Escherichia coli heat-shock proteins IbpA and IbpB affect biofilm formation by influencing the level of extracellular indole

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The development of *Escherichia coli* biofilm requires the differential expression of various genes implicated in cell signalling, stress responses, motility and the synthesis of structures responsible for cell attachment. The *ibpAB* operon is among the stress-response genes most induced during growth of the *E. coli* biofilm. In this study we demonstrated, to our knowledge for the first time, that the lack of IbpAB proteins in *E. coli* cells inhibited the formation of biofilm at the air–liquid interface, although it allowed normal planktonic growth. We showed that *ibpAB* mutant cells experienced endogenous oxidative stress, which might result from a decreased catalase activity. The endogenous oxidative stress in *ibpAB* cells led to increased expression of tryptophanase, an enzyme which catalyses the synthesis of indole. We demonstrated that the formation of biofilm by the *ibpAB* mutant was delayed due to the increase in the extracellular concentration of indole, which is known to play the role of a signal molecule, inhibiting biofilm growth.

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INTRODUCTION

In natural environments, rather than existing as single free-floating (planktonic) cells most bacteria live attached to surfaces in complex biofilm communities (Hall-Stoodley *et al.*, 2004; Harrison *et al.*, 2007). Ordered steps of biofilm development can be defined: reversible attachment to a surface, irreversible attachment, maturation, and the dispersion of cells to a planktonic state (Prüß *et al.*, 2006; Wood, 2009). Mature biofilms often form three-dimensional, complex and dynamic structures with the attributes of a multicellular organism (Hall-Stoodley *et al.*, 2004; Harrison *et al.*, 2007). The environmental conditions (e.g. microscale nutrient and oxygen concentrations) are not homogeneous throughout a biofilm; therefore, during adaptation to the local environment, sessile micro-organisms form subpopulations of cells (microcolonies) which express distinct metabolic pathways, stress responses and other specific activities (Stewart & Franklin, 2008). This physiological heterogeneity in biofilms results from significant changes in gene expression occurring during each step of biofilm development (Prüß *et al.*, 2006). The reprogramming of gene expression is also responsible for differentiation in the phenotype of planktonic and sessile cells. Biofilm cells are often more resistant to various stresses (antibiotics, oxidative stress, heavy metals) than their planktonic counterparts (Fux *et al.*, 2005; Harrison *et al.*, 2007). Therefore, formation of biofilm can be

regarded as a survival strategy of bacteria in hostile environments. However, biofilms can cause serious problems in medicine and industry. The antibiotic-resistant biofilms of pathogenic bacteria may be responsible for many chronic infections (Fux *et al.*, 2005), while in industry, biofilms often cause fouling and the corrosion of metal surfaces (Harrison *et al.*, 2007).

Although the process of biofilm development has been extensively investigated using several bacterial strains, its molecular mechanism is not fully known. Numerous recent studies concerning *Escherichia coli* biofilm have been based mainly on whole-transcriptome profiling. These reports indicate that the temperature, type of medium and other growth conditions determine which genes are induced or repressed during biofilm development (Schembri *et al.*, 2003; Beloin *et al.*, 2004; Ren *et al.*, 2004; Domka *et al.*, 2007). Analysis of the temporal development of *E. coli* K-12 biofilms revealed that differentially expressed genes (biofilm versus planktonic, and biofilm versus planktonic cells) belong to several functional categories and are implicated in stress response, quorum sensing, motility, production of fimbriae, and sulfur and tryptophan metabolism and transport (Domka *et al.*, 2007).

Among the stress-response genes overexpressed in biofilm populations, the *ibpA* and *ibpB* genes have been identified (Schembri *et al.*, 2003; Ren *et al.*, 2004; Junker *et al.*, 2007). The function of the IbpA and IbpB proteins in biofilm development has not yet been established. The *ibpA* and *ibpB* genes form an operon controlled by the σ^{32} and σ^{54} subunits of RNA polymerase (Kuczyńska-Wisńik *et al.*,

Abbreviations: DNP, 2,4-dinitrophenol; DNPH, 2,4-dinitrophenylhydrazine; H₂DCFDA, 2',7'-dichlorofluorescein diacetate; PVC, polyvinyl chloride; SOD, superoxide dismutase.

2001) and are induced under heat- and other stress conditions (Allen *et al.*, 1992; Chuang *et al.*, 1993; Laskowska *et al.*, 2003; Pérez *et al.*, 2007; Nobre *et al.*, 2009). The IbpA and IbpB proteins share about 50 % homology and belong to the ubiquitous family of small heat-shock proteins (sHsps). sHsps are characterized by a relatively low molecular mass (12–30 kDa), an oligomeric structure and a conserved C-terminal domain of approximately 100 amino acid residues (Haslbeck *et al.*, 2005; Nakamoto & Vigh, 2007). This so-called ‘ α -crystallin’ domain is homologous to the mammalian eye lens sHsp (α -crystallin), which prevents protein aggregation and cataract formation in the lens. IbpA and IbpB were identified as components of inclusion bodies (Allen *et al.*, 1992) and aggregates of heat-denatured proteins (Laskowska *et al.*, 1996). *In vitro* studies revealed that IbpA and IbpB form complexes with denatured proteins and facilitate their subsequent refolding by the ATP-dependent DnaK-DnaJ-GrpE chaperone system in cooperation with the AAA⁺ protein ClpB (Veinger *et al.*, 1998; Mogk *et al.*, 2003a, b; Matuszewska *et al.*, 2005). Both IbpA and IbpB are required *in vitro*, to efficiently stabilize denatured protein in a folding-competent state; however, they fulfil different roles in this process. IbpA prevents formation of large aggregates of the substrate, but simultaneously inhibits ClpB-DnaK-dependent refolding. IbpB interacts with aggregates via IbpA and alleviates the IbpA-mediated inhibitory effect (Ratajczak *et al.*, 2009). Deletion of the *ibpAB* operon causes increased aggregation of heat-denatured proteins and reduces cell viability under severe heat stress (Kuczyńska-Wisniak *et al.*, 2002). At lower heat-shock temperatures (45 °C) the phenotype of the *ibpAB* mutation is observed in the triple mutant *ibpABclpB* and in *ibpAB* cells with downregulated DnaK/DnaJ levels (Mogk *et al.*, 2003a). Recently, we found that IbpA and IbpB participate in the defence against oxidative stress induced by copper. In the presence of oxygen, *ibpAB* cells exhibit increased sensitivity to copper ions and accumulate elevated amounts of oxidized proteins (Matuszewska *et al.*, 2008).

The results presented in this paper provide new insights into the role of IbpAB during biofilm development. We demonstrate that the lack of IbpAB proteins in *E. coli* cells results in endogenous oxidative stress and overproduction of extracellular indole, which inhibits the growth of the biofilm at the air–liquid interface.

METHODS

Bacterial strains, growth conditions and plasmids. *E. coli* W3110 [F[−] *lam rpoS(Am) rph-1 Inv(rrnD–rrnE)*] (Colón-González *et al.*, 2004) and its derivatives were used in this study. The W3110 Δ *ibpAB*::cm mutant was generated by P1 transduction (Sambrook *et al.*, 1989) of W3110 with phage grown on MC4100 Δ *ibpAB*::cm (Kuczyńska-Wisniak *et al.*, 2002). The *ibpAB* mutation was verified by Western blotting using anti-IbpAB serum. W3110 Δ *katE731*::kan, W3110 Δ *katG729*::kan and W3110 Δ *tnaA*::Tn10 were constructed by P1 transduction using *E. coli* JW1721-1, JW3914-1 and JC12337 (*E. coli* Genetic Stock Center, Yale University), respectively, as donors.

Both W3110 *katE* and W3110 *katG* strains exhibited decreased (by 30–50 %, depending on the growth state) catalase activity when compared to W3110.

To grow biofilms, the overnight cultures were diluted 1:50 into fresh LB medium supplemented with 0.1 M MOPS pH 7.4 and 0.2 % glucose and incubated for up to 24 h, without shaking, at 30 °C in 96-well polyvinyl chloride (PVC) microtitre plates, using 150 μ l of culture per well. Plasmid pGab was constructed by cloning between the *EcoRI* and *BamHI* sites of plasmid pGB2 (Churchward *et al.*, 1984) a PCR fragment containing the *ibpAB* operon with its own σ^{32} promoter. The PCR fragment was generated with primers 5′-ATGGAATTCAACCATAACTGCA-3′ and 5′-TATCACGGATCCGAACGTGC-3′, using *E. coli* chromosomal DNA as the template, and digested with *EcoRI* and *BamHI*.

Visualization and quantification of the *E. coli* biofilm. At the indicated time points, bacterial growth was determined by measuring the optical density (OD₅₉₅). After removing the planktonic cells from the PVC wells, the biofilm was stained with 0.1 % crystal violet for 10 min (Colón-González *et al.*, 2004). After rinsing with water, the attached crystal violet was eluted with a mixture of 80 % ethanol and 20 % acetone and quantified spectrophotometrically (*A*₅₉₅) to calculate the relative amount of biofilm (*A*₅₉₅/OD₅₉₅).

Enzyme assays. Cells grown in PVC microtitre plates were collected (1.5 ml) at different time points of growth, indicated in the figures, and sedimented. The pellets were washed, resuspended in 300 μ l 10 mM Tris/HCl pH 7.4 and disrupted by sonication (Vibra-Cell sonicator, three 5 s bursts at 20 % power output, with cooling in ice). To determine the catalase activity, the cell extracts were added to 20 mM H₂O₂ and the rate of decrease in *A*₂₄₀ was measured immediately (Jakubowski *et al.*, 2000). Superoxide dismutase (SOD) activity in cell extracts was determined by a colorimetric method using a SOD Assay kit-WST according to the protocol (Fluka Analytical). Tryptophanase activity was assayed according to Choi *et al.* (2005). A 0.2 ml sample of the cell extract was mixed with 0.2 ml of the reaction mixture (2.75 mg sodium pyrophosphate, 19.6 mg disodium EDTA dihydrate and 10 mg BSA in 100 ml 0.05 M potassium phosphate, pH 7.5) and 0.2 ml 20 mM tryptophan. After 1 h incubation at 37 °C the reaction was stopped by adding 2 ml colour reagent solution (14.7 g *p*-dimethylaminobenzaldehyde, 52 ml H₂SO₄ and 948 ml 95 % ethanol) and the *A*₅₄₀ was measured. Strain W3110 *tnaA*::Tn10 was used as a negative control for this measurement.

Extracellular indole assay. The method of Domka *et al.* (2006) was used. To measure extracellular indole concentrations in *E. coli* cultures at different time points of growth (see figures), 2 ml Kovac’s reagent (10 g *p*-dimethylaminobenzaldehyde, 50 ml HCl and 150 ml amyl alcohol) was mixed for 2 min with 5 ml of medium collected after sedimentation of the bacteria. The reaction mixture was diluted 1:10 in HCl/amyl alcohol solution (50 ml HCl and 150 ml amyl alcohol) and the *A*₅₄₀ was measured. The concentration of indole was calculated based on the calibration curve. Strain W3110 *tnaA*::Tn10, which does not produce indole, was used as a negative control.

2′,7′-Dichlorofluorescein oxidation. The level of intracellular oxidation was measured in exponential- and stationary-phase cultures using 2′,7′-dichlorofluorescein diacetate (H₂DCFDA) according to Jakubowski *et al.* (2000). Bacteria were sedimented by centrifugation, washed and resuspended in 50 mM sodium phosphate buffer pH 7.4. 5 mM H₂DCFDA (dissolved in DMSO) was added to a final concentration of 10 μ M and samples were incubated at 30 °C. After 30 min, the bacteria were pelleted, washed, suspended in the buffer and disrupted by sonication. The cell extracts were clarified by centrifugation and fluorescence of the supernatant was measured using a Perkin-Elmer LS-5B spectrofluorimeter (excitation, 490 nm;

emission, 519 nm). The emission values were normalized by the protein concentration.

Immunodetection of IbpAB and oxidized proteins.

Immunodetection of IbpAB in cell extracts was carried out using IbpAB-specific antiserum as primary antibodies (Kuczyńska-Wisnik *et al.*, 2002), with anti-rabbit IgG horseradish peroxidase conjugate (Sigma) and ECL detection reagents (Pierce Biotechnology). The major products of protein oxidation, carbonyl groups, were detected as described by Matuszewska *et al.* (2008). Briefly, crude protein extracts were derivatized with 2,4-dinitrophenylhydrazine (DNPH) and subjected to SDS-PAGE and Western blotting (Sambrook *et al.*, 1989). The DNPH-derivatized proteins were immunodetected using anti-DNP antibodies (Sigma).

RESULTS

The *ibpAB* mutation inhibits formation of *E. coli* W3110 biofilm at the air–liquid interface

To form biofilm, the W3110 WT and *ibpAB* strains were grown at 30 °C in PVC microtitre plates containing LB-MOPS medium. The *ibpAB* mutation did not affect growth (Fig. 1a), but it did influence biofilm formation (Fig. 1b). The wild-type (WT) biofilm appeared after 8 h of growth and after crystal violet staining was visible as a ring at the air–liquid interface of the cultures (Fig. 1b). The amount of WT air–liquid interface biofilm increased during further incubation up to 24 h. Formation of the *ibpAB* biofilm was significantly delayed: after 12 h the amount of *ibpAB* cells attached to the PVC surface was twofold lower than in the WT cultures. However, the relative amounts of the *ibpAB* and WT biofilms reached almost the same levels after 24 h (Fig. 1b) and afterwards decreased gradually at comparable rates (data not shown). The reduction of the WT biofilm during the late stationary phase was in agreement with previously published data (Colón-González *et al.*, 2004). The static cultures cultivated in the microtitre plates probably reach anaerobiosis at the stationary phase. These conditions might be a signal for the detachment of *E. coli* biofilm.

The level of the IbpAB proteins in the WT cells increased gradually (two- to threefold) during the exponential phase of growth and reached its maximum after 8 h (Fig. 1c), approximately at the time when the biofilm became visible. Thereafter, the IbpAB level stayed nearly constant and was comparable in planktonic and biofilm cells (Fig. 1c). The 2D-PAGE used for separation of IbpA from IbpB revealed that at 16 h the IbpA:IbpB molar ratio was 1.5:1 in planktonic as well as in biofilm cells (data not shown).

We have previously found that the lack of IbpAB chaperones increases the sensitivity of *E. coli* to oxidative stress induced by copper ions (Matuszewska *et al.*, 2008); therefore we were also interested in whether the presence of Cu(II) would influence the formation of WT and *ibpAB* biofilms. We found that the development of the WT biofilm was inhibited in the presence of Cu(II). After 8 h the WT biofilm was hardly detectable and its amount was 3.5-fold lower than that formed in the absence of Cu(II)

(compare Fig. 1b and Fig. 1e). An additional reduction of the biofilm was observed in the *ibpAB* culture exposed to Cu(II). In the presence of Cu(II) the *ibpAB* biofilm appeared 4 h later (Fig. 1e) than in the non-treated cultures (Fig. 1b). Moreover, the difference between the WT and *ibpAB* biofilms was more pronounced in cultures exposed to Cu(II) (Fig. 1e) than in the non-treated bacteria (Fig. 1b). It should be noted that since the cultures were incubated without shaking, WT and *ibpAB* cells were grown in the presence of limited amounts of oxygen. Under these microaerobic conditions, in the presence of Cu(II), the *ibpA/B* mutation caused moderate inhibition of *E. coli* growth (Fig. 1d). The above results indicated that under some stress conditions [Cu(II)-induced stress] bacteria lacking IbpAB proteins could grow as free-floating cells at rates comparable with WT cells. However, the ability of the *ibpAB* mutant to form a sessile population – biofilm – was seriously impaired under these conditions. Complementation experiments confirmed that the *ibpAB* mutation was responsible for the observed phenotype. Low-copy-number plasmid pGab, carrying the *ibpAB* operon, restored the ability of the *ibpAB* cells to form biofilm at the air–liquid interface (Fig. 1f).

Planktonic and biofilm *ibpAB* cells contain an increased level of tryptophanase

To reveal the mechanism responsible for the suppression of *ibpAB* biofilm formation, we compared the protein profiles of the WT and *ibpAB* cultures at different stages of biofilm growth. We found that both planktonic and attached *ibpAB* cells accumulated a ~50 kDa protein during the stationary phase of growth (12–16 h) (Fig. 2a). The cell extracts were resolved by 2D-PAGE (data not shown) to isolate the 50 kDa protein, which was identified by mass spectrometry as tryptophanase (TnaA). It is known that the expression of *tnaA* is induced at the stationary phase of growth (Lacour & Landini, 2004), at alkaline pH (Blankenhorn *et al.*, 1999) and during oxidative stress (Pomposiello *et al.*, 2001; Zheng *et al.*, 2001). TnaA converts tryptophan to indole, ammonia and pyruvate. It has been proposed that indole acts as an extracellular signal which inhibits biofilm formation (Lee *et al.*, 2007). Therefore, we hypothesized that the delayed adhesion of the *ibpAB* cells to the PVC surfaces might result from the enhanced activity of TnaA and the increased synthesis of indole. Indeed, we found that the activity of TnaA (Fig. 2b) was significantly (1–5–3-fold) higher in the mutant strain throughout the whole course of the experiment, except the late stationary phase (24 h), when the enzyme activity was comparable in both strains. Enhanced TnaA activity in *ibpAB* cells might be responsible for the increased concentration of extracellular indole – after 16 h its level in the *ibpAB* culture was ninefold higher than in the WT strain (Fig. 2c). Complementation experiments confirmed that the IbpAB proteins influence the level of extracellular indole. WT(pGB2) and *ibpAB*(pGab) cultures produced comparable amounts of extracellular indole (Fig. 2c).

Fig. 1. Lack of IbpAB proteins delays formation of *E. coli* biofilm at the air-liquid interface. *E. coli* WT and *ibpAB* strains were grown in the absence (a–c) or presence (d, e) of 2 mM CuCl₂ in PVC microtitre plates as described in Methods. (a, d) Growth of WT (●) and *ibpAB* (○) cells. (b, e) Biofilm formation. Biofilms were stained with crystal violet at the indicated time points; in (b), for each time point, one representative well of 12 replicate wells is shown (WT, upper row, *ibpAB*, lower row). Relative amounts of the WT (grey bars) and *ibpAB* (white bars) biofilms were estimated as described in Methods. Means ± SD of three independent experiments are shown. In each experiment, 12 wells were averaged to obtain each data point. (c) The level of IbpAB proteins increases during the exponential phase. IbpAB proteins were detected in whole WT cultures (containing both planktonic and biofilm cells, 4 h and 8 h) and in samples containing planktonic (P) or biofilm (B) cells (16 h and 24 h). Aliquots of samples corresponding to the same amounts of bacteria were separated by SDS-PAGE and immunodetected using anti-IbpAB serum and ECL reagents. The IbpA and IbpB proteins migrated in the polyacrylamide gel as one band and were detected by the serum. (f) Complementation of the *ibpAB* mutant using the low-copy-number plasmid pGab and empty plasmid pGB2 as a control. *E. coli* WT(pGB2) (grey bar), *ibpAB*(pGB2) (white bar) and *ibpAB* (pGab) (black bar) were grown in the presence of 2 mM CuCl₂. Relative amounts of biofilm were determined after 16 h.

It has been reported that temperature affects indole signalling in *E. coli* and indole reduces biofilm formation more significantly at 30 °C than at 37 °C (Lee *et al.*, 2008). Therefore, the effect of the *ibpAB* mutation (delayed biofilm formation) should be suppressed at 37 °C. Indeed, we did not observe any substantial differences in the formation of WT and *ibpAB* biofilms at 37 °C (Fig. 3), in spite of the fact that the amount of indole in the *ibpAB*

culture was still some five-to sevenfold higher than in the WT strain (data not shown). Lee *et al.* (2008) demonstrated that the addition of exogenous indole reduces *E. coli* biofilm formation in a dose-dependent manner. Under the conditions applied in our studies we obtained a similar result (data not shown). Taken together, these data suggest that the increased concentration of extracellular indole was responsible for the delay of the *ibpAB* biofilm formation.

Fig. 2. TnaA activity and extracellular indole concentration are increased in *E. coli* cells lacking IbpAB proteins. *E. coli* WT and *ibpAB* cells were grown in PVC microtitre plates for 16 h as described in the legend to Fig. 1. (a) Planktonic (P) and biofilm (B) cells were collected separately and samples containing equal amounts of protein were resolved by SDS-PAGE. The gel was stained with Coomassie brilliant blue. (b) TnaA activity was measured in cell extracts obtained from whole *E. coli* cultures containing both planktonic and biofilm cells, A.U., arbitrary units. Grey bars, WT; white bars, *ibpAB*. (c) The extracellular level of indole was determined in the spent medium, after sedimentation of planktonic cells. Grey bars, WT(pGB2); white bars, *ibpAB*(pGB2); black bars, *ibpAB*(pGab). TnaA activities and extracellular indole levels were normalized to cell density. Means $\pm$ SD of three independent experiments are shown.

ibpAB cells are exposed to endogenous oxidative stress

Our next aim was to identify the signal responsible for the increased synthesis of TnaA and extracellular indole in

Fig. 3. The *ibpAB* mutation does not inhibit air-liquid biofilm growth at 37 °C. *E. coli* WT (grey bars) and *ibpAB* (white bars) were grown at 37 °C in PVC microtitre plates as described in the legend to Fig. 1. At the indicated time points the relative amount of biofilm was determined as described in Methods. The data correspond to the means $\pm$ SD of three independent experiments.

ibpAB cells. The fact that (1) IbpA and IbpB participate in the protection of *E. coli* cells against oxidative damage (Kitagawa *et al.*, 2000, 2002; Matuszewska *et al.*, 2008) and (2) the TnaA level increases during oxidative stress (Pomposiello *et al.*, 2001; Zheng *et al.*, 2001) prompted the hypothesis that cells lacking IbpAB proteins are exposed to endogenous oxidative stress which leads to the overproduction of TnaA and indole. To investigate this possibility, we first compared the level of oxidation of H₂DCFDA in WT and *ibpAB* cells. The oxidation of H₂DCFDA depends on the level of reactive oxygen species and reduced glutathione; therefore, it is commonly used to detect general oxidative stress in cells (Jakubowski *et al.*, 2000; Echave *et al.*, 2003). We found that, independently of the phase of growth, the oxidation of H₂DCFDA was enhanced in the *ibpAB* strain by 30–40 %, as compared to WT cells (Fig. 4a), confirming that *E. coli ibpAB* was exposed to endogenous oxidative stress. Further evidence supporting this hypothesis was provided by immunodetection of oxidized proteins in WT and *ibpAB* cells. Elevated protein oxidation was observed in *ibpAB* cells up to 6 h after inoculation (Fig. 4b). The protein band which appeared in the second hour of culture in the *ibpAB* cells, but was absent in the WT strain, corresponded to the oxidized EF-G protein, according to the literature (Tamarit *et al.*, 1998). The amount and pattern of carbonylated proteins were not changed significantly after the sixth hour of culture (data not shown). Thus, in the absence of IbpAB

Fig. 4. The *ibpAB* strain is challenged with endogenous oxidative stress. *E. coli* WT (grey bars) and *ibpAB* (white bars) were grown as described in the legend to Fig. 1. (a) At the indicated time points, the oxidation of H₂DCFDA was measured as arbitrary units of fluorescence intensity. The data correspond to the means $\pm$ SD of three independent experiments. The observed difference between WT and *ibpAB* strains was found to be significant ($P < 0.05$) in a paired two-tailed *t* test. (b) Protein carbonyl groups were detected in the same samples. After derivatization with DNPH (see Methods), aliquots containing equal amounts of protein were resolved by SDS-PAGE and analysed using an anti-DNP antibody.

some *E. coli* proteins became more sensitive to oxidation during the early exponential phase. However, the damage caused by endogenous oxidative stress did not affect the growth of *E. coli ibpAB* (Fig. 1a).

Next, we measured two enzymic activities in WT and *ibpAB* cells: those of catalase and superoxide dismutase (SOD), which participate in antioxidant defence and neutralize H₂O₂ and superoxide ions, respectively. Interestingly, in the *ibpAB* strain catalase activity was decreased by 25–30 %, when compared to WT cells (Fig. 5a), while SOD activity was not affected (Fig. 5b). It should be pointed out that in the complementation tests, catalase activity in the *ibpAB*(pGab) culture reached the levels detected in the WT cells. These results suggest that inefficient catalase activity in the *ibpAB* mutant might be responsible for the elevated endogenous oxidative stress (H₂O₂) and increased level of oxidized proteins. This, in

Fig. 5. The *ibpAB* mutation affects catalase activity in *E. coli* cells. *E. coli* strains were grown in PVC microtitre plates as described in Methods. (a) Catalase activity was determined in cell extracts obtained from cultures of *E. coli* WT(pGB2) (grey bars), *ibpAB*(pGB2) (white bars) and *ibpAB*(pGab) (black bars), which contained both planktonic and biofilm cells. (b) SOD activity was determined in extracts of WT (grey bars) and *ibpAB* (white bars). Means $\pm$ SD of three independent experiments are shown. A.U., arbitrary units.

turn, may lead to the overproduction of TnaA and extracellular indole, resulting in biofilm suppression. To verify this hypothesis we tested the ability of catalase mutant strains to form biofilm. *E. coli* has two catalase enzymes, HPI and HPII (Visick & Clarke, 1997). HPI, the product of the *katG* gene, is induced during exponential growth in response to low concentrations of hydrogen peroxide, and at the stationary phase in a σ^S -independent way. The *katE* gene, which codes for HPII, is not peroxide-inducible, but is activated at the transition from the exponential to the stationary phase by RNA polymerase containing the σ^S subunit. We found that the lack of HPI catalase, but not HPII, resulted in the delay of *E. coli* biofilm formation at the air–liquid interface (Fig. 6a).

Moreover, increased TnaA activity (Fig. 6b) and an elevated level of extracellular indole (Fig. 6c) were detected in the mid-exponential-phase *katG* cultures. The enzyme activity and production of extracellular indole were only slightly affected by the *katE* deletion (Fig. 6b, c). These data indicated that the lack of IbpAB proteins resulted in diminished HPI activity, leading to the overproduction of extracellular indole and inhibition of biofilm formation.

Fluidization of *E. coli* membranes by benzyl alcohol inhibits biofilm formation

We found that in the presence of Cu(II), the inhibitory effect of the *ibpAB* mutation on biofilm growth was stronger than in non-stressed cultures (compare Fig. 1d and Fig. 1b). It is known that copper stress influences permeability of membranes (Suwalsky *et al.*, 1998). It was also reported that the *ibpAB* mutation increases membrane fluidity and permeability (Nakamoto & Vigh, 2007); therefore Cu(II) can cause more severe damage to the *ibpAB* cell membranes, leading to further inhibition of biofilm formation. It is possible that the altered physical state of the membranes contributes to the suppression of *ibpAB* biofilm growth in the absence of Cu(II) also. Interestingly, we found that non-lethal doses of a membrane fluidizer, benzyl alcohol (Shigapova *et al.*, 2005), increased the level of extracellular indole (although it did not influence TnaA activity) in the WT culture. Simultaneously, benzyl alcohol significantly suppressed biofilm formation (Fig. 7). This result suggests that the fluidization and permeabilization of *E. coli* membranes caused by the lack of IbpAB proteins (Nakamoto & Vigh, 2007) may contribute to reduced biofilm formation by *ibpAB* cells.

DISCUSSION

E. coli biofilm development is a complex process which requires the coordinated expression of more than 100 genes (reviewed by Prüß *et al.*, 2006; Wood, 2009). Gene expression profiles in *E. coli* biofilms have identified various stress-response genes as being important during the growth of the biofilm. The stress-response genes overexpressed in biofilms are involved in the SOS response (*dinI*, *dinP*, *dinG*, *sbmC*, *recN*, *sulA*), envelope stress (*pspABCDE*, *cpxABR*, *rpoE*), general stress response (*rpoS*, *ycfR*) and heat stress (*dnaK*, *dnaJ*, *clpB*, *ibpA*, *ibpB*) (Schembri *et al.*, 2003; Beloin *et al.*, 2004; Ren *et al.*, 2004; Junker *et al.*, 2007; Domka *et al.*, 2007). Compared to the role of the surface proteins directly involved in cell attachment, little is known about the function of molecular chaperones in biofilm development. It was reported that DnaK, DnaJ and GrpE are required for the synthesis of flagella (Shi *et al.*, 1992). DnaK and DnaJ are also involved in the regulation of synthesis of cyclic lipopeptides, putisolins, which possess surfactant activity and play an important role in the formation of *Pseudomonas putida* biofilm (Dubern *et al.*, 2005).

Fig. 6. The *katG* mutation inhibits formation of *E. coli* biofilm. *E. coli* WT (grey bars), *katE* (white bars) and *katG* (black bars) strains were grown in PVC microtitre plates as described in Methods. (a) At the indicated time points, relative amounts of biofilm were estimated. (b) TnaA activity was measured in cell extracts obtained from cultures containing both planktonic and biofilm cells. A.U., arbitrary units. (c) The level of extracellular indole was determined in spent medium collected after the sedimentation of planktonic cells. TnaA activities and indole levels were normalized to cell density. Means $\pm$ SD of three independent experiments are shown.

Fig. 7. Benzyl alcohol increases the level of extracellular indole and inhibits biofilm formation. The *E. coli* WT strain was grown in the presence of increased concentrations of benzyl alcohol in PVC microtitre plates as described in Methods. After 3 h, TnaA activity (dark grey bars) and extracellular concentrations of indole (light grey bars) were measured. Biofilm was detected by crystal violet staining after 16 h of incubation (photographs). Means $\pm$ SD of three independent experiments and representative wells are shown. A.U., arbitrary units.

In this study we focused on the role of the IbpAB proteins, which have been previously reported to protect *E. coli* cells against heat and oxidative stresses (Kuczyńska-Wisniak *et al.*, 2002; Matuszewska *et al.*, 2008). The results presented in this paper demonstrate that the lack of IbpAB proteins in *E. coli* cells allows normal planktonic growth but inhibits the formation of biofilm at the air–liquid interface. We propose that an increased concentration of extracellular indole in the Δ *ibpAB* culture is responsible for this inhibitory effect. Recently, it has been proposed that indole plays the role of an extracellular signal (Wang *et al.*, 2001), inhibiting development of *E. coli* biofilms (Lee *et al.*, 2008). However, other studies in which different *E. coli* strains and media were used suggested that indole may increase biofilm formation ability (Di Martino *et al.*, 2003; Collet *et al.*, 2007). Intriguingly, indole may also function as an interspecies signal which increases the formation of the biofilms of *Pseudomonas aeruginosa* and *P. fluorescens*, bacteria which do not produce this signal (Lee *et al.*, 2007). Indole is a product of the reaction catalysed by tryptophanase, an increased level of which was detected in *ibpAB* cells (Fig. 2a, b). It is known that *tnaA* expression can be induced in the presence of reactive oxygen species (Pomposiello *et al.*, 2001; Zheng *et al.*, 2001). We showed that cells lacking the IbpAB proteins are exposed to endogenous oxidative stress, which might lead to enhanced expression of the *tnaA* gene. Using H_2DCFDA as a probe, we detected an increased level of general oxidative stress (Fig. 4a) and found elevated amounts of oxidized proteins

in *ibpAB* cells (Fig. 4b). These results indicated that the mutant was indeed exposed to endogenous oxidative stress. Determination of the two main enzyme activities involved in the antioxidant protection (catalase and SOD) revealed that the *ibpAB* strain also exhibited a decreased catalase activity (Fig. 5). Since it was reported that IbpAB proteins protect various enzymes against oxidative damage (Kitagawa *et al.*, 2002; Matuszewska *et al.*, 2008), it is possible that these proteins also prevent inactivation of catalase(s). The method used in this work for the quantification of catalase activity did not allow us to distinguish which of the two *E. coli* catalases, HPI (the product of the *katG* gene) or HP II (coded by the *katE* gene), was affected by the *ibpAB* mutation. Further studies revealed that the lack of HPI, but not HP II, delayed air–liquid interface biofilm formation (Fig. 6a), accompanied by increased TnaA activity and overproduction of extracellular indole (Fig. 6b, c) in mid-exponential-phase cultures. A significant increase in the level of extracellular indole in the *katG* culture was observed only at the eighth hour of culture (Fig. 6c), which may indicate that mechanisms were initiated in *katG* cells that counteract further overproduction of extracellular indole. Hence, the effect of the *katG* mutation on biofilm growth (Fig. 6a) was less evident than in the case of *ibpAB* cells (Fig. 1b). It should be noted that HPI is produced at the exponential and stationary phase, whereas HP II is activated at the transition from the exponential to the stationary phase (Visick & Clarke, 1997), i.e. at the late stage of biofilm growth. Therefore, it is not surprising that the lack of HP II did not influence the early steps of biofilm development at mid-exponential phase (Fig. 6a).

The above results suggested that detoxification of intracellular H_2O_2 (a by-product of aerobic metabolism) by HPI was impaired in the absence of IbpAB. How the endogenous oxidative stress (H_2O_2) led to the induction of *tnaA* and overproduction of extracellular indole in *ibpAB* cells remains unclear. A similar induction mechanism seems to exist in the WT strain exposed to exogenous H_2O_2 . We found that WT cells grown in the presence of 5 mM H_2O_2 exhibited higher (by 25 %) TnaA activity and produced twofold more indole compared to non-stressed WT cultures. Furthermore, H_2O_2 did not affect growth of the strain, but significantly delayed WT biofilm formation (data not shown). The inhibitory effect of oxidative and heavy metal stresses on biofilm formation was also found in *E. coli* cultures exposed to paraquat, Cd(II) (data not shown) and Cu(II) (Fig. 6). It is important to note that Zhang *et al.* (2007) observed the opposite effect of oxidative and heavy metal stress on *E. coli* biofilm. They reported that H_2O_2 and Cd(II) strongly stimulated growth of *E. coli* BW25113 biofilm at 37 °C, in non-buffered LB-medium. Interestingly, this enhanced biofilm formation was accompanied by decreased indole synthesis. Therefore, the data published by Zhang *et al.* (2007) and our results demonstrate that oxidative and heavy metal stress may affect (increase or decrease) biofilm

formation by influencing the extracellular indole concentration. The differences between their studies and ours could result from the use of different *E. coli* strains and growth conditions.

We demonstrated that the inhibitory effect of the *ibpAB* mutation on biofilm formation was stronger in the presence of Cu(II) than in non-treated cultures (compare Fig. 1e and Fig. 1b). The mechanism of copper cytotoxicity is complex and includes the generation of reactive oxygen species and perturbations of membrane permeability (Suwalsky *et al.*, 1998; Kershaw *et al.*, 2005). We hypothesize that membrane damage may be one of the reasons for the inhibition of biofilm formation under Cu(II)-induced stress. Perturbed membrane permeability may impair synthesis, export and maintenance of various cell surface molecules and structures (flagella, type 1 fimbriae, curli and extracellular polymeric substances) implicated in different stages of biofilm formation (Van Houdt & Michiels, 2005). Moreover, the activity of inner-membrane proteins AcrEF and Mtr, responsible for the export and import of indole, respectively (Wood, 2009), could also be affected, leading to an increased concentration of extracellular indole. It is known that *ibpAB* mutation decreases membrane stability (Nakamoto & Vigh, 2007); therefore Cu(II) can cause more severe damage to *ibpAB* cell membranes, reflected in the additional inhibition of biofilm formation. Our experiment with the membrane fluidizer benzyl alcohol provided further evidence that the altered physical state of membranes contributes to the suppression of biofilm growth. We demonstrated that non-lethal doses of benzyl alcohol increased the extracellular level of indole and significantly suppressed biofilm formation (Fig. 7).

In summary, we have demonstrated that the *IbpAB* proteins accelerate formation of *E. coli* biofilm by protecting cells against endogenous oxidative stress. In the absence of *IbpAB*, *E. coli* cells experience oxidative stress and overproduce TnaA and extracellular indole. Increased membrane fluidity and permeability in the *ibpAB* mutant may also contribute to the increased extracellular concentration of indole, which reduces biofilm formation.

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