# A previously uncharacterized gene, *yjfO* (*bsmA*), influences *Escherichia coli* biofilm formation and stress response

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Bacteria growing as surface-adherent biofilms are better able to withstand chemical and physical stresses than their unattached, planktonic counterparts. Using transcriptional profiling and quantitative PCR, we observed a previously uncharacterized gene, *yjfO* to be upregulated during *Escherichia coli* MG1655 biofilm growth in a chemostat on serine-limited defined medium. A *yjfO* mutant, developed through targeted-insertion mutagenesis, and a *yjfO*-complemented strain, were obtained for further characterization. While bacterial surface colonization levels (c.f.u. $cm^{-2}$) were similar in all three strains, the mutant strain exhibited reduced microcolony formation when observed in flow cells, and greatly enhanced flagellar motility on soft (0.3 %) agar. Complementation of *yjfO* restored microcolony formation and flagellar motility to wild-type levels. Cell surface hydrophobicity and twitching motility were unaffected by the presence or absence of *yjfO*. In contrast to the parent strain, biofilms from the mutant strain were less able to resist acid and peroxide stresses. *yjfO* had no significant effect on *E. coli* biofilm susceptibility to alkali or heat stress. Planktonic cultures from all three strains showed similar responses to these stresses. Regardless of the presence of *yjfO*, planktonic *E. coli* withstood alkali stress better than biofilm populations. Complementation of *yjfO* restored viability following exposure to peroxide stress, but did not restore acid resistance. Based on its influence on biofilm maturation and stress response, and effects on motility, we propose renaming the uncharacterized gene, *yjfO*, as *bsmA* (biofilm stress and motility).

## INTRODUCTION

When growing as surface-adherent, biofilm communities, bacteria are typically quite resistant to a variety of adverse environmental conditions, including antimicrobial agents, pH extremes and oxidative stresses (reviewed by Costerton *et al.*, 1987). Biofilms have been implicated in a number of problems, including many infections, industrial fouling, and corrosion (Costerton *et al.*, 1987; McLean *et al.*, 1996).

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Abbreviation: TEM, transmission electron microscopy.

The gene array data discussed in this paper have been deposited in Gene Expression Omnibus (GEO) under accession number GSE18362.

Several detailed studies have been conducted on biofilms to ascertain the differences between planktonic and biofilm bacteria. In Gram-negative bacteria, these studies include large-scale mutant screens using microtitre (O'Toole & Kolter, 1998b; Prigent-Combaret *et al.*, 1999) and competition culture assays (Junker *et al.*, 2006), promoter identification using *in vivo* expression technology (Finelli *et al.*, 2003), proteomic analyses (Sauer *et al.*, 2002), and transcription profiling (Domka *et al.*, 2007; Hancock & Klemm, 2007; Junker *et al.*, 2007; Ren *et al.*, 2004; Schembri *et al.*, 2003; Whiteley *et al.*, 2001a). The use of these techniques has allowed the identification of genes important for biofilms, such as those associated with cell signalling involving acylated homoserine lactones (Herzberg *et al.*, 2006; Lee *et al.*, 2007a), osmotic stress and reduced oxygen (Prigent-Combaret *et al.*, 1999), and the global regulators *rpoS* (Adams & McLean, 1999), *relA* and *spoT* (Balzer & McLean, 2002).

Many of the genes expressed at higher levels in biofilms than in planktonic cells encode proteins of unknown function. A number of these are now being associated with various biofilm phenotypes. In *Pseudomonas aeruginosa*, *ndvB* is responsible for the tobramycin resistance of biofilms (Mah *et al.*, 2003). In *Escherichia coli*, *ariR* (*ymgB*) is needed for biofilm formation and for full expression of acid resistance genes in biofilms (Lee *et al.*, 2007b). The *E. coli bhsA* (*ycfR*) gene is needed for stimulation of biofilm formation by stresses such as hydrogen peroxide, low pH and heat stress (Zhang *et al.*, 2007). In the present study, we observed the uncharacterized gene *yjfO* (b4189) to be upregulated. This same gene has been found to be upregulated in at least two previous transcriptional profiling studies (Beloin *et al.*, 2004; Junker *et al.*, 2007). Here, we show that *yjfO* mutants are altered in biofilm structure and cell motility, and in their ability as biofilms to respond to pH and oxidative stresses.

## METHODS

**Bacterial strains and media.** The strains and plasmids used in this study are listed in Table 1. The insertion mutation in strain B4189 was confirmed upon receipt by PCR. Cultures were maintained on Luria–Bertani (LB) agar (MG1655), LB supplemented with 50 μg kanamycin $ml^{-1}$ (mutant strain), or LB supplemented with 50 μg kanamycin $ml^{-1}$ and 100 μg ampicillin $ml^{-1}$ (complemented strain). For long-term preservation, overnight cultures were frozen at −80 °C using glycerol [final concentration 12.5 % (v/v)] as a cryoprotectant. Prior to experimentation, cultures were revived from frozen stock, cultured overnight on LB agar, and then transferred to MOPS serine medium (Neidhardt *et al.*, 1974). This minimal medium, used in other biofilm studies (Junker *et al.*, 2007; Schembri *et al.*, 2003), contained serine (1 mg $ml^{-1}$) as the carbon source. Additional amino acids (Ile, Arg, Gly, His, Leu, Met, Phe, Val and Thr, each at 40 μg $ml^{-1}$) were also present. All cultures were grown at 37 °C, using laboratory facilities at Texas State University.

**Chemostat culture.** The biofilm culture chemostat apparatus has been previously described (Whiteley *et al.*, 1997). Briefly, it consists of a chemostat from which the culture can be circulated through a biofilm culture device (in this case 4 m Tygon laboratory tubing or a flow cell). For culturing, the chemostat was filled with MOPS serine medium, as described above (Neidhardt *et al.*, 1974), inoculated with 1 ml of an overnight culture of *E. coli* and grown at 37 °C as a batch culture for 24 h, after which continuous culture was initiated with a peristaltic pump at a dilution rate of 0.025 $h^{-1}$. The culture was allowed to equilibrate for one full generation (40 h). At this point, biofilm growth was initiated as a second pump continuously circulated the chemostat culture through the 4 m length of attached tubing (100 ml $h^{-1}$) for 96 h. Bacteria attached to the tubing were considered to be the biofilm culture, whereas unattached cells represented the planktonic culture.

**Cell harvesting.** For planktonic cell harvesting, 200 ml culture was mixed with an equal volume of ice-cold (−20 °C) stop solution [5 % (v/v) water-saturated phenol in ethanol] to stop endogenous nuclease activity, and then placed into nuclease-free 50 ml centrifuge tubes (Falcon) (Arnold *et al.*, 2001). Cells were harvested by centrifugation (3200 ***g***, 4 °C for 20 min), frozen (−80 °C), and then transported in liquid nitrogen to Texas A&M University for RNA extraction and analysis.

For biofilm cell harvesting, the biofilm-colonized tubing was removed from the chemostat, drained to remove planktonic cells, and then filled with ice-cold stop solution. Following this, the tubing was cut into 2 cm sections, each section was cut in half and placed into a sterile Petri plate, and biofilm cultures were scraped from the tubing with a sterile scalpel into 200 ml ice-cold stop solution. The scraped tubing sections were then placed into 200 ml ice-cold stop solution and sonicated in a bath sonicator (Sonicor Instrument Corporation) at 60 Hz for 10 min to further dislodge biofilm cells. Biofilm cells were then harvested from the stop solution by centrifugation, frozen, and transported to Texas A&M University for RNA extraction and analysis as described above.

**Biofilm growth measurements.** In order to measure biofilm and planktonic cell growth, duplicate chemostat cultures were established for each strain as described above. Following growth, the culture was analysed by dilution plating to enumerate the planktonic populations. For biofilm growth, the biofilm-colonized tubing was removed and rinsed with sterile medium, to remove loosely attached cells. The tubing was cut into five 2 cm sections, placed into sterile PBS, sonicated at 60 Hz for 5 min, and vortexed for 2 min to dislodge biofilm cells (McLean *et al.*, 1999). Cell numbers were then measured by dilution plating onto LB agar.

**RNA processing and gene array analysis.** RNA extraction and purification were conducted at Texas A&M University using a previously described hot phenol extraction protocol (Arnold *et al.*, 2001) and enzymic purification. [$^{33}$P]CTP (New England Biochemical) was used to label cDNA during reverse transcription (Arnold *et al.*, 2001). Gene array analysis was conducted using the Sigma Genosys macroarray protocol previously described by Arnold *et al.* (2001). For differential expression patterns to be considered significant, a minimum twofold change in expression level (compared with the background) during both the original and replicate run was needed.

**Real-time (quantitative) PCR.** Transcriptional profiling results for *yjfO* were validated using real-time PCR (Ju *et al.*, 2007). RNA was purified as described above, and reverse-transcribed to generate cDNA using a high-capacity cDNA archive kit (Applied Biosystems) using the manufacturer's protocol. The primers used for quantitative PCR were: TM-B4189-204F (5′-ACC GCC AGT AAC GGA CCA T-3′) and TM-B4189-313R (5′-CTA ATG CGT CAT CCG GAG AAC-3′), and 5′-/5(6)-FAM/CCA TCG TGC TTA CGC TAC CTA TTC GCT GTA/36-TAMTph/-3′ (Integrated DNA Technologies) was used as

**Table 1.** *E. coli* strains and plasmids used in this study

| Strain | Characteristics | Source and reference |
|---|---|---|
| MG1655 (DS291) | Wild-type (F$^{-}$ $\lambda^{-}$ *rph-1*) | M. Cashel, NIH (Hernandez & Cashel, 1995) |
| B4189 (FB22940) | MG1655 *yjfO*::Tn*5*(KAN-I-SceI) at position 167 in minus orientation | F. R. Blattner, University of Wisconsin (Kang *et al.*, 2004) |
| pMW201 | pGEM-T containing *yjfO* | This study |

a probe [5(6)-FAM=5′6-carboxyfluorescein; 36TAMpH=3′TAMRA]. No template and no reverse transcriptase controls were used. cDNA products were measured using an Applied Biosystems Prism 7500 real-time PCR system. Real-time analysis was conducted in duplicate.

**Epifluorescence microscopy.** To compare biofilm formation of the various strains, each strain was grown in a chemostat coupled to a three-chamber flow cell (Stovall Life Science). The chemostat was inoculated and equilibrated as described above. For biofilm growth, the culture was pumped through the flow cell at 100 ml $h^{-1}$. After 96 h biofilm growth, the flow cell was removed from the chemostat and each chamber was washed with 5 ml sterile water to remove planktonic cells. Cells were stained with 20 μM Syto 9 (Invitrogen) for 30 min, and then rinsed with 5 ml sterile water. Biofilms were viewed using a Nikon Eclipse 80 I microscope. Images were obtained using a Nikon DXM 1200F digital camera and images were analysed using Image-Pro Plus (version 5.1) (Mirza *et al.*, 2007). Adobe Photoshop CS3 (version 10.0.1) was used to convert the images to greyscale, invert the image (to make bacteria appear dark against a light background), and optimize contrast. Each flow cell experiment was carried out in triplicate.

**Transmission electron microscopy (TEM).** In order to observe whether the presence or absence of *yjfO* affected cell surface stability or flagella production, cells were examined by negative-stain preparations with TEM. Overnight cultures were grown on agar plates and then a loop-full of culture was suspended in a water droplet on Parafilm. The cell suspension was then mixed with 1 % (w/v) uranyl acetate, placed on a Formvar-coated grid, and examined by TEM (Merchant *et al.*, 2007). TEM films were scanned (2700 dots per inch) using a photographic high-resolution scanner, and the images were inverted and optimized for contrast using Adobe Photoshop CS3 as described above.

**Twitching and flagellar motility assays.** In order to elicit differences in twitching motility between the strains, the methods outlined by Semmler *et al.* (1999) were employed. Each strain was stabbed into the centre of an LB agar plate containing 1 % (w/v) agar and incubated at 37 °C in an inverted position. To examine flagella-based motility, cultures were stab-inoculated into the centre of an LB soft agar (0.3 %, w/v, agar) plate (O'Toole & Kolter, 1998a) and the plates incubated in an upright position at 37 °C. Colony diameter due to twitching motility and flagella motility was measured after 16 h incubation.

**Hydrophobicity assay.** Strains were tested for their ability to partition into hexane from an aqueous suspension as described by Zhang *et al.* (2007).

**Biofilm stress assays.** We evaluated the bacterial responses to pH, hydrogen peroxide and elevated temperature stresses using the experimental strategy of Zhang *et al.* (2007). For this purpose, each strain was again cultured in a chemostat, coupled to laboratory tubing as described above. In order to evaluate biofilm stress responses, the biofilm-colonized tubing was removed and cut into 2 cm pieces. Sensitivity to acidic and alkaline conditions was assessed by placing five pieces of tubing into pH 2.5 or pH 12 MOPS serine medium, which was subsequently incubated at 37 °C for 20 min. To determine viability following exposure to hydrogen peroxide, five pieces of tubing were incubated for 5 min at 37 °C in 20 mM $H_2O_2$. To assess viability following exposure to heat, five pieces of tubing were placed into MOPS serine medium and incubated for 10 min at 65 °C. Following incubation, the tubing was placed into PBS, which was subsequently sonicated at 60 Hz for 5 min and vortexed for 2 min. Each sample was serially diluted and plated on LB. A total of three biological replicates were performed for each stress measurement.

**Planktonic stress assays.** In order to determine strain viability following exposure to numerous environmental stressors, the methods outlined by Zhang *et al.* (2007) were employed. All strains were incubated in MOPS serine medium at 37 °C with shaking at 100 r.p.m. to $OD_{600}$ 0.3. To assess viability at altered pH, 2 ml culture was incubated for an additional hour at 37 °C without shaking in pH 2.5 or pH 12 MOPS serine medium. To determine culture viability following exposure to hydrogen peroxide, 1 ml culture was incubated with 20 mM $H_2O_2$ at 37 °C without shaking for 15 min. For heat sensitivity, 5 ml of each strain was removed and heated for 20 min at 65 °C. Following each treatment, cultures were serially diluted and plated on LB, and incubated at 37 °C for 24 h. A minimum of three biological replicates were performed.

**Data analysis.** On the basis of dilution plating, the planktonic and biofilm cell concentrations were calculated as c.f.u. $ml^{-1}$ and c.f.u. $cm^{-2}$, respectively. For statistical analyses, the data were log-transformed (Whiteley *et al.*, 2001b) and the data analysed by one-way ANOVA, with a minimum threshold of significance of $P<0.05$. Where applicable, all pair-wise comparisons were analysed by the Holm–Sidak method using SigmaStat v3.0 (Systat Software). Several measurements gave no detectable survival and in these cases, the c.f.u. data for that particular sample were assigned a value of 1 c.f.u. (the $\log_{10}$ of an undetectable sample was therefore zero). A minimum of three biological replicates was performed for each measurement. SigmaPlot v8.0 (Systat) was used to plot the results.

**Complementation of *E. coli* mutant.** The *yjfO* gene was amplified from MG1655 using the primers *yjfO*-F1 (5′-GATGTGGG-TTACGCTTTCGT-3′), *yjfO*-R1 (5′-CCACTGTCCTGTCACGATG-3′) using AmpliTaq Gold (Applied Biosystems). Purified *yjfO* gene products were cloned into the pGEM-T Easy Vector (Promega) to generate pMW201, where *yjfO* could be transcribed from the *lac* promoter through induction with 0.1 mM IPTG. The resulting plasmid was subsequently electroporated into the corresponding mutant strain. Verification of the inserted gene was accomplished through restriction digestion using *Eco*RI and plasmid sequencing. IPTG was present in the MOPS serine medium during chemostat experiments with the complemented strain.

## RESULTS

### Gene array

In order to investigate differences in biofilm and planktonic culture gene expression, we conducted transcriptional profiling on 96 h biofilms of *E. coli*. We found that 43 genes were differentially expressed between biofilm and planktonic culture. In the literature, the number of genes that are differentially expressed within biofilms in comparison with planktonic cultures ranges from a low of approximately 0.5 % of the genome (Whiteley *et al.*, 2001a) to a high of almost 20 % (Hancock & Klemm, 2007; Ren *et al.*, 2004). The values obtained in the present study (43 genes) (GenBank accession number GSE18362) represent approximately 1 % of the genome (Blattner *et al.*, 1997) and are certainly consistent with other reports. As noted elsewhere (Beloin *et al.*, 2004; Ren *et al.*, 2004; Schembri *et al.*, 2003), many of the differentially regulated genes were uncharacterized. Several initially uncharacterized genes, identified in earlier transcriptional profiling investigations, have since been shown to be important in biofilm functions (Domka *et al.*, 2007; Zhang *et al.*, 2007). Under our experimental conditions, expression of *yjfO* (b4189) was $3.3 \pm 0.3$-fold higher in biofilms than in planktonic culture, as measured by transcriptional profiling. Upregulation of *yjfO* in biofilms was confirmed by quantitative PCR [$\Delta$ threshold cycle ($C_t$) $5.0 \pm 0.5$]. Two previous studies have also shown *yjfO* to be upregulated in biofilms (Beloin *et al.*, 2004; Junker *et al.*, 2007).

## Biofilm formation

To investigate the role of *yjfO* in biofilm formation, we circulated a steady-state *E. coli* culture through a three-chambered flow cell for 96 h. The flow cell was removed after 96 h, stained with 20 μM Syto 9 and viewed using a Nikon Eclipse 80 I microscope at $\times 100$ magnification. Clumping and microcolony formation were observed in the wild-type (MG1655) biofilms (Fig. 1a). This phenotype was absent in the *yjfO* mutant (Fig. 1b), but could be restored upon genetic complementation (Fig. 1c). In order to measure adherent cell populations, we grew each of the strains in a chemostat coupled to 4 m Tygon laboratory tubing for biofilm culture (Whiteley *et al.*, 1997). Following 96 h of biofilm growth, the tubing was removed and assayed for colonization by dilution plating as described above. No statistically significant difference was noted in planktonic or adherent cell populations among any of the strains (Fig. 2). Based on these observations we concluded that *yjfO* expression is important in microcolony formation and biofilm maturation processes, but not necessary for planktonic growth or initial surface adhesion (Beloin *et al.*, 2004; Sauer *et al.*, 2002).

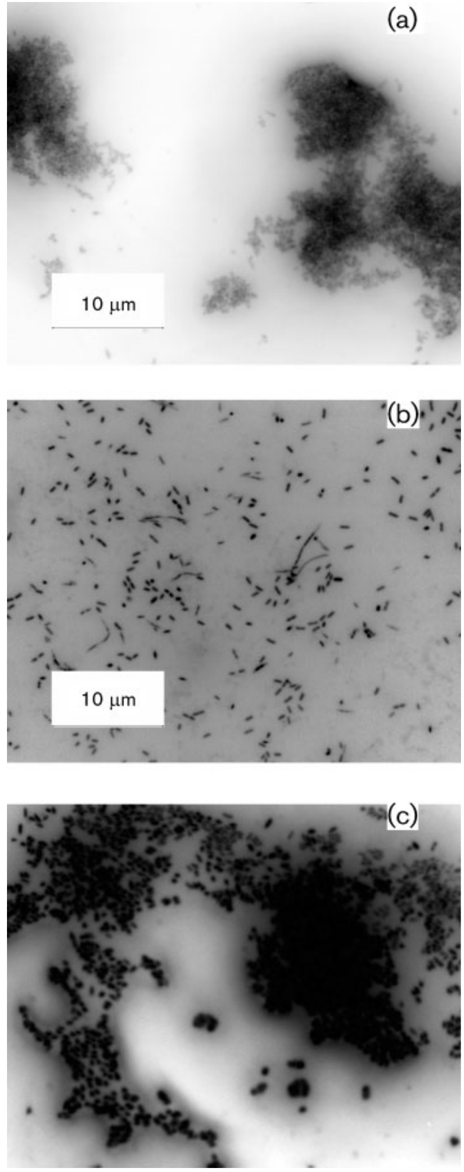


**Fig. 1.** Representative epifluorescence microscopy images of 96 h biofilms from chemostat cultures grown in serine-limited MOPS medium: MG1655 (a), *yjfO* (b4189) (b) and complemented *yjfO* (c). The magnification is the same in all figures.

## Motility and ultrastructure

As twitching motility has been shown to be important for microcolony formation (O'Toole & Kolter, 1998a), we investigated whether the phenotype of the *yjfO* deletion could be explained by loss of this characteristic. Although there were slight differences in colony expansion (Fig. 3a), these differences were not significant when analysed by one-way ANOVA ($P=0.63$), indicating that *yjfO* had no effect on twitching motility. In contrast, flagella-based motility was enhanced in the *yjfO* mutant and the complemented but not induced strain ($P<0.001$ compared with the wild type), but could be restored to wild-type levels when the *yjfO*-complemented strains were induced by IPTG (Fig. 3b). Using TEM, we examined negatively stained preparations of the various strains for the presence of flagella and found an indication of enhanced flagella in some of the *yjfO* mutants (Fig. 4). In future studies, we will explore the mechanism(s) for this *yjfO*-enhanced flagella-based motility.

Based on its sequence, the YjfO protein is predicted to be a lipoprotein (Rudd *et al.*, 1998), and as such has the potential to be associated with cell membranes. Cell preparation during negative-stain TEM involves suspending the bacteria in a heavy metal solution (to provide electron contrast), and then exposing the stained, unfixed cells to high vacuum during TEM examination. Other EM preparation approaches, such as conventional embedding or freeze substitution (Graham & Beveridge, 1990), employ chemical fixation and gradual dehydration, processes which stabilize and protect membranes and other structures from the high vacuum and electron beam in TEM. As fixation and gentle dehydration protocols are not used during routine negative-stain TEM preparation, one would anticipate that membranes lacking a key structural component would be much more prone to vacuum-

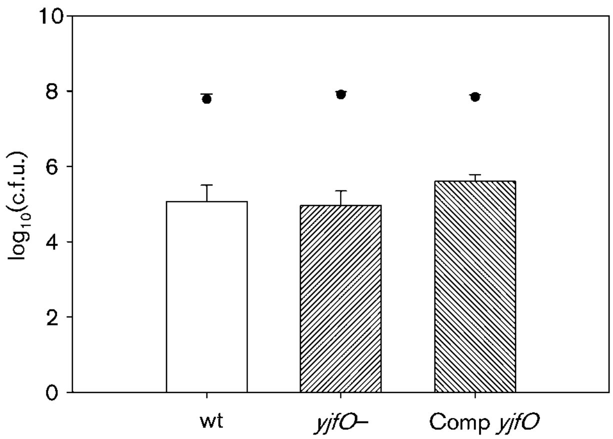


**Fig. 2.** Planktonic (scatter plot) and biofilm growth (bar graph) of MG1655, *yjfO* (b4189) (*yjfO*–) and complemented *yjfO* (Comp *yjfO*) after 96 h chemostat growth. Values in all figures are expressed as $\log_{10}$(c.f.u. ml$^{-1}$) for planktonic cultures and $\log_{10}$(c.f.u. cm$^{-2}$) for biofilm cultures (±SEM). No significant differences were seen among the planktonic populations or biofilm populations of the three strains.

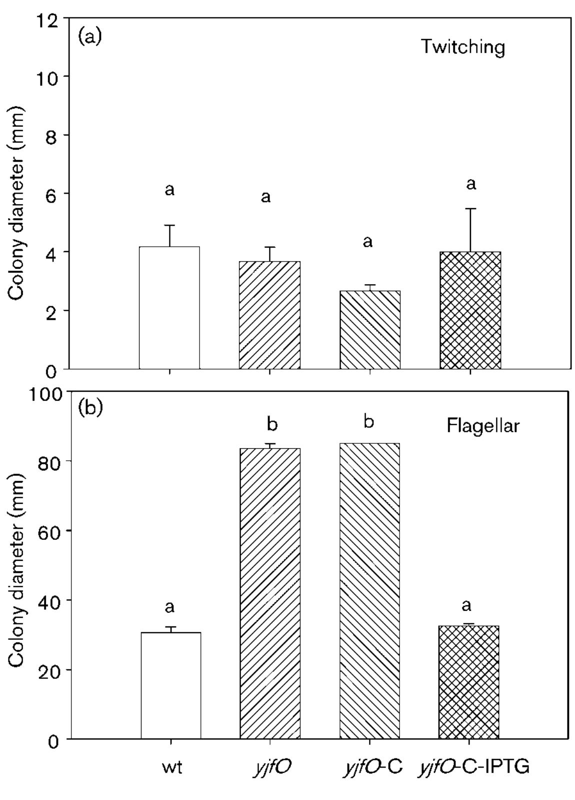


**Fig. 3.** Twitching (a) and flagella-based (b) motility in MG1655 (wt), *yjfO* mutant (*yjfO*) and *yjfO*-complemented strains in the absence (*yjfO*-C) and presence (*yjfO*-C-IPTG) of IPTG induction. Values with the same letter, in all figures, are not significantly different ($P<0.05$).

induced damage. However, we did not observe any major differences in the cell envelope membranes, regardless of the presence (Fig. 4a, c) or absence of *yjfO* (Fig. 4b). However, we cannot rule out subtle, membrane-associated changes due to *yjfO* solely on the basis of negative-stain TEM examination.

## Cell surface hydrophobicity

In order to assess the effect of *yjfO* on cell surface hydrophobicity, aqueous suspensions of the *E. coli* strains were mixed with an equal volume of hexane, and the fraction of cells remaining in the aqueous layer was measured as described elsewhere (Zhang *et al.*, 2007). Following this procedure, the percentage of cells (±SE) remaining in the aqueous phase was: wild-type 86.5 (0.5), *yjfO* 81.5 (0.5), complemented *yjfO* without IPTG 82.5 (0.5), and complemented and IPTG-induced *yjfO* 92 (3). Based on these results, *yjfO* appears to have had a limited effect on cell surface hydrophobicity in these experiments. However, recent work by Q. Ma and T. K. Wood (unpublished results, personal communication) has shown that *yjfO* overexpression in another *E. coli* strain results in greatly increased hydrophobicity. As a result, we cannot completely rule out a contribution of *yjfO* to cell surface hydrophobicity.

## Planktonic and biofilm stress assays

In order to determine the viability of each strain following exposure to commonly encountered environmental stresses, chemostat-grown biofilm populations were exposed to acid, base, hydrogen peroxide (oxidative stress) or heat stress. As shown in Fig. 2, no statistically significant differences in overall cell numbers were noted between the strains in the absence of stress, thus allowing for standardization and efficient determination of biofilm cell viability following exposure to each stressor.

Exposure of 96 h biofilms to pH 2.5 MOPS serine medium (acid stress) resulted in a decrease in overall viability for all strains assayed. In the planktonic populations (Fig. 5a), the acid-treated wild-type cells were reduced in number in comparison with the untreated control; however, the difference was only marginally significant ($P=0.099$). The other two populations (*yjfO* mutant and complemented strain) were significantly reduced in comparison with the untreated control, with the *yjfO* strain showing the biggest reduction and the complemented strain showing a sensitivity intermediate between those of the wild-type and the *yjfO* mutant. However, the acid-treated planktonic strains did not differ significantly with respect to each other. In contrast to the planktonic results, the viability patterns in the biofilm cultures following acid stress were quite different (Fig. 6a), with all populations differing significantly from each other. In comparison with the acid-treated wild-type biofilms, *yjfO* mutant viability was reduced approximately 200-fold. The viability of the

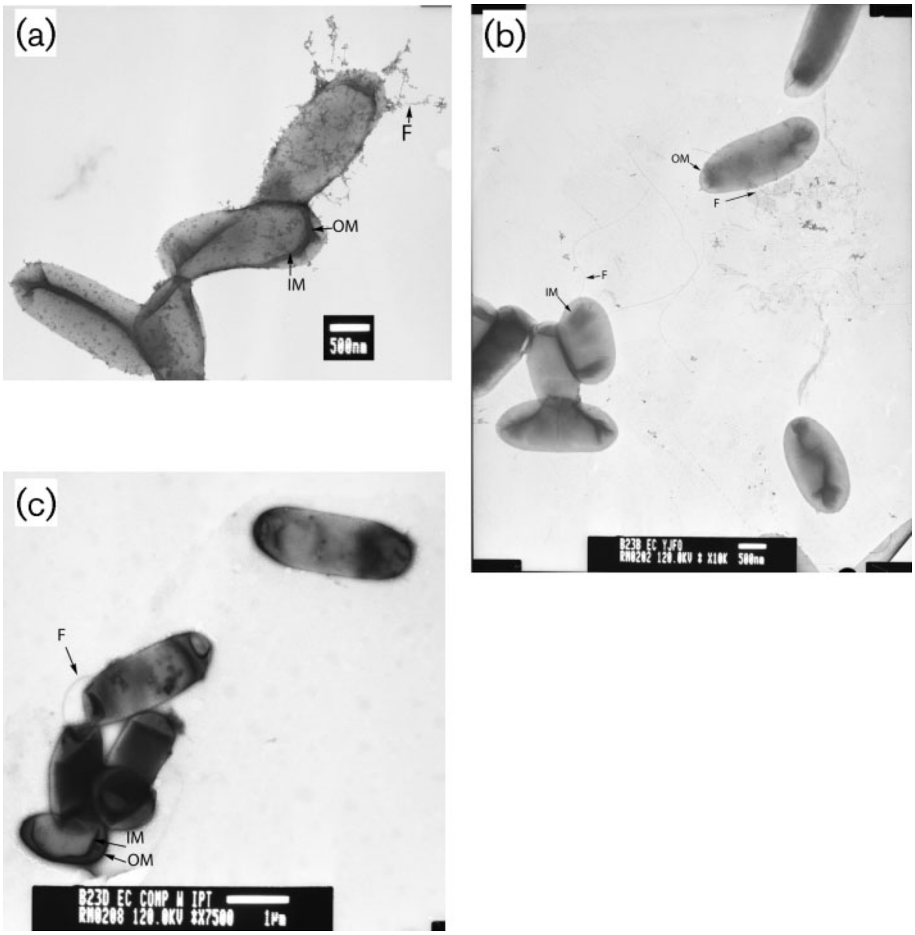


**Fig. 4.** TEM micrographs of negative-stain preparations of *E. coli* MG1655 (a), *yjfO* (b), and *yjfO*-complemented and IPTG-induced strains (c). Flagella (F), outer (OM) and inner (IM) membranes are indicated. Bars: 0.5 μm (a, b), 1.0 μm (c).

complemented strain was reduced even further (approximately sixfold in comparison with the mutant). The failure of pMW201 to complement the acid-sensitive phenotype of the mutant suggests that the *yjfO* insertion mutation has polar effects on expression of the adjacent downstream gene *yjfN*. Given that the complemented strain survives less well than the mutant, it is also possible that high levels of YjfO increase sensitivity to low pH.

Exposure of 96 h biofilms to pH 12 MOPS serine medium (base stress) resulted in decreased viability of *E. coli* biofilms (Fig. 6b). Here, all three strains (wild-type, mutant and complemented strain) showed a statistically similar reduction in viability. In contrast, there was no significant stress-induced change in viability in planktonic cultures (Fig. 5b). The increased susceptibility of biofilm populations to an environmental stress (in this case, alkaline pH), in comparison with planktonic populations, is highly unusual in that the converse is generally the case (Costerton *et al.*, 1987; Davey & O'Toole, 2000). We will investigate this issue in future studies.

Exposure of planktonic and biofilm populations to oxidative stress (20 mM $H_2O_2$) resulted in a statistically significant decrease in viability only in the *yjfO* mutant biofilm, which was complemented by the reintroduction of the *yjfO* gene (Fig. 6c). In planktonic cultures, there was a modest, and statistically insignificant, reduction in the viability of the *yjfO* mutant (Fig. 5c). We interpret these results as demonstrating that *yjfO* is involved in the protection of biofilms against oxidative stress.

In contrast to the above-mentioned stresses, we observed no significant differences in planktonic (Fig. 5d) or biofilm (Fig. 6d) culture viability following exposure to heat (65 °C).

## DISCUSSION

*E. coli* is routinely found in soil, water and intestinal mucus, all of which impose unique stresses on the organism in both biofilm and planktonic modes of growth (Fabich *et al.*, 2008). In intestinal mucus, *E. coli* encounters

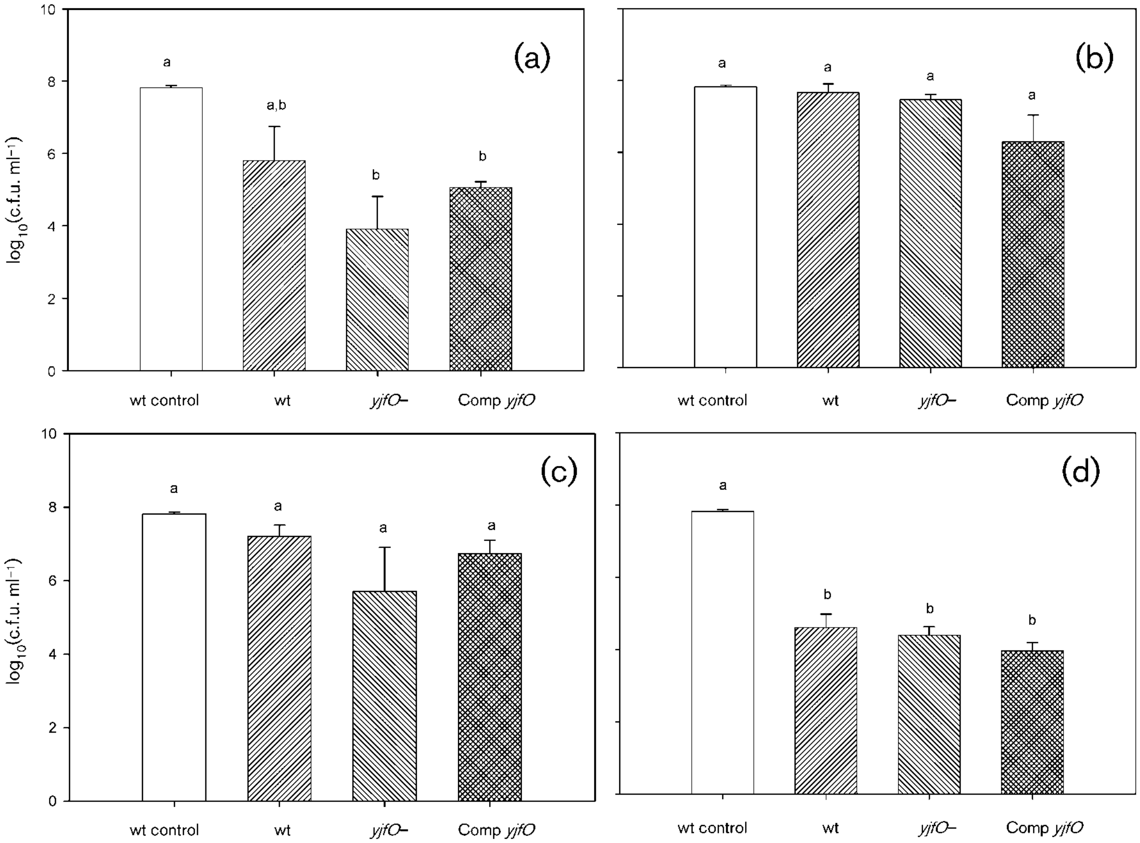


**Fig. 5.** Survival of *E. coli* planktonic cells following exposure to acid (a), base (b), oxidative stress (c) and heat stress (d). Strains in this figure are designated untreated wild-type control (wt control), treated MG1655 (wt), *yjfO* (b4189) (*yjfO*−), and complemented and induced *yjfO* (Comp *yjfO*). The value for the untreated control corresponds to the average of the wild-type planktonic cells without exposure to any stressor and is included for viability comparisons.

acetate and other volatile fatty acids, produced as metabolic by-products of the resident flora (Arnold *et al.*, 2001). These organic acids are capable of traversing the membrane into the bacterial cell, thus inducing acid stress (Arnold *et al.*, 2001). Typically, these weak acids decrease bacterial viability, yet strains of *E. coli*, including MG1655 (Arnold *et al.*, 2001), are capable of combating them. Acid resistance is one factor that allows *E. coli* to efficiently colonize and inhabit the intestinal tract. In this same largely anaerobic environment, *E. coli* would be exposed to transient concentrations of reactive oxygen species from mucosal innate defences (McLean *et al.*, 1988). In contrast, *E. coli* does not experience alkaline conditions (above pH 8) in its normal environment, and so the low resistance to alkaline stress is anticipated. The surprising observation in the current study was the increased resistance of planktonic populations (Fig. 5b) to alkali stress when compared with biofilm populations (Fig. 6b). Also, heat susceptibility was unaffected by biofilm (Fig. 6d) or planktonic growth (Fig. 5d). Although biofilm growth is normally associated with stress resistance (Costerton *et al.*, 1987; Davey & O'Toole, 2000), our current study certainly shows exceptions to this phenomenon.

In this study, we focused on *yjfO*, a member of the *yhcN* family. When first described (Rudd *et al.*, 1998), the *yhcN* family consisted of nine parahomologous, uncharacterized genes (*yjfO*, *yahO*, *ybiJ*, *ybiM*, *ycfR*, *ydgH*, *yhcN*, *yjfN* and *yjfY*) of unknown function. Members of this family are predicted to have evolved from a common ancestor, based on the presence of a signal peptide and a shared motif in their N and C termini (Rudd *et al.*, 1998). One of these genes, *ycfR*, has been shown to be upregulated in biofilm cultures and to be associated with biofilm stress responses (Zhang *et al.*, 2007). On that basis, Zhang *et al.* (2007) have proposed that this gene be renamed to *bhsA* for influencing biofilms through hydrophobicity and stress response. In the present study, *yjfO* does contribute to the biofilm stress response but unlike *ycfR* does not appear to contribute to cell surface hydrophobicity.

Cell aggregation and microcolony formation of surface-adherent bacteria via twitching motility and other

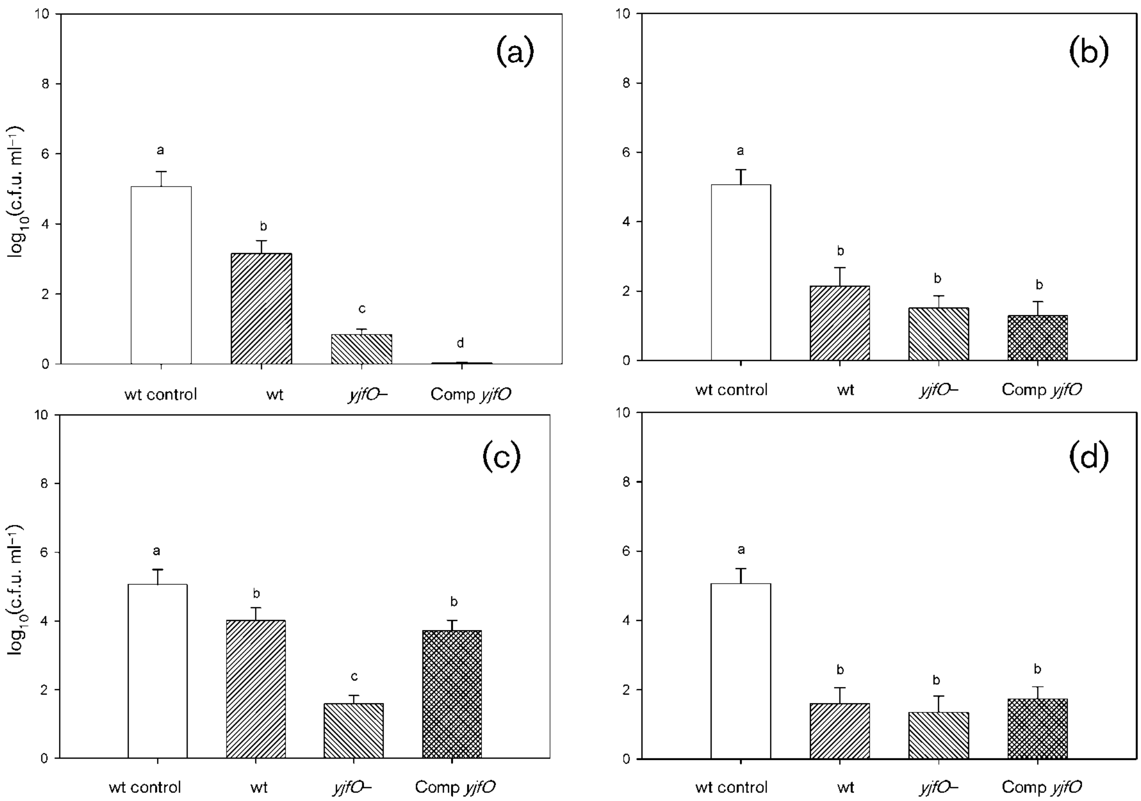


**Fig. 6.** Survival of *E. coli* biofilm cells following exposure to acid stress (a), base stress (b), oxidative stress (c) and heat stress (d). For strain designations, see legend to Fig. 5. The value for the untreated control corresponds to the average of the wild-type biofilm cells without exposure to any stressor and is included for viability comparisons.

processes are hallmarks of early biofilm maturation (O'Toole & Kolter, 1998a; Sauer *et al.*, 2002). In the current study (Fig. 1), we noted that wild-type *E. coli* formed microcolonies within flow cells. This feature was absent in the *yjfO* mutant biofilm, although it was restored upon genetic complementation. However, adherent cell concentrations were similar (Fig. 2). Aside from twitching and flagella motility (O'Toole & Kolter, 1998a), other characteristics involved in cell aggregation include cell–cell adhesion, cell signalling (Domka *et al.*, 2006) and hydrophobic interactions (McEldowney & Fletcher, 1986). The most striking feature of *yjfO* deletion was the loss of microcolony formation (Fig. 1) and greatly enhanced flagella motility (Fig. 3). Other investigators have shown the importance of microcolonies, water channels and other biofilm structures in the resistance of the component organisms to various stresses (Matz *et al.*, 2004; Pamp & Tolker-Nielsen, 2007). Although flagella motility has been shown to be important in biofilm structure development (Wood *et al.*, 2006), our present study suggests that unchecked flagella motility disrupts microcolonies, a feature that certainly provides a plausible explanation for the greatly reduced biofilm stress response. Due to its contribution to biofilm stress response as well as its contribution to flagella motility, we propose renaming *yjfO* as *bsmA* (biofilm stress and motility).

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