

Coordinate gene regulation by fimbriae-induced signal transduction

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Fimbriae are thread-like polymers displayed in large amounts on the bacterial surface and used by many pathogens to attach to receptors on host tissue surfaces. Fimbriae contain disulfide bridges, contrary to many *Escherichia coli* surface proteins produced in bulk amounts. Here we investigate whether fimbriae expression can affect expression of other genes. Analysis of gene expression in two *E. coli* strains, differing in the *fim* locus, indicated the *flu* gene to be affected. The *flu* gene encodes the antigen 43 (Ag43) surface protein, specifically involved in bacterial aggregation, and microcolony and biofilm formation. Ag43 production is repressed by the global regulator OxyR, which monitors the cell's thiol–disulfide status. Only the thiol form of OxyR represses Ag43 production. We demonstrate that production of several different disulfide-containing fimbriae results in the abolition of Ag43 production. No effect was observed in an *oxyR* mutant. We conclude that fimbriae expression *per se* constitutes a signal transduction mechanism that affects a number of unrelated genes via the thiol–disulfide status of OxyR. Thus, phase variation in fimbrial expression is coordinated with the expression of other disease- and colonization-related genes.
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Introduction

The ability of bacteria to recognize and adhere to a specific surface is a fundamental aspect of microbial ecology and pathogenesis. Bacterial adhesins confer specific recognition and adhesion to a diverse spectrum of target molecules, ranging from components of tissue or cell surfaces to inorganic materials such as dental enamel (Klemm and Schembri, 2000). Type 1 fimbriae are thin, rigid, adhesive surface organelles found on many *Escherichia coli* strains and other members of the Enterobacteriaceae (Klemm and Krogfelt, 1994). On a fimbriated bacterium, several hundred of these organelles radiate peritrichously from the surface to a distance of ~1 µm. Type 1 fimbriae exert their adhesive properties by virtue of the FimH adhesin, a minor organelle component located at the tip of the fimbriae (Choudhury *et al.*, 1999). The FimH adhesin recognizes mannose-containing glycoproteins that are present on many mammalian host tissues,

such as the surface of the urinary tract. This enables the bacteria to target, attach to and eventually to colonize the uroepithelium (Connell *et al.*, 1996; Sauer *et al.*, 2000). Type 1 fimbriation has been correlated with urovirulence in *E. coli*, the causative agent of >80% of urinary tract infections in humans.

Expression of type 1 fimbriae is phase variable due to a flip–flop-type control system based on an invertible DNA switch, located immediately upstream of the major subunit gene. A promoter located in the switch drives the expression of the *fim* genes (Olsen and Klemm, 1994). On or off orientation of the switch results in a fimbriated or bald phenotype, respectively. Two tyrosine-class recombinases, FimB and FimE, catalyse the inversion of the switch (Klemm, 1986). Only the FimB recombinase efficiently promotes off-to-on switching, while FimE primarily catalyses on-to-off inversion (Klemm, 1986; Gally *et al.*, 1996). A number of global regulators including integration host factor (IHF) (Blomfield *et al.*, 1997), the leucine-responsive regulatory protein (Lrp) (Blomfield *et al.*, 1993) and H-NS (Olsen and Klemm, 1994; Olsen *et al.*, 1998; Schembri *et al.*, 1998) also affect inversion of the *fim* switch.

Another prominent surface protein of *E. coli* subject to phase variation is the product of the *flu* gene (also termed *agn*), antigen 43 (Ag43) (Diderichsen, 1980; Henderson and Owen, 1999). The expression of *flu* is phase variable due to the concerted action of the Dam methylase (activation) and OxyR (repression) (Henderson and Owen, 1999; Haagmans and van der Woude, 2000). OxyR is involved in the cell's oxidative stress response and also monitors the cellular thiol–disulfide status (Zheng *et al.*, 1998; Åslund and Beckwith, 1999; Debarbieux and Beckwith, 1999; Storz and Imlay, 1999). OxyR can exist in both a reduced and an oxidized form in which two cysteine groups are oxidized to form a disulfide bridge. Unlike other genes in the OxyR regulon that are activated by the oxidized form of OxyR (Storz and Imlay, 1999), only the reduced form is able to repress *flu* efficiently (Henderson and Owen, 1999; Haagmans and van der Woude, 2000).

Ag43 is a self-recognizing surface-displayed protein (~50 000 copies per cell) that confers autoaggregation of bacteria by an intercellular handshake mechanism (Hasman *et al.*, 1999). Expression of Ag43 causes a characteristic frizzy colony morphology type on solid media and settling of cells from standing liquid cultures (Hasman *et al.*, 2000). Recently, Ag43 was demonstrated to be involved in bacterial biofilm formation (Danese *et al.*, 2000; Kjærgaard *et al.*, 2000; O'Toole *et al.*, 2000). Interestingly, Ag43-mediated autoaggregation is abolished by the presence of type 1 fimbriae on the cells (Hasman *et al.*, 1999). Apparently, the presence of these ~1-µm-long, rigid organelles on the cell surface impedes the close cell contact required for the Ag43–Ag43

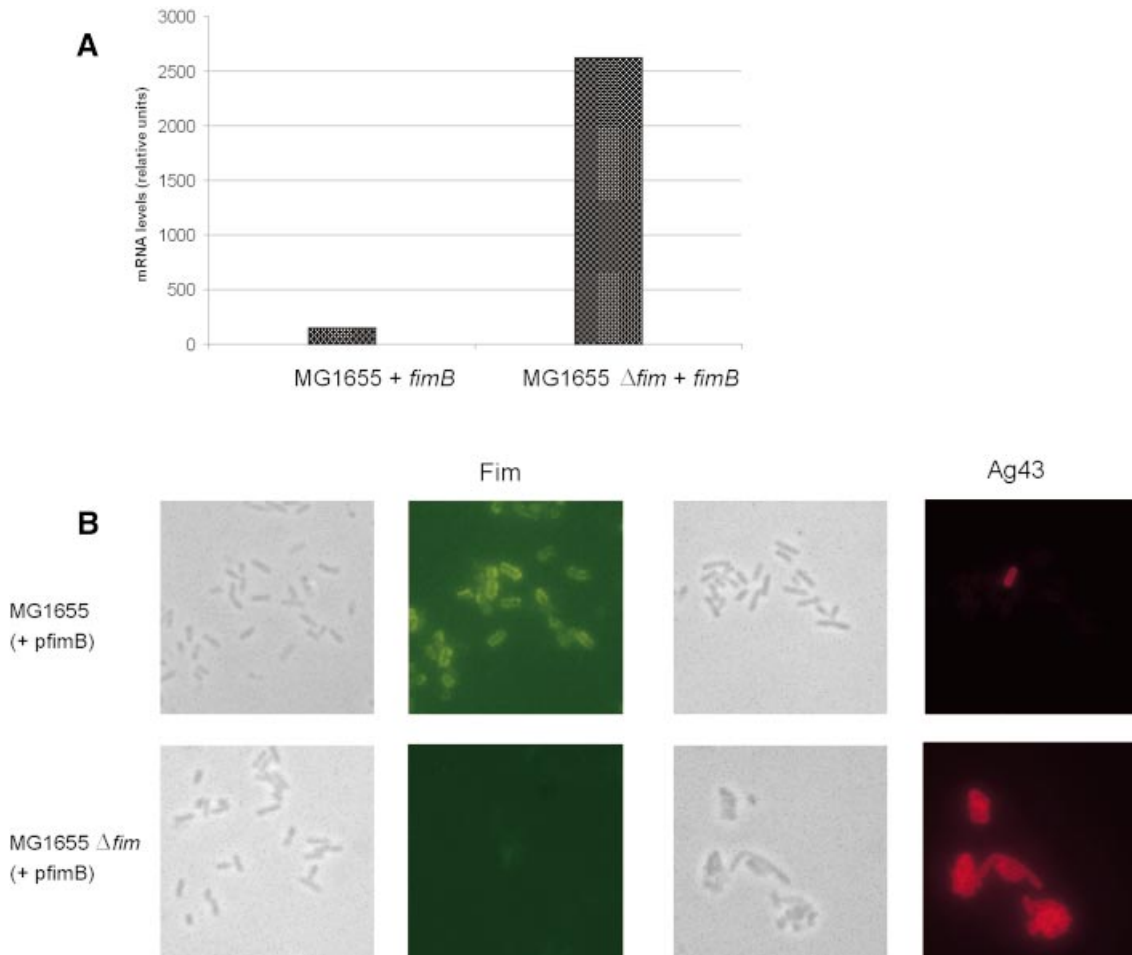


Fig. 1. (A) Comparison of *flu* gene transcription levels in *E.coli* strains MG1655(*pfimB*) and MG1655 Δ *fim*(*pfimB*) (a derivative in which the *fim* genes have been deleted). Quantification of mRNA levels was performed by direct scanning of DNA chips. (B) Phase contrast and immunofluorescence microscopy of *E.coli* strains MG1655(*pfimB*) and MG1655 Δ *fim*(*pfimB*) employing anti-Fim and anti-Ag43 serum, respectively.

handshake function; thus, Fim is phenotypically dominant to Ag43.

Adhering to a particular tissue or cell surface by fimbriae-mediated attachment heralds a significant event in the life cycle of a bacterium. It is therefore implicit that expression of such target recognition organelles is of fundamental importance in a bacterium's interplay with the environment and might influence the expression of other genes required in the new habitat. Furthermore, a type 1 fimbriated cell has up to 500 fimbriae on the surface, i.e. 0.5 million structural proteins, representing ~8% of the total cellular protein and a significant resource drain for the cell. With this in mind, we have investigated whether the fimbriate state of a bacterium influences the expression of other non-related genes by inter-system cross-talk.

Results

Deletion of the *fim* gene cluster affects transcription of *flu*

The advent of genome-wide DNA microarrays for analysis of gene expression and, specifically, a DNA chip based on the genome of the *E.coli* K-12 reference strain MG1655 (Selinger *et al.*, 2000) provided an attractive way of analysing expression of genes in *E.coli* that might be

affected by the fimbriation status of the cell. An isogenic pair of strains consisting of *E.coli* strain MG1655 and its Δ *fim* sibling were transformed with a plasmid, pPKL9 (*pfimB*), encoding the *fimB* recombinase, to ensure optimal expression of the *fim* gene cluster. PCR monitoring of the *fim* switch in MG1655(*pfimB*) revealed it to be in the on orientation (it is absent in MG1655 Δ *fim*; data not shown). Phenotypically, the two strains represent the extremes in fimbrial phase variation, being either fimbriated or bald, respectively. Total mRNA was isolated from each of the two strains and investigated by DNA microarray analysis. Comparison of the transcript signals of strains MG1655(*pfimB*) and MG1655 Δ *fim*(*pfimB*) revealed that the transcription of the *flu* gene, diametrically positioned to the *fim* locus on the *E.coli* chromosome, was increased ~20-fold when *fim* was absent (Figure 1A). We previously found that Ag43-mediated autoaggregation is abolished by the presence of type 1 fimbriae on the cells; thus, Fim is phenotypically dominant to Ag43 (Hasman *et al.*, 1999). However, the dramatic change in transcription of the *flu* gene observed in the DNA microarray experiments suggested a possible interaction between *fim* and *flu* at the transcriptional level. Furthermore, when we re-examined our MG1655(*pfimB*) and MG1655 Δ *fim*(*pfimB*) strains by fluorescence microscopy employing either

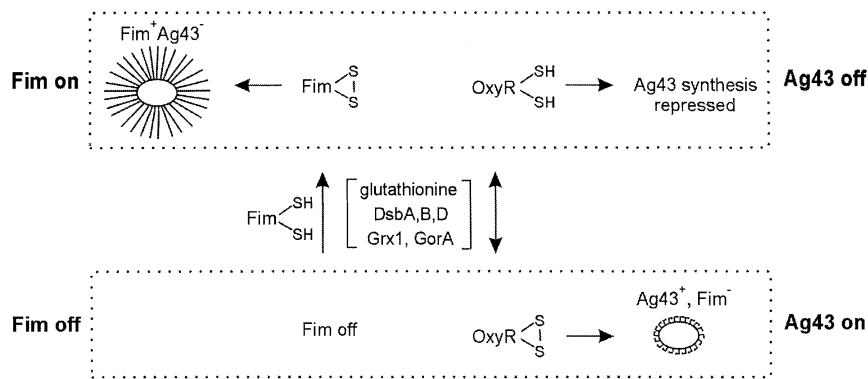


Fig. 2. Model describing the coordination of the phase-variable Fim and Ag43 phenotypes via OxyR-relayed thiol–disulfide signal transduction. In the fimbrial phase on state, disulfide bond formation of fimbrial subunit proteins takes place in the periplasm catalysed by DsbA, -B and -C (Raina and Missiakas, 1997; Åslund and Beckwith, 1999; Debarbieux and Beckwith, 1999). The ultimate oxidative potential is linked to the cytoplasmic pool of glutathione via the cytoplasmic membrane-located DsbB (Raina and Missiakas, 1997; Debarbieux and Beckwith, 1999). The thiol–disulfide status of glutathione is monitored by OxyR (Zheng *et al.*, 1998; Storz and Imlay, 1999), which under these conditions is driven to the reduced state and acts as an active repressor of the *flu* gene. Other auxiliary enzymes are indicated. In the fimbrial phase off state, OxyR can exist as both a reduced and oxidized form, with the result that repression of Ag43 synthesis is relieved.

Fim- or Ag43-specific antisera, Ag43 expression was only observed in the MG1655 Δ *fim*(p*fim*B) strain (Figure 1B).

Production of disulfide-containing fimbriae affects Ag43 phenotype and implicates OxyR

The expression of *flu* is phase variable due to the concerted action of the Dam methylase (activation) and OxyR (repression) (Henderson and Owen, 1999; Haagmans and van der Woude, 2000). OxyR is involved in the cell's oxidative stress response and also monitors the cellular thiol–disulfide status (Zheng *et al.*, 1998; Åslund and Beckwith, 1999; Debarbieux and Beckwith, 1999; Storz and Imlay, 1999). OxyR can exist in both a reduced and an oxidized form in which two cysteine groups are oxidized to form a disulfide bridge. Unlike other genes in the OxyR regulon that are activated by the oxidized form of OxyR (Storz and Imlay, 1999), only the reduced form is able to repress *flu* efficiently (Henderson and Owen, 1999; Haagmans and van der Woude, 2000). OxyR can be invoked to mediate signal transduction between *fim* and *flu* according to the following model. The nascent structural components of type 1 fimbriae all contain multiple cysteine (-SH) residues. When fimbriae are being made, several hundred thousand copies of the structural components are transported to the surface with the concomitant formation of S–S disulfide bridges; in effect, a net oxidation that must be accompanied by a similar reduction in order to maintain the cellular status quo (Raina and Missiakas, 1997). Thus, fimbrial biosynthesis and the accompanying S–S bridge formation might be invoked to influence the oxidation level of OxyR and drive it toward the reduced state, which is an active repressor of the *flu* gene. Fimbriae formation, accompanied by massive disulfide bridge formation, might therefore constitute a signal that represses the expression of the *flu* gene via the thiol–disulfide status of OxyR (Figure 2).

In order to investigate this issue further, Ag43 expression as a function of fimbriation was probed by immunofluorescence microscopy (Figure 3). A dramatic difference in Ag43 expression was observed when MG1655 Δ *fim* was

transformed with plasmids pPKL4 (encoding the *fim* gene cluster) and pPAP5 (encoding the *pap* gene cluster responsible for P fimbriae production), respectively. Virtually none of the cells of the two fimbriae-producing recombinants expressed Ag43, whereas the MG1655 Δ *fim* control did. The architecture of P fimbriae and type 1 fimbriae is similar, i.e. both organelles are polymers composed of disulfide bridge-containing proteins. The fact that production of P fimbriae causes abolition of Ag43 production is consistent with our model that production of these organelles communicates with the *flu* gene via the thiol–disulfide state of OxyR. Production of a third disulfide-containing fimbrial system, F1C, was also shown to have the same negative effect on Ag43 production (data not shown).

Fimbriation does not affect Ag43 production in an oxyR background

To examine directly the role of OxyR in communicating the cellular fimbriae status to *flu*, we monitored the effect of fimbriae production by immunofluorescence microscopy with the same set of fimbriae-encoding plasmids in an *oxyR* mutant. Here no effect of fimbriation on Ag43 production was observed, arguably due to disrupted communication between the systems in the absence of OxyR (Figure 3). It should also be noted that the expression control systems of type 1, P and F1C fimbriae are totally different and unlikely to play any role in the observed cross-talk with *flu*.

Production of flagella does not affect Ag43 phenotype

Production of flagella is equivalent to fimbriae production in that a similar amount of protein is exported to the bacterial surface. However, the major flagellar component, FliC, does not contain any cysteine residues and flagella production would therefore not be expected to influence the cellular thiol–disulfide status monitored by OxyR. Flagella production in Enterobacteriaceae is controlled ultimately by the *flhDC* master control locus (Macnab,

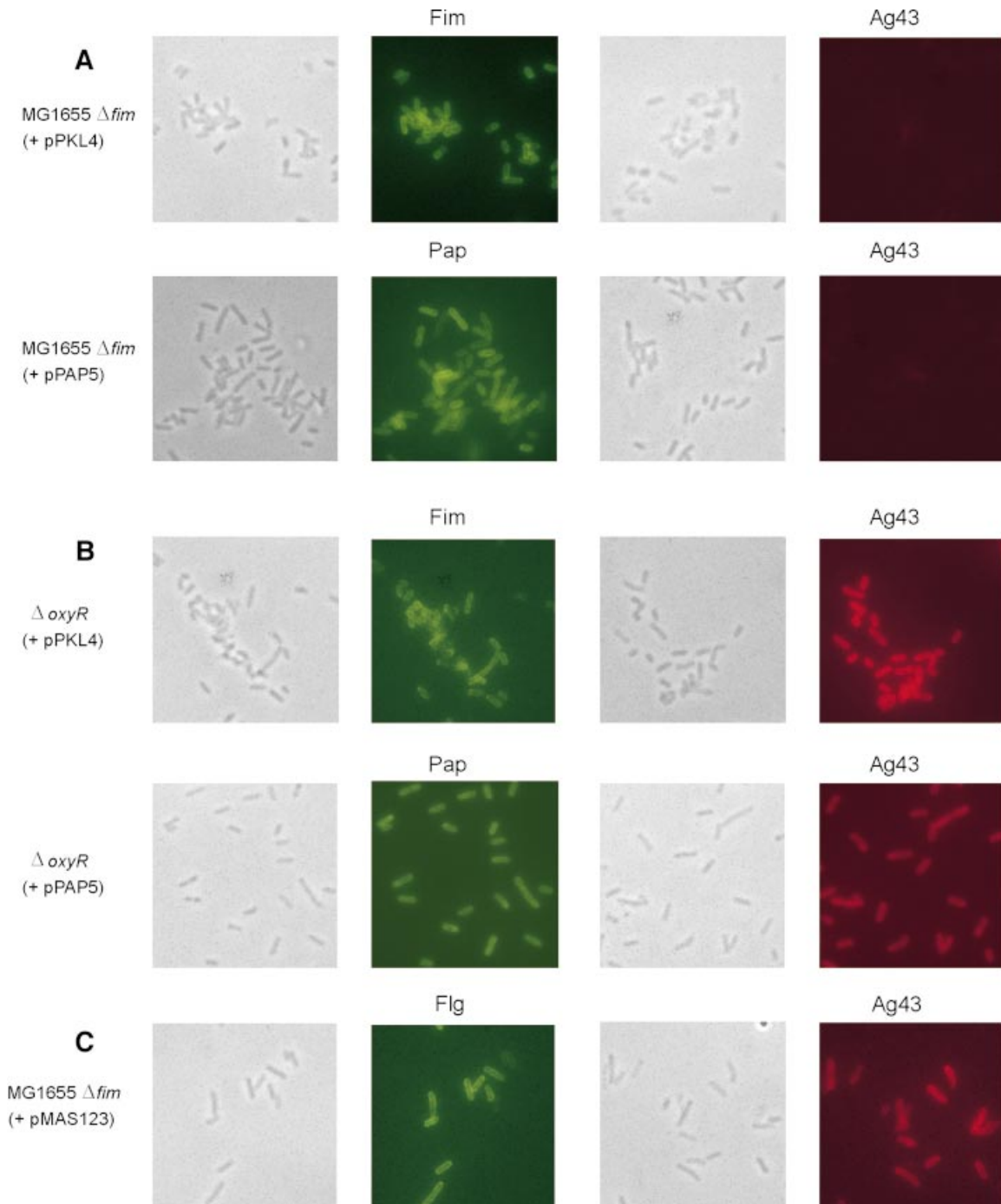


Fig. 3. Phase contrast and immunofluorescence microscopy of *E. coli* Δfim (A) or $\Delta oxyR$ (B) strains expressing type 1 (pPKL4) or P (pPAP5) fimbriae employing anti-Fim, -P or -Ag43 serum, respectively. (C) Phase contrast and immunofluorescence microscopy of *E. coli* strain MG1655 Δfim (pMAS123) induced for overexpression of flagella employing anti-flagella and anti-Ag43 serum, respectively.

1992). Indeed, when we introduced a plasmid, pMAS123, encoding the *flhDC* locus into MG1655 Δfim , a high level of flagellation was observed by immunofluorescence microscopy, whereas Ag43 production was unaffected (Figure 3). This suggested that only production of disulfide-containing organelles such as type 1 and related fimbriae could affect the cellular thiol–disulfide status, whereas large-scale production of surface structures basically devoid of cysteine residues, such as flagella, had no influence. Furthermore, it is comforting to note that

Ag43, in spite of its large size (~110 kDa), does not contain any cysteine residues (Henderson and Owen, 1999) and expression and export of Ag43 would not affect the cellular thiol–disulfide status. In fact, relatively few *E. coli* proteins that are exported in bulk amounts contain cysteine residues. Indeed the two major outer membrane porins of *E. coli*, OmpC and OmpF, do not contain any cysteines, and would therefore not affect the OxyR-based signal transduction system described here; type 1 and related fimbriae appear unique in this respect.

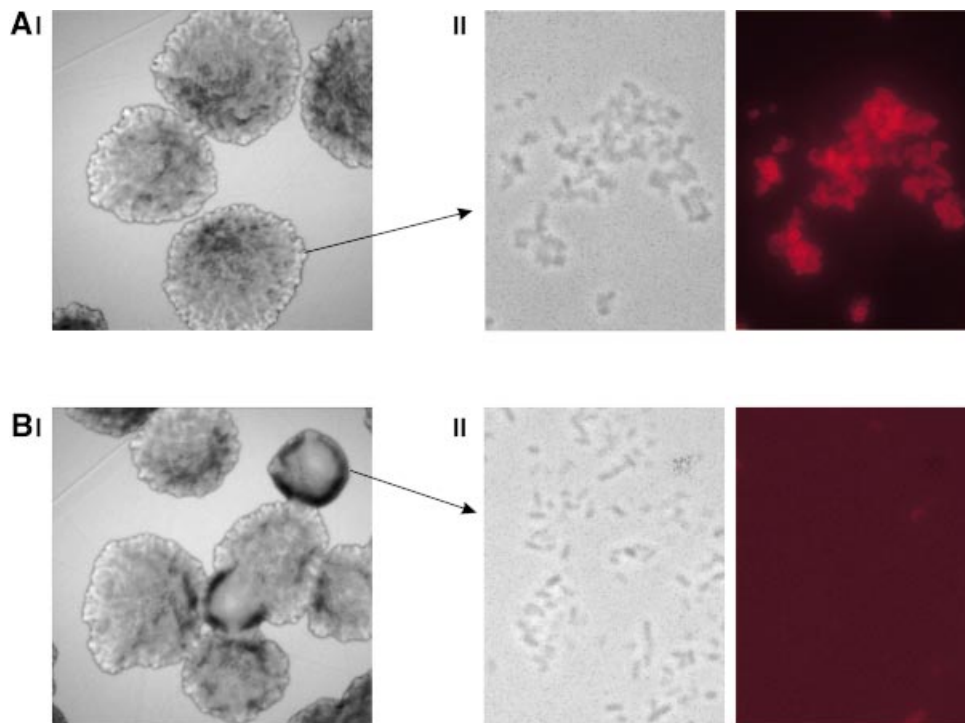


Fig. 4. Colony morphology and surface expression of Ag43 by MG1655 Δ *fim*(*pfimB*) in the absence (A) and presence (B) of the reducing agent DTT. (I) Phase contrast microscopy of colonies. (II) Phase contrast and immunofluorescence microscopy of cells employing anti-Ag43 serum. Exposure of cells from Ag43-positive (form 1) colonies to DTT resulted in ~10 times the number of Ag43-negative (form 3) colonies.

Thiol–disulfide signal transduction can be driven by simple reducing agents

In our model, fimbrial biosynthesis and the accompanying disulfide bridge formation influence the cellular thiol–disulfide status and drive it toward a more reduced state with a simultaneous OxyR^{ox}→OxyR^{red} shift causing down-regulation of the *flu* gene. In line with this, we speculated whether simple reducing agents could influence the cellular thiol–disulfide status in a similar way and influence Ag43 production. As previously mentioned, expression of Ag43 is phase variable; Ag43 production results in a characteristic frizzy colony morphology (Hasman *et al.*, 2000). MG1655 Δ *fim*(*pfimB*) can give rise to two different colony morphology forms: form 1 (Ag43⁺) with a frizzy surface and form 3 (Ag43⁻) with a smooth surface. We speculated that it would be possible to influence Ag43 expression by altering the cellular thiol–disulfide status by adding a simple reducing agent such as dithiothreitol (DTT) to the cells. This would, albeit crudely, mimic production of disulfide-containing fimbriae with an OxyR^{ox}→OxyR^{red} shift and might cause detectable down-regulation of the *flu* gene. Aliquots of cells from a form 1 colony were plated out on either normal LB plates or LB plates containing DTT. Progeny on normal LB plates were virtually all of form 1; however, ~10% (104/953) of the colonies on DTT plates were form 3 (Figure 4). Form 1 and form 3 colonies were verified to consist almost entirely of Ag43⁺ and Ag43⁻ cells, respectively (Figure 4). This result suggests that Ag43 production can indeed be influenced by manipulation of the redox potential of the growth medium. Also, it lends strong credence to our model invoking biosynthesis of disulfide-containing surface structures such as

fimbriae to constitute a signal that alters the cellular thiol–disulfide status, thereby affecting OxyR and, in turn, Ag43 production.

Discussion

Escherichia coli is the most frequent cause of urinary tract infection (UTI), and most isolates are able to express several different adhesins that mediate specific binding to the uroepithelium with different receptor specificities. The most common fimbriae on UTI strains are type 1 and P fimbriae, and >80% of the strains are able to express either or both fimbrial types (Nowicki *et al.*, 1984; Gander and Thomas, 1987); ~8% are able to express F1C fimbriae (Pere *et al.*, 1987). Expression of type 1, P and F1C fimbriae is phase variable, although the underlying regulatory systems are completely different; more specifically, type 1 fimbriae expression is based on DNA inversion, whereas phase variation of P fimbriae depends on a combination of Dam methylation and the PapB and PapI regulators (Forsman *et al.*, 1989; Braaten *et al.*, 1994). F1C expression is similar to that of P fimbriae. Interestingly, expression of P and type 1 fimbriae seems to be coordinated through inter-system cross-talk via PapB in which P fimbriation dominates (Xia *et al.*, 2000). The expression of the Ag43 self-recognizing adhesin is also phase variable through the concerted action of Dam methylase and OxyR. The present results add another dimension of expressional coordination and molecular cross-talk among these phase-variable adhesins.

Our data indicate that production of disulfide-containing fimbriae in *E. coli* constitutes an *en bloc* signal that causes repression of the Ag43-encoding *flu* gene via a novel

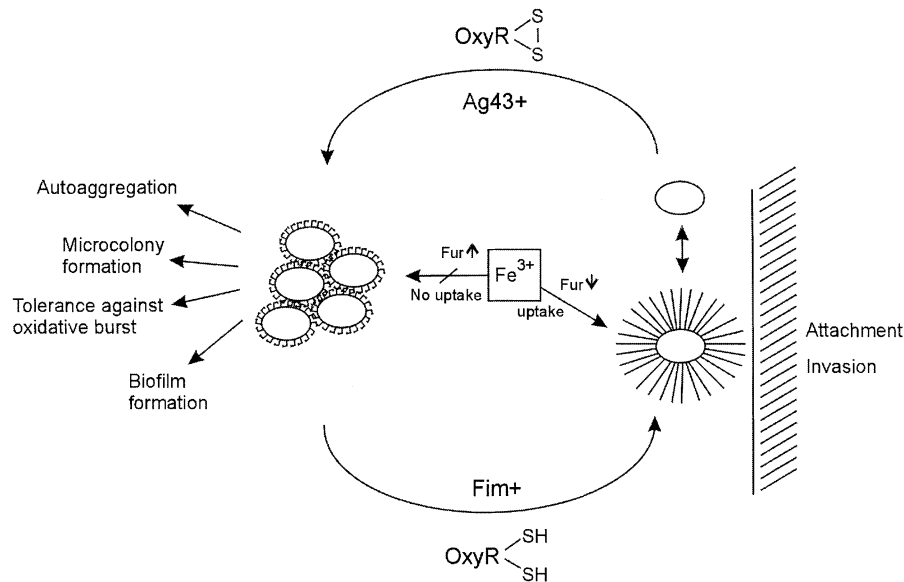


Fig. 5. Model describing the thiol–disulfide-induced niche-specific adaptation of *E.coli* in host environments. The expression of fimbriae and Ag43 leads to diametrically opposed virulence-associated cellular processes.

pathway invoking the thiol–disulfide status of OxyR: in effect, a one-directional signalling system where fimbriation dominates. From a cellular economy perspective, this makes sense because fimbrial display has been shown to abrogate the Ag43-mediated cell aggregating function (Hasman *et al.*, 1999). Arguably, there is no reason to produce copious amounts of Ag43 when the associated cell–cell aggregating phenotype is blocked. Production of fimbriae is phase variable and affected by various environmental cues (Klemm, 1994). Fimbriae confer specific adhesion to receptors on mammalian tissue surfaces, and many strains are able to express several different types of fimbriae. On the other hand, Ag43 expression is involved intimately in bacterial aggregation, and microcolony and biofilm formation (Danese *et al.*, 2000; Kjærgaard *et al.*, 2000; O’Toole *et al.*, 2000). Bacterial biofilms constitute an efficient protective state against host defences (Costerton *et al.*, 1999).

The on↔off phase-variable expression of type 1 fimbriae, P fimbriae and Ag43 might appear at first sight as random phenomena but are now known to be influenced by various environmental cues. Here we report that these processes are highly coordinated with a hierarchic structure: P fimbriation is dominant to type 1 fimbriation and fimbriation in general is dominant to Ag43 expression. The fimbrial phase on allows bacterial attachment to various epithelial targets, and the differential expression of type 1 and P fimbriae through inter-system cross-talk allows differential target and niche selection. The fimbrial phase off allows expression of Ag43, which enhances microcolony and biofilm formation. Finally, the Ag43 phase off allows bacterial dispersal and might lead to colonization of additional sites, thus completing the cycle (Figure 5).

It has not escaped our attention that the fimbriate status of the cell ($Fim^+ \leftrightarrow Fim^-$) can influence a wide range of cellular parameters via the outlined OxyR-mediated signal transduction mechanism, allowing rapid adaptation to new environmental niches (Figure 5). In this respect, it is

noteworthy that the oxidized (disulfide) form of OxyR is known to activate the expression of several genes involved in the oxidative stress response (Zheng *et al.*, 1998; Storz and Imlay, 1999). Leukocytes are known to produce oxidative bursts upon bacterial attachment; however, OxyR-activated gene products such as the KatG catalase are ideal to counter this oxidative threat. Furthermore, the oxidized form of OxyR activates global regulators such as *fur* (Zheng *et al.*, 1999). Free iron is limited in mammalian body fluids, but bacteria have evolved efficient systems for iron uptake (Guerinot, 1994). Fur is the global cellular repressor of ferric ion uptake from the environment (Crosa, 1997). A fimbriated phenotype, promoting the reduced state of OxyR, would be concomitant with reduced activity of the *fur* gene and derepression of Fe^{3+} uptake systems. Interestingly, the present fimbriae–OxyR signal transduction mechanism might be invoked to explain the reported enigmatic influence of P fimbriation on iron uptake in *E.coli* (Zhang and Normark, 1996). Disulfide-containing fimbriae are produced by a wide range of bacteria and OxyR is ubiquitous in bacteria. The present findings might therefore have implications for our general understanding of bacterial signal transduction in environments such as the human body.

Materials and methods

Bacterial strains and plasmids

Escherichia coli K-12 reference strain MG1655 was used in this work. A Δfim derivative of MG1655 was constructed using the temperature-sensitive plasmid pMAS115 (Kjærgaard *et al.*, 2000). Plasmid pFimB (alias pPKL9) contains the *fimB* recombinase gene under transcriptional control of the tetracycline gene promoter (Klemm, 1986) and was used to ensure ‘on’ orientation of the *fim* switch. Plasmids pPKL4 (type 1 fimbriae), pPAP5 (P fimbriae) and pPKL143 (F1C fimbriae) have been described previously (Lindberg *et al.*, 1984; Klemm, 1986; Klemm *et al.*, 1994). Plasmid pMAS123 contains the *E.coli* *fliHDC* flagellar master control genes placed under transcriptional control of the inducible arabinose promoter in the expression vector pBADHisA.

Global gene expression analysis

Cells from glycerol stocks were grown overnight in LB broth at 37°C with shaking. These cultures were then diluted 1:100 and grown under the same conditions to an OD₆₀₀ of 0.9. Cells were harvested and RNA was extracted using Qiagen RNeasy columns. Enrichment of mRNA and microarray analysis were performed according to the Affymetrix Expression Analysis Technical Manual. The *E.coli* GeneChip microarrays were purchased from Affymetrix, Santa Clara, CA. Microarray experiments demonstrating the negative effect of type 1 fimbriation on Ag43 expression and the overexpression of Ag43 in the absence of type 1 fimbriae were demonstrated in two independent experiments and essentially the same results were obtained.

Fluorescence microscopy

Cells were grown as described above. In the case of induction for flagella production, cells were grown to an OD₆₀₀ of 0.4 and then induced for 4 h with 0.2% arabinose. Antibodies against type 1 fimbriae, P fimbriae, F1C fimbriae, Ag43 and flagella were employed to assess surface presentation of corresponding organelles/proteins by immunofluorescence microscopy. As secondary antibody, a fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-labelled anti-rabbit or mouse serum was used. Cell fixation, immunolabelling and microscopy were performed as previously described (Hasman *et al.*, 1999).

DDT induction of Ag43 phase variation

Ag43-expressing (form 1) colonies of MG1655Δ*fim*(p*fimB*) were resuspended in phosphate-buffered saline (PBS) and diluted to a concentration of 10² c.f.u./ml. The cells were incubated in the presence of 100 mM DTT at room temperature for 30 min and then plated onto LB agar plates containing DTT. These plates were prepared by spreading 100 μl of a 1 M DTT solution onto the surface of the plate just prior to the addition of bacteria. Plates were then incubated overnight at 37°C. As a control, Ag43-expressing colonies were subjected to exactly the same treatment but in the absence of DTT. Colony morphology was viewed with a Carl Zeiss Axioplan epifluorescence microscope and digital images were captured with a 12-bit cooled slow-scan charge-coupled device camera (KAF 1400 chip; photometrics; Tuscon, AZ) controlled by the PMIS software (Photometrics).

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