DAF- and collagen-binding properties of chimeric Dr fimbriae

Beata Zalewska,¹ Janusz Stangret,² Katarzyna Bury,¹
Marek Wojciechowski,³ Józef Kur¹ and Rafał Piątek¹

Correspondence
Rafał Piątek
wejraf@o2.pl

¹Department of Microbiology, Gdańsk University of Technology, ul. G. Narutowicza 11/12, 80-952 Gdańsk, Poland

²Department of Physical Chemistry, Gdańsk University of Technology, ul. G. Narutowicza 11/12, 80-952 Gdańsk, Poland

³Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, ul. G. Narutowicza 11/12, 80-952 Gdańsk, Poland

Display of heterologous proteins on the surface of micro-organisms has become an increasingly used strategy for a range of applications in microbiology, biotechnology and vaccinology. In this study, the potential of the major structural protein DraE of *Escherichia coli* Dr fimbriae as a display system for heterologous sequences was tested. One copy of a heterologous sequence mimicking a small P_k epitope of simian virus 5 was inserted into the *draE* gene, replacing the N-terminal region of the surface-exposed domain 2 as previously done with the glycoprotein D of herpes simplex virus type 1. The exposure of chimeric proteins on the bacterial surface was detected by immunofluorescence microscopy. Insertion of the heterogenic peptides had no detectable effect on the Ig-barrel structure of the DraE fimbrial subunits, as confirmed by FTIR spectroscopy. Additionally, the affinity of the chimeric fimbriae for DAF and type IV collagen was similar to that of the wild-type Dr fimbriae.

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INTRODUCTION

Many bacteria live adhered to surfaces in complex communities, rather than existing as free-swimming isolated cells. The ability of bacteria to recognize and adhere to a specific surface is a fundamental aspect of microbial pathogenesis and ecology. Most bacteria can express several adhesins, enabling them to recognize and attach to a very diverse spectrum of molecules on target surfaces (Krogfelt, 1991). In general, bacterial adhesins are organized as thin, thread-like organelles referred to as fimbriae, although they can also exist as non-polymeric protein species on the cell surface (Krogfelt, 1991; Klemm & Schembri, 2000).

Fimbrial adhesins are produced in large amounts (up to about 500 copies per cell) that make them attractive for surface display of heterologous protein segments. This can be achieved by genetic fusion of DNA segments encoding for example immunologically relevant sectors of various foreign peptides into permissible positions of fimbrial structural genes. The resulting chimeric proteins are displayed on the bacterial cell surface and constitute candidate vaccines. The technology has also been used for surface

display of random peptide libraries as integral parts of the fimbriae (Klemm & Krogfelt, 1994; Klemm & Schembri, 2000). The repetitive nature of the helically arranged subunits results in the display of the same epitopes 10² to 10³ times on each fimbrial thread, or 10⁵ to 10⁶ times on each bacterial surface, rendering fimbriae major immunogens of fimbriated killed or live bacterial vaccines. A vaccine based on wild-type fimbriae has been successfully used, for example to prevent enterotoxigenic *Escherichia coli* diarrhoeal infections in farm animals (Moon & Bunn, 1993). The results obtained confirmed the high immunogenicity of fimbriae in live vaccines and purified proteins.

E. coli cells express various proteins on the bacterial surface that mediate attachment to mammalian receptors. *E. coli* strains bearing Dr fimbriae, or the related adhesins afimbrial adhesin I (AFA-I), afimbrial adhesin III (AFA-III) or F1845 adhesin, are associated with urinary tract infections (UTI), in particular cystitis, gestational pyelonephritis and diarrhoeal disease (Giron *et al.*, 1991; Johnson, 1991; Levine *et al.*, 1993; Nowicki *et al.*, 1994). Despite similar genetic organization, the phenotypic expression in the Dr family of adhesins is associated with either fimbrial or afimbrial morphology. The *dra* gene clusters share a highly conserved region, including the *draA*, *draB*, *draC*, *draD*, and *draF* genes. Unlike these genes, the structural adhesin-encoding gene, designated *draE*, is highly heterogeneous

Abbreviations: DAF, decay-accelerating factor; FTIR, Fourier transform infrared; gD, glycoprotein D; HSV-1, herpes simplex virus type 1; MRHA, mannose-resistant haemagglutination; SV5, simian virus 5; TRITC, tetramethylrhodamine isothiocyanate.

within the Dr family of adhesins. Members of this family bind to the Dr^a blood-group antigen present on the decay-accelerating factor (DAF; CD55), a complement regulatory and signalling molecule (Nowicki *et al.*, 2001). Dr haemagglutinin (a major structural subunit of Dr fimbriae) is the only member of the Dr family adhesins that has the unique ability to bind to two receptors – DAF and type IV collagen (Westerlund *et al.*, 1989; Carnoy & Moseley, 1997). The binding of Dr fimbriae (expressed at the bacterial cell surface) to the short consensus repeat 3 domain (SCR-3) of the DAF receptor leads to the internalization of Dr⁺ *E. coli* into epithelial cells (Nowicki *et al.*, 1993). The DAF-binding site of the DraE subunit contains amino acids located in the central part of the protein. The latest research has revealed that residues 63–81 are involved in DAF recognition (Van Loy *et al.*, 2002; Selvarangan *et al.*, 2004). Additionally, the interactions of DraE adhesin with the 7s domain (tetramer) of type IV collagen localized at the basement membrane renal interstitium, which underlies all epithelia, may facilitate colonization and development of persistent renal infections caused by *E. coli* Dr⁺ strains. Replacement of a single amino acid at the 113 position of DraE results in loss of the type IV collagen-binding property (Carnoy & Moseley, 1997; Selvarangan *et al.*, 2004). Furthermore, previous investigations have shown that the Dr adhesins AfaE-I, AfaE-III, DraE and DaaE adhere to CEA and related cell adhesion molecules (CEACAM1 and CEACAM6). Recognition of CEA and CEACAM6 is accompanied by tight attachment of the bacteria to elongated cell surface microvilli (Korotkova *et al.*, 2006).

The potential of the major structural protein DraE of *E. coli* Dr fimbriae has been used to display a 14 aa peptide of a small P_k epitope (GKPIP_NPLLGLDST) localized within the N-terminal common domain of the V/P proteins derived from simian virus 5 (SV5). The main function of the V protein of SV5 is degradation of the STAT1 protein, an essential component of the IFN- α/β and IFN- γ signalling cascade, through a proteasome-mediated pathway in human cells but not in murine cells. The exact molecular details of how V blocks IFN signalling and targets STAT1 for proteasome-mediated degradation have yet to be determined, but it seems likely that a small fragment of the P_k epitope (GKPIP_NPLL) may be critical for the interaction of protein V with the host cell protein(s) involved in this process (Sun *et al.*, 2004; Young *et al.*, 2001). The ability to display the foreign epitopes on the surface of bacteria can be used in the future for development of recombinant vaccines, such as purified fimbrial proteins. Both epitopes were inserted into the DraE adhesin in place of the N-terminal region of the surface-exposed domain 2 (from V28 to A38) located between C19 and C51, conserved among the Dr family adhesins. Initially, the insertion position of heterologous epitopes was chosen based on mutagenesis studies (Carnoy & Moseley, 1997) and computer analysis of surface-exposed domains (Emini *et al.*, 1985), but this was later confirmed by the atomic resolution

structure of the AfaE-III afimbrial subunit, which is closely related to the DraE fimbrial subunit (Anderson *et al.*, 2004). The construction of potential chimeric fimbrial vaccines should be preceded by research focused on biological and physico-chemical properties of fimbrial subunits after insertion of immune-relevant peptides. Therefore, the present study aimed at characterizing the DAF and type IV collagen-binding phenotypes of DraE fimbrial subunits with inserted heterologous epitopes. Whole-cell extracts and fimbrial fractions were purified from *E. coli* BL21(DE3) strains expressing wild-type Dr (pCC90) or Dr-V5/HSV (pCC90D54stop-E-V5/E-HSV) chimeric fimbriae, and were then tested in *in vitro* binding assays. Our investigation demonstrated that the adherence phenotype of DraE was retained also when various sectors of foreign proteins were inserted. Additionally, the chimeric fimbriae had an affinity for DAF and type IV collagen that was similar to that of the wild-type Dr fimbriae. The epitopes inserted into DraE adhesin did not affect the ability of the subunits to polymerize into fimbrial structures. Size-exclusion chromatography, immunofluorescence microscopy, SDS-PAGE, and Western blotting of fimbrial samples not denatured thermally confirmed this. The FTIR spectroscopy analyses of isolated fimbrial fractions showed that insertion of heterogenic peptides had no detectable effect on the Ig-barrel structure of the DraE subunits.

METHODS

Bacterial strains, plasmids, enzymes and reagents. The over-expression was carried out in *Escherichia coli* BL21(DE3) (Novagen). Bacterial cells were grown in Luria broth (LB) without glucose or on Luria agar (LA) plates (containing 1.5 % agar) supplemented with the appropriate antibiotics (Sigma).

Plasmid pCC90, corresponding to the *dra* operon with its promoter region and regulatory genes upstream of the *draB* gene deleted, and pCC90D54stop, with a mutated *draE* gene (the GAC triplet of the *draE* gene encoding Asp-54 was replaced with a stop codon), were provided by S. Moseley, University of Washington, Seattle (Carnoy & Moseley, 1997).

Plasmid pET30Ek/LIC, an expression vector with a strong T7 promoter, a kanamycin resistance gene and a pBR322 origin of replication, was from Novagen.

Plasmid pDraE-HSV3, encoding DraE adhesin with an inserted glycoprotein D (gD) epitope (QPELAPEDPED) of herpes simplex virus type 1 (HSV-1), was constructed as described previously (Zalewska *et al.*, 2003).

Plasmid pDraE-V5, encoding DraE adhesin with an inserted V5 epitope, was constructed on the basis of a pET30Ek/LIC expression vector as previously described for the HSV-1 gD epitope (Zalewska *et al.*, 2003).

The plasmid DNAs were isolated from *E. coli* cultures using the Mini-prep Plus kit (A&A Biotechnology). After enzymic reactions, the DNAs were purified using the Clean-Up kit (A&A Biotechnology). Restriction enzymes were purchased from New England BioLabs. The reagents for PCR were obtained from DNA-Gdańsk II, and other reagents were purchased from Sigma.

Antisera. Rabbit anti-Dr adhesin antibodies raised against purified native Dr fimbriae (Pham *et al.*, 1997) have been described previously. HSV-Tag mouse monoclonal immunoglobulin G1 (IgG1) antibodies with specificity and affinity for the 11 aa peptide derived from HSV-1 gD were from Novagen. Mouse monoclonal anti-V5 antibodies [with a specificity and affinity for the 14 aa P_k epitope present on the P and V proteins of the paramyxovirus of simian virus 5 (SV5)] conjugated to horseradish peroxidase were purchased from Invitrogen. Anti-rabbit IgG (whole molecule) antibodies conjugated to horseradish peroxidase and tetramethylrhodamine isothiocyanate (TRITC) were purchased from Sigma. Anti-mouse IgG (whole molecule) antibodies conjugated to fluorescein isothiocyanate (FITC) were purchased from Sigma.

PCR amplification and cloning of the *sygdraE-V5* gene. The *draE* gene with its signal sequence, encoding DraE adhesin, was amplified in two DNA fragments (I and II) on a template of pCC90, as previously described for insertion of an HSV-1 epitope (Zalewska *et al.*, 2003). The primers used in the amplification were designed based on the sequencing data obtained (GenBank accession no. AF329316).

For amplification of DNA fragment I, the following primers were used: 1-DraE-HSV forward primer (Zalewska *et al.*, 2003) and 2-DraE-V5 reverse primer *GGTGGAGTCCAGGCCGAGCAGCGGGTTCGGGATCGGTTTGCCGGTCAGGTACCAACCCGTACCTGCAC-TC* (the italicized sequence encodes a V5 epitope, and the bold part is complementary to the nucleotide sequence at the 5' end of the *draE* gene). For amplification of DNA fragment II, the following primers were used: 3-DraE-V5 forward primer *GGCAAACCGATCCCGAAC-CCGCTGCTCGGCTGGACTCCACCGCACCGCACCAATAGGGCCGGTACCGTGCAAGCG* (the italicized sequence encodes a V5 epitope, and the bold part is complementary to the nucleotide sequence at the 3' end of the *draE* gene) and 4-DraE-HSV reverse primer (Zalewska *et al.*, 2003). The DNA fragments obtained were hybridized and reamplified by PCR in a Perkin-Elmer 2400 thermocycler. The *sygdraE-V5* gene PCR product was digested with *NdeI* and *HindIII*, purified using the DNA Clean Up kit, and cloned directionally into the *NdeI* and *HindIII* sites of the pET30Ek/LIC vector. The resulting recombinant plasmid was designated pDraE-V5. This plasmid was confirmed by restriction analysis and sequenced using the ABI Prism 377 automatic sequencing system. The nucleotide sequence was analysed using the GeneDoc and CLUSTAL_X computer programs.

Purification of chimeric fimbriae (Dr-V5 or Dr-HSV) and wild-type fimbriae (Dr). Recombinant plasmid pDraE-V5/pDraE-HSV3 encoding DraE-syg adhesin (with the signal sequence) with a V5/HSV-1 epitope was introduced into *E. coli* BL21(DE3)/pCC90D54stop (DraE⁻ mutant). The recombinant *E. coli* BL21(DE3)/pCC90D54stopE-V5/E-HSV cells expressing Dr-V5/Dr-HSV fimbriae were grown on LA plates with the appropriate antibiotics (100 µg ampicillin ml⁻¹ and 20 µg kanamycin ml⁻¹) at 37 °C for 24 h. After incubation, 100 ml LB medium (supplemented with the appropriate antibiotics and with a 20% lactose solution to a final concentration of 0.4% to induce DraE-V5/DraE-HSV expression) was inoculated with single bacterial colonies scraped from the LA plates and grown with agitation at 37 °C for 24 h. Then the cultures were harvested by 20 min centrifugation at 1000 g. The supernatant was decanted, and the pellet was redissolved in PBS (3 ml). After that, the Dr-V5/Dr-HSV fimbrial proteins were isolated by heat shock treatment and ammonium sulfate precipitation, and then dialysed against PBS. Finally, the fimbriae were purified by gel-filtration chromatography on a Superdex 200 10/300 GL column (Amersham Biosciences) in PBS. They were eluted in the dead volume that contained high-molecular-mass fimbrial polymers. The eluted fimbriae were analysed by SDS-PAGE, and the protein concentration was determined by the Bradford method.

For the SDS-PAGE analysis, fimbrial samples were heated in Laemmli buffer at 98 °C for 5 min, or were incubated with Laemmli buffer at 25 °C for 10 min, but not denatured thermally.

The recombinant *E. coli* BL21(DE3)/pCC90 strain was used as a positive control for Dr fimbriae expression. The procedure for isolating DraE protein from the bacterial cell surface was the same as for the chimeric proteins. The recombinant strain *E. coli* BL21(DE3)/pCC90D54stop (not expressing Dr fimbriae at the cell surface) was used as a negative control.

Samples for IR analysis. The native and chimeric fimbriae samples in PBS were concentrated to a final concentration of 2 mg ml⁻¹ using Amicon Ultra-15 centrifugal filter devices (50 000 MWCO; Millipore). The samples to be lyophilized were frozen in liquid nitrogen and processed in a Christ Alpha 2-4 freeze-drier (Osterode) at a pressure of 10 µm of Hg and a condenser temperature of -60 °C for 18 h. The dried fimbrial samples were suspended in deionized H₂O to a final concentration of 25 mg ml⁻¹.

IR measurements and analysis. FTIR spectra were recorded on a Nicolet 8700 spectrometer (Thermo Electron Co.), using the Specac Golden Gate single-reflection diamond-heated ATR accessory. For each spectrum, 256 scans were made with a selected resolution of 4 cm⁻¹ and the spectrometer source was on Turbo mode during measurements. The temperature of the accessory top-plate was kept constant at 25 ± 1 °C by the electronic temperature controller (Specac West 6100+). The spectrometer and the ATR accessory were purged with dry nitrogen to prevent water vapour contamination of the spectra.

The advanced ATR correction algorithm introduced in the OMNIC 6.2 software for Nicolet FTIR spectrometers was used to correct the ATR spectra. The algorithm accounts for the relative band intensity caused by the dependence of the depth to which the sample is penetrated by the infrared beam upon wave number as well as the shift of band to lower wave numbers caused by dispersion of the refractive index and also the deviation from Beer's law caused by non-polarization effects. In order to use this ATR correction, four inputs are required: (1) the refractive index of the sample (the value for water was used), (2) the refractive index of the ATR crystal, (3) the angle of incidence, and (4) the number of bounces.

The reference water spectrum was subtracted from the protein sample spectra to obtain a straight and horizontal background line in the range of 1900–1720 cm⁻¹. The difference spectra were resolved into analytical band components (Gaussian and Lorentzian product function) using the band-fitting procedure.

The spectra were handled and analysed using the commercial programs GRAMS/32 (Galactic Industries Corporation) and RAZOR (Spectrum Square Associates) run under GRAMS/32.

Western blot analysis. The samples were mixed with sample buffer and run in 15% (w/v) bis-acrylamide gels containing SDS. The proteins were electroblotted onto nitrocellulose membranes and incubated with rabbit anti-Dr serum at a 1:5000 dilution or mouse anti-V5/HSV antibodies at a 1:2500 dilution. Blots were visualized according to Zalewska *et al.* (2003).

ELISA. The ability of purified chimeric Dr-V5/HSV fimbriae to bind to type IV collagen was analysed in an enzyme immunoassay. Initially, the wells of the microtitre plate were coated with type IV collagen (from human placenta) (Sigma) at a concentration of 20 µg ml⁻¹ and incubated at 4 °C overnight. They were then washed three times with PBS and blocked with 1% BSA in PBS for 2 h at 37 °C. The blocking solution was removed and 100 µl of different concentrations (1, 2, 3, 4 and 5 µg ml⁻¹) of the purified Dr fimbriae and Dr-V5/HSV chimeric fimbriae in PBS buffer were added to collagen-coated

coverslips and incubated for 1 h at 37 °C. After incubation, the plate was washed four times with PBS and the bound proteins were detected with rabbit polyclonal anti-Dr antibodies at a 1 : 500 dilution for 40 min at 37 °C. The bound anti-Dr antibodies were detected with polyclonal goat anti-rabbit horseradish peroxidase conjugate (Sigma) at a 1 : 5000 dilution for 40 min at 37 °C. All antibodies were diluted in PBS containing 0.1 % Tween 20. The bound antibodies were quantified using Sigma Fast *o*-phenylenediamine substrate as instructed by the manufacturer (Sigma), and measured in an ELISA plate reader at 490 nm wavelength. The experiment was performed in duplicate.

Agglutination of erythrocytes. Agglutination of erythrocytes by Dr⁺ *E. coli* strains is the result of binding to DAF. A binding assay performed with fimbrial preparations of Dr-HSV showed no agglutination reaction of erythrocytes expressing the DAF receptor (Zalewska *et al.*, 2003). The inserted HSV-1 epitope could result in less stable fimbriae that can break easily from the cell surface and cannot sustain mannose-resistant haemagglutination (MRHA) due to shearing stress. For that reason, agglutination of cells expressing DAF could be difficult to observe. Previous studies by Das *et al.* (2005) showed that alanine substitutions for amino acids in the hydrophilic surface-exposed domain 2 did not affect the binding of the Dr adhesin to DAF. This suggests that the chosen insertion position of foreign epitopes localized from V28 to A38 of the DraE fimbrial subunit should not affect the bacterial adherence to DAF (Van Loy *et al.*, 2002). To verify the results obtained, a whole-cell binding assay was done, since this can be more sensitive than the binding assay of purified fimbriae. For this experiment, a human erythrocyte suspension was prepared from blood group O whole human blood donated by a healthy volunteer. *E. coli* cells expressing wild-type Dr fimbriae or chimeric Dr-V5/HSV suspended in PBS (adjusted to a final OD₆₀₀ of 0.4) were mixed with an equal volume (20 µl) of a 3 % (v/v) suspension of human erythrocytes in PBS with 2 % methyl α -D-mannoside on glass slides. Twofold dilutions of *E. coli* were rotated for 5 min on ice, and the haemagglutination titre was recorded. An erythrocyte suspension mixed with the Dr-54stop mutant was used as a negative control.

Cell lines. HeLa cells were maintained in minimal essential medium (MEM) supplemented with 10 % (v/v) fetal calf serum (Sigma) and penicillin-streptomycin solution (Sigma) in a 5 % CO₂ atmosphere at 37 °C. The cell line was passaged using 0.25 % trypsin containing EDTA (Sigma).

Immunofluorescence microscopy of surface-exposed DraE-V5 fimbrial subunits. *E. coli* BL21(DE3)/pCC90D54stop-E-V5 cells from cultures grown on LA plates at 37 °C for 24 h were harvested and washed gently in PBS. Bacterial suspensions (100 µl; 10⁵–10⁶ cells ml⁻¹) were incubated at room temperature for 1 h with 50 µl of a 1 : 500 dilution (anti-Dr fimbriae) or 1 : 250 dilution (anti-V5 epitope) in PBS of the primary antibodies. The reaction mixtures were then washed three times with PBS containing 10 % (v/v) glycerol, and incubated with 50 µl of a 1 : 25 dilution (anti-rabbit IgG TRITC conjugate for the DraE protein) or 1 : 50 dilution (anti-mouse IgG FITC conjugate for the V5 epitope) in PBS of secondary antibodies at room temperature for 1 h. After the washings, bacterial suspensions (10 µl) were loaded on glass slides and observed with an immunofluorescence microscope (Olympus BX-60).

Immunofluorescence analysis of the binding between HeLa cells and *E. coli* strains expressing native Dr fimbriae and chimeric Dr-V5/HSV. HeLa cells were placed in the wells of a six-well plate with glass coverslips and grown in the appropriate medium for 24 h. The cells were then washed three times with PBS and fixed with 5 % formaldehyde for 10 min. After fixation, *E. coli* strains (grown overnight on LB medium) expressing wild-type Dr fimbriae

or chimeric Dr-V5/HSV suspended in PBS (adjusted to a final OD₆₀₀ of 0.4) were added to a culture plate containing HeLa cells grown on glass coverslips and incubated for 1 h at 37 °C. Then the cells were washed three times with PBS and incubated for 45 min at 37 °C with rabbit anti-Dr polyclonal antibody diluted 1 : 500 in PBS, followed by three washings with PBS. After the washings, cells were incubated with anti-rabbit IgG TRITC conjugate diluted 1 : 500 in PBS for 1 h at 37 °C, followed by three washings with PBS. Finally, the glass coverslips were examined for bound bacteria using an Olympus BX-60 immunofluorescence microscope. *E. coli* BL21(DE3)/pCC90D54stop was used as a negative control. The experiment was performed in duplicate.

RESULTS

Construction of a pDraE-V5 recombinant plasmid

The recombinant plasmid pDraE-V5 encoding the DraE fimbrial subunit (with its signal sequence) with a P_k epitope present on the P and V proteins of SV5 was obtained by cloning of a reamplified PCR product into the *Nde*I and *Hind*III sites of the pET30Ek/LIC vector. The sequence encoding the 14 aa P_k epitope (GKPIPNNPLLGLDST) was introduced into the *draE* gene in place of the N-terminal region of the surface-exposed domain 2 (VAKTRGQLTDA), as previously described for the HSV-1 gD epitope derived from HSV-1 (Zalewska *et al.*, 2003). Analysis of the AfaE-dsc structure (98 % amino acid identity to DraE) showed that this region encompassed residues located in the loop connecting the A2 and B β -strands (Fig. 1). The first two residues, V28 and A29, of

Fig. 1. Localization of DAF- and collagen-binding regions in the structure of AfaE-III-dsc. Ribbon representation of AfaE-III-dsc (PDB code 1RXL) with the indicated residues (shown in red) replaced by an epitope sequence in chimeric fimbriae. Residues implicated in DAF binding (Van Loy *et al.*, 2002; Anderson *et al.*, 2004) and collagen recognition (Carnoy & Moseley 1997; Korotkova *et al.*, 2006) are shown in green and blue, respectively. The β strands A2 and B are labelled. View B is rotated 180° counterclockwise with respect to view A.

the exchange region from the C-terminal end of the A2 β -strand.

Expression of chimeric Dr-V5 fimbriae and immunological detection of an inserted P_k epitope

Expression of the chimeric Dr-V5 fimbriae was performed as described in Methods. Fimbriae were isolated from the bacterial cell surface by shearing and separation on a Superdex 200 10/300 GL column. Chimeric Dr-HSV and wild-type Dr fimbriae were isolated for comparison. The concentrations of the proteins were determined by the Bradford method. We obtained about 4 mg of DraE-V5, comparable to the amounts obtained for DraE-HSV and DraE. The ability of *E. coli* BL21(DE3) harbouring plasmids pDraE-V5 and pCC90D54stop to express chimeric Dr-V5 fimbriae was analysed by immunoblotting, haemagglutination and immunofluorescence microscopy as previously described for Dr-HSV fimbriae (Zalewska *et al.*, 2003). As a negative control, an *E. coli* strain containing the *dra* gene cluster with a mutated *draE* gene (Dr-D54stop mutant) was used. In Western blots the rabbit anti-Dr polyclonal antibody and mouse monoclonal anti-V5 antibody recognized chimeric DraE-V5 proteins (Fig. 2). Detection of an inserted HSV-1 epitope has previously been described (Zalewska *et al.*, 2003). We also found that chimeric fimbriae with inserted epitopes (V5 of SV5 and gD of HSV-1) isolated from the cell surface were stable in Laemmli buffer at the ambient temperature at a level comparable to native Dr fimbriae. This property of the fimbrial fractions allowed analysis by SDS-PAGE and Western blotting (with anti-Dr serum and antibodies against inserted epitopes) with

samples not denatured thermally. A similar approach has been used to characterize other pili and fimbriae (Zavialov *et al.*, 2002). The chimeric Dr-V5 and Dr-HSV fimbriae were similar to the wild-type Dr fimbriae and were visualized as high-molecular-mass multimers forming distinct bands of decreasing size (Fig. 2). This shows that the epitopes inserted into the DraE fimbrial subunits have not affected the ability of the subunits to polymerize into high-molecular-mass structures. Immunofluorescence staining (anti-Dr-anti-TRITC and anti-V5-anti-FITC) of *E. coli* expressing chimeric Dr-V5 fimbriae showed that the specific antibodies recognized the chimeric DraE-V5 protein (Fig. 3). Altogether, these results demonstrated cell-surface expression of the heterologous P_k epitope inserted into a DraE fimbrial subunit like the HSV-1 gD epitope.

IR spectroscopy of native and chimeric fimbriae

FTIR spectroscopy is a powerful technique used in the study of protein conformation, especially for recognizing the formation of secondary structures and their dynamics (Haris & Severcan, 1999). The IR spectra of proteins between 1700 and 1500 cm⁻¹ are characterized by two major bands associated with amide I and amide II vibrations. The amide I band is mainly associated with the stretching vibration of the C=O of the amide groups (Arrondo & Goni, 1999). The C=O stretching vibration is affected differently by dipole interactions and hydrogen bonds in different secondary structures. Thus, the shape of the amide I band reflects the secondary structure of the analysed proteins. Fig. 4(a) shows the IR spectra in the range from 1800 to 1300 cm⁻¹ of native and chimeric (gD, V5) Dr fimbriae. The spectra in this region of amide bands

Fig. 2. Immunoblot analysis of fimbrial polymerization into high-molecular-mass structures. Fimbrial preparations from recombinant *E. coli* strains were analysed with anti-Dr (anti-fimbrial) (a) and anti-V5 (anti-epitope) antibodies (b). Fimbrial preparations were separated by 12% SDS-PAGE and transferred onto nitrocellulose. Fimbriae were isolated from *E. coli* BL21(DE3)/pCC90D54stop (lanes 1 and 2), *E. coli* BL21(DE3)/pCC90D54stop-E-V5 (lanes 3 and 4), *E. coli* BL21(DE3)/pCC90D54stop-E-HSV (lanes 5 and 6), and *E. coli* BL21(DE3)/pCC90 (lanes 7 and 8). Samples in lanes 1, 3, 5 and 7 were denatured at 98 °C; samples in lanes 2, 4, 6 and 8 were not thermally denatured. Lane M shows PageRuler Prestained Protein Ladder Plus (Fermentas) for 10, 15, 27, 35, 55, 70, 100, 130 and 250 kDa. The chimeric and wild-type fimbriae were visualized as high-molecular-mass multimers forming distinct bands of decreasing size. The arrows indicate a monomer form of the DraE protein.

Fig. 3. Surface display of chimeric fimbriae examined by immunofluorescence microscopy (IFM) of *E. coli* strains. Bacteria were incubated with primary antiserum, washed and incubated with secondary antiserum conjugated with TRITC or FITC, washed and subjected to microscopy. (a) IFM of *E. coli* BL21(DE3)/pCC90D54stop incubated with a rabbit anti-Dr serum and anti-rabbit serum conjugated with TRITC (negative control of fimbrial expression). (c) IFM of *E. coli* BL21(DE3)/pCC90D54stop incubated with a mouse anti-V5 serum and anti-mouse serum conjugated with FITC (negative control of fimbrial expression). (e) IFM of *E. coli* BL21(DE3)/pCC90D54stop-E-V5 incubated with a rabbit anti-Dr serum and anti-rabbit serum conjugated with TRITC. (g) IFM of *E. coli* BL21(DE3)/pCC90D54stop-E-V5 incubated with a mouse anti-V5 serum and anti-mouse serum conjugated with FITC. (i) IFM of *E. coli* BL21(DE3)/pCC90 incubated with a rabbit anti-Dr serum and anti-rabbit serum conjugated with TRITC (positive control of fimbrial expression). (k) IFM of *E. coli* BL21(DE3)/pCC90 incubated with a mouse anti-V5 serum and anti-mouse serum conjugated with FITC. (b, d, f, h, j and l) Phase-contrast micrographs of the bacterial cells shown in (a), (c), (e), (g), (i) and (k), respectively. Micrographs were taken with an Olympus BX-60 microscope at a magnification of $\times 10\,000$.

I and II are almost identical, with the maximum absorbance at 1631 and 1532 cm^{-1} , respectively. The shape and the value of the maximum band of amide I show that the fimbrial samples analysed were mainly composed of β -structures. Band-fitting of the spectra decomposed the amide I band of fimbrial samples into two characteristic components (Fig. 4b). The main component at 1629 cm^{-1} , identical in native and chimeric fimbriae (gD, V5), was attributed to amide groups involved in extended β -sheet structures (Subirade *et al.*, 1994). The second component of the native and the chimeric fimbriae spectra was observed at 1663 and 1661 cm^{-1} , respectively. These bands are located in the spectral region attributed to turns and unordered structures (Arrondo & Goni, 1999). The β -sheet and turns/unordered components accounted for about 84 % and 16 % of the amide I band area, respectively. These

FTIR data are in agreement with published structures of AfaE-dsc and DraE proteins (Anderson *et al.*, 2004; Pettigrew *et al.*, 2004). The IR data presented showed that the insertion of the heterogenic epitopes into the A2-B loop of the DraE subunit did not perturb the Ig-barrel structure of the adhesin to any detectable extent.

Agglutination of erythrocytes

Haemagglutination by *E. coli* strains expressing Dr fimbriae is the result of binding to DAF on the surface of erythrocytes. Therefore equal volumes of the whole-cell extracts of *E. coli* strains expressing chimeric Dr-V5 or Dr-HSV fimbriae and the erythrocyte suspension were mixed in PBS to examine the MRHA phenotype. The whole-cell extract of *E. coli* strains expressing wild-type Dr fimbriae was used

Fig. 4. IR analysis of chimeric and native Dr fimbriae. FTIR spectra of Dr (solid line) and chimeric (gD, V5) (dashed line) aqueous solutions at 25 °C. (a) Original spectra; absorbance has been scaled to the same value at the maximum of the amide I band for a better comparison. (b) Superposition of band components resulting from the band-fitting procedure; the band components for the amide I region are shown together with their band positions.

as a positive control. This experiment showed that the whole-cell extracts of the *E. coli* strains expressing chimeric Dr-V5 or Dr-HSV fimbriae mediated agglutination of erythrocytes expressing DAF receptor comparable to that seen for the wild-type Dr fimbriae (Fig. 5A). Additionally, all *E. coli* strains showed haemagglutination titres of 1:128, comparable to that of the wild-type Dr adhesin.

Adherence to the DAF receptor of *E. coli* strains expressing chimeric fimbriae

To examine the specificity of binding to the DAF receptor of the *E. coli* strains expressing chimeric Dr-V5 or Dr-HSV fimbriae, a HeLa cell line was used. *E. coli* strains expressing chimeric Dr-V5 or HSV (grown overnight on LB medium), suspended in PBS (adjusted to a final OD₆₀₀ of 0.4), were added to a culture plate containing HeLa cells grown on glass coverslips. Bound bacterial cells were detected with a rabbit anti-Dr polyclonal antibody and an anti-rabbit IgG TRITC conjugate. The binding patterns of the chimeric Dr-V5 and Dr-HSV adhesins to HeLa cells were similar to that of wild-type Dr adhesin (Fig. 5B). The results achieved corroborated the data obtained previously indicating that the replacement of amino acids in domain 2 did not affect the binding of *E. coli* strains expressing chimeric fimbriae to the DAF receptor.

Analysis of type IV collagen binding by chimeric Dr-V5 and Dr-HSV fimbriae

The isolated and purified Dr-V5 and Dr-HSV chimeric fimbriae were compared with respect to their ability to bind type IV collagen in an enzyme immunoassay. Varying concentrations of fimbrial fractions (1–5 µg ml⁻¹) were incubated in wells of a microtitre plate coated with 2 µg of type IV collagen. Bound fimbriae were detected with a rabbit anti-Dr polyclonal antibody (Fig. 6). The binding phenotype was not dependent on the protein concentrations. A control sample prepared in the same manner from *E. coli* BL21(DE3)/pCC90D54stop did not bind the type IV collagen.

DISCUSSION

Surface display of foreign epitopes on bacteria can be useful in many applications, such as recombinant vaccines, reagents for diagnostics, whole-cell biocatalysts and bioadsorbents (Georgiou *et al.*, 1997). Over the last decade, a range of immune-relevant sectors of foreign proteins have been successfully displayed on the bacterial cell surface by a large variety of fimbriae and flagella. Fimbriae are adhesive organelles that enable bacteria to target and colonize specific host cells and tissues. They are very attractive candidates for epitope surface display due to their long, thread-like polymeric structures, and to the fact that they are found on bacterial surfaces in amounts up to 500 copies per cell (Stentebjerg-Olesen *et al.*, 1997; Klemm & Schembri, 2000). Each fimbrial fibre is composed of hundreds of structural subunits, which allows an amplification of inserted epitopes to thousands of copies. Chimeric fimbrial vaccines can be used as live recombinant vaccines and purified proteins. Isolating fimbrial subunits from the bacterial surface is very easy. The display of foreign epitopes on the surface of bacteria seems to be important for a strong immune response from live bacterial vaccines. Previous investigations demonstrated that heterologous antigen exposition on the bacterial cell surface elicited a strong immune response (Leclerc *et al.*, 1994a, b).

This study demonstrated the adherence and IR spectral properties of DraE fimbrial subunits after insertion of foreign epitopes in place of the N-terminal region of the surface-exposed domain 2. Studies focusing on the biological and physico-chemical properties of fimbrial subunits after insertion of heterologous epitopes have to our knowledge never been done before. The effective display of peptide segments on Dr fimbriae expressed at the bacterial cell surface can be used for the development of a potential fimbrial vaccine. Dr fimbriae are highly immunogenic proteins, inducing protective antibodies that inhibit bacterial adhesion and colonization. Recent research by Goluszko *et al.* (2005) proved the immunological properties of the wild-type Dr fimbriae: the vaccination of C3H/HeJ mice with purified Dr antigen elicited an excellent humoral response that reduced the mortality associated

Fig. 5. DAF-binding properties of chimeric Dr fimbriae. (A) Agglutination of erythrocytes. *E. coli* cells expressing wild-type Dr fimbriae or chimeric Dr-V5/HSV were mixed with an equal volume of a suspension of human erythrocytes. Agglutination was observed visually and photographed. Agglutination of erythrocytes by Dr⁻ *E. coli* (Dr-D54stop mutant) (a), Dr⁺ *E. coli* (b), Dr-V5⁺ *E. coli* (c) and Dr-HSV⁺ *E. coli* (d). (B) Binding by Dr, Dr-V5 and Dr-HSV fimbriae to DAF in HeLa cells. HeLa cells were fixed, incubated with *E. coli* strains and processed for immunofluorescence labelling using rabbit anti-Dr serum and secondary antiserum conjugated with TRITC. *E. coli* BL21(DE3) represents a negative control (a). Pictures (b), (c) and (d) show adhesion to the DAF receptor mediated by *E. coli* BL21(DE3)/pCC90 (positive control), *E. coli* BL21(DE3)/pCC90D54stop-E-V5 and *E. coli* BL21(DE3)/pCC90D54stop-E-HSV strains, respectively. Micrographs were taken with an Olympus BX-60 microscope at a magnification of $\times 10\,000$.

with an experimental urinary tract infection. This was associated with an inhibition of Dr⁺ *E. coli* strains binding to surface receptors in the bladder and kidney.

Fig. 6. Type IV collagen-binding assay: binding of purified wild-type Dr fimbriae and chimeric Dr-V5 and Dr-HSV fimbriae to type IV collagen. Varying concentrations of fimbrial preparations (1–5 µg ml⁻¹) were incubated in microtitre plates coated with 2 µg ml⁻¹ of type IV collagen from human placenta. Bound fimbriae were detected using anti-Dr serum. All fimbrial fractions bound to type IV collagen independent of the concentration. Each bar represents the mean $\pm$ SEM from two independent experiments.

Both of the analysed epitopes (gD of HSV1 and V5 of SV5) were inserted into the DraE fimbrial subunit in place of a region between V28 and A38. The residues from K30 to A38 form an A2-B loop and are not involved in stabilization of the β -barrel structure of DraE. Interestingly, the residues V28 and A29 that constitute the C-terminal end of the 2 β -strand (Fig. 1) are also exposed to the solvent. The calculated average (averaged over all NMR models of the AfaE-III-dsc structure) solvent-accessible surfaces (SAS) of these two residues side chains are 25 % and 55 % respectively, which clearly indicates that V28 does not belong to the group of residues building the hydrophobic core of the protein and that its replacement should not disturb the structure of the DraE.

The selected insertion position of the foreign epitopes into the DraE subunit was not involved with the transport machinery, subunit-subunit interactions or bioassembly of the fimbrial structure, which confirmed the usefulness of the above strategy. The surface-exposed epitopes of gD or V5 were recognized by monoclonal antibodies against the 11 or 14 aa peptide domains and serum specific to DraE adhesin. FTIR spectroscopy analysis indicated that the structure of DraE fimbrial subunits with inserted heterologous epitopes did not undergo any detectable perturbation. In the case of the chimeric fimbriae we did not observe any band shift corresponding to the type- β

structures of native Dr fimbriae (Fig. 4). These results revealed that the insertion of heterologous epitopes did not disturb the network of hydrogen bonds stabilizing the DraE Ig-barrel structure.

The DraE fimbrial subunit is a prototype among the Dr family adhesins, and binds to three receptors: DAF, CEA and type IV collagen. The bacterial colonization of the urinary tract involves binding to the surface receptors and entry into uroepithelial cells. Binding to collagen may enhance the colonization and contribute to the dissemination of urinary infection (Goluszko *et al.*, 2005). Therefore, the potential application of chimeric fimbriae as recombinant vaccines requires the determination of the DraE receptor specificity after insertion of a foreign epitope. The experiments performed here indicated that chimeric Dr fimbriae mediated DAF-dependent agglutination of human erythrocytes (Fig. 5A). *E. coli* strains expressing chimeric fimbriae showed haemagglutination titres of 1:128, comparable with that of the wild-type Dr adhesin. The results obtained were then confirmed by the whole-cell binding assay, which demonstrated that bacterial cells expressing chimeric fimbriae adhered to the DAF receptor in cell lines expressing DAF (examined by immunofluorescence microscopy) (Fig. 5B). The available data revealed that the amino acid residues of the DraE protein involved in DAF binding may be delineated and are located on one side of the molecule in its central part (Fig. 1A). This binding region is formed mainly by the residues from β -strands A1, A2, B, C2, E, F and G_d and is separated from the insertion region by about 10 Å (Van Loy *et al.*, 2002; Anderson *et al.*, 2004). This significant three-dimensional separation of the DAF-binding region from the A2-B loop region could explain the observed native-like adhesion abilities of chimeric Dr fimbriae (Fig. 1A).

The binding of Dr fimbriae to the DAF receptor leads to the internalization of Dr⁺ *E. coli* into epithelial cells (Nowicki *et al.*, 1993). The invasive region may involve selected amino acids in domain 2 (Lea *et al.*, 1999; Anderson *et al.*, 2004; Das *et al.*, 2005). In light of the above results, future experiments will focus on analyses of the invasive abilities of chimeric fimbriae.

Previously it has been shown that DraE mutations at positions T88 and I111 affect the type IV collagen binding of the DraE adhesin (Carnoy & Moseley, 1997). Recent research revealed that residues P40, P43, I114 and Y115 are also implicated in collagen binding by DraE fimbrial subunits. The mutations P40A, P43V, I114A and Y115A resulted in a complete loss of collagen recognition by DraE (Korotkova *et al.*, 2006). The mentioned residues form the hydrophobic surface of DraE, which is involved in the binding not only of type IV collagen but also of a recently discovered carcinoembryonic antigen (CEA) (Korotkova *et al.*, 2006). Residues P40 and P43 follow the insertion site and are located in the A2-B loop in a part proximal to the B strand (Fig. 1B). Therefore it was very important to determine the collagen-binding phenotype of the chimeric

fimbriae. The collagen-binding assay showed that chimeric Dr-V5 and Dr-HSV fimbriae restored the capacity to bind to the type IV collagen. Bound bacterial cells were detected with rabbit anti-Dr serum. It was demonstrated that adhesive chimeric subunits DraE-V5/DraE-HSV were present on the bacterial surface as polymeric structures, and that they mediated bacterial attachment to DAF and type IV collagen receptors. Hence it can be concluded that display of immunologically relevant sectors of foreign peptides on Dr fimbriae offers new opportunities to test the potential beneficial adhesion for mucosal immunization and protection. The binding properties of the chimeric Dr fimbriae can be used for future testing of the potential adjuvant effects of these molecules. An additional advantage of the system described is its high valence caused by the huge number of proteins displayed on the bacterial surface. This allows amplification of the inserted epitope to thousands of copies.

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