Mat fimbriae promote biofilm formation by meningitis-associated *Escherichia coli*

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The *mat* (or *ecp*) fimbrial operon is ubiquitous and conserved in *Escherichia coli*, but its functions remain poorly described. In routine growth media newborn meningitis isolates of *E. coli* express the meningitis-associated and temperature-regulated (Mat) fimbria, also termed *E. coli* common pilus (ECP), at 20 °C, and here we show that the six-gene (*matABCDEF*)-encoded Mat fimbria is needed for temperature-dependent biofilm formation on abiotic surfaces. The *matBCDEF* deletion mutant of meningitis *E. coli* IHE 3034 was defective in an early stage of biofilm development and consequently unable to establish a detectable biofilm, contrasting with IHE 3034 derivatives deleted for flagella, type 1 fimbriae or S-fimbriae, which retained the wild-type biofilm phenotype. Furthermore, induced production of Mat fimbriae from expression plasmids enabled biofilm-deficient *E. coli* K-12 cells to form biofilm at 20 °C. No biofilm was detected with IHE 3034 or MG1655 strains grown at 37 °C. The surface expression of Mat fimbriae and the frequency of Mat-positive cells in the IHE 3034 population from 20 °C were high and remained unaltered during the transition from planktonic to biofilm growth and within the matured biofilm community. Considering the prevalence of the highly conserved *mat* locus in *E. coli* genomes, we hypothesize that Mat fimbria-mediated biofilm formation is an ancestral characteristic of *E. coli*.

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INTRODUCTION

Escherichia coli is a multifaceted bacterium that colonizes the mammalian intestine as a harmless commensal but also causes a repertoire of intestinal and extraintestinal infectious diseases (Dobrindt, 2005) and survives in the environment (Savageau, 1983). *E. coli* from the mother and/or the surrounding environment are generally among the earliest colonizers in the oxygenous neonatal gut. During the first months of life a succession of bacterial populations progresses to a complex, more stable and adult-like microbial community dominated by strict anaerobes (Adlerberth & Wold, 2009). However, some *E. coli* strains are able to persist as a member of the normal microbiota and constitute a major portion of the facultative intestinal flora on adult mucosal surfaces. *E. coli* also frequently colonizes anatomical locations outside the gastrointestinal tract, e.g. vagina and urinary bladder (Obata-Yasuoka *et al.*, 2002; Rosen *et al.*, 2007). The population structure of *E. coli* is

largely clonal (Ochman & Selander, 1984; Selander *et al.*, 1986), and isolates are frequently categorized into distinct pathogroups according to specific combinations of phenotypic traits. Based on multilocus enzyme electrophoresis, *E. coli* strains fall into four main phylogenetic groups (designated A, B1, B2 and D), each containing varying proportions of different pathogroups and non-pathogens. *E. coli* strains resident in childhood and adult microbiota commonly include pathogenic variants (Sarff *et al.*, 1975; Siitonen, 1992), predominantly belonging to the virulence-associated phylogenetic group B2 (Nowrouzian *et al.*, 2005; Obata-Yasuoka *et al.*, 2002; Zhang *et al.*, 2002). Despite their primarily asymptomatic colonization, these B2 strains are able to induce various diseases, such as neonatal meningitis, urinary tract infections, sepsis and pneumonia, after entering into extraintestinal sites in both normal and compromised hosts (Picard *et al.*, 1999; Russo & Johnson, 2000). This implies that the normal flora is an important reservoir for extraintestinal pathogenic *E. coli* strains.

The evolutionary success of *E. coli* relies in part on the ability to express surface proteins that mediate cellular interactions on biotic and abiotic surfaces. Expression of meningitis-associated and temperature-regulated (Mat) fimbriae was originally detected in the genetically conserved O18ac : K1 : H7 clonal group of human newborn meningitis

Abbreviations: CV, crystal violet; ECP, *E. coli* common pilus; Mat, meningitis-associated and temperature-regulated; NMEC, newborn meningitis *Escherichia coli*; SIP, surface-influenced planktonic.

The GenBank/EMBL/DDBJ accession number for the *mat* operon region of the *E. coli* IHE 3034 chromosome reported in this paper is HM102365.

E. coli (NMEC) isolates cultivated at low temperature (Pouttu *et al.*, 2001). Recently, expression of the fimbria, also called *E. coli* common pilus (ECP; Rendón *et al.*, 2007), was found to be wide spread in pathogenic and non-pathogenic *E. coli* strains and to contribute, to a variable extent, to *E. coli* adherence onto cultured epithelial cells (Blackburn *et al.*, 2009; Lasaro *et al.*, 2009; Rendón *et al.*, 2007; Saldaña *et al.*, 2009); however, the adhesin or the receptor have not yet been identified. The *mat* (or *ecp*) genes are wide spread and conserved in *E. coli*, and it appears that their regulatory mechanisms are modulated by multiple environmental cues and differ among clonal groups of *E. coli* (Blackburn *et al.*, 2009; Pouttu *et al.*, 2001; Rendón *et al.*, 2007).

A matrix-enclosed, organized-community lifestyle in a biofilm is a common mode of growth for many bacterial species that inhabit diverse environments (Hall-Stoodley *et al.*, 2004). Biofilm formation allows bacteria to survive in stressful conditions and facilitates transmission to new niches. *E. coli* have developed a spectrum of sophisticated and finely controlled pathways to promote growth in such

surface-bound communal structures (Beloin *et al.*, 2008; Van Houdt & Michiels, 2005). The early stages of biofilm development often involve employment of flagella-mediated motility for reversible and initial attachment onto a surface (Pratt & Kolter, 1998; Wood *et al.*, 2006). Production of other surface organelles, including F pili, type 1 fimbriae and curli, is needed for irreversible attachment to strengthen the adhesion (Ghigo, 2001; Pratt & Kolter, 1998; Reisner *et al.*, 2003; Vidal *et al.*, 1998). In this study, we analysed the *mat* cluster of NMEC for its contribution to biofilm formation and describe a function of Mat fimbriae as a biofilm-promoting factor.

METHODS

Strains, plasmids and growth conditions. *E. coli* strains and plasmids used in this study are listed in Table 1. All cultivations were performed without agitation at 20 or 37 °C in Luria–Bertani (LB) broth or in M63 medium [minimal M63 salts (Pardee *et al.*, 1959) supplemented with 0.4% glucose and 1% Casamino acids]. When necessary, media were supplemented with ampicillin 100 µg ml⁻¹,

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

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i> strains		
IHE 3034-Rif	Rifampicin-resistant IHE 3034, O18:K1:H7	Pouttu <i>et al.</i> (2001)
IHE 3034-90	IHE 3034-Rif <i>matB</i> :: <i>cat</i>	Pouttu <i>et al.</i> (2001)
IHE 3034-91	IHE 3034-Rif Δ <i>matB</i> (+61 to +528 bp from the ATG of <i>matB</i>)	This study
IHE 3034-92	IHE 3034-Rif Δ <i>matC</i> (+61 to +609 bp from the ATG of <i>matC</i>)	This study
IHE 3034-93	IHE 3034-Rif Δ <i>matD</i> (+61 to +2466 bp from the ATG of <i>matD</i>)	This study
IHE 3034-94	IHE 3034-Rif Δ <i>matE</i> (+61 to +1584 bp from the ATG of <i>matE</i>)	This study
IHE 3034-95	IHE 3034-Rif Δ <i>matF</i> (+151 to +696 bp from the ATG of <i>matF</i>)	This study
IHE 3034-96	IHE 3034-Rif Δ <i>matBCDEF</i> (+61 bp from the ATG of <i>matB</i> to +753 bp from the ATG of <i>matF</i>)	This study
IHE 3034-97	IHE 3034-Rif Δ <i>matBCDEF</i> complemented with <i>matBCDEF</i>	This study
IHE 3034-Sm	Streptomycin-resistant IHE 3034, O18:K1:H7	Pouttu <i>et al.</i> (1999)
IHE 3034-2	IHE 3034-Sm <i>fimA</i> :: <i>cat</i>	Pouttu <i>et al.</i> (1999)
IHE 3034-8	IHE 3034-Sm <i>sfaA</i> ::Gm	Pouttu <i>et al.</i> (1999)
IHE 3034-80	IHE 3034-Sm <i>fliC</i> :: <i>cat</i>	This study
IHE 3034-59	IHE 3034-Sm <i>fimA</i> :: <i>cat sfaA</i> ::Gm	Pouttu <i>et al.</i> (1999)
IHE 3034-79	IHE 3034-Sm <i>fimA</i> :: <i>cat sfaA</i> ::Gm <i>fliC</i> :: <i>sat</i>	Pouttu <i>et al.</i> (2001)
IHE 3034-99	IHE 3034-Sm <i>fimA</i> :: <i>cat sfaA</i> ::Gm <i>fliC</i> :: <i>sat</i> Δ <i>matB</i>	This study
ABU38	Asymptomatic bacteriuria strain	Lindberg <i>et al.</i> (1975)
MG1655	K-12	Blattner <i>et al.</i> (1997)
S17-1 λ pir	<i>recA thi pro hsdR</i> RP4-2-Tc::Mu Km::Tn7 λ pir	Simon <i>et al.</i> (1983)
Plasmids		
pGP704	Suicide vector, <i>pir</i> -dependent	Miller & Mekalanos (1988)
pCVD442	Suicide vector, <i>pir</i> -dependent, <i>sacB</i>	Donnenberg & Kaper (1991)
pSE380	Expression vector, <i>trc</i> promoter	Invitrogen
pMAT6	IHE 3034 <i>matBCDEF</i> in pSE380	Pouttu <i>et al.</i> (2001)
pMAT10	IHE 3034 <i>matB</i> in pSE380	Pouttu <i>et al.</i> (2001)
pMAT12	IHE 3034 <i>matC</i> in pSE380	This study
pMAT13	IHE 3034 <i>matD</i> in pSE380	This study
pMAT14	IHE 3034 <i>matE</i> in pSE380	This study
pMAT15	IHE 3034 <i>matF</i> in pSE380	This study
pMAT20	MG1655 <i>matBCDEF</i> in pSE380	This study

chloramphenicol 25 µg ml⁻¹, gentamicin 30 µg ml⁻¹, rifampicin 75 µg ml⁻¹, streptomycin 100 µg ml⁻¹, streptothricin 100 µg ml⁻¹ or tetracycline 12.5 µg ml⁻¹. Induction of *mat* genes in recombinant *E. coli* strains was done with 5 µM IPTG.

Construction of chromosomal mutants. In-frame deletion of the entire *matBCDEF* region and each putative *mat* gene on the chromosome of the spontaneously Rif-resistant strain IHE 3034-Rif was performed by site-specific mutagenesis with the *pir*-dependent suicide vector pCVD442 essentially as described by Mobley *et al.* (1993). Briefly, the 5' and 3' flanking sequences of each deletion site were amplified by PCR from IHE 3034 chromosomal DNA with *Pfu* polymerase (Promega), fused with each other by recombinant PCR and then inserted between the *SacI* and *XbaI* sites of the pCVD442. The resulting plasmids were introduced into IHE 3034-Rif by conjugation using the donor strain S17-1 λ *pir*. After sucrose selection, the allelic exchanges were screened by PCR, and the correct deletions were verified by sequencing before testing of the mutants. Primers for the mutagenesis were designed on the basis of the *mat* region sequence of *E. coli* IHE 3034 (accession number HM102365), which was determined by using a commercially available sequencing service (DNA Sequencing and Genomics Laboratory, University of Helsinki, Finland). To obtain a *fimA sfaA fliC matB* quadruple mutant, the *matB* gene was deleted from the Sm-resistant strain IHE 3034-79, a *fimA sfaA fliC* derivative, as described above. The *fliC* gene of IHE 3034-Sm was inactivated by allelic exchange with a *fliC::cat* cassette using the *pir*-dependent suicide vector pGP704 essentially as described before (Pouttu *et al.*, 1999).

In the *matBCDEF* mutant strain, the deletion was complemented by insertion of the wild-type alleles back into the chromosomal deletion site by reverse allelic exchange using pCVD442.

Cloning of *mat* genes. For *in trans* complementation of the individual *mat* deletion derivatives, plasmids encoding MatC, MatD, MatE and MatF were constructed in the inducible pSE380 vector as described earlier (Pouttu *et al.*, 2001). Briefly, the genes were amplified by PCR from IHE 3034 chromosomal DNA with *Pfu* polymerase (Promega), cloned into the *EcoRI*–*SalI* site of pSE380 under the inducible *trc* promoter, and the nucleotide sequences were verified by sequencing. Plasmid construct pMAT20 was similarly made by cloning *matBCDEF* genes from MG1655 chromosomal DNA into pSE380, and confirmed by partial sequencing. The plasmids were introduced into the different *E. coli* hosts by CaCl₂ transformation or by electroporation (Sambrook & Russell, 2001).

Biofilm formation. Biofilm formation on plastic surfaces was measured using the microtitre plate assay system, performed basically as described by Genevaux *et al.* (1996). Briefly, the cells were grown for 48 h in M63 medium at 20 or 37 °C, diluted in M63 medium (1:200, v/v) and then 160 µl of each suspension was added into the wells of a PVC microtitre plate (Falcon). After 48 h of growth at 20 or 37 °C, adherent cell layers were washed twice with PBS, pH 7.1, dried, stained with 0.1% crystal violet (CV) for 10 min and again washed twice with PBS and dried. To quantify biofilm formation the CV stain was solubilized in ethanol/acetone (80:20, v/v), diluted 1:4 in ethanol/acetone (80:20, v/v) and the absorbance at 595 nm was measured in an ELISA recorder (Multiskan EX, Thermo Scientific). The mean absorbance from six wells was calculated for each assay. We also used this method to quantify biofilm formation on polystyrene microtitre plates (Nunc) and glass coverslips (Menzel-Gläser).

Initial attachment. To assess the initial attachment of IHE 3034-Rif derivatives onto PVC surfaces, the strains were grown in static M63 medium for 48 h at 20 °C, diluted in M63 to an OD₆₀₀ value of 0.1, and then 500 µl of each suspension was added to a 24-well plate containing sterile PVC tabs (1 × 1 cm²) and incubated at 20 °C. After

various time intervals the tabs were rinsed twice with PBS, fixed with 3.5% paraformaldehyde in PBS for 10 min, immunostained (see below) and examined by epifluorescence microscopy (Olympus BX50). The numbers of bacteria in 20 randomly chosen microscopic fields of 10³ µm² were determined. The initial attachment to PVC was also tested by using the CV-based method of O'Toole & Kolter (1998), with the following modifications. The strains were grown statically in M63 medium for 48 h at 20 °C, diluted in fresh M63 to an OD₆₀₀ value of 0.1 or 1.0, and 160 µl of each suspension was inoculated into the wells of a 96-well microtitre plate. After incubation for 1 h at 20 °C, the adhered cells were washed twice with PBS and quantified by CV staining, as described above.

Detection of Mat fimbriae expression by whole-cell ELISA.

Polyclonal rabbit antiserum against Mat fimbriae of *E. coli* was available from previous work (Pouttu *et al.*, 2001). Bacteria were grown statically in glass flasks as planktonic cultures and collected by centrifugation. In order to investigate the effect of biofilm growth on Mat fimbriae expression, cells were also grown in the wells of PVC microtitre plates. The non-attached planktonic cells were first removed from the wells [referred to as surface-influenced planktonic (SIP) cells], and surface-attached biofilm cells were harvested from the wells after washing by scraping cells from the surface and resuspending them in PBS. The amount of Mat fimbriae on bacterial cells was assessed by whole-cell ELISA according to the procedure of de Ree *et al.* (1986). Briefly, bacterial cells were adjusted to equal concentrations by resuspending in PBS to an OD₆₀₀ value of 0.25, and microtitre plates (Nunc) were coated with 100 µl of the bacterial suspension per well and dried overnight at 37 °C. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T) and wells were blocked with 100 µl of PBS containing 2% BSA at room temperature for 2 h. After washing three times with PBS-T, 100 µl of anti-Mat fimbria antibodies diluted from 10⁻³ to 10⁻⁷ in PBS containing 1% Tween 20 (PBS-T2) was added to wells in duplicate and incubated at 37 °C for 2 h. The plates were then washed three times with PBS-T, and 100 µl of alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (Dako) diluted 1:750 in PBS-T2 was added and incubated at room temperature for 2 h. Finally, the plates were washed three times with PBS-T, 100 µl phosphatase substrate (Sigma) at 1 mg ml⁻¹ in diethanolamine/magnesium chloride buffer (Reagent) was added and the absorbance at 405 nm of each well was measured after 30 min incubation at 37 °C by an ELISA recorder (Multiskan EX, Thermo Scientific).

Indirect immunofluorescence. For detection of Mat fimbriae expression by indirect immunofluorescence as described by Pouttu *et al.* (2001), bacterial cells were suspended in PBS and immobilized on glass slides, fixed with 3.5% paraformaldehyde in PBS for 10 min, washed and left to react for 30 min with anti-Mat fimbria antibodies diluted 1:50 in PBS containing 1% BSA. After washing three times with PBS, the slides were incubated for 30 min with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) diluted 1:1000 in PBS containing 1% BSA. After washing and mounting in Fluorescent Mounting Medium (Dako), the slides were examined in an epifluorescence microscope (Olympus BX50) equipped with a filter for FITC. The proportions of Mat-positive bacteria in six randomly chosen microscopic fields of 10³ µm² were determined.

Western blotting. Bacterial cells were adjusted to equal concentrations by resuspending in PBS to an OD₆₀₀ of 0.8. Pelleted cells from 750 µl of the bacterial suspension were resuspended in 20 µl H₂O, HCl-treated with 2 µl 1 M HCl, boiled for 2 min and neutralized with 2 µl 1 M NaOH. Denatured samples were mixed with half of their volume of SDS-PAGE loading buffer and boiled for 5 min. Samples of 15 µl were subjected to 12% SDS-PAGE, transferred onto a PVDF membrane (GE Healthcare) and then Mat fimbrial proteins were

reacted with primary anti-Mat fimbria antibodies (diluted 1:500 in PBS containing 0.1% Tween 20), followed by the secondary peroxidase-conjugated anti-rabbit IgG (1:2500; GE Healthcare), and enhanced chemiluminescence detection reagents (GE Healthcare) according to the manufacturer's instructions. To visualize the bands, the membrane was exposed to X-ray film (Agfa).

Serum agglutination. Bacterial agglutination in anti-Mat antiserum, diluted 10^{-2} in PBS, was performed on glass slides by a routine procedure (Rhen *et al.*, 1983).

RESULTS AND DISCUSSION

Effects of non-polar mutations on Mat expression

The chromosomal 7 kb *mat* gene cluster of O18ac:K1:H7 meningitis isolate *E. coli* IHE 3034 contains six putative genes, of which *matA*, *matB* and *matC* have earlier been shown to affect Mat fimbria biogenesis (Pouttu *et al.*, 2001). *matB* (orthologues also known as *yagZ* and *ecpA*) encodes the major fimbriin, which is the only known structural element of the Mat fimbria (Pouttu *et al.*, 2001). To define more precisely the genes of the *mat* cluster that participate in Mat fimbria biogenesis, we inactivated separately, by in-frame deletions, the entire *matBCDEF* region and each of these putative *mat* genes of the spontaneously Rif-resistant strain IHE 3034-Rif. Whole-cell ELISA using polyclonal anti-Mat fimbria antiserum as primary antibodies showed a decreased surface expression of Mat fimbriae in the deletion mutant strains cultivated at 20 °C (Fig. 1a). As a negative control for Mat fimbriae expression, the analysis included the *matB::cat* derivative of IHE 3034-Rif (Pouttu *et al.*, 2001). Plasmids encoding MatB, MatC, MatD, MatE and MatF (Table 1) were used to complement the corresponding *mat* deletions, and the *matBCDEF* mutant strain was complemented by insertion of the genes back into the chromosomal deletion site. After growth at 20 °C, each complemented strain showed a Mat expression level comparable to that of the wild-type strain (Fig. 1b) confirming that the individual mutations had no polar effects on expression. No Mat fimbriae were detected in bacteria grown at 37 °C (Fig. 1a, b, lower panels). These results indicated that the expression of the *matB–F* genes is required for Mat fimbria biogenesis in *E. coli* IHE 3034, in addition to *matA*, which was shown by Pouttu *et al.* (2001) to be required for synthesis of Mat fimbriae.

Biofilm formation by meningitis *E. coli* IHE 3034 involves Mat fimbriae

The capacity of strain IHE 3034-Rif and its derivatives to form a monospecies biofilm on PVC plastic surfaces was quantified using CV staining of bacteria after 48 h static incubation in the biofilm-promoting M63 medium at 20 or 37 °C. High biofilm-forming ability is associated with asymptomatic bacteriuria *E. coli* strains (Ferrières *et al.*, 2007); therefore the test included for comparison the strain ABU38, which in our previous work has proven to be

Fig. 1. Surface expression of Mat fimbriae by IHE 3034 strains. The parent strain IHE 3034-Rif, Mat fimbria-negative *matB::cat* derivative, *mat* deletion mutants (a) and complemented *mat* deletion mutants (b) were grown in LB at 20 °C (upper panels) or at 37 °C (lower panels) and analysed by whole-cell ELISA with anti-Mat fimbria serum as primary antibodies. The deleted genes and complementations are indicated in the inserts (upper panels). In the upper panel (a) $\Delta matBCDEF$ (Δ) overlaps with *matB::cat* ($\square$), $\Delta matB$ ($\blacktriangledown$) and $\Delta matC$ ($\blacklozenge$). The data represent means and standard deviations of two independent assays.

efficient in biofilm formation (unpublished). The commensal-like *E. coli* strain ABU38 belongs to phylogenetic lineage B1, has few virulence-associated genes and is unable to express type 1 fimbriae (Zdziarski *et al.*, 2008). This strain also differs from strain IHE 3034 in Mat fimbriae expression, since no agglutination was detected with anti-Mat fimbria antiserum. The biofilm analysis demonstrated a strong biofilm formation by IHE 3034-Rif after growth at low temperature, but not at 37 °C, in contrast to the phenotype of ABU38 (Fig. 2a). Thus the mechanisms of biofilm production by the group B2 strain IHE 3034-Rif and the group B1 strain ABU38 are different. No biofilm was formed by the Mat fimbriae-deficient *matBCDEF* mutant strain (Fig. 2a, b) nor the individual *mat* deletion mutant strains (Fig. 2a). The apparent differences in biofilm-forming abilities are not due to the differential growth in the M63 medium, as the mutants exhibited growth rates comparable to that of the parent strain (data not shown). As shown in Fig. 2(a), single-copy chromosomal complementation of the *matBCDEF* deletion completely restored the biofilm-forming phenotype. When biofilm formation by IHE 3034-Rif was quantified over time at 20 °C, a steady plateau was seen after 24 h of incubation, while ABU38 had already formed the highest-density biofilm at 16 h and diminished thereafter (Fig. 2b). Parallel results were obtained when polystyrene was used as a template instead of PVC, whereas IHE 3034-Rif

Fig. 2. Biofilm formation by IHE 3034-Rif and MG1655 *mat* derivatives. The biofilm-forming ability of the strain ABU38 is shown for comparison. Bacteria were grown in M63 medium in PVC microtitre plate wells, and biofilm-associated cells were visualized by CV staining. The data represent means and standard deviations of three independent experiments. (a) Biofilm formation by IHE 3034-Rif derivatives after growth for 48 h at 20 or 37 °C. The left panel shows the quantification of biofilms and the right panels show representative samples of the macroscopic biofilm structures formed in the wells by IHE 3034-Rif and the *Mat* fimbriae-deficient *matBCDEF* mutant. (b) Kinetics of biofilm production during 48 h of growth at 20 °C. (c) Quantification of biofilm formation by MG1655 derivatives after growth for 48 h at 20 or 37 °C. (d) Induced *Mat* fimbriae expression of MG1655 derivatives after growth for 48 h in M63 medium at 20 or 37 °C analysed by whole-cell ELISA with anti-*Mat* fimbria serum as primary antibodies. The whole-cell ELISA data represent means and standard deviations of two independent assays.

of *Mat* fimbriae promotes formation of biofilm in the non-NMEC host MG1655, we introduced the plasmid pMAT6, encoding *Mat* fimbriae from IHE 3034, into MG1655. The overexpression from the plasmid resulted in the production of *Mat* fimbriae at both 20 and 37 °C (Fig. 2d) and biofilm growth at low temperature (Fig. 2c).

Comparison of the *mat* cluster sequence from MG1655 with IHE 3034 shows several non-synonymous nucleotides and hence amino acid variation in the predicted *Mat* proteins. Previous studies have demonstrated that the minor allelic variation of the type 1 fimbrial adhesin FimH affects tissue tropism through changes in the mannose receptor specificity (Pouttu *et al.*, 1999; Sokurenko *et al.*, 1995, 1998) and influences the ability to promote mannose-sensitive biofilm formation (Schembri & Klemm, 2001). These findings prompted us to test the functional properties of the MG1655 *Mat* fimbria variant. The *matBCDEF* genes from MG1655 were cloned under the inducible *trc* promoter in pSE380. When the resulting plasmid pMAT20 was transformed into MG1655, we observed *Mat* fimbriae expression (Fig. 2d) and biofilm formation (Fig. 2c) equivalent to that detected in the strain harbouring pMAT6. The same result was obtained when the two plasmids were expressed separately in the IHE 3034-Rif or the IHE 3034 *matBCDEF* background (data not shown). The results show that there is no critical impact of the allelic variations on the function of the encoded proteins. Furthermore, the results illustrate that the *mat* genes are functional in MG1655, thus suggesting that the repression of *Mat* fimbriae expression in MG1655 is a consequence of differences in regulatory mechanisms, similar to the silencing of the highly conserved curli operons (Römling *et al.*, 1998) in a large number of *E. coli* strains (Hammar *et al.*, 1995; Olsén *et al.*, 1993; Uhlich *et al.*, 2001). Interestingly, despite the induced *Mat* expression, no clear biofilm formation was detected at 37 °C, indicating that under our test conditions the

developed biofilm poorly on glass surfaces (data not shown), implying that biofilm formation by IHE 3034-Rif is dependent on substratum hydrophobicity. Our results are in line with the recent observation that *Mat* fimbriae do not mediate biofilm formation on glass surfaces at 37 °C in the probiotic *E. coli* Nissle 1917 strain (Lasaro *et al.*, 2009).

The *mat* operon in the genome of *E. coli* MG1655 (K-12) is 97.8% identical in sequence to that in the IHE 3034 genome, but MG1655 is incapable of *Mat* fimbriae expression in LB (Pouttu *et al.*, 2001) or M63 medium (Fig. 2d). As expected, strain MG1655 had no ability to form a biofilm under the growth conditions employed in this study (Fig. 2c). To assess whether induced production

expression of Mat fimbriae is not enough for biofilm formation at 37 °C. Our results demonstrate that the presence of an expressed *mat* gene cluster is correlated with the ability of *E. coli* to produce a temperature-dependent biofilm, and we hypothesize that the lack of Mat-fimbriae-dependent biofilm at 37 °C results from downregulation of other surface structures (White-Ziegler *et al.*, 2008) interacting with the Mat fimbriae. These factors remain to be identified.

Flagella and other fimbriae of IHE 3034 are not critical for biofilm formation at low temperature

Strain IHE 3034 expresses the mannoside-binding type 1 fimbriae, the sialic acid-binding S-fimbriae and flagella (Selander *et al.*, 1986) in addition to Mat fimbriae. Since various *E. coli* surface structures participate in biofilm formation (Van Houdt & Michiels, 2005), we compared surface filaments for their impact on biofilm formation by IHE 3034-Sm. No change in biofilm formation was observed by the single type 1 fimbrial *fimA*, S-fimbrial *sfaA* or flagellar *fliC* mutants, or with the *fimA sfaA fliC* triple mutant. However, the *fimA sfaA fliC matB* derivative, also deficient in Mat fimbriae production, failed to form a biofilm (Fig. 3), indicating that under these test conditions Mat fimbriae are a principal mediator of biofilm formation by IHE 3034.

Fig. 3. Biofilm formation by IHE 3034 surface filament mutants. The parental strain IHE 3034-Sm and surface filament mutants (type 1 fimbrial *fimA*, S-fimbrial *sfaA*, flagellar *fliC* and Mat fimbrial *matB*) were grown for 48 h at 20 °C in M63 medium in PVC microtitre plates and the biofilm formation was quantified with CV. The data represent means and standard deviations of three independent experiments.

Mat fimbriae affect early stages of biofilm development and are present in mature biofilms

Biofilm formation initiates as surface attachment and then proceeds to a complex architecture of cell interactions (Stoodley *et al.*, 2002). We next determined the initial attachment of IHE 3034-Rif derivatives onto a PVC surface, first by using the CV-staining method with 1 h incubation time at 20 °C. At low bacterial density, both the wild-type and the *matBCDEF* mutant adhered weakly and no difference was seen between the strains (Fig. 4a), whereas the adherence at higher bacterial density enabled the wild-type IHE 3034-Rif to bind efficiently on the PVC surface. Similar results were obtained by indirect immunofluorescence microscopy analysis of PVC tabs incubated with low-density bacterial suspensions. At early time points (≤ 1 h), the attachment of the *matBCDEF* mutant was indistinguishable from that of the wild-type strain (Fig. 4b). However, after 3 h, a difference between the wild-type and mutant became evident. Over time, IHE 3034-Rif formed a progressively denser layer of cells on the PVC surface, whereas the attachment of the *matBCDEF* mutant remained unchanged, indicating that the mutant was defective in irreversible attachment and thus biofilm formation.

Expression of Mat fimbriae is evidently regulated by phase variation (Pouttu *et al.*, 2001), an adaptive strategy to switch on and off the expression, leading to cell-to-cell variability in a population. The majority of the IHE 3034-Rif cells cultured statically in M63 medium and used to inoculate the wells with PVC tabs were Mat-fimbriated ($69.6 \pm 2.0\%$), and no accumulation of Mat-positive cells was observed during the initial attachment experiment (Fig. 4b). Interestingly, these frequencies of Mat fimbriae-expressing cells were markedly higher than the frequencies observed earlier with IHE 3034-Rif cells cultured in LB (Pouttu *et al.*, 2001), suggesting that the adjustment of the balance between the two fimbriation states responds to environmental signals. This may be responsible for the ~50 % lower level of biofilm formation when M63 was replaced with LB as a growth medium (data not shown).

Formation of a biofilm involves coordinate multifactorial reprogramming of gene expression, including induction or downregulation of fimbriae genes (Domka *et al.*, 2007; Hancock & Klemm, 2007; Schembri *et al.*, 2003). We next assessed whether Mat fimbriae are differentially expressed in sessile biofilm cells of IHE 3034-Rif and their planktonic counterparts. The cells from a glass flask, a substratum that does not support biofilm formation by IHE 3034-Rif, were used as a reference, as an inoculum, and were defined as 'inoculate' cells; the cells in the glass flasks at the end of the incubation periods were termed 'planktonic' cells. The non-attached cells removed from the PVC wells after biofilm growth were defined as 'surface-influenced planktonic (SIP)' cells, according to Steyn *et al.* (2001), and the surface-attached cells harvested from the walls of the PVC wells were termed 'biofilm' cells (see Methods). After 24 or

Fig. 4. Initial attachment and Mat fimbriae expression of IHE 3034-Rif derivatives onto PVC surfaces. (a) Quantification of initial attachment in the PVC microtitre plate wells by CV staining. Bacteria were adjusted in M63 medium to low or high density (OD₆₀₀ 0.1 or 1.0, respectively) and incubated in the wells for 1 h at 20 °C. The data represent means and standard deviations of three independent experiments. (b) Indirect immunofluorescence microscopy analysis of the initial biofilm formation using primary antibodies directed against Mat fimbriae. The bacteria were adjusted in M63 medium to low density (OD₆₀₀ 0.1) and incubated at 20 °C in microtitre plates containing PVC tabs. At the indicated time points, tabs were removed and cells were fixed, immunostained and examined by epifluorescence microscopy. Representative microscopy fields of phase-contrast (left panels) and epifluorescence images (right panels) are shown, and the mean number of attached bacteria (left panels) and the proportion of Mat fimbriated bacteria (right panels) ± SD in 20 and 6 randomly chosen fields of 10³ μm², respectively, is indicated below the images. Arrows indicate adherent, Mat fimbriated bacteria. Scale bar, 5 μm.

48 h of static growth in M63 medium at 20 °C, the mean Mat expression levels of the entire cell populations were quantified by whole-cell ELISA. No changes in the surface expression of Mat fimbriae were apparent on IHE 3034-Rif cells harvested from the different modes of growth (Fig. 5a). We also analysed the cell samples by immunoblotting with anti-Mat antibodies to detect both surface expressed and intracellular fimbrial proteins. Consistent with the ELISA results, the 18 kDa band of the major fimbriin MatB, corresponding to the calculated molecular mass of mature MatB (Pouttu *et al.*, 2001), was expressed in similar amounts in all IHE 3034-Rif samples (Fig. 5b). Similarly, examination of the IHE 3034-Rif cell populations by indirect immunofluorescence microscopy with anti-Mat fimbriae antiserum was in line with these and the initial

Fig. 5. Biofilm formation and Mat fimbriae expression by IHE 3034-Rif and the *matBCDEF* deletion mutant. Expression of Mat fimbriae in planktonic, surface-influenced planktonic (SIP) and biofilm cells of IHE 3034-Rif was analysed by whole-cell ELISA (a) and Western blotting of HCl-treated cellular proteins (b) with anti-Mat fimbriae serum as primary antibodies after growth for 24 h or for 48 h in M63 medium at 20 °C. The whole-cell ELISA data represent means and standard deviations of three independent experiments. Western blotting was assessed twice, and a representative blot is shown.

attachment results, as 74 to 83 % of the cells in the populations expressed the fimbriae (data not shown).

Conclusion

The *E. coli* strains IHE 3034 and ABU38 differ in Mat fimbriae expression and form biofilms that vary in temperature sensitivity. This is a further indication that *E. coli* isolates use multiple mechanisms for biofilm formation (Beloin *et al.*, 2008; Van Houdt & Michiels, 2005). We (Pouttu *et al.*, 2001) and others (Rendón *et al.*, 2007) have found that Mat fimbriae represent the most common fimbrial type in *E. coli* and that the *mat* genes in *E. coli* of different origin are highly conserved (97 to 98 % nucleotide sequence identity). This suggests that the Mat fimbria represents an ancestral fimbrial type in *E. coli* and that its functions are essential for *E. coli* as a species, although still poorly described. The current data indicate that the regulation of Mat fimbriae in different clonal groups of *E. coli* is variable. Enterohaemorrhagic, enterotoxigenic and enteropathogenic *E. coli* strains were shown to produce the fimbriae after growth at 37 °C in tissue culture medium, suggesting that intestinal *E. coli* strains may express the fimbriae in the human intestine (Blackburn *et al.*, 2009; Rendón *et al.*, 2007; Saldaña *et al.*, 2009). Our ongoing studies indicate that IHE 3034 also is able to express the fimbriae at 37 °C, albeit under differential growth conditions (T. A. Lehti, unpublished). The central mechanism for Mat fimbriae expression in IHE 3034 is currently under investigation. Recently, Lasaro *et al.* (2009) reported that the Mat fimbria homologue of the probiotic *E. coli* strain Nissle 1917 is essential for intestinal colonization of the infant mouse. In the mucosal habitats of *E. coli*, multispecies biofilm communities are present on the gut (Palestrant *et al.*, 2004) and vaginal (Swidsinski *et al.*, 2005) epithelia and there is evidence for the *in vivo* community behaviour of commensal and pathogenic *E. coli* (Macfarlane & Macfarlane, 2006; Rosen *et al.*, 2007), suggesting that the structured lifestyle contributes both to normal ecology and to pathogenicity of *E. coli* in the host. Furthermore, the ability of *E. coli* to grow protective biofilms on non-host targets in heterogeneous environmental niches arguably ensures continual transmission to human populations. The results presented in this study show that the expression of the ubiquitous *mat* fimbrial operon enables *E. coli* to display multicellular behaviour at low temperatures. It remains to be established whether Mat-mediated biofilm formation plays a role in environmental survival and/or in host colonization by *E. coli*.

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