG*	Denome *et al*. (1999)
	CS109 ΔPBP5,7 (CS204-1)	F$^+$ λ$^-$ *glnV rpoS rph ΔdacA ΔpbpG*	Denome *et al*. (1999)
	CS109 ΔPBP4,5,7 (CS315-1)	F$^+$ λ$^-$ *glnV rpoS rph ΔdacB ΔdacA ΔpbpG*	Denome *et al*. (1999)
Pseudomonas aeruginosa	PAK	Wild-type strain, laboratory strain Serogroup 06	Bradley (1980)
	PAO1	Wild-type strain, laboratory strain Serogroup 05	Hancock and Carey (1979)
Plasmids			
	pUCP20	3898 bp Shuttle vector encoding TEM-1, Amp^r	West *et al*. (1994)
	pUCP20Gm	4698 bp pUCP20 with SmaI-flanked gentamicin cassette inserted into unique ScaI site within *bla*, Amp^S, Gm^r	Chiang and Burrows (2003)
	pUCP20Gm (AmpC)	1201 bp DNA fragment amplified from pNU359 and containing *ampC* from *Enterobacter cloacae*, cloned in pUCP20Gm digested with EcoRI and HindIII, Amp^r, Gm^r	This study
	pUCP20Gm (BlaS)	972 bp DNA fragment amplified from pMON13, containing *blaS* from *Stenotrophomonas maltophilia*, cloned in pUCP20Gm digested with EcoRI and HindIII, Amp^r, Gm^r	This study
	pUCP20Gm (Oxa-3)	2400 bp DNA fragment from pMON922 and containing *oxa-3* from *Pseudomonas aeruginosa*, released with XbaI and PstI, cloned in pUCP20Gm digested with XbaI and PstI, Amp^r, Gm^r	This study
	pUCP20Gm (Oxa-3*)	pUCP20Gm (Oxa-3) with S72A, T73A mutations within Oxa-3, Amp^S, Gm^r	This study
	pUCP20Gm (Oxa-3**)	pUCP20Gm (Oxa-3) mutated to have a termination codon at position 59, Amp^S, Gm^r	This study
	pUCP20Gm (TEM-1)	1075 bp DNA fragment amplified from pBR322 and encoding TEM-1, cloned in pUCP20Gm digested with EcoRI and HindIII	This study
	pUCP26	4977 bp Shuttle vector encoding TetA, Tet^r	West *et al*. (1994)
	pBR322	4363 bp cloning vector, Amp^r, Tet^r	Balbas *et al*. (1986)

Species or plasmid	Strain	Relevant characteristics	Reference or source
	pBR322Gm	pBR322 with SmaI-flanked gentamicin cassette inserted into unique ScaI site within *bla*, Tet^r	This study
	pBR322-T1	pBR322 with S70A mutation within Tem-1, Amp^S, Tet^r	This study
	pMON13	L-1 Metallo-β-Lactamase, Km^r Ap^r; 1.6 kb HindIII–SalI fragment of pMON01 in $pBGS18^+$	Sanschagrin *et al*. (1998)
	pMON922	Class D β-lactamase, Ak^r Ap^r Cm^r Km^r Su^r, 5.3 kb BamHI fragment of (OXA-3) in pBGS131	Sanschagrin *et al*. (1995)
	pNU359	AmpC β-lactamase (P99) from *Enterobacter cloacae*, Amp^r	Galleni *et al*. (1988)