Antigen-43-mediated autoaggregation impairs motility in *Escherichia coli*

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Functional interaction between bacterial surface-displayed autoaggregation proteins such as antigen 43 (Ag43) of *Escherichia coli* and motility organelles such as flagella has not previously been described. Here, it has been demonstrated for the first time that Ag43-mediated aggregation can inhibit bacterial motility. Ag43 overexpression produces a dominant aggregation phenotype that overrides motility in the presence of low levels of flagella. In contrast, induction of an increased flagellation state prevents Ag43-mediated aggregation. This phenomenon was observed in naturally occurring subpopulations of *E. coli* as phase variants expressing and not expressing Ag43 revealed contrasting motility phenotypes. The effects were shown to be part of a general mechanism because other short adhesins capable of mediating autoaggregation (AIDA-I and TibA) also impaired motility. These novel insights into the function of bacterial autoaggregation proteins suggest that a balance between these two systems, i.e. autoaggregation and flagellation, influences motility.

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INTRODUCTION

The ability of bacteria to colonize and cause infection is often linked to the expression of specific surface structures. Examples include fimbriae that mediate attachment, flagella that mediate motility, aggregation factors that mediate cell clumping, and a capsule, which provides protection against phagocytosis and host defences. Although the function of these surface structures is often antagonistic, very little is understood regarding the molecular interactions that contribute to their contrasting phenotypes.

Antigen 43 (Ag43) is an autotransporter (AT) protein of *Escherichia coli* that promotes bacterial cell-to-cell aggregation (Diderichsen, 1980; Hasman *et al.*, 1999; Henderson & Owen, 1999; Owen, 1983). It can be expressed on the *E. coli* cell surface in very high numbers (up to 50 000 copies per cell), resulting in a characteristic frizzy colony morphology (Hasman *et al.*, 2000; Henderson & Owen, 1999; Owen, 1992). Ag43-mediated aggregation is a distinct phenotype that can be visualized macroscopically as flocculation and settling of cells in static liquid suspensions. The protein belongs to the AIDA group of AT proteins and exhibits a high degree of homology to several other members of the AT protein family, namely the AIDA-I adhesin involved in diffuse adherence of enteropathogenic *E. coli* and the TibA adhesin of enterotoxigenic *E. coli* (Sherlock *et al.*, 2004, 2005). Ag43 is cleaved into two subunits (α and β), each constituting roughly half of the protein. The β

subunit is believed to be an outer-membrane pore-forming component through which Ag43^z gains access to the bacterial surface. The α subunit, on the other hand, remains attached to the bacterial cell surface via interactions with the β subunit (Henderson *et al.*, 2004). The expression of Ag43 is phase-variable with switching rates of $\sim 10^{-3}$ per cell per generation due to the concerted actions of Dam methylase (positive regulation) and OxyR (negative regulation) (Henderson & Owen, 1999; Schembri & Klemm, 2001; Schembri *et al.*, 2003a; Waldron *et al.*, 2002).

Ag43 dramatically enhances biofilm formation (Danese *et al.*, 2000; Kjaergaard *et al.*, 2000b; Reisner *et al.*, 2003) and is specifically correlated with the biofilm mode of growth (Schembri *et al.*, 2003b). Recent work has demonstrated that Ag43 expression is correlated with biofilm formation by uropathogenic *E. coli* during infection of bladder cells (Anderson *et al.*, 2003) and in enteropathogenic *E. coli* (Torres *et al.*, 2002). Mutations that destroy the function of the gene encoding Ag43 (i.e. *flu*) affect biofilm formation (Reisner *et al.*, 2003; Schembri *et al.*, 2003b). Ag43-mediated clumping also protects bacteria against killing agents like hydrogen peroxide (Schembri *et al.*, 2003a).

In contrast to autoaggregation, motility contributes to colonization of different environmental niches by facilitating the spread of the infecting agent (Josenhans & Suerbaum, 2002). Different modes of surface colonization have been described and are linked to the expression of organelles that allow organisms to swim, swarm and dart. Flagella and type IV pili are prominent organelles that contribute to motility

Abbreviations: Ag43, antigen 43; AT, autotransporter.

(Bardy *et al.*, 2003; Harshey, 2003). Co-ordinated expression of these organelles is achieved by tightly regulated signalling systems that involve the various genes encoding them (up to 50 genes contribute to *E. coli* flagella biosynthesis), which are, in some cases, coupled to quorum sensing systems (Harshey, 2003; Soutourina & Bertin, 2003). Environmental conditions, including moisture, temperature and nutrient availability, affect motility, and unique peptides or polysaccharides are thought to signal transcriptional activation of motility organelles in some species (Harshey, 2003). The master regulator that controls expression of flagella in *Enterobacteriaceae* is *flhDC* (Eberl *et al.*, 1996; Macnab, 1992; Soutourina & Bertin, 2003).

Autoaggregation mediated by Ag43 and motility appear to play distinct roles in bacterial colonization, survival and spread. However, although autoaggregation and motility are opposing phenotypes predicted to act in an antagonistic manner, functional interactions between aggregation factors and motility organelles have hitherto not been studied. Here, we investigated the dynamics that occur between Ag43 and flagella and demonstrate that autoaggregation driven by Ag43 (and other homologous AT proteins, including AIDA-I and TibA) impairs bacterial motility. Naturally occurring phase variants of *E. coli* that overexpress Ag43 display a severe reduction in motility, suggesting that a balance between these contrasting phenotypes may contribute to the control of bacterial movement.

METHODS

Bacterial strains, plasmids and DNA manipulations. *E. coli* K-12 reference strain MG1655 was used in this work. Additional strains

and plasmids are listed in Table 1. Bacteria were grown from glycerol stocks overnight on Luria–Bertani (LB) agar and in LB broth at 37 °C with shaking unless otherwise stated. Strains containing expression plasmids with an inducible promoter were grown in media supplemented with IPTG as indicated. Ampicillin (100 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) were used to maintain plasmids. DNA purifications, PCR, cloning and transformations were performed according to methods described elsewhere (Sambrook & Russell, 2001). Plasmid purification columns were purchased from Qiagen (Australia) and restriction enzymes from New England Biolabs (Canada).

Semi-solid agar assay for assessment of motility. To evaluate motility in different strains, overnight cultures were prepared in LB broth and resuspended to an OD₆₀₀ of approximately 0.35 (BioSpecmini; Shimadzu). One microlitre of suspension was spotted onto the centre of a fresh 0.25 % LB agar plate (*n*=4), supplemented with the appropriate inducer and antibiotic, using a sterile graded inoculation loop. Plates were incubated at 37 °C for 16–24 h and motility was determined by measuring the distance between the centre of the agar and the leading edge of bacterial growth. Data are shown as the mean diameter (mm) of movement ± SEM for at least three independent experiments.

Transmission electron microscopy (TEM). TEM was used to detect flagella on bacteria grown on semi-solid agar and in broth. A 10 µl bacterial cell suspension (OD₆₀₀ ~ 2.0) was adsorbed onto a glow-discharged Formvar-coated copper grid. After 1 min the grid was washed carefully in sterile distilled water and visualized on a JEOL JEM 1010 transmission electron microscope operated at 80 kV. In each experiment, control strains not carrying relevant plasmids, or grown under the appropriate non-induced (versus induced) conditions, were included for comparison.

Suspension autoaggregation assay. To assess autoaggregation in different strains, we used the suspension clumping assay to monitor bacterial settling over time (Hasman *et al.*, 1999). Overnight LB cultures supplemented with the appropriate antibiotics and inducer were mixed well prior to the start of the assay. Fifty microlitre samples (*n*=4) were taken approximately 0.5 cm below the surface

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference
<i>E. coli</i> strains		
BD1428	K-12 reference strain	Diderichsen (1980)
MG1655	K-12 reference strain	Bachmann (1996)
MS426	MG1655 Δ <i>flhDC</i>	Reisner <i>et al.</i> (2003)
MS427	MG1655 Δ <i>flu</i>	Reisner <i>et al.</i> (2003)
MS641	MG1655 <i>oxyR::kan</i>	Schembri <i>et al.</i> (2003a)
MS680	STEC O26; Ag43 phase-off	This study
MS684	STEC O26; Ag43 phase-on	This study
MS1287	MG1655 Δ <i>motAB</i>	This study
Plasmids		
pFHC2235	<i>dam</i> gene from MG1655 in pUC19	Schembri <i>et al.</i> (2004)
pKKJ128	<i>flu</i> gene from MG1655 in pACYC184	Kjaergaard <i>et al.</i> (2000a)
pMG600	<i>flhDC</i> operon from <i>Serratia</i> in pVLT33	Givskov <i>et al.</i> (1995)
pOS33	<i>aidA</i> gene from pIB264 in pACYC184	Sherlock <i>et al.</i> (2004)
pRMV3	<i>tibA</i> gene from H10407 in pACYC184	Sherlock <i>et al.</i> (2005)
pKD46	λ -Red recombinase expression plasmid	Datsenko & Wanner (2000)
pCP20	FLP synthesis under thermal control	Cherepanov & Wackernagel (1995)

of the liquid cultures at 15 min intervals, transferred to a round bottom microtitre plate (TPP; Europe) and OD₆₀₀ was measured by a microtitre plate reader. Data are shown as the mean absorbance $\pm$ SD, where the degree of autoaggregation is inversely proportional to the turbidity.

Assessing Ag43-mediated autoaggregation as a function of flagella. To analyse whether a balance between Ag43-mediated autoaggregation and flagella expression might influence motility, we examined autoaggregation in the presence of increasing flagella expression. *E. coli* MG1655 was transformed with two plasmids: (i) pKKJ128 (constitutive Ag43 expression); and (ii) pMG600 (IPTG-inducible flagella expression) (Givskov *et al.*, 1995). The effect of increased flagella expression on the clumping activity of Ag43 was assessed by growth in the presence of increasing concentrations of IPTG followed by the autoaggregation assay as above. Similar experiments were performed using overnight liquid cultures inoculated onto the surface of semi-solid agar containing different concentrations of IPTG to study whether a balance between these expression states influences motility. TEM was used to assess flagella expression under the different growth conditions and immunoblotting was used to assess both Ag43 and flagella (FliC) levels.

Immunoblotting. To prepare immunoblots, 100 μ l of an overnight LB broth culture was boiled for 10 min and the protein concentration was determined by using the Bradford assay. Three micrograms of protein were mixed with SDS loading buffer, boiled for 10 min, cooled on ice and then separated on 12.5% Tris-SDS-PAGE gels run at 15 V cm⁻¹ for 60 min in Tris-SDS glycine running buffer, according to methods described in Sambrook & Russell (2001). Proteins were transferred to PVDF membranes (Biotrace) by use of a mini trans-blot electrophoretic apparatus (Bio-Rad) run at 100 V for 60 min. Blots were blocked overnight in 5% skim milk in 0.1% PBS-Tween 20 (PBST) and probed with rabbit anti-Ag43 (1:5000) and anti-flagellin (1:1000) antibodies diluted in 5% skim milk in PBST with 0.1% sodium azide for 1 h at room temperature. Blots were washed three times in PBST and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (1/10 000) for 1 h in 5% skim milk in PBST. Blots were washed three times in PBST and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma) dissolved in distilled water.

Construction of *E. coli* MG1655 Δ motAB mutant. Electro-competent cells of MG1655(pKD46) were prepared by growing the cells at 30 °C in ampicillin-SOB medium followed by washing of the cells extensively in ice-cold 10% glycerol in distilled water (Sambrook & Russell, 2001). Primers MOTABF (5'-TGCAAAATGGCCTGTT-CGGCTTGTGTTTCAGTACCAGCAGGCTGATGCGGTGTAGGCT-GGAGCTGCTTC-3') and MOTABR (5'-GTATATGGATCTGCTGG-CTCTGCTTTATCGGTTGATGGCGAAATCGCGGCCATATGAAT-ATCCTCCTTA-3') were used to amplify a 1.6 kb PCR product from plasmid pKD4, representing the kanamycin resistance cassette from pKD4 and additional 50 bp overhang regions (underlined) at the 5' and 3' ends of the PCR product complementary to the *motA* and *motB* genes in *E. coli* MG1655. The PCR product was purified using a Qiagen PCR clean-up column and transformed into competent MG1655(pKD46). MG1655 Δ motAB transformants carrying the kanamycin cassette on the chromosome in place of *motAB* were selected on kanamycin plates at 37 °C and screened by PCR using kt and k2 primers as described by Datsenko & Wanner (2000), as well as MOTSCF (5'-TCCATGTACGCCAACAGTTCGTCC-3') and MOTSCR (5'-AGCCTTGAGCACTCTATCAACCC-3') in various combinations. The kanamycin cassette was removed from the chromosome of MG1655*motAB::kan* using plasmid pCP20 as described by Datsenko & Wanner (2000). Plasmids pMG600 and pKKJ128 were then introduced into MG1655 Δ motAB by heat shock

to give MG1655 Δ motAB(pMG600), MG1655 Δ motAB(pKKJ128) and MG1655 Δ motAB(pMG600, pKKJ128) which were maintained on kanamycin, chloramphenicol and both antibiotics, respectively.

Assessment of autoaggregation and motility in natural *E. coli* phase variants. Wild-type phase variants of *E. coli* BD1428 and MS680 were selected from LB agar plates based on morphological differences arising from differential expression of Ag43 (see Fig. 6). These colony variants (five of each) were inoculated into 5 ml LB broth, grown overnight at 37 °C, and 10 and 1 μ l samples were used to inoculate 5 ml LB broth for the suspension autoaggregation assay, and semi-solid LB agar for the swimming motility assay, respectively. Data are shown as the mean values $\pm$ SEM ($n=5$) for one independent experiment representative of several.

Statistics. Motility between different strains was compared using the non-parametric Kruskal-Wallis test (SPSS v9.0; SPSS Inc.). *P* values < 0.05 were considered significant. Aggregation was compared using Student's *t*-test.

RESULTS

An *E. coli* oxyR mutant is non-motile

Several studies have shown that mutation or deletion of the *E. coli* stress response regulator gene *oxyR* results in over-expression of Ag43 (Haagmans & van der Woude, 2000; Hasman *et al.*, 1999; Henderson & Owen, 1999; Schembri & Klemm, 2001; Schembri *et al.*, 2003a). As a result, *E. coli* *oxyR* mutants display a strong clumping phenotype. During further characterization of an *E. coli* K-12 *oxyR* mutant (MS641) (Schembri *et al.*, 2003a), we observed that it displayed altered motility characteristics. This prompted us to perform a detailed comparison of the motility phenotype between MS641 and its wild-type (WT) parent strain MG1655. MS641 displayed an acute impairment in swimming ability (Fig. 1a–c) that correlated with increased Ag43-mediated clumping (Fig. 1d). This observation was not strain-dependent since several independently constructed *E. coli* K-12 *oxyR* mutants at our disposal also displayed this phenotype (data not shown). These data implied that the severely impaired motility of MS641 may be due to an abundance of the Ag43 autoaggregator at the bacterial surface. However, because OxyR is a global regulator that affects the activity of numerous bacterial expression pathways, further experiments were performed to exclude extraneous OxyR affects.

Enhanced Ag43 expression through Dam methylation impairs motility

To circumvent interference from OxyR-regulated factors separate from Ag43, we employed the Dam-methylation plasmid pFCH2235. This plasmid contains the *E. coli* *dam* gene cloned directly downstream of the IPTG-inducible *lacZ* promoter. Overexpression of the Dam methylase after IPTG induction results in methylation at the GATC sites located within the *flu* gene promoter and this prevents repression by OxyR (Haagmans & van der Woude, 2000; Wallecha *et al.*, 2002). In *E. coli* MG1655 transformed with pFCH2235, motility was completely ablated, in contrast to the rapid

Fig. 1. Ag43 over-expressing OxyR-deficient *E. coli* MG1655 displays impaired swimming motility compared to the wild-type parent strain. Comparisons between strains were made using a semi-solid agar motility assay. MG1655 is highly motile (a) compared to strain MS641 (b) which lacks functional *oxyR* and therefore produces abundant amounts of the autoaggregator Ag43. The abatement of motility in MS641 is shown in (c: ▲, MS641; ○, MG1655), which is predicted to result from the proficient clumping activity of Ag43 (d).

motility of MG1655 (Fig. 2a). This effect was contingent upon the presence of IPTG, demonstrating the requirement for Dam methylation. This observation supported the notion that impaired motility in MS641 is due to overexpression of Ag43 rather than alternative OxyR-regulated affects. MG1655(pFHC2235) cells at the periphery of growth readily expressed flagella (TEM data not shown).

Fig. 2. Dam methylation in *E. coli* MG1655 enhances Ag43 expression and impedes motility. Transformation of plasmid pFHC2235 containing an IPTG-inducible *dam* methylase gene into *E. coli* MG1655 ablated motility in this strain as compared to the characteristic rapid swimming motility of MG1655. ■, MG1655(pFHC2235); △, MG1655

Ag43-mediated inhibition of motility is independent of regulatory systems

To confirm whether Ag43 directly and specifically acts as an inhibitor of motility, we used the Ag43 constitutive expression plasmid pKKJ128 (Kjaergaard *et al.*, 2000a, b). pKKJ128 was transformed into the previously characterized MG1655Δ*flu* strain MS427 (Reisner *et al.*, 2003), which lacks the ability to express Ag43. When MS427(pKKJ128) was grown on 0.25 % agar it was completely impaired in its ability to swim (Fig. 3), consistent with the notion that Ag43-mediated aggregation prevents motility. Furthermore, since the *flu* gene is constitutively transcribed by the tetracycline gene promoter in pKKJ128, Ag43-mediated inhibition of motility occurs completely independently of the regulatory mechanisms that control Ag43 expression (i.e. OxyR repression and Dam methylation). Taken together, these data demonstrate that, under these growth conditions, Ag43 overexpression causes a dominant aggregation phenotype that impedes motility.

Ag43 homologues TibA and AIDA-I also impair motility

The AIDA-I and TibA AT proteins display approximately 25 % amino acid identity to Ag43, possess similar functional characteristics and promote cell-to-cell aggregation (Sherlock *et al.*, 2004, 2005). To investigate whether these proteins also impair motility in a manner similar to Ag43, we analysed MG1655 transformants containing plasmids leading to the constitutive overexpression of TibA or AIDA-I. Overexpression of TibA and AIDA-I significantly

Fig. 3. Ag43 directly functions as a surface antagonist of motility. *E. coli* MS427(pKKJ128) over-expresses Ag43 and is non-motile in comparison to the control strain MS427(pACYC184), which lacks the ability to express Ag43 (a). ▲, MS427+Ag43; ○, MS427. The inability of MS427(pKKJ128) to swim correlated with an inability to increase expression of flagella, whereas the parent strain MS427 expressed abundant flagella at the outer swimming edge of growth (b).

impaired motility (Fig. 4), an observation consistent with our findings with Ag43.

Increased flagellation can counteract Ag43-mediated inhibition of motility

Based on these observations, we hypothesized that a balance exists between flagellation and autoaggregation and this might influence motility via the dominance of one phenotype over the other. We therefore investigated whether overexpression of flagella could counteract the ability of Ag43 to inhibit motility. A system of controlled flagella expression, as described by Givskov *et al.* (1995), was used in which the master regulator *flhDC* genes are cloned behind an IPTG-inducible promoter on the pVLT33

Fig. 4. The self-aggregating proteins TibA and AIDA-I function as surface antagonists of bacterial motility. Ag43-deficient *E. coli* MG1655 was transformed with pRMV3 (TibA) or pOS33 (AIDA-I) and the resultant strains were significantly less motile compared to the parent strain MS427(pACYC184). ■, MS427+AIDA-I; ◇, MS427+TibA; ○, MS427.

plasmid (pMG600). Using this system, we examined motility of *E. coli* MG1655 containing pMG600 in the presence of increasing amounts of IPTG (i.e. corresponding to increasing amounts of flagella), and *E. coli* MG1655 containing both pMG600 and pKKJ128 for constitutive expression of Ag43. Few flagella were detected in *E. coli* MG1655(pMG600) grown without supplemental IPTG as assessed by TEM, while increased flagellation was observed with increasing concentrations of IPTG (Fig. 5a). Semi-solid agar motility assays demonstrated that impaired motility in MG1655(pKKJ128, pMG600) could be reversed when 10 μ M IPTG was added to the medium to increase flagellation (Fig. 5b).

Increased flagellation also inhibits autoaggregation

Ag43 belongs to a class of short non-organelle-type adhesins and thus aggregation requires close cell–cell contact. To assess whether flagella expression interfered with Ag43-mediated cell–cell aggregation, we investigated if overexpression of flagella could counteract the ability of Ag43 to mediate cell clumping. MG1655(pMG600) transformants containing pKKJ128 clumped strongly compared to the control strain MG1655(pMG600) (data not shown). In conditions of low flagella expression in MG1655(pKKJ128, pMG600), the clumping phenotype mediated by Ag43 could not be overcome (Fig. 5c). However, increased flagellation (100 μ M IPTG) counteracted Ag43-mediated autoaggregation (Fig. 5c). The difference in aggregation was not related to the level of Ag43 expression, since immunoblot analysis demonstrated equivalent amounts of Ag43 protein in conditions of increased flagella synthesis (Fig. 5d). Flagella overexpression also counteracted the autoaggregation phenotype mediated by AIDA-I (plasmid pOS33) and TibA (plasmid pRMV3) (data not shown). Taken together,

Fig. 5. Increased flagellation can counteract Ag43-mediated inhibition of motility and auto-aggregation. (a) TEMs of MG1655(pKKJ128, pMG600) grown in the presence of increasing concentrations of IPTG for increasing flagella expression. Bars, 2 μm. (b) Motility assay demonstrating that MG1655(pKKJ128) was completely non-motile in contrast to MG1655(pMG600) when grown in 10 μM IPTG and assessed at 16 h. Comparison with MG1655(pKKJ128, pMG600) demonstrates that increased flagellation can counteract the inhibition of motility in MG1655(pKKJ128) brought about by overexpression of Ag43. Representative plates corresponding to MG1655(pKKJ128), MG1655(pMG600) and MG1655(pMG600, pKKJ128) are shown on the right. (c) Settling assay demonstrating the effect of increased flagellation on auto-aggregation mediated by Ag43 in MG1655(pKKJ128, pMG600). Low level flagella expression (0.01 μM IPTG, ■) did not affect autoaggregation, whereas increased flagellation (100 μM, ○) impaired Ag43-mediated clumping. (d) Immunoblot analysis demonstrating equivalent levels of expression of Ag43 in MG1655(pMG600, pKKJ128) regardless of the level of flagella expression, which is demonstrated to increase in the presence of increasing concentrations of IPTG.

these data demonstrate that under these growth conditions increased flagellation can counteract autoaggregation driven by surface structures such as Ag43.

The above data can be interpreted in two ways: (i) increased flagellation results in physical shielding of the intimate contact required for Ag43–Ag43 aggregation, or (ii) flagella-mediated motility prevents Ag43–Ag43 interactions as a result of dynamic bacterial cell movement. To test these hypotheses, we constructed a non-motile but flagellated mutant by insertional inactivation of the *motAB* genes (MS1287). When MS1287 was transformed with plasmids pMG600 (*flhDC*⁺) and pKKJ128 (*flu*⁺) and grown under flagella-inducing conditions, it did not aggregate (data not

shown), suggesting that physical interference via induction of hyper-flagellation is able to prevent Ag43-mediated aggregation. We note that this does not preclude the possibility that flagella-driven bacterial movement may also reduce the efficiency of Ag43-mediated aggregation.

Ag43-mediated inhibition of motility occurs in natural phase-variant subpopulations of *E. coli* K-12

Ag43 expression is phase-variable and subpopulations of phase-on and phase-off cells can be identified by distinct differences in colony morphology. The *E. coli* reference strain BD1428 (alias X474) undergoes colony morphology

phase variation switching that gives rise to several different morphotypes (Hasman *et al.*, 2000). Two of these morphotypes are the direct result of Ag43 switching: (i) flat frizzy colonies caused by Ag43 overexpression (i.e. Ag43 phase-on cells), and (ii) flat irregular-shaped colonies with

Fig. 6. Phase variation of Ag43 expression gives rise to naturally occurring sub-populations of *E. coli* with different motility phenotypes. Colony morphology (a), settling assay (b) and motility assay (c) of Ag43 phase-on (▲) and phase-off (□) *E. coli* K-12 BD1428 cells. Ag43-expressing phase-on colonies aggregate and are non-motile, in contrast to their phase-off counterparts which do not aggregate and are highly motile. (d) A wild-type STEC O26 strain selected for phase-off (MS680, ○) and phase-on (MS684, ■) Ag43 expression displayed similar results in aggregation (left panel) and motility (right panel), i.e. Ag43-expressing phase-on STEC O26 aggregate and are non-motile, in contrast to their Ag43 phase-off counterparts, which do not aggregate and are highly motile.

a smooth surface (i.e. Ag43 phase-off cells) (Fig. 6a). To investigate whether the described interactions between autoaggregation and motility occur in natural phase-variant subpopulations of *E. coli*, we examined the motility of selected BD1428 colonies representing the Ag43 phase-on and Ag43 phase-off morphotypes. The Ag43 phase-on and phase-off variants exhibited profound differences in their ability to autoaggregate and swim in the semi-solid agar-motility assay. On the one hand, Ag43 phase-off cells did not aggregate (Fig. 6b) and were highly motile in the semi-solid agar swimming assay (Fig. 6c). Ag43 phase-on cells, however, which overexpress Ag43 (Fig. 6a), clumped strongly (Fig. 6b) and were entirely non-motile (Fig. 6c).

Ag43 phase variants of a wild-type *E. coli* O26 also exhibit differences in motility

To demonstrate if the above results are relevant to wild-type *E. coli*, we selected out phase-on and phase-off Ag43-expressing colonies from a wild-type Shiga toxin-producing *E. coli* (STEC) O26 strain. Ag43 phase-off (MS680) and phase-on (MS684) cells were selected by differences in colony morphology and Ag43 expression was verified by Western blotting with Ag43-specific antiserum (data not shown). In agreement with the results for the K-12 strain BD1428, Ag43 phase-off cells were motile while Ag43 phase-on cells were non-motile (Fig. 6d). These results illustrate that natural phase variation in Ag43 expression directly affects bacterial motility and that this phenomenon occurs naturally in subpopulations of *E. coli*.

DISCUSSION

Bacteria express numerous surface structures that enable them to interact with and survive in changing environments, e.g. flagella to swim and adhesins for attachment to surfaces. These surface components have highly diverse structures, and it must be implicit that they physically interfere with each other in such a way that the activity of one surface structure may sometimes be obstructed by the presence of another. We have previously demonstrated, for example, that both fimbriae and capsule physically block the actions of the considerably shorter Ag43 adhesin in *E. coli* (Hasman *et al.*, 1999; Schembri *et al.*, 2004). Interference between

bacterial surface structures is likely to have important consequences on the interplay between these organisms and their environment, including host–pathogen interactions. Here, we have systematically studied the interactions between these traits (i.e. autoaggregation and motility) and have demonstrated for the first time that bacterial autoaggregation, a phenomenon mediated by Ag43 and other homologous AT proteins, inhibits bacterial motility.

Many bacteria, including *Bordetella pertussis* (Menozzi *et al.*, 1994), *Mycobacterium tuberculosis* (Menozzi *et al.*, 1996), *Staphylococcus aureus* (McDevitt *et al.*, 1994) and *Streptococcus pyogenes* (Frick *et al.*, 2000), form aggregates. This confers an ability to resist various host defences, e.g. complement attack and phagocytosis (Berge *et al.*, 1997; Ochiai *et al.*, 1993), which supports the notion that bacterial autoaggregation is an important virulence mechanism. Formation of aggregates usually takes place through autoaggregation of cells. In some cases, the underlying molecular mechanism is known, and self-recognizing surface proteins are often responsible for autoaggregation. A well-studied example is autoaggregation of *S. pyogenes*, which occurs through the intercellular interactions between pairs of protein H (Frick *et al.*, 2000). Several different systems are independently capable of conferring autoaggregation in *E. coli*. Apart from the well characterized Ag43, AIDA-I and TibA AT proteins, a recent analysis of *E. coli* K-12 revealed several additional cryptic AT-encoding genes whose products are able to mediate aggregation (Roux *et al.*, 2005). Other mechanisms of cell aggregation have also been described. Curli, for example, are extracellular surface organelles formed by the precipitation of secreted soluble subunit proteins into thin fibres (Hammar *et al.*, 1996). Autoaggregation involves intercellular fibre precipitation mediated by a nucleator protein. Bundle forming pili (BFP), on the other hand, are a type IV class of fimbriae produced by enteropathogenic *E. coli* that emanate from the cell surface and align along their longitudinal axes to form bundles of filaments (Bieber *et al.*, 1998; Giron *et al.*, 1991). These are long, flexible, rope-like structures composed almost exclusively of a single repeating structural subunit referred to as BfpA (Giron *et al.*, 1991). Expression of BFP mediates two phenotypes thought to play a role in colonization: autoaggregation in liquid cultures and localized adherence on tissue culture cell monolayers (Bieber *et al.*, 1998). In enteroaggregative *E. coli*, two fimbrial types designated aggregative adherence fimbriae I and II (AAF/I and AAF/II) have been identified (Czczulin *et al.*, 1997; Nataro *et al.*, 1992). These flexible 2–3 nm wide structures mediate prominent autoagglutination of bacterial cells to each other (Nataro *et al.*, 1987; Nataro & Kaper, 1998). Variants of the FimH adhesin of type 1 fimbriae also promote cell aggregation (Schembri *et al.*, 2001; Schembri & Klemm, 2001). Contrary to organelle-mediated aggregation systems, AT proteins are anchored directly to the outer cell membrane. Thus, aggregation mediated by AT proteins results in a more intimate cell–cell contact compared to systems where the intercellular interactions are based on

polymeric structures that protrude further from the bacterial surface, i.e. fimbriae and curli.

Several lines of evidence from the current study strongly suggest that bacterial autoaggregation interferes with motility: (i) *E. coli oxyR* mutants are non-motile, (ii) over-expression of the Dam methylase in *E. coli* relieves OxyR-mediated repression of Ag43 and prevents motility, (iii) constitutive expression of Ag43 prevents motility, (iv) the Ag43 homologues AIDA-I and TibA interfere with motility, and (v) *E. coli* cells expressing Ag43 in a ‘phase-on’ or ‘phase-off’ state exhibit striking non-motile and motile phenotypes, respectively. Here, the link between autoaggregation and motility is shown as a function of Ag43 phase variation where *E. coli* subpopulations can convert from a motile to non-motile phenotype.

Ag43 is predicted to protrude approximately 10 nm from the *E. coli* cell surface. The protein is present in up to 50 000 copies in K-12 strains and current evidence suggests it is localized around the entire cell surface. Our results suggest a model whereby Ag43-mediated aggregation prevents motility when flagella are present in low numbers (associated with a swimming phenotype). Increased flagellation may create a physical barrier that prevents the intimate contact required for Ag43–Ag43 interaction. This interference is consistent with the inhibition of Ag43-mediated aggregation by other surface structures (Hasman *et al.*, 1999; Schembri *et al.*, 2004). Alternatively, cell movement mediated by expression of flagella could prevent the intimate contact necessary for intercellular adhesion to become established by efficient homotypic Ag43 interactions. We tested the later hypothesis by constructing a *motAB* mutant in *E. coli* K-12. The products of *motA* and *motB* are required for flagella rotation, but do not contribute to the synthesis or export of flagella subunit proteins; thus the strain produces flagella, but is non-motile. Our results indicate that the induction of non-motile flagella is able to counteract Ag43-mediated aggregation, although this does not preclude the possibility that flagella-driven bacterial movement may also reduce the efficiency of Ag43-mediated aggregation. One of the implications of these observations is that any mechanism capable of mediating efficient cell–cell aggregation may affect motility.

Ag43 is widespread in *E. coli* and is expressed in many pathogenic strains. A survey of enteropathogenic and urinary tract infectious strains showed that 77 and 60 %, respectively, of these were capable of Ag43 expression (Owen *et al.*, 1996). Moreover, many strains possess duplex or multiple copies of the gene, as seems to be the case in enteropathogenic and enterohaemorrhagic subtypes (Klemm *et al.*, 2004; Roche *et al.*, 2001; Torres & Kaper, 2002). Functionally, Ag43 (and other homologous proteins) promotes bacterial adhesion and biofilm formation, both of which are closely associated with bacterial virulence. Flagella, on the other hand, are motility organelles that enable bacteria to move to different locations (e.g. for nutrient acquisition). Together, these two surface components play a significant role in biofilm

formation with most models predicting that they are expressed at different phases; flagella for motility as well as possibly initial adhesion, and Ag43 for aggregation and the development of microcolonies. Phase variation of Ag43 may represent one mechanism by which bacteria switch between the expression of these two important phenotypes to exploit their full repertoire of survival and virulence capabilities.

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REFERENCES

- Anderson, G. G., Palermo, J. J., Schilling, J. D., Roth, R., Heuser, J. & Hultgren, S. J. (2003). Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* **301**, 105–107.
- Bachmann, B. (1996). Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 2460–2488. Edited by F. C. Neidhardt & R. Curtiss. Washington, DC: American Society for Microbiology.
- Bardy, S. L., Ng, S. Y. & Jarrell, K. F. (2003). Prokaryotic motility structures. *Microbiology* **149**, 295–304.
- Berge, A., Kihlberg, B. M., Sjöholm, A. G. & Björck, L. (1997). Streptococcal protein H forms soluble complement-activating complexes with IgG, but inhibits complement activation by IgG-coated targets. *J Biol Chem* **272**, 20774–20781.
- Bieber, D., Ramer, S. W., Wu, C. Y., Murray, W. J., Tobe, T., Fernandez, R. & Schoolnik, G. K. (1998). Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. *Science* **280**, 2114–2118.
- Cherepanov, P. P. & Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of FLP-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**, 9–14.
- Czeczulin, J. R., Balepur, S., Hicks, S., Phillips, A., Hall, R., Kothary, M. H., Navarro-Garcia, F. & Nataro, J. P. (1997). Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative *Escherichia coli*. *Infect Immun* **65**, 4135–4145.
- Danese, P. N., Pratt, L. A., Dove, S. L. & Kolter, R. (2000). The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. *Mol Microbiol* **37**, 424–432.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Diderichsen, B. (1980). *flu*, a metastable gene controlling surface properties of *Escherichia coli*. *J Bacteriol* **141**, 858–867.
- Eberl, L., Christiansen, G., Molin, S. & Givskov, M. (1996). Differentiation of *Serratia liquefaciens* into swarm cells is controlled by the expression of the *flhD* master operon. *J Bacteriol* **178**, 554–559.
- Frick, I. M., Morgelin, M. & Björck, L. (2000). Virulent aggregates of *Streptococcus pyogenes* are generated by homophilic protein–protein interactions. *Mol Microbiol* **37**, 1232–1247.
- Giron, J. A., Ho, A. S. & Schoolnik, G. K. (1991). An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. *Science* **254**, 710–713.
- Givskov, M., Eberl, L., Christiansen, G., Benedik, M. J. & Molin, S. (1995). Induction of phospholipase- and flagellar synthesis in *Serratia liquefaciens* is controlled by expression of the flagellar master operon *flhD*. *Mol Microbiol* **15**, 445–454.
- Haagmans, W. & van der Woude, M. (2000). Phase variation of Ag43 in *Escherichia coli*: Dam-dependent methylation abrogates OxyR binding and OxyR-mediated repression of transcription. *Mol Microbiol* **35**, 877–887.
- Hammar, M., Bian, Z. & Normark, S. (1996). Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. *Proc Natl Acad Sci U S A* **93**, 6562–6566.
- Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* **57**, 249–273.
- Hasman, H., Chakraborty, T. & Klemm, P. (1999). Antigen-43-mediated autoaggregation of *Escherichia coli* is blocked by fimbriation. *J Bacteriol* **181**, 4834–4841.
- Hasman, H., Schembri, M. A. & Klemm, P. (2000). Antigen 43 and type 1 fimbriae determine colony morphology of *Escherichia coli* K-12. *J Bacteriol* **182**, 1089–1095.
- Henderson, I. R. & Owen, P. (1999). The major phase-variable outer membrane protein of *Escherichia coli* structurally resembles the immunoglobulin A1 protease class of exported protein and is regulated by a novel mechanism involving Dam and OxyR. *J Bacteriol* **181**, 2132–2141.
- Henderson, I. R., Navarro-Garcia, F., Desvaux, M., Fernandez, R. C. & Ala'Aldeen, D. (2004). Type V protein secretion pathway: the autotransporter story. *Microbiol Mol Biol Rev* **68**, 692–744.
- Josenhans, C. & Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *Int J Med Microbiol* **291**, 605–614.
- Kjaergaard, K., Schembri, M. A., Hasman, H. & Klemm, P. (2000a). Antigen 43 from *Escherichia coli* induces inter- and intraspecies cell aggregation and changes in colony morphology of *Pseudomonas fluorescens*. *J Bacteriol* **182**, 4789–4796.
- Kjaergaard, K., Schembri, M. A., Ramos, C., Molin, S. & Klemm, P. (2000b). Antigen 43 facilitates formation of multispecies biofilms. *Environ Microbiol* **2**, 695–702.
- Klemm, P., Hjerrild, L., Gjermansen, M. & Schembri, M. A. (2004). Structure–function analysis of the self-recognizing Antigen 43 autotransporter protein from *Escherichia coli*. *Mol Microbiol* **51**, 283–296.
- Macnab, R. M. (1992). Genetics and biogenesis of bacterial flagella. *Annu Rev Genet* **26**, 131–158.
- McDevitt, D., Francois, P., Vaudaux, P. & Foster, T. J. (1994). Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Mol Microbiol* **11**, 237–248.
- Menozi, F. D., Boucher, P. E., Riveau, G., Gantiez, C. & Loch, C. (1994). Surface-associated filamentous hemagglutinin induces autoagglutination of *Bordetella pertussis*. *Infect Immun* **62**, 4261–4269.
- Menozi, F. D., Rouse, J. H., Alavi, M., Laude-Sharp, M., Muller, J., Bischoff, R., Brennan, M. J. & Loch, C. (1996). Identification of a heparin-binding hemagglutinin present in mycobacteria. *J Exp Med* **184**, 993–1001.
- Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* **11**, 142–201.
- Nataro, J. P., Kaper, J. B., Robins-Browne, R., Prado, V., Vial, P. & Levine, M. M. (1987). Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr Infect Dis J* **6**, 829–831.
- Nataro, J. P., Deng, Y., Maneval, D. R., German, A. L., Martin, W. C. & Levine, M. M. (1992). Aggregative adherence fimbriae I of

enteroaggregative *Escherichia coli* mediate adherence to HEP-2 cells and hemagglutination of human erythrocytes. *Infect Immun* **60**, 2297–2304.

Ochiai, K., Kurita-Ochiai, T., Kamino, Y. & Ikeda, T. (1993). Effect of co-aggregation on the pathogenicity of oral bacteria. *J Med Microbiol* **39**, 183–190.

Owen, P. (1983). Antigens of the *Escherichia coli* cell envelope. In *Electroimmunochemical Analysis of Membrane Proteins*, pp. 347–373. Edited by O. J. Bjerrum. Amsterdam, New York: Elsevier.

Owen, P. (1992). The Gram-negative outer membrane: structure, biochemistry and vaccine potential. *Biochem Soc Trans* **20**, 1–6.

Owen, P., Meehan, M., de Loughry-Doherty, H. & Henderson, I. (1996). Phase-variable outer membrane proteins in *Escherichia coli*. *FEMS Immunol Med Microbiol* **16**, 63–76.

Reisner, A., Haagensen, J. A., Schembri, M. A., Zechner, E. L. & Molin, S. (2003). Development and maturation of *Escherichia coli* K-12 biofilms. *Mol Microbiol* **48**, 933–946.

Roche, A., McFadden, J. & Owen, P. (2001). Antigen 43, the major phase-variable protein of the *Escherichia coli* outer membrane, can exist as a family of proteins encoded by multiple alleles. *Microbiology* **147**, 161–169.

Roux, A., Beloin, C. & Ghigo, J. M. (2005). Combined inactivation and expression strategy to study gene function under physiological conditions: application to identification of new *Escherichia coli* adhesins. *J Bacteriol* **187**, 1001–1013.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schembri, M. A. & Klemm, P. (2001). Coordinate gene regulation by fimbriae-induced signal transduction. *EMBO J* **20**, 3074–3081.

Schembri, M. A., Kjaergaard, K., Sokurenko, E. V. & Klemm, P. (2001). Molecular characterization of the *Escherichia coli* FimH adhesin. *J Infect Dis* **183**, S28–31.

Schembri, M. A., Hjerrild, L., Gjermansen, M. & Klemm, P. (2003a). Differential expression of the *Escherichia coli* autoaggregation factor antigen 43. *J Bacteriol* **185**, 2236–2242.

Schembri, M. A., Kjaergaard, K. & Klemm, P. (2003b). Global gene expression in *Escherichia coli* biofilms. *Mol Microbiol* **48**, 253–267.

Schembri, M. A., Dalsgaard, D. & Klemm, P. (2004). Capsule shields the function of short bacterial adhesins. *J Bacteriol* **186**, 1249–1257.

Sherlock, O., Schembri, M. A., Reisner, A. & Klemm, P. (2004). Novel roles for the AIDA adhesin from diarrheagenic *Escherichia coli*: cell aggregation and biofilm formation. *J Bacteriol* **186**, 8058–8065.

Sherlock, O., Vejborg, R. M. & Klemm, P. (2005). The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* is self recognizing and induces bacterial aggregation and biofilm formation. *Infect Immun* **73**, 1954–1963.

Soutourina, O. A. & Bertin, P. N. (2003). Regulation cascade of flagellar expression in Gram-negative bacteria. *FEMS Microbiol Rev* **27**, 505–523.

Torres, A. G. & Kaper, J. B. (2002). Pathogenicity islands of intestinal *E. coli*. *Curr Top Microbiol Immunol* **264**, 31–48.

Torres, A. G., Perna, N. T., Burland, V., Ruknudin, A., Blattner, F. R. & Kaper, J. B. (2002). Characterization of Cah, a calcium-binding and heat-extractable autotransporter protein of enterohaemorrhagic *Escherichia coli*. *Mol Microbiol* **45**, 951–966.

Waldron, D. E., Owen, P. & Dorman, C. J. (2002). Competitive interaction of the OxyR DNA-binding protein and the Dam methylase at the antigen 43 gene regulatory region in *Escherichia coli*. *Mol Microbiol* **44**, 509–520.

Wallecha, A., Munster, V., Correnti, J., Chan, T. & van der Woude, M. (2002). Dam- and OxyR-dependent phase variation of *agn43*: essential elements and evidence for a new role of DNA methylation. *J Bacteriol* **184**, 3338–3347.