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OmpA influences *Escherichia coli* biofilm formation by repressing cellulose production through the CpxRA two-component system

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Summary

Previously we discovered that OmpA of *Escherichia coli* increases biofilm formation on polystyrene surfaces (González Barrios *et al*., *Biotechnol Bioeng*, 93:188–200, 2006a). Here we show OmpA influences biofilm formation differently on hydrophobic and hydrophilic surfaces since it represses cellulose production which is hydrophilic. OmpA increased biofilm formation on polystyrene, polypropylene, and polyvinyl surfaces while it decreased biofilm formation on glass surfaces. Sand column assays corroborated that OmpA decreases attachment to hydrophilic surfaces. The *ompA* mutant formed sticky colonies, and the extracellular polysaccharide that caused stickiness was identified as cellulose. A whole-transcriptome study revealed that OmpA induces the CpxRA two-component signal transduction pathway that responds to membrane stress. CpxA phosphorylates CpxR and results in reduced *csgD* expression. Reduced CsgD production represses *adrA* expression and results in reduced cellulose production since CsgD and AdrA are responsible for 3,5-cyclic diguanylic acid and cellulose synthesis. Real-time polymerase chain reaction confirmed *csgD* and *adrA* are repressed by OmpA. Biofilm and cellulose assays with double deletion mutants *adrA ompA*, *csgB ompA*, and *cpxR ompA* confirmed OmpA decreased cellulose production and increased biofilm formation on polystyrene surfaces through CpxR and AdrA. Further evidence of the link between OmpA and the CpxRA system was that overproduction of OmpA disrupted the membrane and led to cell lysis. Therefore, OmpA inhibits cellulose production through the CpxRA stress response system, and this reduction in cellulose increases biofilm formation on hydrophobic surfaces.

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Introduction

Cell appendages promote biofilm formation for *Escherichia coli* in a complex manner. For example, flagella and type 1 pili are required for biofilm formation (Pratt and Kolter, 1998). In addition, the outer membrane protein Antigen 43 increases biofilm formation in a fimbriae-independent way and facilitates multispecies biofilm formation (Danese *et al*., 2000a). Curli are another outer surface appendage that increase biofilm formation (Vidal *et al*., 1998). In curli-producing strains, other outer surface appendages, such as colanic acid and flagella, no longer influence biofilm formation (Prigent-Combaret *et al*., 2000). Similarly, the conjugation pilus acts as an adhesion factor for cell–cell and cell–surface interactions which promotes biofilm formation, and this appendage overrides the importance of flagella, type 1 fimbriae, Antigen 43 and curli (Reisner *et al*., 2003). Furthermore, exopolysaccharides (EPS), as well as its components such as colanic acid and cellulose, also affect biofilm formation (Sutherland, 2001). Although colanic acid is critical for biofilm three-dimensional structure formation based on the research of Danese and colleagues (2000b), overproduction of colanic acid inhibits biofilm formation in *E. coli* BW25113 strains (Zhang *et al*., 2008). Cellulose synthesis is also required for *E. coli* 1094 biofilm formation on glass slides (Da Re and Ghigo, 2006). However, in a *csgD*-overexpressing strain of *E. coli* MG1655, cellulose production negatively affects curli-mediated surface adhesion and aggregation on hydrophobic polypropylene (PP) microtitre plates (Gualdi *et al*., 2008).

OmpA is a major protein of the outer membrane in *E. coli* K-12 strains (Chai and Foulds, 1977) with ~100 000 copies per cell (Smith *et al*., 2007). It is a representative protein for outer membrane protein assembly and structure (Kleinschmidt, 2003), is a receptor for bacteriophage including K3, Ox2 and M1, acts as an immune target and is involved in adhesion (Smith *et al*., 2007). Due to its abundance in the bacterial outer

membrane, and its interesting structure with four short loops that protrude from the cell (Smith *et al*., 2007), we hypothesized that it may play a role in bacterial biofilm formation. Previously, we discovered that OmpA promotes biofilm formation in *E. coli* K-12 on polystyrene (PS) surfaces (González Barrios *et al*., 2006a), and Orme *et al*. found that OmpA is overproduced during biofilm formation using both laboratory and clinical strains of *E. coli* (Orme *et al*., 2006). OmpA binds to silicon nitride surfaces (Lower *et al*., 2005), and we found OmpA participates in the regulation pathway through which conjugative plasmids increase biofilm formation (Yang *et al*., 2008). In *Acinetobacter baumannii*, OmpA plays a role in biofilm formation on plastic as well as in the bacterial attachment to biotic surfaces such as *Candida albicans* filaments and A549 human alveolar epithelial cells (Gaddy *et al*., 2009). At the genetic level, OmpA is negatively regulated by the σ^E-dependent small RNA MicA (Johansen *et al*., 2008).

The Cpx stress response system is a two-component signal transduction system that includes a sensor kinase CpxA and a response regulator CpxR (DiGiuseppe and Silhavy, 2003). CpxA kinase autophosphorylates at His-248 using ATP after sensing envelope stress, then transfers this phosphate to a conserved Asp-51 of CpxR to form phosphorylated CpxR (CpxR–P) (Siryaporn and Goulian, 2008). The Cpx system has been linked to biofilm formation since the expression of Cpx-regulated genes is induced during initial adherence of *E. coli* to abiotic surfaces (Otto and Silhavy, 2002). Mutations in *cpxA* also reduce biofilm formation by affecting microbial adherence to solid surfaces (Dorel *et al*., 1999), and the mechanism for this biofilm reduction is inactivation of the Cpx pathway which results in induction of CsgD which promotes curli synthesis as well as cellulose production under low temperatures (Dorel *et al*., 1999; Prigent-Combaret *et al*., 2001; Gualdi *et al*., 2008).

The regulation of cellulose production by CsgD is complex. CsgD stimulates transcription of *adrA* (Zogaj *et al*., 2001), which encodes a putative transmembrane protein with a GGDEF domain (Römling *et al*., 2000). AdrA then activates cellulose production post-transcriptionally by interacting with the cellulose synthesis operons *bcsABZC* and *bcsEFG*, as well as by producing an activator of cellulose biosynthesis (Gerstel and Römling, 2003). In addition to the CsgD/AdrA pathway, there are alternative pathways for cellulose production in both *E. coli* (Da Re and Ghigo, 2006) and *Salmonella* sp. (García *et al*., 2004) that also involve 3,5-cyclic diguanylic acid (c-di-GMP). For example, *E. coli* 1094 uses YedQ for regulating cellulose production (Da Re and Ghigo, 2006), and in *Salmonella* sp., cellulose synthesis is independent of AdrA, but dependent on STM1987 (García *et al*., 2004). In the probiotic *E. coli* strain Nissle 1917, neither the CsgD/AdrA pathway nor YedQ is required for cellulose production, although cellulose production is still regulated by c-di-GMP (Monteiro *et al*., 2009).

We find here that OmpA influences biofilm formation in a surface-dependent manner (increases biofilm formation on plastic but decreases biofilm formation on glass). Hence, our goal was to explore the genetic basis of how OmpA influences biofilm formation in this divergent manner. Whole-transcriptome analyses were performed to identify pathways influenced by OmpA in biofilm formation, and we found the Cpx system is activated by OmpA. In addition, we found cellulose production increases upon deletion of *ompA*, which in turn leads to the surface-dependent biofilm formation due to the hydrophilic nature of this polymer.

Results

OmpA increases biofilm formation on hydrophobic surfaces and decreases biofilm formation/attachment on hydrophilic surfaces

We investigated whether deletion of *ompA* would affect biofilm formation on different surfaces (hydrophilic and hydrophobic surfaces) since this deletion decreased biofilm formation on hydrophobic PS surfaces (González Barrios *et al*., 2006a). Here, upon deleting *ompA*, a 10- to 20-fold reduction of biofilm formation was found on PS, polyvinyl chloride (PVC) and PP surfaces (Fig. 1A). This dramatic reduction in biofilm formation on hydrophobic surfaces was complemented by expressing *ompA* from pCA24N_*ompA* with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 1A) under conditions where these results were not influenced by cell density differences.

In contrast, under the same conditions, the *ompA* mutant made ~10-fold more biofilm than the wild-type strain on glass (hydrophilic) surfaces (Fig. 1B). We also tested the impact of deleting *ompA* via sand columns in order to study attachment to a hydrophilic surface (Fig. 2); deleting *ompA* increased the percentage of attached cells by as much as 10-fold at 21 min. As expected, the *fimA* mutant (negative control) had less attachment than the wild-type strain (Van Houdt and Michiels, 2005) (Fig. 2). Hence, OmpA reduces cell attachment to hydrophilic surfaces.

Furthermore, the deletion of *ompA* did not change cell growth; the specific growth rate for the *ompA* mutant was $1.65 \pm 0.04\ h^{-1}$ while that of the wild-type strain was $1.63 \pm 0.04\ h^{-1}$. The effect of adding IPTG was also tested, and 0.1 mM IPTG did not affect cell growth or biofilm formation for the *ompA* mutant. Hence, OmpA influences biofilm formation in a surface-dependent fashion that is not related to growth but instead is dependent on the hydrophobicity of the abiotic surface.

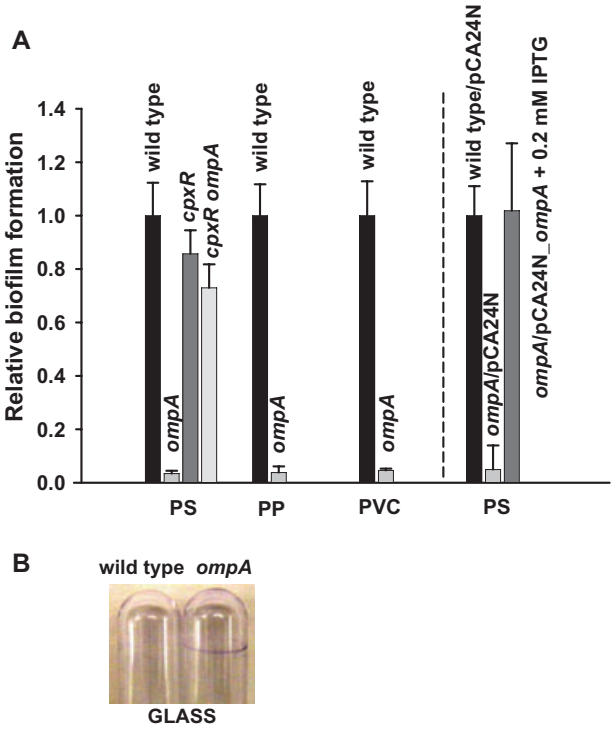

Fig. 1. Relative normalized biofilm formation (total biofilm/growth) in LB at 37°C after 24 h for the *ompA* mutant versus the BW25113 wild-type strain in 96-well plates constructed of polystyrene (PS), polyvinyl chloride (PVC) and polypropylene (PP), for the *cpxR* and *cpxR ompA* mutants in 96-well plates constructed of PS, and for the wild type/pCA24N, *ompA*/pCA24N and *ompA*/pCA24N_*ompA* strains with 0.2 mM IPTG in 96-well plates constructed of PS (A). Data are the average of 10 replicate wells from two independent cultures, and one standard deviation is shown. Biofilm formation for the BW25113 wild-type strain and the *ompA* mutant in LB at 37°C after 48 h in glass culture tubes (B).

Overproduction of OmpA leads to cell lysis

While deleting *ompA* did not affect cell growth, overproducing OmpA in shake flasks led to cell lysis (Fig. S1), which indicates OmpA expression increases envelope stress. With the same level of induction (0.1 mM IPTG), cells aggregated in overnight cultures of *ompA*/pCA24N_*ompA* while no cell clumping was seen in the *ompA*/pCA24N strain (vector control).

To corroborate the lysis seen upon visual inspection, cell lysis was quantified by measuring the genomic DNA released into the culture supernatants by BW25113 *ompA*/pCA24N and *ompA*/pCA24N_*ompA*. Upon overproducing OmpA using 0.1 mM IPTG for 12.5 h, 48 ± 5% cell lysis occurred with *ompA*/pCA24N_*ompA* versus 2.5 ± 0.9% cell lysis for the *ompA*/pCA24N strain. Hence, overproduction of OmpA leads to a 20-fold increase in cell lysis. These results were verified using an independent measure of cell lysis, via release of intracellular β-galactosidase activity. Since *E. coli* BW25113 does not have β-galactosidase activity due to mutated *lacZ*, we used *E. coli* MG1655/pCA24N and MG1655/pCA24N_*ompA*. Upon overproducing OmpA using 0.05 mM IPTG, β-galactosidase activity increased 250-fold in the supernatant compared with that of the MG1655/pCA24N strain and similar levels of cell lysis were found compared with the genomic DNA method (25 ± 9% cell lysis compared with 0.1 ± 0.1% cell lysis).

In addition, we also checked the influence of OmpA on other phenotypes. No differences between the wild-type strain and the *ompA* mutant were found for cell swimming motility, pH of planktonic cultures, EPS production and colanic acid formation.

OmpA induces cpxP *expression*

To further explore the role of OmpA in biofilm formation, a whole-transcriptome analysis of the *ompA* mutant versus the wild-type strain was performed using biofilm cells collected from a hydrophobic (PS) surface after 15 h of incubation (Table S1). *cpxP* was found to be the most repressed gene by deleting *ompA* (1.9-fold and 3.3-fold based on the two independent experiments). This result was corroborated by quantitative, reverse transcription-polymerase chain reaction (qRT-PCR) with biofilm cells from a third independent culture which showed *cpxP* was repressed by 2.7-fold. Hence, OmpA induces *cpxP* expression.

OmpA represses cellulose production

Colonies of the *ompA* mutant were found to be sticky compared with the wild-type strain, which indicated that the composition of the extracellular matrix may be changed by deleting *ompA*. Thus we tested colony morphology for the *ompA* mutant relative to the wild-type

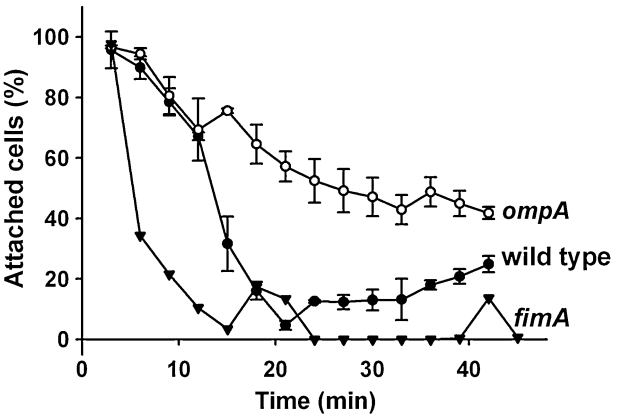

Fig. 2. Attachment to sand columns for the wild-type strain and the BW25113 *ompA* mutant at 37°C in LB medium. The BW25113 *fimA* mutant was used as fimbriae minus negative control. Data for the wild-type strain and the *ompA* mutant are the average of two independent cultures, and one standard deviation is shown.

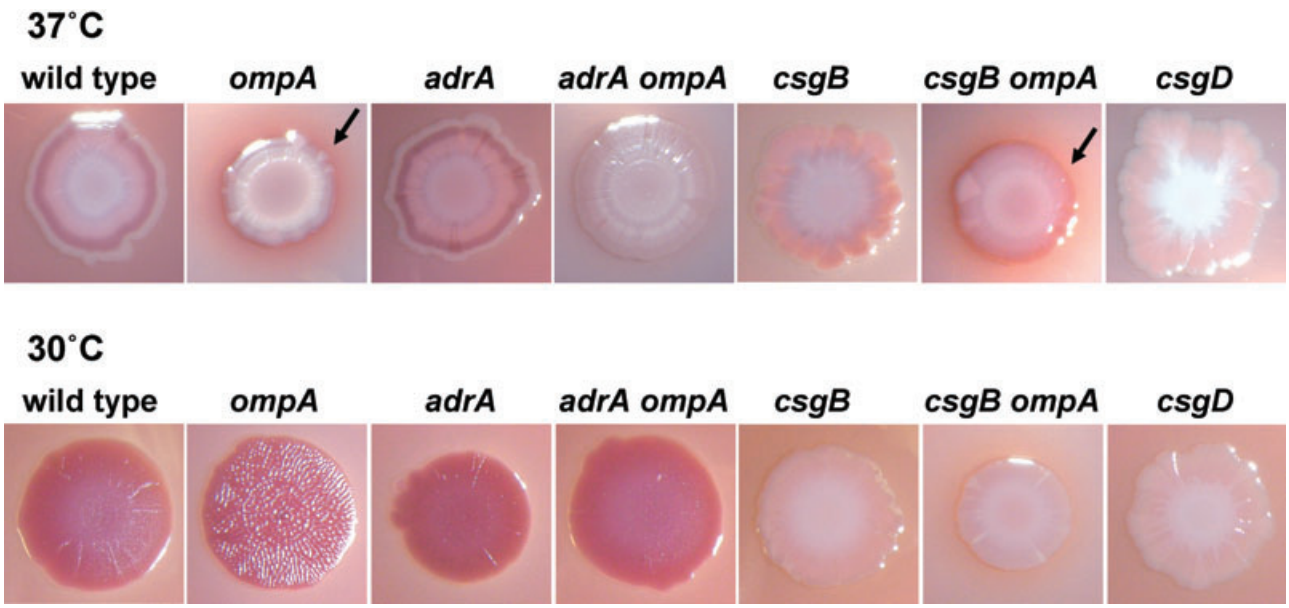

Fig. 3. Colony morphology of the BW25113 wild-type strain, the *ompA* mutant, the *adrA* mutant, the *adrA ompA* mutant, the *csgB* mutant, the *csgB ompA* mutant and the *csgD* mutant on Congo red plates at 37°C and 30°C. Black arrows point to the red zone of cellulose production around the *ompA* and *csgB ompA* mutants. Each panel is 2 cm by 2 cm in actual size on agar plates.

strain on Congo red plates at 37°C (Fig. 3) and found a red circle was formed around the *ompA* colony, which indicates deleting *ompA* may lead to the overproduction of some extracellular matrix that binds Congo red.

In *E. coli*, Congo red binds to both cellulose and curli (Da Re and Ghigo, 2006). To distinguish whether it is cellulose or curli that were overproduced in the *ompA* mutant, double mutants *adrA ompA* and *csgB ompA* (*csgB* encodes the curlin nucleator protein) were constructed by P1 transduction, and colony morphologies for these two strains were also tested with Congo red plates at 37°C. The *csgB ompA* mutant formed the same red EPS circle *outside* the colony as the *ompA* single mutant formed (Fig. 3), which indicates that without the curli gene *csgB*, the *ompA* mutation still increases Congo red binding to substances around its colony. Hence, the Congo red-binding substance formed upon deleting *ompA* was not curli. The *adrA ompA* mutant lacked the outer red EPS circle, which indicates deleting *adrA* decreased Congo red binding. Hence, deleting *ompA* increases primarily cellulose production.

At 30°C (Fig. 3), the *ompA* mutant had a red, rough surface, which was due to the overproduction of primarily curli at this low temperature (Gualdi *et al*., 2008) since the wild-type strain, *ompA* mutant, *adrA* mutant and *adrA ompA* mutant all had red colonies due to curli formation whereas the curli-deficient strains *csgB*, *csgB ompA* and *csgD* mutants had white colonies. Only the *ompA* mutant had a rough surface while the wild-type strain, the *adrA* mutant, and the *adrA ompA* mutant all had red, smooth surfaces which indicates increased cellulose production leads to a rough surface when curli are expressed at the same time.

Congo red-binding and calcofluor-binding assays were then performed for quantification of the cellulose production in planktonic cultures at 37°C, a temperature where curli are not generally formed (Fig. 4). Congo red is indicative of both curli formation and cellulose production while calcofluor is indicative primarily of cellulose production (Da Re and Ghigo, 2006). Both assays showed the consistent result that the *ompA* mutation increases cellulose production by six- to over sevenfold. Also from this assay we can see that cellulose production for the wild-type strain and the *adrA* mutant was similar, which means cellulose production in the wild-type strain is usually repressed under these conditions (Gualdi *et al*., 2008).

To confirm that the cellulose production in the *ompA* mutant was indeed much higher than in the wild-type strain, we detected cellulose on the exterior of the cells directly by measuring the glucose evolved upon its digestion with cellulase. Upon addition of cellulase, the glucose generated by digesting cellulose from the *ompA* mutant was 12 $\pm$ 4-fold higher than from the wild-type strain. The amount of cellulose produced by the wild-type strain in this assay was 0.011 $\pm$ 0.001 mg cellulose per mg protein, which is consistent with our calcofluor-binding result (0.02 $\pm$ 0.01 mg cellulose per mg protein).

To verify that the increase in cellulose upon deletion of *ompA* seen for planktonic cells also holds for biofilm cells, we repeated these three cellulose assays using biofilm cells collected from glass wool. As with planktonic cells, we found the biofilm cells of the *ompA* mutant had much

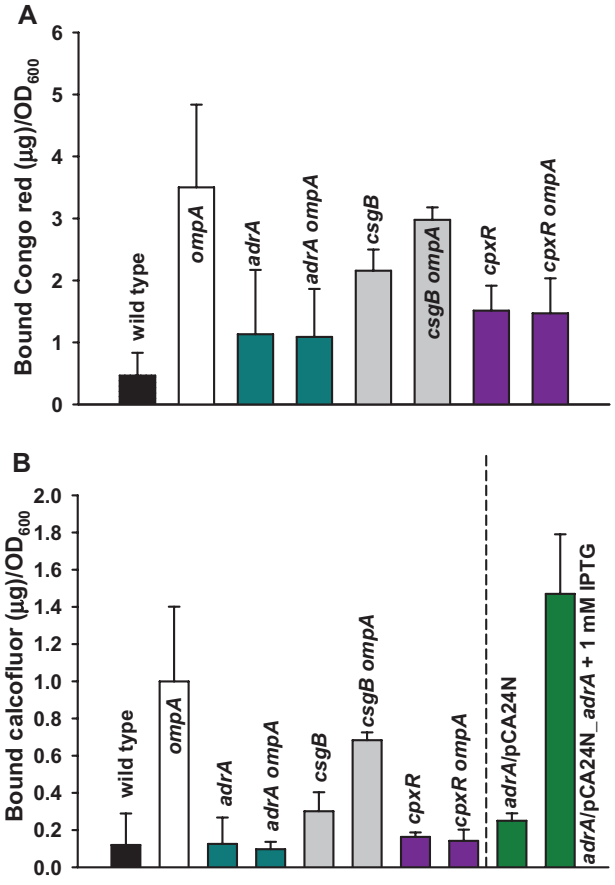

Fig. 4. Cellulose production of planktonic cells using Congo red for the wild-type strain, the BW25113 *ompA* mutant, the *adrA* mutant, the *adrA ompA* mutant, the *csgB* mutant, the *csgB ompA* mutant, the *cpxR* mutant and the *cpxR ompA* mutant at 37°C in LB (A). Cellulose production of planktonic cells using calcofluor for the wild-type strain, the *ompA* mutant, the *adrA* mutant, the *adrA ompA* mutant, the *csgB* mutant, the *csgB ompA* mutant, the *cpxR* mutant, the *cpxR ompA* mutant, the *adrA*/pCA24N strain and the *adrA*/pCA24N_*adrA* strain at 37°C in LB (B). *adrA* was induced with 1 mM IPTG. Data are the average of two independent cultures, and one standard deviation is shown.

higher cellulose production than the wild-type strain: ~4-fold using the Congo red-binding assay, ~6-fold using the calcofluor-binding assay and ~22-fold using the cellulase digestion assay.

Short-time induction of OmpA represses csgD *and* adrA *expression*

Since cellulose production was induced by deleting *ompA* but none of the cellulose-related genes had their expression changed in the whole-transcriptome studies with 15-h-old biofilm cells from PS surfaces, we hypothesized that the expression of cellulose genes may be induced in the exponential phase of the *ompA* culture and reduced to the wild-type levels after entering stationary phase, so they were not detected in the whole-transcriptome studies performed with stationary-phase cells. Thus we performed qRT-PCR using exponential-phase cultures of *ompA*/pCA24N_*ompA* and *ompA*/pCA24N (OmpA was induced with 0.1 mM IPTG for 30 min in exponential-phase cultures with the turbidity at 600 nm around 0.5) and found the expression of *csgD* and *adrA* was repressed 4.0-fold and 3.5-fold due to the expression of *ompA*. We used housekeeping gene *rrsG* as an internal reference for the qRT-PCR and calculated the changes of gene *csgD* and *adrA* expression based on the *rrsG* expression level. CsgD and AdrA synthesize c-di-GMP (Da Re and Ghigo, 2006), which binds to BcsAB (cellulose synthase) to increase cellulose production (Zogaj *et al*., 2001). Hence, OmpA represses *csgD* and *adrA* expression leading to less cellulose production, and deleting *ompA* should result in increased cellulose production as was seen (Figs 3 and 4).

ompA expression was only induced for 30 min with IPTG in this qRT-PCR experiment and the cell viability was not changed within this short time since Fig. S1A shows that the turbidity at 600 nm for the OmpA overproduction strain starts to decrease after 2 h of induction with IPTG (IPTG was added at 2.5 h and the turbidity at 600 nm started to decrease after 4.5 h). This result was confirmed by a time-course β-galactosidase activity measurement using culture supernatants of the MG1655/pCA24N and MG1655/pCA24N_*ompA* strains that showed significant cell lysis occurred after 2 h of induction with 0.05 mM IPTG (data not shown). Hence, OmpA causes cell lysis only when the cell density and *ompA* expression reaches some threshold level (after 2 h of induction by IPTG). Therefore, this qRT-PCR result was not affected by cell viability.

Cellulose inhibits biofilm formation on polystyrene plates

To corroborate our hypothesis that cellulose inhibits biofilm formation on plastic due to its hydrophilic nature, we overproduced AdrA with the *adrA*/pCA24N_*adrA* strain (Table 1) and assayed biofilm formation on PS plates. With 1 mM IPTG, overproducing AdrA decreased biofilm formation by more than 10-fold on hydrophobic surfaces (Fig. 5). This reduction in biofilm formation was accompanied by a fivefold increase in cellulose production upon inducing AdrA (Fig. 4B). Hence, cellulose inhibits biofilm formation on PS plates.

OmpA influences cellulose production and biofilm formation through CpxR

Since *cpxP* transcription was repressed in the *ompA* mutant cells that formed biofilm on PS (Table S1) and since *cpxP* transcription depends almost exclusively on CpxR activity (Danese and Silhavy, 1998), we hypoth-

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

Strain/plasmid	Genotype	Source
Strain		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner (2000)
MG1655	F$^-$ λ$^-$ *ilvG rfb-50 rph-1*	Blattner *et al.* (1997)
BW25113 *ompA*	BW25113 Δ*ompA* Ω Km^r	Baba *et al.* (2006)
BW25113 *cpxR*	BW25113 Δ*cpxR* Ω Km^r	Baba *et al.* (2006)
BW25113 *adrA*	BW25113 Δ*adrA* Ω Km^r	Baba *et al.* (2006)
BW25113 *csgB*	BW25113 Δ*csgB* Ω Km^r	Baba *et al.* (2006)
BW25113 *csgD*	BW25113 Δ*csgD* Ω Km^r	Baba *et al.* (2006)
BW25113 *fimA*	BW25113 Δ*fimA* Ω Km^r	Baba *et al.* (2006)
BW25113 *cpxR ompA*	BW25113 Δ*cpxR* Δ*ompA* Ω Km^r	This study
BW25113 *adrA ompA*	BW25113 Δ*adrA* Δ*ompA* Ω Km^r	This study
BW25113 *csgB ompA*	BW25113 Δ*csgB* Δ*ompA* Ω Km^r	This study
Plasmid		
pCP20	Ap^r, Cm^r; temperature-sensitive replication and thermal induction of FLP recombinase	Cherepanov and Wackernagel (1995)
pCA24N	Cm^r; *lacI*^{q}, pCA24N	Kitagawa *et al.* (2005)
pCA24N_*ompA*	Cm^r; *lacI*^{q}, pCA24N p$_{T5\text{-}lac}$::*ompA*	Kitagawa *et al.* (2005)
pCA24N_*adrA*	Cm^r; *lacI*^{q}, pCA24N p$_{T5\text{-}lac}$::*adrA*	Kitagawa *et al.* (2005)

Km^r, Cm^r and Ap^r denote kanamycin, chloramphenicol and ampicillin resistance respectively.

esized that OmpA production may activate the two-component CpxRA system which increases CpxR phosphorylation. To study the relationship between OmpA and the CpxRA system and also their relationship with cellulose production and biofilm formation, we constructed a *cpxR ompA* double mutant via P1 transduction and measured its cellulose production (Fig. 4) and biofilm formation (Fig. 1A). The results show that without CpxR, deletion of *ompA* does not change cellulose production or biofilm formation. Hence OmpA represses cellulose production and increases biofilm formation on PS by activating CpxR.

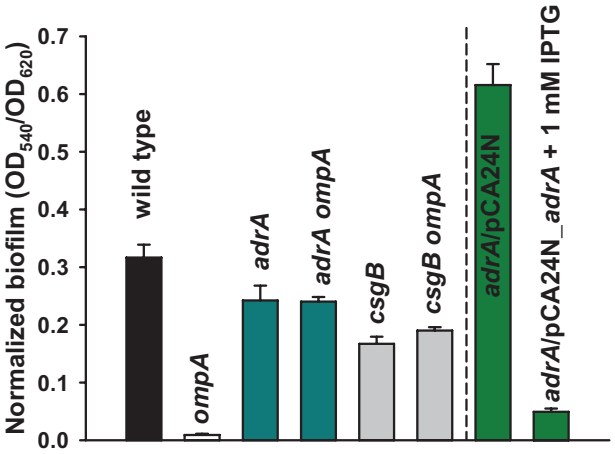

Fig. 5. Normalized biofilm formation (total biofilm/growth) in polystyrene 96-well plates in LB at 37°C after 15 h. Data are the average of 10 replicate wells from two independent cultures, and one standard deviation is shown. *adrA* was induced with 1 mM IPTG.

Discussion

In this study, we show OmpA influences biofilm formation through the Cpx stress response system which reduces cellulose production (summarized in Fig. 6) and results in more biofilm formation on hydrophobic surfaces and less biofilm formation on hydrophilic surfaces. The lines of evidence that support this are: (i) OmpA increases biofilm formation on hydrophobic surfaces and decreases biofilm formation on hydrophilic surfaces without affecting growth (Fig. 1), (ii) OmpA reduces attachment on hydrophilic surfaces (Fig. 2), (iii) OmpA overproduction leads to cell lysis (Fig. S1) indicating envelope stress, (iv) OmpA induces *cpxP* expression (Table S1) and qRT-PCR corroborated that OmpA induces expression of *cpxP* indicating an activation of the CpxRA signal transduction pathway (Danese and Silhavy, 1998), (v) the BW25113 *cpxR ompA* double

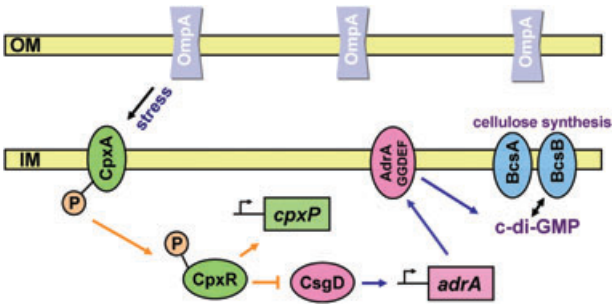

Fig. 6. Hypothesized mechanism for cellulose production via OmpA. OmpA appears to activate the CpxRA two-component system which forms CpxR–P. CpxR–P induces *cpxP* transcription and represses *csgD* which leads to the repression of *adrA*. AdrA is responsible for c-di-GMP expression and cellulose production. → indicates induction and ⊥ indicates repression.

mutant has a bigger colony size than either the *cpxR* or *ompA* single mutants (this phenotype was also observed in our study) which indicates a positive relationship between CpxR and OmpA as Typas and colleagues (2008) reported, (vi) OmpA represses cellulose production around colonies on the Congo red plate (Fig. 3, 37°C), in planktonic cells as well as in biofilm cells as shown three ways: via a Congo red assay, a calcofluor assay (Fig. 4), and a cellulase-digestion assay in which glucose was measured, (vii) double mutant *csgB ompA* has similar colony morphology with the *ompA* mutant, indicating it is cellulose, not curli, that was induced by deleting *ompA* (Fig. 3), (viii) double mutants *adrA ompA* and *cpxR ompA* fail to increase cellulose production compared with the *ompA* single mutant, while the *csgB ompA* double mutant has more cellulose production than the *csgB* mutant (Fig. 4), indicating a connection among OmpA, AdrA and CpxR for regulating cellulose production, (ix) qRT-PCR shows OmpA represses *csgD* and *adrA* transcription which should reduce cellulose, (x) overproduction of AdrA increases cellulose production (Fig. 4B) and decreases biofilm formation on PS plates (Fig. 5) which matches the *ompA* mutant phenotype with high cellulose production and low biofilm formation on PS plates, and (xi) double mutants *adrA ompA* (Fig. 5) and *cpxR ompA* (Fig. 1A) have no change in biofilm formation compared with the *adrA* and *cpxR* single mutants, which means OmpA functions through AdrA and CpxR for controlling biofilm formation.

The Cpx-signalling pathway responds to stress from misfolded proteins from the inner membrane (Raivio and Silhavy, 1999) as well as to a lipoprotein located on the outer membrane, NlpE (Otto and Silhavy, 2002). Here we discovered the Cpx-signalling pathway also responds to the most abundant outer membrane protein, OmpA. Expression of *cpxP* was repressed in the *ompA* mutant (Table S1) and these results were corroborated by qRT-PCR. Transcription of *cpxP* is a result of CpxRA system activity and CpxR phosphorylation (Danese and Silhavy, 1998; Wolfe *et al*., 2008). In addition, cell lysis by overproduction of OmpA clearly indicates membrane stress occurred. Hence we hypothesize that the production of OmpA can directly or indirectly activate the CpxRA two-component stress response system.

CpxR negatively regulates expression of *csgB* and *csgD* (Dorel *et al*., 2006). For the regulation of *csgD*, OmpR binds to the *csgD* promoter and activates *csgD* expression, whereas CpxR–P binds to its own recognition site on the *csgD* promoter which overlaps the OmpR binding site and represses the *csgD* expression (Jubelin *et al*., 2005). This repression is triggered by osmolarity and curli overproduction (Prigent-Combaret *et al*., 2001). CsgD stimulates synthesis of AdrA, a protein with a GGDEF domain, which is responsible for c-di-GMP synthesis and which is required for cellulose production (Zogaj *et al*., 2001; Gualdi *et al*., 2008). Since cellulose biosynthesis genes *bcsABZC* are constitutively transcribed, cellulose synthesis depends only on AdrA for synthesizing c-di-GMP which can control cellulose production by binding with BcsB, which works with BcsA as a cellulose synthase (Zogaj *et al*., 2001; Gualdi *et al*., 2008). Our qRT-PCR result for *csgD* and *adrA* shows that with overproduction of OmpA, expression of both *csgD* and *adrA* was repressed, resulting in a reduction of cellulose production. Hence OmpA decreases cellulose production by inhibiting the CsgD/AdrA pathway. There may also be some alternative regulation pathways (Da Re and Ghigo, 2006) such as YedQ for cellulose production that are influenced by OmpA.

OmpA increases biofilm formation on hydrophobic surfaces (PS, PVC and PP), decreases biofilm formation on hydrophilic surfaces (glass), and decreases attachment to sand by decreasing cellulose production. We explain this interesting observation based on the hydrophilic property of cellulose, a polysaccharide consisting of a linear chain of several hundred to over 10 thousand β $(1 \rightarrow 4)$ linked D-glucose units. AdrA is required for *Salmonella typhimurium* attachment to glass surfaces (Römling *et al*., 2000), and in *Salmonella enteritidis*, cellulose increases biofilm formation on glass tubes (Solano *et al*., 2002). Therefore, cellulose promotes biofilm formation on glass, and we saw this with the *ompA* mutant that produces more cellulose in that there was more biofilm formation on glass (Fig. 1B) and more attachment to sand (Fig. 2). We also found that increasing cellulose production by overproducing AdrA (Fig. 4B) decreases biofilm formation on hydrophobic PS plates (Fig. 5). Hence, cellulose decreases biofilm formation on PS, and it is repression of cellulose synthesis that causes OmpA to work differently on different surfaces.

Factors other than cellulose production can also influence biofilm formation, including curli formation, which is also regulated by CsgD (Römling *et al*., 2000). The interaction between cellulose and curli makes their influence on biofilm formation complex. Cellulose decreases curli-mediated biofilm formation on PP microtitre plates (Gualdi *et al*., 2008), which can be explained by the hydrophilic property of cellulose; however, similar results were obtained also on glass, a hydrophilic surface. Curli help adherence of *E. coli* to intestinal epithelial cells, and cellulose alone has no effect, but coexpression of cellulose and curli decreases this adherence (Wang *et al*., 2006). In addition, with pure water contact angle measurements, coexpression of cellulose and thin aggregative fimbriae leads to formation of a highly hydrophobic network while cellulose alone shows hydrophilic properties in *S. typhimurium* (Zogaj *et al*., 2001). In our biofilm and cellulose studies, we found the *csgB* mutant, deficient

for curli formation, produces more cellulose than the wild-type strain and other mutants such as *cpxR* and *adrA* (Fig. 4). The *csgB ompA* double mutant produced high amounts of cellulose, lost the ability to decrease biofilm on PS plates (Fig. 5), and its phenotype became similar to the *csgB* single mutant for biofilm formation. Hence we can make two hypotheses: (i) the deficiency of curli formation can lead to more cellulose production, or (ii) cellulose cannot decrease biofilm on PS surfaces in the absence of curli.

OmpA exists in *E. coli* and in many enterobacteria. It is located on the outer membrane of bacterial cells, and functions as an adhesin and as an invasin (Smith *et al*., 2007). The position of OmpA indicates its importance for adhesion and biofilm formation and also indicates that the regulation of OmpA may occur post-transcriptionally, which cannot be detected using transcription-level genetic tools such as whole-transcriptome studies and qRT-PCR. Hence, in addition to CpxRA, whose activation by OmpA is reflected in an increase in *cpxP* transcription, there may be additional regulatory pathways that OmpA works through to alter biofilm formation.

Experimental procedures

Bacterial strains, media, growth conditions and growth rate assay

All the strains and plasmids used in this study are listed in Table 1. *Escherichia coli* K-12 BW25113 and its isogenic mutants (Baba *et al*., 2006) were obtained from the Genome Analysis Project in Japan (Mori *et al*., 2000) and were used for all experiments except the β-galactosidase cell lysis experiments in which *E. coli* MG1655 was used. Plasmid pCA24N_*ompA*, carrying *ompA* under control of the $P_{T5\text{-}lac}$ promoter with tight regulation via the *lacI*^{q} repressor, as well as the empty plasmid pCA24N, were also obtained from the Genomic Analysis Project in Japan (Kitagawa *et al*., 2005). Expression of *ompA* and *adrA* was induced by 0.05–1 mM IPTG (Sigma, St Louis, MO, USA).

Luria–Bertani medium (LB) (Sambrook *et al*., 1989) was used to culture all the *E. coli* cells. Kanamycin (50 μg ml^{-1}) was used for pre-culturing the isogenic knockouts. Chloramphenicol (30 μg ml^{-1}) was used for the strains harbouring pCA24N and its derivatives. The specific growth rates of the *E. coli* wild-type strain and the *ompA* mutant were determined by measuring the turbidity at 600 nm for two independent cultures of each strain as a function of time with values less than 0.7. All experiments were performed at 37°C except for some of the colony morphology studies which were conducted with Congo red at both 30°C and 37°C.

Crystal violet biofilm assay

The biofilm assay was performed in 96-well PS, PP and PVC plates as reported previously (Kim *et al*., 2009). Briefly, cells were inoculated at an initial turbidity at 600 nm of 0.05 and grown for 15 or 24 h without shaking, and then the cell density and total biofilm were measured using crystal violet staining. Each data point was averaged from at least 12 replicate wells (six wells from each of two independent cultures). For biofilm formation on glass, 1 ml of culture at a turbidity of 0.05 at 600 nm was loaded into 85 mm sterile glass culture tubes and incubated for 48 h without shaking, and then 1 ml of 0.1% crystal violet was added for 20 min to stain the biofilm. At least two independent cultures were used for each strain with at least three tubes used for each culture.

EPS and colanic acid assays

The amount of total EPS and colanic acid was determined as described previously (Zhang *et al*., 2008). Briefly, about 60 mg of cell colony mass from overnight agar plates was boiled in water for 10 min. The supernatant was then used for an anthrone-H_2SO_4 assay to determine EPS concentrations, and colanic acid was determined by measuring the amount of fucose using sulfuric acid and cysteine hydrochloride as reagents. Each assay was performed with two independent cultures for each strain.

Sand column assay

The sand column assay was performed as described previously (Landini and Zehnder, 2002) with modifications to prevent damage to fimbriae (Kim *et al*., 2009). Rather than re-suspending in PBS buffer, cells were directly inoculated into 250 ml of LB medium with an initial turbidity of 0.05 at 600 nm and grown to a turbidity of 0.5–0.6. Then cells were added directly to the sand column (a 12 cm syringe column filled with 18 g of sterile sea sand) at a flow rate of 0.5 ml min^{-1}. Fourteen fractions (1.5 ml each) were collected, and the fraction of attached cells was calculated as 1 – efflux turbidity/input turbidity. The fimbriae minus strain BW25113 *fimA* mutant was used as a negative control.

Cell swimming motility assay and pH

Cell swimming motility was performed as previously described (Sperandio *et al*., 2002; Domka *et al*., 2006). Overnight cultures were used to inoculate the plates. At least five plates were used for each independent culture, and two independent cultures were used for each strain. The pH of the supernatants of the overnight cultures was measured after removing cells by centrifuging at 16 000 *g* for 10 min.

P1 transduction

Transduction with P1 bacteriophage was used to construct the *cpxR ompA*, *adrA ompA* and *csgB ompA* mutants using the Rapid Gene Knockout method (Maeda *et al*., 2008). Briefly, the kanamycin resistance gene (Km^r) was first removed by FLP recombinase (expressed from pCP20) from the Δ*cpxR* Ω Km^r, Δ*adrA* Ω Km^r and Δ*csgB* Ω Km^r mutants, then bacteriophage P1 was grown with BW25113 Δ*ompA* Ω Km^r and the lysate was used for transduction into the three resulting kanamycin-sensitive strains. All six of the mutations

were verified by colony PCR with primer sites located upstream and downstream of the deleted gene (Table S2). Single mutants were used as positive controls.

Cellulose assay using Congo red and calcofluor

To detect curli/cellulose production in the *ompA* mutant using bacterial colonies, 2 μl of overnight culture (with 50 μg ml^{-1} kanamycin for mutants) was spotted onto LB plates (no NaCl) containing 0.004% Congo red and 0.002% Brilliant Blue (Da Re and Ghigo, 2006). Plates were incubated for 24 h at 37°C and 48 h at 30°C. Red colonies indicated the binding of Cong red.

To quantify cellulose production for planktonic cells, the Congo red method of Lee and colleagues (2007) was used with some modifications; similarly, calcofluor was also used to measure cellulose. Cells from 2 ml of a 14–15 h culture were centrifuged and re-suspended in 1 ml of 1% tryptone with 40 μg ml^{-1} Congo red or 16 μg ml^{-1} calcofluor and incubated for 2 h at 250 r.p.m. Bacterial bound Congo red or calcofluor was removed by centrifugation for 5–10 min at 17 000 *g*, and the amount of unbound Congo red or calcofluor was determined by measuring the absorbance of the supernatant at 490 nm for Congo red and at 350 nm for calcofluor.

Cellulose assay using cellulase

Colonies of the wild-type strain and the *ompA* mutant were collected from the surface of LB agar plates after overnight incubation at 37°C. For each strain, around 50 mg of cells were transferred into 400 μl 2.5 M 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer (pH 5.5) with or without 6 U ml^{-1} cellulase (*Aspergillus niger*, MP Biomedicals) and incubated at 37°C for 16 h. Each sample was adjusted with 2.5 mM MES buffer to a turbidity of 40 at 600 nm and centrifuged. The glucose amount in the supernatant was measured using the glucose (HK) assay kit (Sigma-Aldrich) and compared with the signal obtained with no cellulase treatment.

Cellulose assay using biofilm cells

To quantify cellulose production in biofilm cells of the *ompA* mutant, cells were grown in 250 ml of LB medium with 7 g glass wool for 14–15 h. The glass wool was washed twice with 0.85% NaCl and sonicated for 2 min at 22 W (FS3 sonicator, Fisher Scientific) in 100 ml of cold 0.85% NaCl buffer to release the cells which were centrifuged at 10 000 *g* for 2 min (J2-HS centrifuge, Beckman, Palo Alto, CA, USA). The Congo red-binding assay, calcofluor-binding assay and cellulase treatment + glucose determination assay were then performed with these glass wool biofilm cells as indicated above.

Cell lysis assays

Genomic DNA was used as an indicator of cell lysis. BW25113 *ompA*/pCA24N and *ompA*/pCA24N_*ompA* were inoculated into 25 ml of LB medium with an initial turbidity of 0.05 at 600 nm, and IPTG (0.1 mM) was added to both cultures after incubating for 2.5 h with 250 r.p.m. shaking. After a total of 15 h of incubating, 1.8 ml was sampled and centrifuged. The supernatant was purified with phenol/chloroform/isoamyl alcohol followed by precipitating with an equal volume of isopropanol in 1/10 volume of 3 M NaOAc. The genomic DNA concentration was measured by quantitative PCR using primers for *purA* (encodes the subunit of adenylosuccinate synthetase), and the percentage of cell lysis was calculated as the ratio of genomic DNA concentration in the supernatant relative to that of the sonicated sample in which all genomic DNA was released. This experiment was performed with two independent cultures for each strain.

β-Galactosidase was also used as in indicator of cell lysis. This assay was performed as described previously (Rice *et al*., 2007) except the β-galactosidase was generated from a chromosomal copy of *lacZ*$^+$ by the addition of IPTG. MG1655/pCA24N and MG1655/pCA24N_*ompA* were inoculated into 25 ml of LB medium with an initial turbidity of 0.05 at 600 nm, and IPTG (0.05 mM) was added to both cultures after incubating for 2.5 h with 250 r.p.m. shaking. One millilitre of culture was harvested after 15 h and centrifuged for 2 min at 17 000 *g*. The β-galactosidase activity of the supernatant and sonicated cell pellet was measured as previously described (Wood and Peretti, 1991), and the percentage of cell lysis was calculated as the ratio of β-galactosidase activity in the supernatant relative to that of the sonicated cell pellet. This experiment was performed with four independent cultures for each strain.

RNA isolation from biofilms

Thirty grams of PS foam shavings of approximately 1 mm diameter were submerged in 250 ml of medium and autoclaved. Overnight cultures (2.5 ml) of the wild-type strain and the *ompA* mutant were inoculated into 250 ml of medium. After 15 h of incubation, the PS foam was washed twice in 200 ml of cold 0.85% NaCl buffer to remove planktonic cells, and the biofilm cells were removed by sonication at 22 W (FS3 sonicator, Fisher Scientific) in 200 ml of cold 0.85% NaCl buffer. The buffer containing biofilm cells was then centrifuged at 10 000 *g* for 2 min at −2°C (J2-HS centrifuge, Beckman, Palo Alto, CA, USA). This cell pellet was re-suspended in RNA*later* (Ambion) or RNAprotect (Qiagen), transferred to pre-chilled bead beater tubes and centrifuged for 15 s. Cells were lysed with 0.1 mm Zirconia/Silica beads (Biospec Products, OK, USA) in a bead beater (Ren *et al*., 2004a), and total RNA was isolated using a Qiagen RNeasy Mini Kit.

Whole-transcriptome analysis

The *E. coli* GeneChip Genome 2.0 array (Affymetrix, P/N 900551) was used to analyse differential gene expression for the *ompA* mutant versus the wild-type strain for biofilm cells on PS. The *E. coli* GeneChip Genome 2.0 array contains 10 208 probe sets for open reading frames, rRNA, tRNA, and intergenic regions in four *E. coli* strains (MG1655, CFT073, O157:H7-Sakai and O157:H7-EDL933). The cDNA synthesis, fragmentation and hybridizations were performed as described previously (González Barrios *et al*., 2006b). Frag-

mented and labelled cDNA was hybridized for 16 h at 45°C, and global scaling was applied to make the average signal intensity 500. The probe array images were inspected for any image artefacts. Background values, noise values and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA controls were used to monitor the labelling process, and signals of the *araA* and *rhaA* deleted genes of BW25113 (Table 1) were low. If the gene with the larger transcription rate did not have a consistent transcription rate based on the 11–15 probe pairs (*P*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *P*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and if their fold change is higher than the standard deviation for the whole genome (Ren *et al*., 2004b). The expression data were deposited in the NCBI Gene Expression Omnibus and are accessible through Accession No. GSE14064.

qRT-PCR

qRT-PCR was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Total RNA 200 ng was used for the qRT-PCR reaction using the SuperScriptTM III Plantinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA). Primers used are listed in Table S2. The housekeeping gene *rrsG* was used to normalize the gene expression data. The annealing temperature was 60°C for all of the genes in this study.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Partial whole-transcriptome profiles to determine the impact of OmpA on biofilm formation. Fold changes between polystyrene biofilm samples of the BW25113 *ompA* mutant *vs.* the BW25113 wild-type strain at 37°C after 15 h of incubation in LB are shown with two biological replicates PS-1 and PS-2. The GEO accession number is GSE14064. Important fold changes are shown in bold.

Table S2. Primers used for qRT-PCR and double mutant verification.

Fig. S1. Growth for the BW25113 *ompA*/pCA24N and *ompA*/pCA24N_*ompA* strains in LB at 37°C. Data are from two independent cultures (A). Cell clumping for *ompA*/pCA24N and *ompA*/pCA24N_*ompA* overnight cultures in LB at 37°C after 15 h (B). IPTG (0.1 mM) was added to each culture at 2.5 h to induce OmpA.