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The OmpL porin does not modulate redox potential in the periplasmic space of *Escherichia coli*

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The *Escherichia coli* DsbA protein is the major oxidative catalyst in the periplasm. Dartigalongue *et al.* (*EMBO J.*, 19, 5980–5988, 2000) reported that null mutations in the *ompL* gene of *E.coli* fully suppress all phenotypes associated with *dsbA* mutants, i.e. sensitivity to the reducing agent dithiothreitol (DTT) and the antibiotic benzylpenicillin, lack of motility, reduced alkaline phosphatase activity and mucoidy. They showed that OmpL is a porin and hypothesized that *ompL* null mutations exert their suppressive effect by preventing efflux of a putative oxidizing–reducing compound into the medium. We have repeated these experiments using two different *ompL* null alleles in at least three different *E.coli* K-12 genetic backgrounds and have failed to reproduce any of the *ompL* suppressive effects noted above. Also, we show that, contrary to earlier results, *ompL* null mutations alone do not result in partial DTT sensitivity or partial motility, nor do they appreciably affect bacterial growth rates or block propagation of the male-specific bacteriophage M13. Thus, our findings clearly demonstrate that *ompL* plays no perceptible role in modulating redox potential in the periplasm of *E.coli*.

Keywords: alkaline phosphatase/bacterial motility/disulfide bond formation/DsbA/DTT resistance

Introduction

During the past 10 years, the cumulative efforts of various laboratories have demonstrated the existence of complex oxidative–reductive pathways in the periplasm of *Escherichia coli*. It is known that the product of the *dsbA* gene is the major oxidant in the periplasm of *E.coli*. As a consequence, *dsbA* null mutants exhibit highly pleiotropic phenotypes, most likely due to defects in periplasmic disulfide bond formation (Bardwell *et al*., 1991; Kamitani *et al*., 1992; Jacob-Dubuisson *et al*., 1994; Genevaux *et al*., 1999). These phenotypes include reduction in levels of activity of the periplasmic enzyme alkaline phosphatase (AP), sensitivity to the reducing agent dithiothreitol (DTT), mucoidy, resistance to male-specific bacteriophages such as M13 and lack of both cell motility and biofilm formation. The DsbA protein has been shown to be reoxidized by the integral membrane protein DsbB (Bardwell *et al*., 1993; Dailey and Berg, 1993; Missiakas *et al*., 1993; Raina and Missiakas, 1997; Ritz and Beckwith, 2001; Collet and Bardwell, 2002). Hence, null mutations in *dsbB* result in pleiotropic phenotypes similar to those exhibited by *dsbA* single mutants. In turn, reduced DsbB is oxidized by the respiratory chain (Kobayashi *et al*., 1997; Bader *et al*., 1999).

In addition to the DsbA–DsbB oxidation pathway, there are also disulfide bond isomerization pathways carried out by the DsbC and DsbG proteins. The integral membrane protein DsbD is responsible for reducing the DsbC and DsbG isomerases (Raina and Missiakas, 1997; Ritz and Beckwith, 2001; Collet and Bardwell, 2002).

Null mutations in the *dsbD* gene were identified as exhibiting partial sensitivity to DTT and as partial suppressors of *dsbA* mutations (Missiakas *et al*., 1995). In an effort to identify better suppressors of *dsbA* mutations, the same group reported the isolation of null mutations in the *ompL* gene of *E.coli* using two separate genetic approaches (Dartigalongue *et al*., 2000). The first approach selected directly for insertional mutations in the chromosome of *E.coli dsbA* null mutants that restore wild-type levels of resistance to the reducing agent DTT. Upon subsequent testing, these *dsbA ompL* double null mutant strains were shown to possess full AP activity and to be non-mucoid, resistant to benzylpenicillin and fully motile. The second genetic approach involved screening for insertional mutations in an otherwise wild-type chromosome of *E.coli* that alone confer moderate sensitivity to DTT but do not inactivate any of the previously identified *dsb* genes. One such insertion, mapping in *ompL*, was characterized and also shown to result in a bacterial slow growth phenotype and resistance to the male-specific filamentous bacteriophage M13 (Dartigalongue *et al*., 2000). The *ompL* gene product was overexpressed, purified and shown to encode a porin protein. In contrast with their suppressive effect on *dsbA* null mutants, *ompL* null mutations did not suppress any of the examined defects in the *dsbB* gene. To explain their findings, the authors proposed that, in addition to DsbA, there may be an as yet unidentified low molecular weight compound(s) which is also oxidized by DsbB in the periplasm. It was suggested that this putative powerful oxidized agent(s) completely substitutes for DsbA provided that its leakage into the medium is not facilitated by the OmpL porin. Thus, in this model, the OmpL porin was postulated to be the major efflux mediator of this putative low molecular weight redox agent into the medium.

Attempts by one of us (A.A.S.) to identify the putative redox compound(s) predicted by the above model led to

preliminary observations suggesting that the *ompL* null allele does not suppress a *dsbA* null mutant, forcing us to re-examine this in greater detail. Here, we report a complete failure to reproduce any of the numerous physiological phenotypes previously assigned to *ompL* null mutations.

Results and discussion

Construction and testing of mutant bacterial strains

Throughout this study we used the *dsbA*::mini-Tn*10*Tet^R (SR2262) insertion mutation of Dartigalongue *et al*. (2000). We verified that it is indeed a null mutation of *dsbA* by demonstrating that there is no full-length DsbA antigen in extracts prepared from SR2262 mutant bacteria, using standard western blot methodology (data not shown). We transferred the *dsbA*::mini-Tn*10*Tet^R allele by bacteriophage P1-mediated transduction into three standard, but different, *E.coli* K-12 genetic backgrounds, namely CA8000, MG1655 and MC4100. In all cases, the introduction of the *dsbA*::mini-Tn*10*Tet^R allele was associated with a complete lack of DsbA-specific antigen. Furthermore, as expected, the introduction of the *dsbA*::mini-Tn*10*Tet^R mutation resulted in the previously reported phenotypes of high sensitivity to DTT, lack of bacterial motility, mucoidy, reduced expression of AP activity and resistance to the male-specific bacteriophage M13.

In this work, we also employed the *ompL*::mini-Tn*10*Kan^R (SR1791) mutation used by Dartigalongue *et al*. (2000). We verified that the *ompL*::mini-Tn*10*Kan^R and the *dsbA*::mini-Tn*10*Tet^R mutations are closely linked (~50%) by P1-mediated transduction (data not shown), in agreement with the previously reported findings of Dartigalongue *et al*. (2000). In addition, we constructed our own *ompL* deletion/substitution mutation (Δ*ompL*::Kan^R) by taking advantage of the rapid gene substitution methodology of Yu *et al*. (2000), as described in Materials and methods. We have verified the authenticity of both the *ompL*::mini-Tn*10*Kan^R and the Δ*ompL*::Kan^R alleles by carrying out PCR and DNA sequencing experiments using the appropriate DNA primers (see Materials and methods for details). We showed that the site of insertion of the mini-Tn*10*Kan^R cassette in strain SR1791 is indeed at nucleotide position 553 of the region surrounding *ompL*, as reported by Dartigalongue *et al*. (2000).

Reconstruction of dsbA ompL double mutants

To reconstruct the double mutants, the *dsbA* mutation was first transduced from SR2262 by bacteriophage P1 into the three *E.coli* genetic backgrounds listed above. Subsequently, we introduced either the *ompL*::mini-Tn*10*Kan^R (SR1791) or the Δ*ompL*::Kan^R allele that we constructed into both the *dsbA* single mutants and their corresponding wild-type parents. This resulted in the construction of the following set of isogenic mutants in the three genetic backgrounds: (1) *dsbA*::mini-Tn*10*Tet^R; (2) *ompL*::mini-Tn*10*Kan^R; (3) Δ*ompL*::Kan^R, (4) *dsbA*::mini-Tn*10*Tet^R *ompL*::mini-Tn*10*Kan^R; (5) *dsbA*::mini-Tn*10*Tet^R Δ*ompL*::Kan^R. Again, the *ompL* mutant alleles were confirmed by PCR and DNA sequence analysis and, in the case of *dsbA*, by the lack of DsbA-specific antigen. As detailed below, none of the genetic and biochemical experiments that we carried out revealed any phenotypes associated with the *ompL* null mutations, either by themselves or in combination with the *dsbA* null allele.

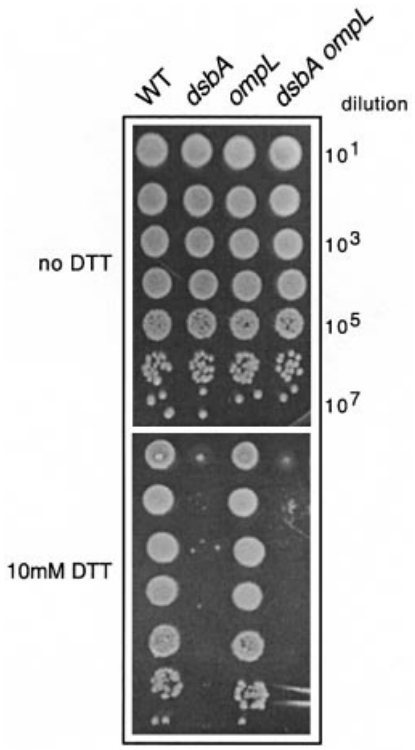

Fig. 1. An *ompL* null mutation does not suppress DTT sensitivity of a *dsbA* null mutant. Ten-fold serial dilutions of exponentially growing bacterial cultures were spotted on LB agar plates containing various DTT concentrations and incubated at 37°C for ~18 h. A representative set of mutants derived from the wild-type strain MG1655 (WT) spotted on LB agar at 37°C with or without 10 mM DTT is shown. The corresponding mutations are indicated at the top of the figure. The *ompL* allele shown here is the *ompL*::mini-Tn*10*Kan^R allele (Dartigalongue *et al*., 2000).

ompL null mutations do not suppress the DTT sensitivity of dsbA null mutants

Having verified the authenticity of the various *dsbA* and *ompL* mutants that we constructed, we proceeded to test their physiological properties. Figure 1 shows that, as expected, the mutant *dsbA* allele alone results in hypersensitivity to the reducing agent DTT, i.e. *dsbA* mutant bacteria are completely unable to form colonies on LB agar containing 10 mM DTT. In contrast, both the wild-type and *ompL* single mutants form normal sized colonies under these conditions (Figure 1; data not shown). At higher concentrations of DTT, we were still unable to observe any differential behavior between the *ompL* null mutants and their corresponding isogenic wild-type parents (data not shown). Surprisingly, in our hands, neither of the two different *ompL* null mutations suppressed the DTT sensitivity of *dsbA* mutants in any of the three different backgrounds used (Figure 1; data not shown). This contrasts sharply with the results of Dartigalongue *et al*. (2000), who identified two out of their three *ompL* null mutations on the basis of complete suppression of the

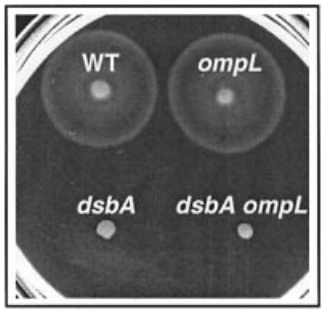

Fig. 2. An *ompL* null mutation does not restore the motility defect of a *dsbA* null mutant. Bacterial motility was monitored after 8 h of incubation at 37°C on soft agar plates. A typical set of results showing the radial growth of the *dsbA* and *ompL* single mutants and of the *dsbA ompL* double mutant compared with the parental strain MG1655 (WT) is presented. The *ompL* allele shown here is the *ompL*::mini-Tn*10*Kan^R allele (Dartigalongue *et al*., 2000).

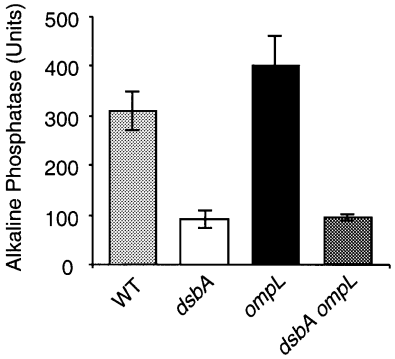

Fig. 3. The defect in AP folding exhibited by a *dsbA* mutation is not suppressed by mutations in *ompL*. AP assays were performed in rich and minimal media as described in Materials and methods. A representative set of results obtained in minimal medium with the wild-type strain MG1655 (WT) and its mutant derivatives is shown. The corresponding mutations are indicated at the bottom of the figure. The assays were done in triplicate and the standard deviation is represented by the error bars. The *ompL* allele shown here is the *ompL*::mini-Tn*10*Kan^R allele (Dartigalongue *et al*., 2000).

DTT sensitivity exhibited by *dsbA* null mutants, finding that the *dsbA ompL* double mutant is as resistant to DTT as the wild-type strain, if not more so.

ompL null mutations do not suppress the motility defect of dsbA mutant bacteria

Owing to the defective formation of disulfide bonds, *dsbA* mutants fail to assemble the P ring of the flagellar hook-basal-body and are therefore non-motile (Dailey and Berg, 1993). Dartigalongue *et al*. (2000) showed that the introduction of any one of their three *ompL* mutations into a *dsbA* null mutant restores full motility. However, when we tested the motility of our MG1655 mutant strains, we clearly found that the *dsbA ompL* double mutants are as defective in motility as the isogenic *dsbA* single mutant (Figure 2; data not shown). This experiment was repeated in the CA8000 motile background and again the *dsbA ompL* double mutants were as non-motile as the *dsbA* single mutant (data not shown). Our conclusion from these motility experiments is that *ompL* null mutations play no detectable role in bacterial motility, either alone or in combination with the *dsbA* null mutation.

ompL null mutations do not influence the levels of AP activity in a dsbA null mutant

Next, we measured the levels of AP activity in our isogenic constructs, both in LB broth ('repressing' conditions for AP activity) and in phosphate-limited minimal medium ('de-repressing' conditions for AP activity). In both cases, neither of the two *ompL* null mutations had any demonstrable effect in modulating the much lower levels of AP activity exhibited by the *dsbA* null mutant (Figure 3; data not shown). Thus, in contrast with the reported results of Dartigalongue *et al*. (2000), an *ompL* null mutation does not restore full or even partial levels of AP activity in the *dsbA* null mutant.

Suppression of other phenotypes associated with dsbA null mutations

Dartigalongue *et al*. (2000) reported that null mutations in the *ompL* gene completely eliminate the mucoid phenotype associated with *dsbA* null mutants. Again, in contrast with their findings, we observed that introduction of an *ompL* null mutation into a *dsbA* null mutant does not influence to any detectable extent the mucoidy exhibited by *dsbA* mutant bacteria in any of the three genetic backgrounds tested (data not shown).

We also examined the effect of an *ompL* null mutation on the sensitivity of a *dsbA* mutant to benzylpenicillin. Contrary to the findings of Dartigalongue *et al*. (2000), we surprisingly found that, in all three genetic backgrounds used, the *dsbA* null mutant was not more sensitive to benzylpenicillin than the isogenic wild-type parent or the *ompL* single mutants. In addition, introduction of an *ompL* null mutation in the *dsbA* mutant did not improve its growth on benzylpenicillin (data not shown).

Previously, it was reported that *dsbA* null mutants are defective in the formation of biofilms (Genevaux *et al*., 1999). This phenotype is related to the defective pili and flagellar biogenesis observed in a *dsbA* mutant (Dailey and Berg, 1993; Jacob-Dubuisson *et al*., 1994; Genevaux *et al*., 1999). As with motility, only the CA8000 and MG1655 parents are capable of biofilm formation. We tested both of these backgrounds for a potential effect of the *ompL* mutations on this *dsbA*-associated phenotype. As shown in Figure 4, the introduction of an *ompL* null mutation does not influence to any detectable extent the failure of *dsbA* mutants to form biofilms.

Phenotypes of ompL single mutants

Dartigalongue *et al*. (2000) enumerated four phenotypes associated with all three of their independently isolated *ompL* null insertion mutations. The first is a partial sensitivity to the reducing agent DTT, which was originally employed as a screen to isolate one of their original *ompL* mutants. As stated above, we did not detect any difference between the *ompL* single mutants and the isogenic wild-type parents in this respect (Figure 1; data not shown). The second phenotype is slow bacterial growth, with an *ompL* single null mutant having a generation time of 70 min compared with 45 min for the wild-type parents at 37°C (the type of medium used was not stated). In contrast, we did not observe any significant difference in the growth rates between the wild-type

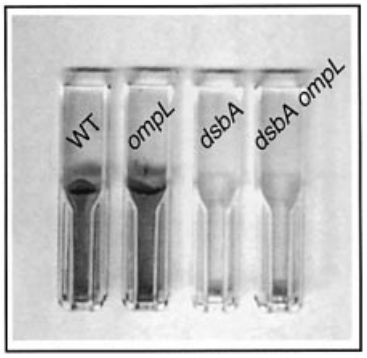

Fig. 4. The altered biofilm formation of a *dsbA* null mutant is not suppressed by an *ompL* null mutation. Biofilm formation was monitored after 12 h of incubation in LB medium at 37°C using polystyrene as the attachment surface. A representative set of experiments obtained with the wild-type strain MG1655 (WT), the *dsbA* and *ompL* single mutants and the *dsbA ompL* double mutant is presented. The *ompL* allele shown here is the *ompL*::mini-Tn*10*Kan^R allele (Dartigalongue *et al*., 2000).

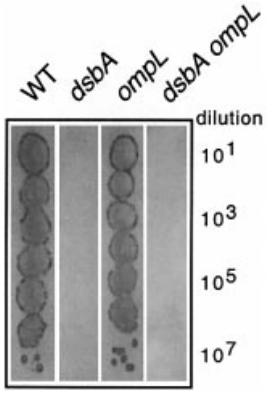

Fig. 5. The *ompL* null mutants are not resistant to bacteriophage M13 infection. Lawns of isogenic derivatives of JM101 were prepared on L-agar plates in the presence of IPTG and X-gal. Ten-fold serial dilutions of bacteriophage M13mp7 were spotted on the lawns followed by incubation of the plates at 37°C for ~18 h. A representative set of results obtained with the parent JM101 (WT), the *dsbA* and *ompL* single mutants and the *dsbA ompL* double mutant is shown. The *ompL* allele shown here is the *ompL*::mini-Tn*10*Kan^R allele (Dartigalongue *et al*., 2000).

parents and the *ompL* null mutants in either LB or minimal media (Figure 1; data not shown). The third phenotype associated with *ompL* null mutations is a block on the growth of the male-specific bacteriophage M13. Again, contrary to previous results (Dartigalongue *et al*., 2000), we did not observe any M13 resistance in the *ompL* null mutants in either the JM101 or CA8000 male backgrounds (Figure 5; data not shown). The failure to block bacteriophage M13 is particularly incomprehensible, given that complementation of this particular phenotype was used to originally clone and subclone the wild-type *ompL* gene (Dartigalongue *et al*., 2000). In addition, Figure 5 shows that the introduction of an *ompL* null mutation into a *dsbA* mutant does not suppress M13 resistance to any extent. Lastly, as stated above, we did not observe any reduction in the motility of single *ompL* mutants (Figure 2; data not shown), contrary to the reported results of Dartigalongue *et al*. (2000).

To explain their findings, Dartigalongue *et al*. (2000) suggested that a small, as yet unidentified, molecule(s), capable of substituting for DsbA, is oxidized by DsbB and that the oxidized and/or reduced forms of this hypothetical molecule are normally effluxed into the medium by the OmpL porin. Hence, in the absence of the OmpL porin, the molecule accumulates in the periplasmic space, thus completely complementing the lack of DsbA in a *dsbA* null mutant.

We have no experimental reason to doubt that the OmpL protein is a bona fide porin (established in the laboratory of Dr Hiroshi Nikaido; Dartigalongue *et al*., 2000). In addition, some of the features of the model proposed by Dartigalongue *et al*. (2000) may still turn out to be correct. However, taking all our genetic and biochemical results together, we conclude that null mutations in the *ompL* gene result in none of the remarkable phenotypes previously assigned to them, and that they play no demonstrable role in the suppression of the pleiotropic phenotypes associated with a *dsbA* null mutation.

The disparity between our results and those of Dartigalongue *et al*. (2000) may be due to several factors. Potential pitfalls associated with this type of genetic experiment may include closely linked uncharacterized suppressor mutations in some of the strains used, mix-ups or undetected contamination of strains. However, given the many discrepancies in the execution and reporting of the genetic experiments of Dartigalongue *et al*. (2000), including a serious lack of concordance between statements made in the Materials and methods section and the Results section, we are unable to offer a single logical scientific explanation for the failure of these authors to correctly assign any of the OmpL phenotypes to their various bacterial constructs.

Materials and methods

Bacterial and bacteriophage strains

We used the standard *E.coli* K-12 strains CA8000, MG1655 and MC4100 for this study. The MC4100 strain (Casadaban, 1976) is female and non-motile. The MG1655 strain, used in the *E.coli* genome sequencing project (Guyer *et al*., 1980; Blattner *et al*., 1997), is motile and female. The CA8000 strain (Hayes, 1953) is motile and male, and thus is the only one of the three capable of propagating bacteriophage M13 and its derivatives. Strain JM101 (Messing, 1979) (a kind gift from Dr Dominique Belin, University of Geneva) is male and was used primarily because it carries the chromosomally encoded *lacZ*ΔM15 mutation that is complemented by the LacZ α fragment encoded by bacteriophage M13mp7. The *in vivo* reconstituted LacZ activity enables the easy visualization of M13 plaques in the presence of IPTG (0.1 mM final) and X-gal (0.003% final). The strains SR2262 (MC4100 *dsbA*::mini-Tn*10*Tet^R) and SR1791 (CA8000 *ompL*::mini-Tn*10*Kan^R) were obtained from Dr Satish Raina (University of Geneva) (Dartigalongue *et al*., 2000). Construction of the deletion/substitution allele of *ompL* was performed in strain DY378 (Yu *et al*., 2000) and is described below. Bacteriophage P1L4 (originally from Dr Lucien Caro, University of Geneva) was used for transduction experiments. Bacteriophage M13mp7 (Messing *et al*., 1981) (the kind gift of Dr William Kelley, University of Geneva) was used to test strains for M13 resistance.

Media and chemicals

LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl pH 7) and LB agar (LB broth containing 1% agar) were supplemented when necessary with either kanamycin (50 μg/ml) or tetracycline (15 μg/ml). Other chemicals and media are described under the appropriate methods.

Construction of an ompL deletion/substitution mutation

We took advantage of the methodology described by Yu *et al*. (2000) to create a complete deletion of the *ompL* gene. The 693 nucleotide long *ompL* (also named *yshA*) open reading frame was substituted by the kanamycin-resistance (Kan^R) cassette from plasmid pKD4 (Datsenko and Wanner, 2000) to score its inheritance easily. To do this, we generated the following two primers: primer LKOR, 5′-**ctggtgggtggcggcctccctacgtttaaaaaatggacttatt**catatgaatatcctccttag, whose 5′ end carries homology to the nucleotide region immediately upstream of the *ompL* start codon (in bold type) and whose 3′ end carries the appropriate homology to one end of the Kan^R cassette of the plasmid pKD4; and primer LKOF, 5′-**gtaggccggataaggcgtttacgccgcatccggcagtttcaca**gtgtaggctggagctgcttcg, whose 5′ end carries homology to the nucleotide region immediately downstream of the *ompL* stop codon (in bold type) and whose 3′ end carries the appropriate homology to the other end of the Kan^R cassette of the plasmid pKD4.

PCR amplification of pKD4 with these two primers resulted in a DNA fragment encoding the kanamycin-resistance cassette flanked by sequences homologous to the regions immediately upstream and downstream of *ompL*. The purified DNA fragment was crossed into the chromosome of DY378 using the protocol of Yu *et al*. (2000). The *ompL* deletion/substitution on the chromosome was verified by PCR amplification of the *ompL* region using external primers located 88 nucleotides upstream (primer LCR: 5′-cgggcttccgtaacgttataaatg) and 112 nucleotides downstream (primer LCF: 5′-gatgctggcggctagagctta) of the *ompL* gene. The PCR products were subsequently sequence-verified using the same primers. The deletion was further confirmed by Southern blot analysis using full-length *ompL* as a DNA probe (Sambrook *et al*., 1989). This Δ*ompL*::Kan^R strain was used as a donor in subsequent bacteriophage P1 transduction experiments.

P1 transduction experiments

The various mutant alleles used in this study were moved from strain to strain by employing bacteriophage P1-mediated transduction, essentially as described by Miller (1992), and selecting at 37°C for the appropriate drug resistance on LB agar plates supplemented with 5×10^{-3} M sodium citrate.

AP activity measurements

Assays were performed with mid-log phase bacterial cell cultures grown at 37°C in either LB broth (repressing conditions for the *phoA* operon) or minimal 121 salts (Miller, 1992) supplemented with 0.2% glucose and limiting for PO_4 (de-repressing conditions for the *phoA* operon) as described previously (Brickman and Beckwith, 1975). AP activity was measured as described previously (Brickman and Beckwith, 1975; Michaelis *et al*., 1983).

DTT sensitivity experiments

A freshly prepared 1 M stock solution of DTT (Eurobio) in H_2O was diluted appropriately into molten LB agar to give the desired final concentrations. The solidified DTT agar plates were used within 1 h of solidification to minimize DTT oxidation by air. Exponentially growing cultures were serially diluted 10-fold, and 5 µl of each dilution was deposited on the plates. The dilutions were allowed to dry and the plates were then incubated at 37°C for ~18 h.

Benzylpenicillin sensitivity experiments

A 30 mg/ml stock solution of benzylpenicillin (potassium salt; Sigma PEN-K, 1596 units penicillin-G base per mg) in H_2O was diluted appropriately into molten LB agar to give the desired final concentration. The same dilutions of exponentially growing cultures used for the DTT sensitivity experiments were tested on the benzylpenicillin plates at the same time. The plates were incubated at 37°C for ~18 h.

Growth rate, motility and biofilm measurements

Growth rates were assayed in LB broth and in 121 salts supplemented with 0.2% glucose (Miller, 1992) at 37°C under constant shaking. At regular time intervals, the optical density (OD) of the cultures was measured at a wavelength of 600 nm. Motility assays were performed essentially as described by Macnab (1986). Two microliters of each culture grown to an $OD_{600\ nm}$ of ~1.0 were applied to freshly prepared soft agar plates (1% tryptone, 0.5% NaCl, 0.3% agar). Plates were incubated at 37°C for 8 h. Under these conditions, *dsbA* null mutants exhibit no significant motility as judged by the diameter of the bacterial growth zone. Biofilm experiments were carried out for 12 h at 37°C in LB broth using polystyrene surfaces as described previously (Genevaux *et al*., 1999).

Immunoblot analysis

Whole-cell extracts were prepared as described previously (Kelley and Georgopoulos, 1997), and proteins were separated in 12% (w/v) polyacrylamide–SDS gels, transferred to nitrocellulose membrane (Schleicher and Schuell) and probed with rabbit monoclonal anti-DsbA antibodies (the kind gift of Dr Joen Luirink, Department of Molecular Microbiology, Free University of Amsterdam). Goat anti-rabbit horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc.) was used as the secondary antibody. Blots were developed by enhanced chemiluminescence according to the manufacturer (Amersham Pharmacia Biotech).

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