Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in *Enterobacteriaceae* strains

Mette Burmølle,¹ Martin Iain Bahl,¹ Lars Bogø Jensen,²
Søren J. Sørensen¹ and Lars Hestbjerg Hansen¹

Correspondence

Lars Hestbjerg Hansen
hestbjerg@bi.ku.dk

¹Department of Microbiology, University of Copenhagen, 1307 Copenhagen K, Denmark

²Unit for Antimicrobial Resistance, The National Food Institute, DTU, Denmark

The conjugative plasmid pOLA52, which confers resistance to olaquinox and other antimicrobial agents through a multidrug efflux pump, was investigated for its ability to promote biofilm formation in *Escherichia coli*. Screening of a transposon-mutagenized pOLA52 clone library revealed several biofilm-deficient mutants, which all mapped within a putative operon with high homology to the *mrkABCD* operon of *Klebsiella pneumoniae*, where these genes are responsible for type 3 fimbriae expression, attachment to surfaces and biofilm formation. Biofilm formation in microtitre plates and in urinary catheters of clones containing pOLA52 with a disrupted putative *mrk* operon was reduced by more than 100-fold and 2-fold, respectively, compared to mutants with an intact *mrk* operon. The conjugative transfer rate of pOLA52 was also significantly lower when the *mrk* operon was disrupted. Through reverse transcriptase analysis, it was demonstrated that the genes contained in the putative *mrk* operon were linked and likely to be expressed as a single operon. Immunoblotting with type 3 fimbriae (MrkA)-specific antibodies further verified expression of type 3 fimbriae. When transferred to other, potentially pathogenic, members of the family *Enterobacteriaceae*, including *Klebsiella pneumoniae*, *Salmonella Typhimurium*, *Kluyvera* sp. and *Enterobacter aerogenes*, pOLA52 facilitated increased biofilm formation. pOLA52 is believed to represent the first example of a conjugative plasmid encoding type 3 fimbriae, resulting in enhanced conjugation frequencies and biofilm formation of the plasmid-harboring strain.

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INTRODUCTION

The plasmid pOLA52 was exogenously isolated from swine manure and demonstrated to confer resistance to the antimicrobial agent olaquinox (Sørensen *et al.*, 2003). This compound was extensively used in pig farming as a growth promoter in the European Union until 1999 and is still in use in other countries. The resistance mechanism of pOLA52 was found to depend on a resistance-nodulation-cell-division (RND) family efflux system (Hansen *et al.*, 2004). Apart from olaquinox, the pump exports chloramphenicol and ethidium bromide across the membrane of *Escherichia coli* cells (Hansen *et al.*, 2004) and recently experiments have demonstrated reduced susceptibility to other antibiotics, detergents and disinfectants (Hansen *et al.*, 2007). Independent of the efflux pump system, pOLA52 confers resistance to the β -lactam antibiotic ampicillin (Hansen *et al.*, 2004, 2007).

Plasmid pOLA52 was recently sequenced (to be published elsewhere; A. Norman, Q. She, S. J. Sørensen & L. H. Hansen) and annotation revealed a putative operon, totalling 5.6 kb, consisting of five genes highly homologous to the *mrkABCD* genes contained in the *mrk* operon of *Klebsiella pneumoniae* (Allen *et al.*, 1991). In *K. pneumoniae*, the *mrk* operon encodes type 3 fimbriae, which are involved in attachment to and biofilm formation on biotic and abiotic surfaces, including different human cell types (Hornick *et al.*, 1991; Livrelli *et al.*, 1996; Schurtz *et al.*, 1994; Tarkkanen *et al.*, 1997). The *mrkA* and *mrkD* genes encode the major structural component of the type 3 fimbriae and an adhesin, respectively (Allen *et al.*, 1991; Jagnow & Clegg, 2003; Langstraat *et al.*, 2001). The genes *mrkB* and *mrkC*, which encode proteins essential for fimbrial expression, are thought to be part of the fimbrial assembly process via the chaperone-usher pathway (Allen *et al.*, 1991; Gerlach *et al.*, 1988; Sauer *et al.*, 2000). The *mrkF* gene encodes the MrkF protein, which is thought to be involved in stabilizing intact fimbriae (Allen *et al.*, 1991).

Abbreviations: CV, crystal violet; RND, resistance-nodulation-cell division.

K. pneumoniae is an opportunistic pathogen, which is often associated with infections in the human respiratory and urinary tract. Because cell attachment is an essential step in infection, the type 3 fimbriae may be crucial for *K. pneumoniae* virulence (Livrelli *et al.*, 1996; Williams & Tomas, 1990). In several other members of the family *Enterobacteriaceae*, adhesion-mediating fimbriae and pili are believed to be essential for virulence (Hornick *et al.*, 1991). The *mrkABCDF* genes of pOLA52 are the first type 3 fimbriae-encoding genes reported to be present on a plasmid. Previously, plasmid-borne fimbrial genes, different from the *mrk* genes, have been reported to be involved in aggregative adherence of *E. coli* to mucosal cells, causing traveller's diarrhoea and persistent diarrhoea among infants, especially in the developing world (Levine, 1987; Vila *et al.*, 2000), and also to contribute to the pathogenicity of *E. coli* O157 (Brunner *et al.*, 2001).

Biofilms are recognized as surface-attached bacteria embedded in a self-produced matrix, composed mainly of polysaccharides, but also containing proteins and nucleic acids (Sutherland, 2001). Factors important for biofilm formation include production of extracellular polymers and cell surface-attached structures including flagella, fimbriae and curli (Castonguay *et al.*, 2006; Klausen *et al.*, 2006). Pili involved in conjugation of Gram-negative bacteria have also been shown to promote biofilm formation in several studies (Dudley *et al.*, 2006; Ghigo, 2001; Reisner *et al.*, 2003, 2006). Ghigo (2001) demonstrated how biofilm formation of an *E. coli* strain was dependent on whether this strain contained a conjugative plasmid and expressed the conjugative pili. Dudley *et al.* (2006) recently demonstrated that thin type IV pili, different from the thicker conjugative pili, but also involved in conjugation, promoted cell–cell attachment as well as attachment to abiotic surfaces and thereby promoted biofilm formation.

Bacteria organized in biofilms cause many human infections. They attach to human cell tissue, to teeth, and to medical devices such as prosthetic heart valves and urinary catheters (Donlan & Costerton, 2002). Biofilms on urinary catheters often involve *Enterobacteriaceae* such as *E. coli*, *K. pneumoniae* and *Enterobacter aerogenes* (Donlan & Costerton, 2002). These biofilm-associated infections are hard to cure due to an increased resistance of the biofilm bacteria to antibiotics and phagocytosis by inflammatory cells, and thus represent a large medical problem (Donlan & Costerton, 2002; Fux *et al.*, 2005; Jefferson, 2004). Because of the increased protection of bacteria in biofilm, they should achieve a higher fitness in this life form compared to the free-living, planktonic state (Jefferson, 2004). A selective pressure may therefore exist, favouring the prevalence of genes encoding biofilm-associated traits, like plasmid-encoded pili or fimbriae.

In our laboratory we have observed an increased biofilm phenotype in an *E. coli* CSH26 strain harbouring plasmid pOLA52. The aim of this study was to investigate whether

the putative type 3 fimbriae-encoding operon, with homology to the *mrk* operon from *K. pneumoniae*, is expressed and could be responsible for the biofilm phenotype.

METHODS

Strains and plasmids used in this study. The strains and plasmids used in this study are listed in Table 1.

Media and culture conditions. Bacteria were grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989). Solid medium contained 15 g agar l⁻¹. Bacterial cultures were incubated at 37 °C; LB broths were incubated on a rotary shaker at 250 r.p.m. Phosphate-buffered saline (PBS) was used for washing cells and preparing serial dilutions (Sambrook *et al.*, 1989). The antibiotics kanamycin (Kan, 50 µg ml⁻¹), ampicillin (Amp, 100 µg ml⁻¹), tetracycline (Tet, 20 µg ml⁻¹) and rifampicin (Rif, 100 µg ml⁻¹) were used when required.

Construction of a clone library with a Kan^R entranceposon randomly inserted in pOLA52. A knockout library of pOLA52 was created. First, pENTRANCEPOSON (Kan^R) (Finnzymes) was digested with *Bgl*II restriction endonuclease. The entranceposon was then excised from an agarose gel as a 1.2 kb fragment and purified using the QIAEX II Gel Extraction kit (Qiagen). Two micrograms of a plasmid preparation of pOLA52 (Plasmid Mini AX kit, A&A Biotechnology) was incubated with 20 ng entranceposon and 1 µl MuA transposase in MuA transposase buffer according to the manufacturer's protocol (Finnzymes). The transposition reaction was diluted 10-fold in sterile deionized water, electroporated into *E. coli* Genehogs (Invitrogen), and successful transpositions of the entranceposon into pOLA52 were selected for on solid LB medium containing Kan. Three hundred transformants, *E. coli* Genehogs/pOLA52::Kan^R, each representing a different, randomly distributed transposition event in pOLA52, were then frozen in microtitre plates in 17% glycerol at –80 °C for later use.

Screening of the clone library for the ability to form biofilm. A total of 88 of the transformants described above were randomly selected and screened for their ability to form biofilm in microtitre plate wells, by use of a previously established method in which cell attachment and biofilm formation are quantified by staining with crystal violet (CV) (O'Toole & Kolter, 1998). *E. coli* CSH26 with and without pOLA52 were included as reference strains. The 88 clones were inoculated from glycerol stocks directly into microtitre plates containing 200 µl LB broth and grown with agitation (200 r.p.m.) for 24 h. Staining and quantification of attached cells were performed as previously described (Burmølle *et al.*, 2006), using an EL 340 microplate reader (Bio-Tek Instruments) for absorbance measurements at 595 nm.

Of the 88 clones, 7 were biofilm negative. The biofilm-forming ability of these clones and that of 12 randomly selected biofilm-positive clones was verified by performing the assay in four replicates for each strain. Equal densities (OD₆₀₀ ± 10%) of the bacteria were diluted 10-fold in LB in microtitre plate wells to a total volume of 200 µl. The plates were incubated as described above. Wells containing only LB were used as negative controls and the absorbance measurements of these wells were subtracted from the experimental measurements.

Sequencing of biofilm-negative and -positive clones. Of the 19 clones tested above, the 7 biofilm-negative and 4 randomly selected, biofilm-positive clones were chosen for sequencing to identify the exact location of the entranceposon in the pOLA52 derivatives as

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Strains		
<i>Escherichia coli</i> Genehogs		Invitrogen
<i>Escherichia coli</i> Genehogs ^{Rif}	Spontaneous Rif ^R mutant	This study
<i>Escherichia coli</i> CSH26	Requires proline and leucine for growth (<i>ara</i> Δ (<i>lac-pro</i>) <i>thi</i>)	Miller (1972)
<i>Escherichia coli</i> MC4100	Prototroph	Silhavy <i>et al.</i> (1984)
<i>Salmonella</i> Typhimurium DT27		Olsen <i>et al.</i> (2004)
<i>Klebsiella pneumoniae</i> DSA712		Dorthe Sandvang*
<i>Kluyvera</i> sp. MB101		Burmølle <i>et al.</i> (2005)
<i>Enterobacter aerogenes</i> DSM30053		DMSZ†
Plasmids		
pOLA52	Biofilm positive	Sørensen <i>et al.</i> (2003)
pOLA52- <i>bla</i> ::Kan ^R	Biofilm positive, Amp ^S Kan ^R	This study
pOLA52- <i>oqx</i> B::Kan ^R (H1)	Biofilm positive, Kan ^R	This study
pOLA52- <i>mrk</i> C::Kan ^R (H5)	Biofilm negative, Kan ^R	This study
pLOW2	Low-copy plasmid, Kan ^R	Hansen <i>et al.</i> (1997)
pLOW2:: <i>mrkA</i> -F	Low-copy plasmid, Kan ^R	This study
pLOW2::Tet ^R - <i>mrkA</i> -F	Low-copy plasmid, Kan ^R Tet ^R	This study

*Dorthe Sandvang, Novozymes, Denmark.

†Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

illustrated in Fig. 1. pOLA52::Kan^R plasmids from the 11 chosen clones were purified as described above. From these purifications, 12 μ l plasmid DNA (0.1–1.0 μ g) was digested with *Nla*III in reactions of 20 μ l at 37 °C for 60 min, followed by heat inactivation of *Nla*III at 65 °C for 25 min. Then, 1 μ l T4 ligase (10 units) and 2 μ l ATP (10 mM) was added and ligation was performed at 25 °C for 60 min, followed by heat inactivation of T4 ligase at 65 °C for 25 min. PCRs were performed with primers Ent(Kan)FW (5'-TTATTCGGTCGA-AAAGGATCCG-3') and Ent(Kan)Rev (5'-TCCCGTCAAGTCAG-CGTA-3') and the PCR products were visualized by agarose gel electrophoresis. Sequencing reactions (DYEnamic ET dye terminator cycle sequencing kit, MegaBACE) containing 0.1 μ g purified PCR product and 10 pmol primer Ent(Kan)FW in total volumes of 10 μ l

were conducted. Sequencing was performed using a MegaBACE 1000 sequencer (Molecular Dynamics). To locate the position of the Kan^R cassette, the sequences obtained were compared to the plasmid sequence (A. Norman, Q. She, S. J. Sørensen & L. H. Hansen, unpublished). Two clones were chosen for further analysis: the biofilm-positive *E. coli* Genehogs/pOLA52-*oqx*B::Kan^R (H1) and the biofilm-negative *E. coli* Genehogs/pOLA52-*mrk*C::Kan^R (H5); see Table 1.

Complementation of biofilm formation. To investigate whether the biofilm formation was solely caused by the expression of the *mrk* operon, the absence of the biofilm phenotype was complemented in *E. coli* Genehogs and in strain H5 containing *mrk*C-mutagenized

Fig. 1. The Kan^R cassette contains several *Nla*III recognition sites; therefore *Nla*III digestion of the Kan^R mutagenized plasmid will, when the products are ligated, result in many different circular fragments. Some of these fragments contain part of the Kan^R cassette and part of the original plasmid, and they will also contain the primer annealing sites. PCRs will therefore result in amplification of these circular fragments, and by sequencing of the PCR products, the location of the Kan^R insertion can be determined.

pOLA52. Two primers, mrkFW (5'-AAGTGCACGGGGGAGACT-TACCCGAAATCT-3') and mrkRev (5'-GCAGCGGCCGATTCC-CGGCTTTTAAAC-3'), were designed to amplify the entire *mrkA-F* operon as a 6680 bp fragment with an *Apa*LI site at one end and a *Not*I site at the other (restriction sites are underlined). Following double digestion of both PCR product and vector with *Apa*LI and *Not*I, the *mrk* operon was directionally cloned into pLOW2 (Hansen *et al.*, 1997) and transformed into *E. coli* GeneHogs. The kanamycin resistance of pLOW2 was knocked out by an Entranceposon (Tet^R) insertion into the *aph*(3') gene of the resulting pLOW2-*mrkA-F* plasmid as described above and according to the protocol of the manufacturer (Finnzymes, Finland). This resulted in a selective marker, tetracycline resistance, different from the kanamycin resistance on the pOLA52 derivative in H5. The position of the Entranceposon (Tet^R) in the *aph*(3') gene was verified by the phenotypic loss of kanamycin resistance and by sequencing. Then the H5 strain as well as *E. coli* GeneHogs without the plasmid were transformed with the Tet^R pLOW2::Tet^R-*mrkA-F* plasmid and subjected to the microtitre-biofilm assay described above.

Biofilm formation on urinary catheters. *E. coli* GeneHogs/pOLA52-*oqx*B::Kan^R (H1) and *E. coli* GeneHogs/pOLA52-*mrkC*::Kan^R (H5) were tested for their ability to form biofilms on medical urinary catheters. The strains were grown overnight in LB broth (containing Kan), and the cell density was adjusted (OD₆₀₀ ± 10%). From these cultures, 50 µl was transferred to glass tubes containing 5 ml LB and three pieces of urinary catheter tubing (AquaPlate Glycerine Silicone, Ref. 178305; Rüsch, Kernen, Germany), cut vertically to eliminate channels without bacterial movement and horizontally to obtain 1 cm pieces. Catheter tubing in LB, but not exposed to bacteria, was used as negative control and the experiment was performed in triplicate. The tubes were incubated with gentle shaking for 24 h at 37 °C. The cell suspension was then discarded and the catheter tubing pieces were rinsed twice with 10 ml PBS, stained for 20 min in CV (1%) and washed twice with PBS. Two millilitres of ethanol was added to dissolve the CV bound to the attached cells and A₅₉₅ was measured. The absorbance value of the negative control was subtracted from the measurements.

Exposure to inhibitory compounds. An Amp^S clone from the *E. coli* GeneHogs/pOLA52-*bla*::Kan^R library was isolated as previously described (Hansen *et al.*, 2007). Overnight cultures of this strain (*E. coli* GeneHogs/pOLA52-*bla*::Kan^R), *E. coli* GeneHogs/pOLA52-*oqx*B::Kan^R (H1) and *E. coli* GeneHogs/pOLA52-*mrkC*::Kan^R (H5) were diluted 100-fold in microtitre plate wells containing LB medium. The inhibitory compounds tetracycline (Tet) and hydrogen peroxide (H₂O₂) were added to obtain the following final concentrations: Tet, 0.5, 2, 10 and 20 µg ml⁻¹; H₂O₂, 35, 175, 875 and 1750 µg ml⁻¹. Wells without inhibitory compounds were included, and wells containing only LB served as background. The total volume of each well was 200 µl. Each treatment was performed in triplicate wells. The plate was incubated with shaking (200 r.p.m.) at 37 °C. After 1 h, the respiratory indicator 2,3,5-triphenyltetrazolium chloride (TTC) was added to each well at a final concentration of 0.01%, and the A₄₉₀ was measured after 48 h in an EL 340 microplate reader (Bio-Tek Instruments). The activity in wells exposed to inhibitory compounds was then related to that of the equivalent strain without exposure to inhibitory compounds.

RT-PCR analysis of the putative *mrk* operon. Reverse transcription-PCR (RT-PCR) was performed to verify transcription of the putative *mrk* operon and the linkage of the genes contained in the operon at the mRNA level. Due to the length of the putative *mrk* operon, the RT reactions were performed by use of three reverse primers, annealing at various positions on the operon, followed by nested PCRs to increase the specificity. The PCR primers were designed to demonstrate the linkage of the genes *mrkA-mrkB*,

mrkB-mrkC, *mrkC-mrkD* and *mrkD-mrkF*, respectively. Total RNA was purified from 1.5 ml of overnight culture of *E. coli* GeneHogs (no plasmid) and *E. coli* GeneHogs/pOLA52-*oqx*B::Kan^R (H1) (Genelute Bacterial Total RNA Purification kit, Sigma). The purified RNA was then subjected to DNase digestion and subsequent DNase inhibition, by use of a DNA-free kit (Ambion) as recommended. This RNA was used as template in RT (Expand Reverse Transcriptase kit, Roche). A total of 8 µg was used in a total of six (three for each strain) 20 µl RT reactions (65 °C for 10 min, subsequent cooling on ice, followed by 43 °C for 60 min) containing 20 pmol of the primers mrkFrev1 (5'-ATATTCTGCGCTCCATCC-3'), mrkCrev (5'-CGCTCTGACGAT-AGCCTT-3') or mrkBrev (5'-CATCGGCGCCATAGTCAT-3'). Next, four nested PCR reactions (Phusion, Finnzymes) were performed, using 1 µl of RT product (10-fold diluted in H₂O) as template, as follows. The RT product obtained from the reverse primer mrkBrev was used as template in a nested PCR performed with the primers mrkBrevnst1 (5'-GCGGGGTGATAATAAACG-3') + mrkAfw (5'-GCGGCCAGGTTAATTCT-3'). The mrkCrev-RT product was used as template in a nested PCR with primers mrkCrevnst1 (5'-ACCAGGTTGTCTTTGACG-3') + mrkBfw (5'-GCAGTGAAAGTGGCCGT-3'), and the RT product obtained from mrkFrev1 served as template in two nested PCRs with the primer sets mrkFrev2 (5'-ACCGCGATCGTGGACTTCC-3') + mrkDfw (5'-TTA-GCACCTCGTTCTCCG-3'), and mrkDrev (5'-CGGAGAACGA-GGTGCTAA-3') + mrkCfw (5'-AAGGCTATCGTCAGAGCG-3'). Control nested PCRs, similar to those described above, were performed using the DNase-treated RNA (with twice the RNA concentration used in the RT-PCR reactions) as template to verify that the RNA was DNA-free.

Immunoblotting with MrkA-specific antibodies. A colony immunoblot was performed to verify that the expression of the putative *mrk* operon produced intact type 3 fimbriae. Rabbit antiserum raised against the *K. pneumoniae* type 3 fimbriae major subunit MrkA (Di Martino *et al.*, 2003) was provided as a gift from Arlette Darfeuille-Michaud, Pathogénie Bactérienne Intestinale, USC-INRA 2018 CBRV, Clermont Ferrand, France. From overnight LB broth cultures of *E. coli* GeneHogs (no plasmid), *E. coli* GeneHogs/pOLA52-*oqx*B::Kan^R (H1) and *K. pneumoniae* DSA712, 5 µl was spotted onto a nitrocellulose filter placed on an LB agar plate, grown for 16 h and dried at 80 °C for 2 h. Then the membrane was blocked in 50 ml 5% skim milk (Difco) for 1 h on a slowly rotating platform shaker at room temperature. The rabbit antiserum was added at a 1:500 dilution in 5% skim milk solution and incubated for 1.5 h on the platform shaker at room temperature, after which the membrane was washed three times for 10 min in TBS buffer (125 mM NaCl, 25 mM Tris pH 8.0, 0.05% Tween 20). After washing, the filter was incubated for 1 h at room temperature with peroxidase-labelled goat anti-rabbit immunoglobulin G (DAKO) in TBS at 2000-fold dilution. The membrane was washed again three times for 10 min in TBS buffer and resuspended in 50 ml fresh TBS buffer. For the detection of expressed MrkA protein, 20 mg 4-chloro-1-naphthol (Sigma) and 0.2% H₂O₂ were added and the membrane was allowed to develop for 30 min.

Conjugation frequency. Plasmids pOLA52-*oqx*B::Kan^R and pOLA52-*mrkC*::Kan^R, from strains H1 and H5, respectively, were transferred separately to spontaneous Rif^R *E. coli* GeneHogs cells (H1^{Rif} and H5^{Rif}) by conjugation and these transconjugants were used as donor cells in the following conjugation experiment. Single colonies of the donor strains were transferred to separate tubes containing 5 ml LB broth supplemented with Rif and Kan and incubated to an OD₆₀₀ of 0.4, after which the cultures were washed twice in LB broth (5000 g, 5 min) and the OD₆₀₀ was adjusted to 0.2. The recipient *E. coli* MC4100 strain was prepared analogously; however, this strain was grown in the absence of antibiotics and the wash-steps were omitted. Triplicate transfer experiments were

conducted by mixing 40 µl of either the H1^{Rif} or H5^{Rif} donor strain with 160 µl of the recipient strain in micro-centrifuge tubes, incubated at 37 °C overnight without shaking. Serial dilutions of the mating mixtures were prepared and the following solid media were used for selective plating: LB-agar containing Rif+Kan, minimal media plates (de Liphay *et al.*, 2001) containing glucose and minimal media plates containing glucose and Kan for selection of donors, recipients+transconjugants and transconjugants respectively.

Biofilm formation of potentially pathogenic *Enterobacteriaceae* strains±pOLA52. The plasmid (pOLA52-*bla*::Kan^R) from the Amp^S clone, isolated above, was purified and transferred to *E. coli* CSH26 by electroporation, followed by a conjugative transfer to *Salmonella* Typhimurium DT27, *Klebsiella pneumoniae* DSA712, *Kluyvera* sp. MB101 and *Enterobacter aerogenes* DSM30053, as previously described (Hansen *et al.*, 2007). These strains, with and without pOLA52, were grown overnight in LB±Kn; OD₆₀₀ was measured, and the cultures were diluted in LB to assure similar cell densities of each strain pair (identical species±plasmid). *E. coli* CSH26±pOLA52 was used as control. Then, 20 µl samples were transferred to microtitre plate wells, containing 180 µl LB, in four well replicates. The plate was incubated, and the attached cells were stained and quantified as described above. The biofilm formation of each strain devoid of plasmid was then related to that of its plasmid-containing counterpart.

RESULTS AND DISCUSSION

The sequencing of pOLA52 revealed a gene cluster with high homology to the chromosomal *mrk* operon of *K. pneumoniae*, which encodes type 3 fimbriae (Allen *et al.*, 1991). In *K. pneumoniae*, expression of type 3 fimbriae is essential for attachment to abiotic and biotic surfaces and for biofilm formation (Di Martino *et al.*, 2003; Jagnow & Clegg, 2003; Langstraat *et al.*, 2001; Tarkkanen *et al.*, 1997). The latter includes attachment of *K. pneumoniae* to human epithelial and urinary bladder cells, which may be involved in the common human infections caused by *K. pneumoniae* (Tarkkanen *et al.*, 1997).

The complete *mrk* operon of *K. pneumoniae* contains six genes, of which five homologues are present in pOLA52 (Allen *et al.*, 1991). The remaining gene, *mrkE*, has been suggested to encode a product involved in regulation of expression of type 3 fimbriae; however, strains lacking the *mrkE* gene expressed type 3 fimbriae at levels similar to the wild-type, which suggests that MrkE is not essential for fimbriae expression (Allen *et al.*, 1991). The five translated open reading frames showed an 81–95 % identical amino acid sequence when compared to the *mrk* operon from *K. pneumoniae* (A. Norman, Q. She, S. J. Sørensen & L. H. Hansen, unpublished).

Construction of clone library and screening for loss of biofilm formation phenotype

Due to the close homology between the pOLA52 gene cluster and the *K. pneumoniae* *mrk* operon, we hypothesized that pOLA52 would promote biofilm formation, and this was confirmed by preliminary screenings for biofilm formation by *E. coli* CSH26±pOLA52. In order to evaluate

what specific genes or operons of pOLA52 contributed to biofilm formation when expressed by *E. coli* Genehogs, 88 clones containing pOLA52 with random Kan^R insertions were screened for their ability to form biofilm. Of the 88 *E. coli* Genehogs/pOLA52::Kan^R transformants, 7 appeared to be deficient in biofilm formation. When tested in quadruplicate, on average an 80-fold reduction in biofilm formation was observed in these 7 strains when compared to the 4 biofilm-forming clones selected for sequencing, described later (Fig. 2). Thus, specific genes encoded by pOLA52 induced biofilm formation.

Plasmids were subjected to *Nla*III digestion, T4 ligation and PCR as illustrated in Fig. 1. The insertion of the entranceposon was localized to the putative *mrk* operon of pOLA52 in all of the seven biofilm-negative *E. coli* Genehogs/pOLA52::Kan^R transformants by sequencing (Fig. 3). In each of the four biofilm-positive transformants, the insertion mapped in genes encoding proteins with known functions (*topB*, *toxB*, *soj* and *oqxB*), outside the putative *mrk* operon. This strongly indicates that the putative *mrk* operon, homologous to that encoding type 3 fimbriae in *K. pneumoniae* (Allen *et al.*, 1991), is essential for the biofilm-inducing capability of pOLA52.

Biofilm formation on abiotic surfaces

Two clones, *E. coli* Genehogs/pOLA52-*oqxB*::Kan^R (H1) with the transposon inserted in the *oqxB* gene, encoding the efflux pump, and *E. coli* Genehogs/pOLA52-*mrkC*::Kan^R (H5) with the transposon inserted in the *mrkC* gene homologue, were chosen as representatives of biofilm and non-biofilm phenotypes, respectively. When the two clones were grown in microtitre plates and stained with CV, there was more than a 100-fold, statistically

Fig. 2. Biofilm formation of seven biofilm-negative (B3, C5, D6, E2, E4, G5, H5) and four (randomly picked) biofilm-positive (B8, G6, G8, H1) *E. coli* Genehogs/pOLA52::Kan^R clones. *E. coli* Genehogs without plasmid pOLA52 (NP) was included as a negative control. The cells were incubated in microtitre plate wells for 24 h, followed by quantification of biofilm formation by staining of attached cells and matrix with CV and spectrophotometric absorbance measurements (A₅₉₅). Columns represent means ± SD for four replicates.

Fig. 3. Distribution of the Kan^R insertion in the seven biofilm-negative clones (B3, C5, D6, E2, E4, G5, H5), all localizing in the putative *mrk* operon. The Kan^R insertions in the four biofilm-positive clones all localized outside this operon. The insertions were mapped by *Nla*III digestion of the mutagenized plasmid followed by ligation, PCR amplification and sequencing.

significant (Student's *t*-test, $P < 0.01$) difference in biofilm formation (Fig. 2).

The ability to form biofilm in microtitre plates could be fully complemented when *E. coli* Genehogs and strain H5 were transformed with plasmid pLOW2::Tet^R-*mrkA*-F. CV staining showed attachment to microtitre plates at levels slightly higher than those seen with the H1 derivative (data not shown), probably due to increased expression levels or copy number differences between the pLOW2 and the pOLA52 derivatives.

A twofold reduction (statistically significant, Student's *t*-test, $P < 0.01$) in biofilm formation was observed for H5, containing a disrupted putative *mrk* operon, compared to that of H1, when the two clones were allowed to attach to silicone urinary catheters [biofilm formation (A_{595}) = 0.2 and 0.09 for H1 and H5, respectively]. This implies that bacterial strains containing pOLA52 may have enhanced opportunities for establishment of biofilms on this surface and other medical devices.

Both *E. coli* Genehogs/pOLA52-*bla*::Kan^R and H1 were significantly more resistant than H5 to 0.5 µg Tet ml⁻¹ after 48 h. Compared to the non-exposed equivalent strains, the activity of H5 (biofilm-negative) was reduced by 43.3 %, but by only 22.6 % and 15.8 % for H1 and *E. coli* Genehogs/pOLA52-*bla*::Kan^R, respectively. The three higher concentrations of Tet resulted in near-total inhibition of all three strains. There was no significant difference in the activity when the strains were exposed to any of the concentrations of H₂O₂ tested. The reason for Tet to result in a significant difference in activity compared to H₂O₂ may be the differences in size and diffusion ability of the two compounds, as H₂O₂ is smaller and may move more freely in a biofilm. Neither of the compounds is excluded by the multidrug efflux pump encoded by pOLA52 (Hansen *et al.*, 2007), but to ensure that the lack of the pump did not affect the observed results, *E. coli* Genehogs/pOLA52-*bla*::Kan^R was included in the study.

These results indicate that the biofilm mode of growth protects bacteria from some antibacterial compounds, and strains capable of biofilm formation may therefore obtain a

higher fitness under some conditions than those incapable of biofilm formation. Thus, the consequences of conjugative transfer of pOLA52 to potentially pathogenic bacteria may be severe, as they may cause hard-to-cure infections on various surfaces.

Expression of the *mrk* operon

RT-PCR analysis of the putative *mrk* operon was performed to determine whether the *mrk* genes were transcribed as a single mRNA and thereby likely to be organized as an operon. As shown in Fig. 4, we obtained PCR products when using H1 as template in four RT-PCRs, pair-wise linking the *mrk* genes and indicating organization as an operon, regulated and transcribed by a single promoter. Using a promoter prediction server (www.fruitfly.org/seq_tools/promoter.html), several putative promoters were found to be situated in an IS1 insertion sequence upstream from the operon, which might be involved in the transcription of the *mrkABCDF* genes.

In *K. pneumoniae*, the *mrk* operon encodes type 3 fimbriae, which have previously been detected by immunoblotting with rabbit antiserum against the major subunit MrkA (Di Martino *et al.*, 2003). This was used in this study to verify the expression of type 3 fimbriae on the surface of *E. coli* Genehogs. As expected, both *E. coli* Genehogs/pOLA52-*oqx*B::Kan^R (H1) and *K. pneumoniae* DSA712 showed

Fig. 4. RT-PCR analysis of *mrkA*-F transcription in *E. coli* Genehogs/pOLA52-*oqx*B::Kan^R (H1); PCR amplification products of either RNA (-) as a negative control or reverse-transcribed (RT) DNA (+) were visualized in a 1.2% agarose gel. *mrkA*-B, RT product generated with *mrkB*rev and amplified with PCR primers *mrkB*revnst1 and *mrkA*fw; *mrkB*-C, RT product generated with *mrkC*rev and amplified with PCR primers *mrkC*revnst1 and *mrkB*fw; *mrkC*-D, RT product generated with *mrkD*rev and amplified with PCR primers *mrkD*revnst1 and *mrkC*fw; *mrkD*-F, RT product generated with *mrkF*rev and amplified with PCR primers *mrkF*revnst1 and *mrkD*fw.

positive reactions to the anti-MrkA antibody, and *E. coli* Genehogs did not (data not shown). The results of the RT-PCR analysis and the immunoblotting strongly suggest that the *mrk* operon, contained in pOLA52, encodes type 3 fimbriae, which are expressed by *E. coli* Genehogs and promote biofilm formation.

Effect of type 3 fimbriae on conjugation frequency of *E. coli*/pOLA52

The frequency of conjugative transfer events, calculated as transconjugants per donor, was reduced approximately 3750-fold for the pOLA52-*mrkC*::Kan^R plasmid derivative (transfer frequency 2.4×10^{-5}) compared to the pOLA52-*oqx*B::Kan^R plasmid derivative (transfer frequency 9.0×10^{-2}). This reduction was statistically significant (Student's *t*-test, $P < 0.01$). Thus, by encoding fimbriae, pOLA52 increases the frequency of its own conjugational transfer, and thereby the plasmid promotes its spread into other bacterial populations. This may enhance the possibility of pOLA52 transfer to potential pathogenic bacteria, which will then acquire the ability to attach to surfaces and become more resistant to antimicrobial agents.

The enhanced conjugation frequency may indicate that, as in *K. pneumoniae*, the type 3 fimbriae, encoded by pOLA52, mediate attachment to abiotic as well as biotic surfaces, the biotic here being intraspecific cell-cell attachment leading to a higher conjugation rate. The ability of type 3 fimbriae to enhance conjugation frequency has not to our knowledge been previously described; however, Dudley *et al.* (2006) recently reported a similar observation for plasmid-encoded type 4 pili in *E. coli*. These pili were different from the thicker conjugation pili, but their presence resulted in higher conjugation rates, and enhanced attachment to abiotic (polystyrene and glass coverslips) and to biotic (human epithelial cells) surfaces. Another possible explanation for the observed increased conjugation frequency could be that the type 3 fimbriae (or component(s) thereof) somehow stabilize or aid the conjugation apparatus of the plasmid in the liquid broth. This would require interactions between proteins of the two systems or that the general structural change in the membrane due to the type 3 fimbriae stabilizes the conjugation pili. The reason for higher conjugation frequency of cells expressing the type 3 fimbriae remains to be investigated.

The roles of MrkA and MrkD in biofilm formation

In the seven clones deficient in biofilm formation, the Kan^R insertion was localized to the putative *mrk* operon, specifically in the *mrkA*, *mrkC* or *mrkD* genes. The MrkD adhesin encoded by *mrkD* has previously been reported to mediate *K. pneumoniae* biofilm formation on extracellular matrix- and collagen-coated surfaces (Jagnow & Clegg, 2003), whereas biofilm formation on abiotic plastic

surfaces (microtitre plates) was facilitated by the structural fimbrial component MrkA, but independent of MrkD (Langstraat *et al.*, 2001). In contrast to this, our results indicate important roles of both the *mrkA* and *mrkD* gene products in biofilm formation in polystyrene microtitre plate wells, as transposon insertion in either gene was shown to abolish biofilm formation. Here we show that the insertions in three of the knockout clones are positioned in the *mrkD* gene. The *mrkD* mutants could also abolish the expression of *mrkF*, but previous studies have shown that the absence of MrkF still allows expression of fimbriae. This is indicative of the roles of MrkA, MrkB and MrkC as proteins necessary for the presentation and positioning of the MrkD adhesin in the outer membrane and of the MrkD protein as responsible for the actual adhesion. Further complementation studies are needed to verify this hypothesis. It is possible that the discrepancy is due to the expression of the *mrk* genes in *E. coli* in this study, whereas the other studies were performed in *K. pneumoniae*, or to the fact that we are here studying homologues of the MrkA and MrkD proteins.

Conjugative transfer of pOLA52 to other members of the *Enterobacteriaceae*, and induction of biofilm formation by these strains

Type 3 fimbriae are often produced by potentially pathogenic members of the *Enterobacteriaceae* (Adegbola & Old, 1983; Allen *et al.*, 1991; Old & Adegbola, 1985). We successfully transferred pOLA52 to four such members: *S. Typhimurium*, *K. pneumoniae*, *Kluyvera* sp. and

Fig. 5. Biofilm formation of members of the family *Enterobacteriaceae*: *Salmonella Typhimurium* DT27, *Klebsiella pneumoniae* DSA712, *Kluyvera* sp. MB101 and *Enterobacter aerogenes* DSM30053, with (grey bars) and without (white bars) pOLA-*bla*::Kan^R. *Escherichia coli* CSH26 ± pOLA52 was used as control. The cells were incubated in microtitre plate wells for 24 h, followed by quantification of biofilm formation by staining of attached cells and matrix with CV and spectrophotometric absorbance measurements (A₅₉₅). Columns represent means ± SD for four replicates.

Enterobacter aerogenes. As shown in Fig. 5, statistically significant increases in biofilm formation were observed in all strains harbouring plasmid pOLA52-*bla*::Kan^R (6.5–114-fold induction; Student's *t*-test, *P*<0.01). Although still statistically significant, the increase in biofilm formation was less pronounced in *K. pneumoniae* when compared to the remaining strains, most likely because this strain contains the *mrk* operon on the chromosome, and therefore expresses type 3 fimbriae regardless of harbouring pOLA52. These results are strongly indicative of pOLA52-encoded type 3 fimbriae expression of these *Enterobacteriaceae* strains, enhancing their attachment and biofilm formation, which may lead to increased virulence. It remains to be investigated whether the type 3 fimbriae, expressed from pOLA52, constitute any advantage in adherence to animal cells. We will test this, using model animal systems, in the near future.

Concluding remarks

The results of this and previous studies reveal some disturbing characteristics of pOLA52, regarding human health. The plasmid encodes a multidrug efflux pump of the RND family, capable of exporting a wide range of antibiotics, detergents and disinfectants (Hansen *et al.*, 2004, 2007). Furthermore, the *bla* gene of the plasmid encodes β -lactamase, conferring resistance to β -lactam antibiotics (Hansen *et al.*, 2004). The plasmid also encodes type 3 fimbriae, promoting attachment to abiotic and biotic surfaces, biofilm formation and its own spread by increased conjugative transfer rates. To our knowledge, this is the first example of adhesive fimbriae and a multidrug efflux pump being encoded on a single, conjugative plasmid. The ability to form biofilm and export a variety of chemicals will, in many environments, increase the fitness of the host cell, as demonstrated in this study with exposure to Tet. These traits are therefore likely to contribute to selectively stabilizing the presence of pOLA52 in its present hosts, but also to spread of the plasmid to new hosts. As demonstrated here, pOLA52 can easily be transferred from *E. coli* to other, potentially pathogenic members of the *Enterobacteriaceae*, which may increase the virulence of these recipient strains. Infections caused by a pathogenic bacterial strain that has acquired pOLA52 may therefore be difficult to cure. Previously we have found several *E. coli* isolates from pigs harbouring the *oqxAB* genes on plasmids (Hansen *et al.*, 2005). It remains to be investigated if these plasmids also encode the ability to express type 3 fimbriae. The fact that pOLA52 originates from swine manure renders this scenario worryingly realistic.

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