A Subfamily of Dr Adhesins of Escherichia coli Bind Independently to Decay-accelerating Factor and the N-domain of Carcinoembryonic Antigen*

Received for publication, June 14, 2006, and in revised form, July 17, 2006 Published, JBC Papers in Press, August 1, 2006, DOI 10.1074/jbc.M605681200

Natasha Korotkova1, Ernesto Cota1, Yuri Lebedin1, Severine Monpouet2, Julie Guignot1, Alain L. Servin1, Steve Matthews1, and Steve L. Moseley1

From the 1Department of Microbiology, University of Washington, Seattle, Washington 98195-7242, the 4Division of Molecular Biosciences, Imperial College London, South Kensington, London SW7 2AZ, United Kingdom, 4Xema-Medica Co., 105043 Moscow, Russia, and 3Institut National de la Santé et de la Recherche Médicale, Unité 736, Faculté de Pharmacie, Paris XI, F-92296 Châtenay-Malabry, France

Escherichia coli expressing the Dr family of adhesins adheres to epithelial cells by binding to decay-accelerating factor (DAF) and carcinoembryonic antigen (CEA)-related cell surface proteins. The attachment of bacteria expressing Dr adhesins to DAF induces clustering of DAF around bacterial cells and also recruitment of CEA-related cell adhesion molecules. CEA, CEACAM1, and CEACAM6 have been shown to serve as receptors for some Dr adhesins (AfaE-I, AfaE-III, DraE, and DaaE). We demonstrate that AfaE-I, AfaE-V, DraE, and DaaE adhesins bind to the N-domain of CEA. To identify the residues involved in the N-CEA/DraE interaction, we performed SPR binding analyses of naturally occurring variants and a number of randomly generated mutants in DraE and N-CEA. Additionally, we used chemical shift mapping by NMR to determine the surface of DraE involved in N-CEA binding. These results show a distinct CEA binding site located primarily in the A, B, E, and D strands of the Dr adhesion. Interestingly, this site is located opposite to the β-sheet encompassing the previously determined binding site for DAF, which implies that the adhesion can bind simultaneously to both receptors on the epithelial cell surface. The recognition of CEACAMs from a highly diverse DrCEA subfamily of Dr adhesins indicates that interaction with these receptors plays an important role in niche adaptation of E. coli strains expressing Dr adhesins.

The Dr family of adhesins of Escherichia coli is associated with diarrhea and urinary tract infections (UTI),2 in particular gestational pyelonephritis and recurring cystitis (1–3). This family includes AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr hemagglutinin (DraE), Dr-II, DaaE, and NfaE-I (4). Dr adhesins recognize as receptors decay-accelerating factor (DAF), and the DrCEA subfamily recognizes carcinoembryonic antigen (CEA)-related cell adhesion molecules (CECAM) (5, 6). DAF is a complement regulatory protein present on a variety of epithelial surfaces, including gastrointestinal mucosa, exocrine glands, renal pelvis, ureter, bladder, cervix, and uterine mucosa (7).

The CEACAM family is a group of highly glycosylated homotypic/heterotypic cell surface intracellular adhesion molecules, which includes CEA, CEACAM1, CEACAM3, CEACAM4, CEACAM6, CEACAM7, and CEACAM8 (8). It has been reported recently that E. coli expressing some Dr adhesins, AfaE-I, AfaE-III, DraE, and DaaE (the DrCEA subfamily), adhered to Chinese hamster ovary (CHO) cells expressing CEA and that E. coli expressing AfaE-III, DraE, and DaaE adhered to CHO cells expressing CEACAM1 and CEACAM6 (9). These adhesins also elicit the recruitment of CEACAM1, CEA, CEACAM6, and CEACAM3 to the sites of adherent bacteria (9). Recognition of CEA and CEACAM6 but not CEACAM1 is accompanied by tight attachment of the bacteria to elongated cell surface microvillus-like extensions. This cellular response results from activation of Rho GTPase (Cdc42) and phosphorylation of ezrin/radixin/moesin (9).

The CEA family is a member of the Ig superfamily (8, 10). Each CEA family member consists of an N-terminal Ig variable-like domain. At the amino acid level, the N-terminal domain exhibits greater than 90% identity with other members of the CEA subgroup. The N-terminal domain may be followed by up to six IgC2 domains (A1, B1, A2, B2, A3, and B3) (8), which are all present in CEA. CEACAM1, CEACAM3, and CEACAM4 are inserted into cellular membrane via a carboxyl-terminal transmembrane and cytoplasmic domain, whereas CEA, CEACAM6, CEACAM7, and CEACAM8 are anchored to the membrane via glycosylphosphatidylinositol. These molecules are expressed on numerous cells, including epithelial, endothelial, and myeloid cells (11). Within the family, CEACAM1, a signaling receptor, is the most widely expressed in distinct human tissues, being present in granulocytes, monocytes, and epithelial cells in different organs, including colonic and respiratory epithelia (8, 12, 13).

It has been reported that E. coli and Salmonella enterica bind CEACAM molecules via their mannosyl residues (14–16). Sev-
Dr Adhesins Bind Independently to DAF and N-domain of CEA

eral microorganisms, including *Neisseria meningitidis*, *Neisseria gonorrhoea*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, target members of the CEA/CAM family via the proteinaceous component of the N-terminal domain (17–22). *Neisseria* spp. bind CECAM molecules via the structurally related Opa proteins, whereas in the case of *H. influenzae* and *M. catarrhalis* the ligands appear to be distinct from this family (22, 23). Targeting of CECAM molecules by *Neisseria* leads to cellular invasion and passage across polarized monolayers (24).

In this study, we provide evidence that DrCEA subfamily of adhesins, including AfaE-I, AfaE-III, AfaE-V, DraE, and DaaE, bind to the N-domain of CEA. Using a combined NMR and mutagenesis approach, we identified amino acids of Dr/Afa-III adhesins and CEA involved in the interactions. We demonstrate that Dr/CEA interaction is sensitive to Cm inhibition due to direct disruption of the CEA-binding surface of the adhesin. Using NMR, we also show that CEA and DAF binding sites do not overlap and that DAF does not inhibit binding to CEA.

**EXPERIMENTAL PROCEDURES**

Bacterial strains were grown in LB or Super Broth (SB) medium at 37 °C. Derivatives of pUC-Cm were grown in the presence of 25 μg/ml chloramphenicol (Cm). Derivatives of pET-21d and pCC90-D54stop were grown in 100 μg/ml ampicillin or carbenicillin. *E. coli* DH5α (Invitrogen) and BL21 (DE3) (Novagen, San Diego, CA) were hosts for the plasmids. The purification of *E. coli* chromosomal DNA, plasmid isolation, *E. coli* transformation, restriction enzyme digestion, and ligation were carried out as described (25). Enzymes were purchased from New England Biolabs (Beverly, MA) and used as recommended by the manufacturer.

CHO cell transfectant clones that express human CEA or the vector alone were used (9). Cells were cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 400 μg/ml hygromycin B and cultured according to standard tissue culture techniques. All constructs were confirmed by sequencing using the Big Dye Terminator method and ABI sequencing (PerkinElmer Life Sciences).

Cloning, Expression, and Purification of the N-domain of CEA, CEACAM1, CEACAM6, CEACAM8, and CEA-CEACAM8 Chimera—The sequences corresponding to the mature amino acid sequences of N-domain of CEA, CEACAM1, CEACAM6, and CEACAM8 were PCR-amplified using cDNA clones (IMAGE ID 5184800, 4540619, 3640231, 4618311; ATCC) as templates and inserted into pET-21d (Novagen). CEA-CEACAM8 chimera was synthesized using the PCR-based splicing by overlap extension using CEA and CEACAM8 cDNA clones as templates and the following oligonucleotide pairs: CEAF, 5′-catgcggacagctcactttgcata-3′; CEACAM8-CEAr, 5′-gattgctgtatgcaggcctggggtagcttgttgagttc-3′; CEACAM8r, 5′-gaagttttaaagctccggatctagcgcagaa-3′; CEA-CEACAM8f, 5′-gaactcaacaagctcactttgcatactttgcatacttc-3′. The chimera was then amplified by adding CEAf and CEACAM8r primers to the PCR mix. The hybrid molecule was cloned into pET-21d (Novagen). The proteins were expressed in *E. coli* BL21 (DE3) and purified from inclusion bodies. The inclusion bodies were dissolved overnight in buffer containing 30 mM Tris/HCl (pH 8.5), 150 mM NaCl, 1 mM EDTA, and 8 M urea. One volume of the protein sample was added slowly to 20 volumes of buffer containing 50 mM CHES (pH 9.2) and 500 mM arginine, and the sample was left overnight at 4 °C. The refolded protein was concentrated by ultrafiltration and purified by gel filtration using a Superdex 75 column (Amersham Biosciences) in 30 mM Tris/HCl (pH 8.5), 150 mM NaCl. Calibration of the column was performed using gel filtration markers (Amersham Biosciences).

Purification of CEA N-A3 Domain—CEA containing N and A3 domains and an oligohistidine tag at the N-terminal end (CEA N-A3) was expressed in *Pichia pastoris* as described by You et al. (26) (*P. pastoris* strain generously provided by Dr. John E. Shively, Beckman Research Institute of Hope, Duarte, CA). CEA N-A3 was purified from the supernatant of induced cultures by Ni2+—nitrilotriacetic acid chromatography as described previously (26, 27). For SPR experiments, the protein was then purified by gel filtration chromatography using a Superdex 200 column (Amersham Biosciences) in HBS-E buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA).

Purification of Dr Family Fimbriae—Genes encoding Dr adhesins were amplified by using the following primers: for DraE and AfaE-III, DraE-BamHI (cgagctccagaggtatatatagaaataatgcatactgcgcecc) and DraE-PstI (caggagctctgccagt-tattgcagccgatcc); for AfaE-V, DraE-BamHI primer and AfaE-V-PstI (caggagctctgccagtatcactgcgctagcccggt); for NfaE, NfaE-BamHI (cgagctccagaggtatatatagaaataatgcatactgat) and NfaE-PstI (caggagctctgccagtatcactgcgctagcccggt). Products were digested with BamHI and PstI and inserted into BamHI and PstI restriction sites of pUC-Cm. The gene encoding DaaE adhesin was amplified by using the following primers: DaaE-BamHI (cgagctccagaggtatatatagaaataatgcatactgcgcecc) and DaaE-EcoRI (gaattcttagttcgtccagtaacccc). Product was digested with BamHI and EcoRI and inserted into BamHI and EcoRI restriction sites of pUC-Cm. The gene encoding AfaE-1 adhesin was amplified by using the following primers: AfaE-1-EcoRI (gaagttttaaagctccggatctagcgcagaa) and AfaE-1-PstI (caggagctctgccagtatcactgcgctagcccggt). Product was digested with EcoRI and PstI and inserted into EcoRI and PstI restriction sites of pUC-Cm. The resulting plasmids were transformed into *E. coli* 191A (pCC90-D54stop). This strain contains the necessary genes of the *dra* operon for fimbrial expression, with a premature stop codon at codon 54 within *draE*. Dr fimbriae were purified from recombinant strains as described previously (28). For SPR analysis, fimbriae were purified by gel filtration chromatography using a Superdex 200 column (Amersham Biosciences) in HBS-E buffer.

Purification of DAF—DAF24 contains short consensus repeats 2–4 and an oligohistidine tag at the C-terminal end and was purified from recombinant *P. pastoris* kindly provided by Susan Lea (Oxford University), as previously described (29).

Surface Plasmon Resonance Studies—SPR measurements were carried out in HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% P-20 surfactant (BIACore AB, Uppsala, Sweden)) using a BIACore 2000 system (BIACore AB). To analyze the interaction between Dr fimbriae and N-domains of CECAM receptors, native fimbriae were immobilized on a CM5 research grade sensor chip (BIACore AB) by amine coupling chemistry using the manufacturer’s protocols. Immobilization of 500 response units (RU) resulted in optimal responses. The N-domain of each CECAM was dissolved in running
buffer and analyzed using a $10^2$ dilution series (i.e. 3–300 μM). Analyte was injected over the surface at a flow rate of 20 μl/min for 2 min. For competition experiments, DAF$_{234}$ (30 μM final concentration) or Cm (1 mM final concentration) was added to the running buffer. The affinities of the interactions were studied under steady-state conditions. Average equilibrium responses were measured for six or seven concentrations of CEACAM receptor. Raw sensograms were corrected using the double subtraction protocol (30) and by subtracting both the reference flow cell response and the average of eight buffer injections. The resulting data were analyzed with BIAevaluation 3.0 software (BIAcore AB) to globally fit the data and derive equilibrium constants describing the intermolecular interactions. The reported $K_D$ values are the average of at least three independent experiments. Error estimates were propagated from the S.E. of the association constant ($K_a$).

**Chemical Shift Mapping for the AfaE-dsc and N-CEA Interaction**—The recombinant *E. coli* strain expressing AfaE-III-dsc has been described previously (31). A $^{15}$N-labeled sample of AfaE-III-dsc was produced in minimal medium, containing 0.07% $^{15}$NH$_4$Cl and 0.2% C-glucose, supplemented with 50 μg/ml ampicillin. AfaE-III-dsc was purified under denaturing conditions using the binding of the N-terminal hexahistidine tag to nickel-bound agarose beads as described previously (31). Purified protein was refolded by dialysis into 50 mM sodium acetate buffer, pH 5.0, and concentrated to ~0.2 mM for NMR.

N-CEA in the same buffer was introduced at several molar ratios until saturation was achieved, and two-dimensional $^{15}$N-$^1$H HSQC experiments were recorded at each stage under identical experimental conditions. $^1$H-$^{15}$N resonance shift changes and line widths were monitored by analysis of the two-dimensional $^1$H-$^{15}$N HSQC spectra.

**PCR Mutagenesis of DraE**—DraE was subjected to PCR mutagenesis using the GeneMorph II Random Mutagenesis Kit as directed by the manufacturer (Stratagene, La Jolla, CA). The pCC90 vector containing draE (32) was used as a template with following primers: 5ranmut (5'-cccgcggcgtgctggttgggtaagagcgc-3') and 3ranmut (5'-ccgcgcccgcgtgctggttgggtaagagcgc-3'). Transformants containing mutant derivatives of draE were selected and were described previously (28).

**Selection for CEA Binding-deficient Mutants**—CEA N-A3 protein was used to coat seven 6-well microtiter plates at a concentration of 5 μg/ml in bicarbonate buffer at 37 °C for 1 h. Anti-Dr rabbit antiserum (32) was dissolved in bicarbonate buffer at a 1:100 dilution and used to coat 6-well microtiter plates at 37 °C for 1 h. The wells were blocked with 0.01 M phosphate-buffered saline, pH 7.2 (PBS), containing 1% bovine serum albumin for 15 min at 37 °C. Transformants containing the mutant draE derivatives were resuspended in 12 ml of PBS to an $A_{540}$ of 0.025. 2 ml of cell suspension were added to each well coated with CEA and allowed to bind for 45 min at 37 °C. The supernatant containing unbound bacteria was collected and added to a fresh plate coated with CEA. These steps were repeated successively for a total of six times. The final supernatant was added to a microtiter plate coated with anti-Dr antiserum and allowed to bind for 45 min at 37 °C. The unbound bacteria were removed by 7–9 washes with PBS, and 2 ml of SB medium were added to each well. The plate was incubated with shaking at 37 °C for 1 h. The resulting bacterial suspension was plated on LB medium containing ampicillin, Cm, and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal). White colonies were selected and examined for erythrocyte binding by a mannose-resistant hemagglutination assay (MRHA) (see below). Those transformants that were MRHA$^+$ were analyzed for CEA binding.

**MRHA**—MRHA with human erythrocytes of blood group O was performed as previously described (28).

**CEA Binding Growth Assay**—Those transformants that were MRHA$^+$ were examined for the ability to bind CEA. CEA N-A3 was used to coat 96-well microtiter plates as described above. 100 μl of an overnight culture of each transformant, $A_{540} = 4.0$, were added to individual wells and allowed to bind for 45 min at 37 °C. The unbound bacteria were removed with PBS, and then 150 μl of SB medium were added to each well. The plate was incubated with shaking at 37 °C for 2 h. The resulting growth was then measured at $A_{540}$. Those transformants that exhibited little or no growth were further analyzed to confirm DraE expression.

**Surface Expression Assay and Sequencing Analysis of Mutants**—To confirm that the transformants express the various mutant adhesin variants at equivalent levels, they were examined for the ability to bind polyclonal antiserum prepared against DraE (32). This assay was performed in the same manner as the CEA binding growth assay with following modifications. Plates were coated with anti-Dr antiserum at 1:200 dilution for 2 h. Those transformants that exhibited growth were selected for sequencing.

**CEA Binding Assay with Radiolabeled Bacteria**—This assay was performed in the same manner as the CEA binding growth assay with the following modifications. Plates were coated with soluble CEA (Xema, Moscow, Russia) or CEA N-A3 at a concentration of 15, 10, 5, 3, 1.5, and 0.75 μg/ml in PBS. The bacterial strains were grown overnight in SB supplemented with 10 μCi/ml [3H]thymidine at 37 °C. The cells were then pelleted and resuspended in PBS and adjusted to $A_{540} = 4.0$. 50 μl of each of the bacterial strain suspensions were added to a microtiter plate well together with 50 μl of PBS containing 0.2% bovine serum albumin and allowed to bind for 1 h at 37 °C. To test the effect of competitors on bacterial binding to CEA, the N-domain of CEA (at a concentration of 80, 40, 20, and 10 μM), DAF$_{234}$ (80, 40, and 20 μM), or Cm (10, 2, 1, 0.5, and 0.25 mM) was added to PBS containing 0.2% bovine serum albumin. Unbound bacteria were removed by washing with PBS, and the wells were dried at 60 °C. The wells were placed in scintillation fluid, and bound bacteria was quantified by scintillation counting.

**Site-directed Mutagenesis of the CEA Gene**—Mutations were introduced into the CEA (N-domain) gene on plasmid pET-21d (this study) by site-directed mutagenesis using the QuikChange kit as directed by the manufacturer (Stratagene). Constructs containing mutations were identified by sequence analysis.

**CHO Cell-binding assay**—CHO cells were split into 24-well plates with glass coverslips and grown to confluence. Before the assay, cells were washed twice with Hanks’ balanced saline solution and incubated with fresh medium without antibiotics and without fetal bovine serum for 1 h. The bacterial strains were...
grown overnight on LB medium and harvested and resuspended in PBS to $A_{540} = 0.6$. The bacterial cells were pelleted and resuspended in the tissue culture medium. Then 0.1 ml of each bacterial strain was added to each well. The adherence assay was performed as described previously (28) and repeated in triplicate.

RESULTS

Adhesins of the DrCEA Subfamily, Including AfaE-I, AfaE-V, DraE, and DaaE. Bind Soluble CEA, CEA N-A3, and CHO Cells Expressing CEA—Previously it has been shown that Dr adhesins, including DraE, AfaE-III, and DaaE, bind and elicit recruitment of CEA, CEACAM1, and CEACAM6 (9). To determine if other members of the Dr family can bind CEA, recombinant E. coli expressing different Dr adhesins was constructed. Dr adhesins were expressed in a background strain containing a mutant $dra$ operon expressing genes necessary for assembly of the Dr fimbriae but not the adhesion itself. Transfected CHO cell lines expressing human recombinant CEA or containing the expression vector alone were used to study the recognition of CEA by bacteria expressing Dr adhesins. None of the tested strains showed binding to CHO cells containing the vector alone. Strains expressing DraE/AfaE-III alleles, DaaE, and AfaE-V were able to bind 60–80% of CHO-CEA cells, whereas the parent strain with a premature stop codon in $draE$ did not bind the cells. The recombinants expressing AfaE-I demonstrated poor binding to CHO-CEA cells, whereas E. coli expressing NfaE did not mediate adherence to this cell line.

To further investigate the CEA-binding phenotype of Dr adhesins, we coated plates with soluble CEA (15, 10, 5, 3, 1.5, and 0.75 $\mu$g/ml) and incubated them with radiolabeled E. coli expressing the adhesins. Strains expressing DraE, DaaE, and AfaE-V adhered strongly to plates coated with CEA at a concentration of 15, 10, 5, and 3 $\mu$g/ml and demonstrated poor binding to CEA at 1.5 $\mu$g/ml (Fig. 1). All tested strains failed to bind CEA at a concentration of 0.75 $\mu$g/ml. Moreover, strains expressing AfaE-I, DraE, DaaE, and AfaE-V also bound to a CEA derivative construct consisting of only the N and A3 domains (data not shown).

Previously observed phenotypes of AfaE-I and NfaE correlated well with the ability of the recombinants to bind to CEA-coated plates (Fig. 1). NfaE did not mediate bacterial binding to CEA and CEA N-A3 plates. When bacteria expressing AfaE-I were incubated with the plates coated with 15 and 10 mg of CEA, this adhesin demonstrated a level of adherence comparable with DraE, DaaE, and AfaE-V. However, AfaE-I mediated a reduced level of binding to plates coated with CEA at a concentration of 5 mg/ml and failed to bind CEA at 3 and 1.5 $\mu$g/ml. These data indicate that the affinity of AfaE-I for CEA is significantly lower than DraE, DaaE, or AfaE-V, but since multiple adhesin subunits contribute an avidity effect to the interaction, their binding can only be compared at low concentrations of CEA. Taken together, these results demonstrate that recognition of CEA is expressed by most adhesins of the Dr family, and N-terminal and A3 domains are sufficient for these interactions.

The N-terminal Domain of CEA Mediates Binding to AfaE-I, AfaE-III, AfaE-V, DraE, and DaaE—Dr adhesins recognize as receptors CEA, CEACAM1, and CEACAM6 and also elicit the recruitment of CEACAM3 around adhering bacteria (9). Since the N-terminal and A3 domains of CEA are sufficient for bacterial adherence and the N-terminal domains of CEACAMS have high sequence similarity (Fig. 2), we hypothesized that the N-domain of CEA, CEACAM1, and CEACAM6 is the target for Dr adhesins. To test our hypothesis, we constructed E. coli recombinants expressing the N-domain of CEA (N-CEA), and isolated the protein from inclusion bodies. In a similar manner, the N-domain of CEACAM8 (N-CEACAM8) was cloned, expressed, and purified. When N-CEA was tested as an inhibitor in the CEA binding assay with radiolabeled strains expressing DraE and AfaE-III adhesins, bacterial adherence was inhibited significantly at a concentration of 40 and 80 $\mu$M N-CEA, suggesting that the adhesins recognize the N-domain on CEA (Fig. 3A).

We examined the affinity of Dr adhesins (AfaE-I, AfaE-III, AfaE-V, DraE, DaaE, and NfaE) to N-CEA and N-CEACAM8 by SPR analysis. N-CEACAM8 has a high sequence similarity with N-CEA (Fig. 2), but CHO cells expressing CEACAM8 are not recognized by Dr adhesins (9). The affinities of the interactions were studied by immobilizing purified adhesin to the sensor surface and flowing CEACAMS over the surface. No change in the resonance signal was detectable when N-CEACAM8 was injected to flow over adhesin-immobilized sensor surface, indicating the absence of detectable binding. No binding was observed when NfaE was immobilized to the sensor surface and N-CEA was used as analyte.
**Dr Adhesins Bind Independently to DAF and N-domain of CEA**

SPR studies indicated that the interactions between AfaE-I, AfaE-III, AfaE-V, DraE, and DaaE adhesins and N-CEA are characterized by very fast on and off rates; therefore, steady-state conditions were used to calculate affinity (Fig. 4A). Interestingly, AfaE-V, which does not mediate MRHA and displays very low affinity to DAF,3 was the strongest binding variant among the tested adhesins. AfaE-I demonstrated the lowest affinity to N-CEA, consistent with the previously observed phenotype (Fig. 4B).

**Effect of Competitors (Cm and DAF) on AfaE-III and DraE Binding to CEA**—It has been shown that the binding of DAF to DraE is inhibited by Cm (3). However, adherence of DAF to AfaE-III, differing from DraE by three nonsynonymous nucleotide changes (D52N, T88M, and I111T), is resistant to Cm (3). X-ray studies of DraE in complex with Cm indicated that Cm interacts with Pro40, Gly42, Pro43, Ile111, Gly113, Ile114, and Tyr115 of DraE, and Cm sensitivity of DraE is caused by direct disruption of the DAF-binding surface of the adhesin (33). To investigate the effect of Cm on DraE/CEA interactions, bacterial cells expressing DraE or AfaE-III were mixed with Cm at a concentration of 10, 2, 1, 0.5, and 0.25 mM and incubated with CEA-coated plates (Fig. 3B). Binding of DraE-expressing bacteria was inhibited by Cm at a concentration of 10, 2, 1, and 0.5 mM, whereas AfaE-III-expressing E. coli was resistant to Cm inhibition. These data suggest that DraE binding to Cm disrupts the CEA-binding surface of the adhesin.

Amino acids involved in DAF/AfaE-III and DAF/DraE interactions were implicated by mutagenesis and NMR studies (28, 31). NMR analysis has suggested that the DAF binding region of AfaE-III involves a large surface comprising domains A1, A2, B, C2, E, F, and Gd (31). To determine if binding of DraE adhesins to DAF disrupts the CEA-binding surface of the adhesin, DAF234 was tested as a competitor in the CEA-binding assay with radiolabeled bacteria. DAF at concentrations of 80, 40, and 20 μM had no effect on bacterial adherence to the CEA-coated plates, implying that the binding domains for DAF and CEA are distinct (data not shown).

In order to confirm the effect of DAF or Cm on binding of N-domain of CEA to fimbrial preparations, we utilized SPR analysis. DAF234 (30 μM) or Cm (1 mM) was added to buffer containing N-CEA and flowed over DraE fimbriae immobilized on the sensor surface. Results are clearly consistent with the CEA binding assay with DraE-expressing bacteria described above. DAF had no effect on the affinity of DraE for N-CEA (KD = 13.1 ± 1.5 μM). However, when DAF was replaced with Cm (1 mM), N-CEA failed to bind the DraE immobilized surface.

**Analysis of CEA Binding Phenotypes of DraE/AfaE-III Clinical Isolates**—To investigate the functional variability of DraE/AfaE-III alleles with regard to CEA binding, we purified naturally occurring DraE-related fimbrial variants and utilized SPR to determine the affinity to N-CEA as described above. We found that three variants, JJB17, 2099, and 293, had low affinity to N-CEA (Fig. 5A). JJB17 and 293 each differ from DraE by a single nonsynonymous mutation, I111T and T88A, respectively. 2099 differs from JJB17 by one mutation, D61G. Four adhesins, 513, G2076, 252, and 297, have increased affinity to N-CEA (Fig. 5A). 513 and 297 differ from DraE by single nonsynonymous mutations, T88M and I85M, respectively. G2076 differs from 513 by one mutation, G68D. 252 differs from DraE by two nonsynonymous mutations, A92T and I111V. Interestingly, AfaE-III, differing from 513 by two nonsynonymous nucleotide changes (D52N and I111T) demon-

---

3 N. Korotkova, E. Cota, Y. Lebedin, S. Monpouet, J. Guignot, A. L. Servin, S. Matthews, and S. L. Moseley, unpublished observations.
FIGURE 4. SPR measurement of N-CEA affinity ($K_D$) to Dr adhesins. A, SPR binding curves of the interaction between N-CEA and DraE fimbriae in steady state. The resulting data were analyzed with BIAevaluation 3.0 software to globally fit data and derive the dissociation constants ($K_D$). A fit of these data is shown. RU, relative units. RE, response equilibrium. B, bar chart showing a comparison of dissociation constants ($K_D$) for different Dr adhesins.
strated N-CEA affinity comparable with DraE. This adhesin acquired two CEA-binding mutations, T88M and I111T. It is possible that the positive effect of the T88M mutation on CEA binding compensates for the negative effect of I111T mutation. Thus, our data suggest that (i) residues Ile111, Thr88, and Ile85 are important for DraE/N-CEA interactions, and (ii) the DraE subgroup accumulates a high number of nonsynonymous substitutions, leading to variation in CEA-binding phenotypes of the adhesins.

Identification of Amino Acids of DraE Involved in CEA Binding by Random Mutagenesis—To independently identify amino acids of the adhesin critical for recognition of CEA and to provide further evidence for our NMR-derived N-CEA binding surface, we performed random mutagenesis of DraE. Several mutant strains were isolated that mediated MRHA but demonstrated very weak binding to CEA-coated plates. We chose to further characterize six mutants that contained single amino acid substitutions within DraE (P40S, P43V, R86G, G113A, Y115F, and Y115A).

To examine the effect of the mutations in DraE upon its binding properties, fimbriae were isolated from the bacterial mutants. The affinities of DraE/CEA interactions were analyzed by SPR as described above. Fig. 5B summarizes the DraE mutations and their effects on CEA binding. All mutants mediated binding to N-CEA, but the mutations significantly weaken the interactions between DraE and CEA. The mutation at position R86G has the largest effect, decreasing the affinity more than 7-fold. The positions of DraE mutations affecting CEA binding are shown in Figs. 6B and 7, A and C.

Identification of the Binding Site for CEA in Dr Adhesin by NMR Chemical Shift Mapping—Our earlier work on the structure and assembly of the Dr adhesins provides a further opportunity to perform NMR titration experiments to directly investigate the binding site for CEA (31). An analysis of amide line width and chemical shift changes for AfaE-III-dsc in the presence of N-CEA was carried out (Fig. 6A). The CEA binding region of AfaE-III-dsc may be delineated, and it lies entirely on one side of the molecule, forming a surface comprising side chains from principal strands A1, B, E, and D and adjacent loop regions (Figs. 6, B and C, and 7, E and F). Key amides perturbed in the presence of N-CEA include Gly6, Lys12, Gln20–Gly24, Ile41, Gly42, Val44–Leu49, Arg54–Val56, Ala60, Gly68, Phe70, Met88, Ser91, Ala92, Asn97, Asp104, Gly106, Trp108, Gly110–Ile112, Ile114, and Ala118.

Identification of Amino Acids of CEA Involved in Dr Adhesin Binding—To investigate the affinity of DraE to other members of CEACAM family we constructed E. coli recombinant strains expressing N-domains of CEACAM1 (N-CEACAM1) and CEACAM6 (N-CEACAM6). N-CEACAM1 affinity for DraE was comparable with N-CEA (Table 1). However, N-CEACAM6 showed weak binding to the adhesin. DraE does not interact with N-CEACAM8, as discussed above. Sequence alignment of the N-domains of CEACAM-related proteins, including CEACAM1, CEACAM3, CEA, CEACAM6, and CEACAM8, revealed exceptional homology (Fig. 2). In order to identify amino acids of CEA N domains involved in CEA/Dr
interactions, we constructed a CEA-CEACAM8 chimera consisting of the residues 1–58 of the N domain of CEA fused to residues 59–110 of the CEACAM8 N-domain and analyzed the ability of the chimeric protein to bind DraE by SPR. We found that the affinities obtained for wild-type and the mutant were the same (Table 1), implying that the N-terminal 58 amino acids of CEA are crucial for the CEA/DraE interaction.

Using homologue scanning mutagenesis of the N-terminal 58 amino acids of CEA, we replaced single residues of CEA with corresponding residues of CEACAM8 and determined the affinity of the mutants to DraE. As is shown in Table 1, mutations in Phe9, Val11, Leu28, Ser32, Val39, Gly51, and Thr52 had no significant effect on DraE/N-CEA interactions. Mutation D40A caused an almost 4-fold drop in the DraE/N-CEA affinity, and two mutations, F29R and Q44R, abolished the interaction.

**DISCUSSION**

Diffusely adhering *E. coli* strains express adhesins of the Dr family and cause symptomatic urinary tract or intestinal infections (4). The Dr family of adhesins is composed of a number of related adhesins with extensive sequence diversity. This variability of Dr adhesins is emerging under strong positive selection and results in the generation of clades within the family and the accumulation of point mutations within the clades. Variability in the Dr family of adhesins results in functional diversity with regard to receptor specificity (9, 32).

*E. coli* expressing Dr adhesins adheres to human cells by recognition of the brush border-associated DAF (4). The attachment of bacteria expressing AfaE-III, DraE, and DaaE to DAF induces clustering of DAF around bacterial cells and also recruitment of the brush border proteins, CEA, CEACAM1, CEACAM3, and CEACAM6 (6, 9). It has been shown that Dr adhesins, including AfaE-III, DraE, and DaaE, are involved in adherence to CEA, CEACAM1, and CEACAM6, thus indicating that these molecules might be important for diffusely adhering *E. coli* colonization (9).

In this study, we have demonstrated that the bacteria expressing AfaE-I, DraE/AfaE-III alleles, AfaE-V, and DaaE recognize the N-domain of CEA. Thus, these studies add to the list of bacterial ligands that target the N-domain of CEA. Adherence to this receptor by apparently structurally distinct proteins of

---

*4* N. Korotkova, S. Chattopadhyay, T. Tabata, V. Beshklebnyaya, V. Vigdorovich, B. Kaiser, R. Strong, D. Dykhuizen, E. Sokurenko, and S. Moseley, submitted for publication.
several bacterial genera points to the importance of CEA for the colonization of pathogens.

We found that the DraE adhesin also recognizes the N-domains of CEACAM1 and CEACAM6, demonstrating low affinity to CEACAM6 and high affinity to CEACAM1. Homology-scanning mutagenesis of N-CEA revealed that Phe29 and Gln44 (and to a lesser extent Asp40) of CEA are required for maximal DraE binding affinity. These residues are located in the exposed loops of the GFCC“C” face of the CEA N-domain, which is not sheltered by carbohydrate, as revealed by the crystal structure of murine CEACAM1 (34, 35), and would be accessible for pathogen binding (Fig. 6D). The solvent-exposed area around Phe29 in CEA is hydrophobic and might be important for contacts with Dr adhesins. It has been shown that other pathogenic bacteria also target this exposed protein face of the N-terminal Ig variable-like domain of CEACAM receptors (18–20, 36).

CEACAM family members can exist as dimers in the plasma membrane of eukaryotic cells, and recombinant N domains of CEA have been shown to form oligomers in solution (37–39). It has also been demonstrated that residues on the GFCC“C” face of the CEA are directly engaged in homophilic cell adhesion (34, 40). Notably, amino acid residues Val39, Asp40, and Gln44 in CEACAM1 have been reported to play an important role in homophilic cell adhesion of CEACAM1 (41, 42), which sug-

| TABLE 1 | Affinity (Kd) of DraE adhesin to N-CEACAMs and N-CEA mutants | Kd (nM) |
|---------|---------------------------------------------------------------|---------|
| N-CEA   | 13.1 ± 2.5                                                   |         |
| N-CEACAM1| 15.3 ± 3.8                                                   |         |
| N-CEACAM6| 30.1 ± 2.8                                                   |         |
| CEA-CEACAM8 chimera | 14.4 ± 2.1 |         |
| F9A     | 18.4 ± 1.5                                                   |         |
| V11A    | 24.9 ± 1.2                                                   |         |
| L28P    | 6.6 ± 0.5                                                    |         |
| F29R    | >200                                                         |         |
| S32N    | 12.8 ± 1.3                                                   |         |
| V39A    | 9.35 ± 1.3                                                   |         |
| D40A    | 48.9 ± 3.1                                                   |         |
| Q44R    | >200                                                         |         |
| G51D    | 12.0 ± 1.4                                                   |         |
| T52N    | 11.0 ± 2.1                                                   |         |

FIGURE 7. DraE/AfaE-III adhesin binding-associated surfaces. Surface representation of AfaE-III-dsc with Cm as a yellow stick representation (31, 33). Orientation of A–C is the same as D–F, respectively. A–C, DAF (28) and CEA binding sites derived from DraE mutagenesis are shown in green and red, respectively. D–F, DAF-specific interactions derived from chemical shift mapping analysis of AfaE-III-dsc shown in blue (31). CEA-specific interactions derived from chemical shift analysis of AfaE-III-dsc are shown in dark red. Chemical shift mapping of interactions common to DAF and CEA is shown in orange (31).
suggests that DraE binding to CEA at least partly overlaps the dimerization surface and therefore is likely to inhibit the CEA homophilic interaction. Structural studies of the CEA-DraE complex are needed to provide detailed information about the mechanism of DraE recognition of the receptor.

Using the recently published NMR and x-ray structures of AfaE/DraE (Protein Data Banks codes 1RXL and 1USQ) (31, 33), we have identified the N-CEA binding site on the DraE surface. NMR chemical shift mapping and line width analysis of backbone amides define a contiguous patch of residues in strands A1, B, E, and D and neighboring loop regions. Furthermore, random mutagenesis and functional analyses of the DraE/AfaE-III alleles identified residues Pro40, Pro43, Arg86, Ile85, Thr88, Ile111, Gly113, and Tyr115 as important for binding to CEA. Fig. 7 illustrates the surfaces mapped by the different approaches. In NMR chemical shift mapping, contiguous stretches of perturbed amide delineate the likely binding surface. The presence of proline residues and overlapped resonances can cause an underreporting, whereas indirect perturbations highlight regions outside the binding interface. Care should also be taken when interpreting mutagenesis experiments, since meaningful substitutions must not severely affect protein stability. The combined use of NMR mapping and mutagenesis provides a powerful and robust approach for identifying protein-protein interfaces. As shown in Fig. 7, the CEA binding region defined by mutagenesis clearly overlaps with that determined by NMR experiments. The mutation-derived and NMR-derived surfaces are on the same face of the molecule and in proximity to the Cm binding pocket, which is consistent with the discovery that Cm inhibits CEA binding by DraE. DAF/DraE interactions are also sensitive to the presence of Cm, and therefore one might expect that DraE binding to CEA would be inhibited by DAF. However, our data demonstrate that DAF has no inhibitory effect on CEA/DraE interactions, and thus consistent with the inability of DAF to inhibit CEA.

Previously, it has been shown that DraE mutations at positions Thr88 and Ile111 affect type IV collagen binding of the adhesin (32). According to our unpublished observations, the amino acids Pro40, Pro43, Ile114, and Tyr115 are also important for DraE/collagen interactions. Mutations P40A, P43V, I114A, and Y115A resulted in a complete loss of recognition of collagen by DraE. Therefore, the hydrophobic surface on DraE that includes these residues is involved in the binding of three molecules, CEA, Cm, and the 7S domain of type IV collagen.

Dr adhesin-expressing E. coli penetrates into epithelial cells utilizing a zipper-like mechanism of internalization (43), although neither the mechanism of internalization nor the roles of specific binding activities of Dr adhesins in internalization have been defined in detail. Apart from the interactions of the Dr adhesins, it has been shown that the Dr invasion (AfaD-related proteins) contribute to the process of internalization (44–47). DAF is concentrated in lipid rafts (43), cell surface invaginations that are believed to be important for signal transduction (48). We hypothesize that the role for DAF in Dr-mediated infection is to promote initial binding to the cell membrane. This hypothesis is supported by the observation that all adhesins of the Dr family bind DAF (4) and that DraE family alleles are adapting to the uropathogenic niche by strong positive selection for mutations in the adhesin that enhance binding to DAF. Bacterial binding to DAF is followed by sequential recruitment of adjacent glycosylphosphatidylinositol-anchored receptors, such as CEA and CEACAM6, the carboxyl-terminal transmembrane-anchored CEACAM1 and CEACAM3, and interactions with surface structures, such as β1 integrins, that participate in internalization and/or signaling events (6, 43).

The role of CEACAMs in E. coli infection mediated by Dr adhesins is not yet clear. However, the occurrence of point mutations in Dr adhesins that affect CEACAM binding, under positive selection and at high frequency, suggests that CEACAMs receptors play an important role in niche adaptation of E. coli. It has been shown that CEACAM receptors are involved in N. gonorrhoeae internalization by epithelial cells (49). Moreover, CEA and CEACAM6 mediate zipper-like internalization of N. gonorrhoeae (49). We suggest that these receptors might have similar function during E. coli colonization, providing tight association between bacteria and cell membrane, which ultimately results in the envelopment of the bacterial body by the cell membrane and sequential bacterial uptake through a zipper-like process.

The ability of DraE to interact simultaneously with two receptors is an intriguing observation. The presence of two distinct receptor binding sites on the adhesin could be the result of two independent evolution processes directed toward preserving both activities. Genetic adaptation to one environment is often associated with loss of fitness in other environments. If the binding sites for two receptors overlap, positive selection associated with the adaptation to the first receptor might lead to the loss of binding to the second receptor. Independent receptor binding sites may be important for Dr-mediated E. coli persistence in the host environment, because they would contribute to the maintenance of lipid raft integrity and provide multiple high affinity interactions that can lead to bacterial internalization.

Acknowledgments—We are grateful to Evgeni Sokurenko, Veronica Chesnokova, and Konstantin Korotkov for helpful criticism of the manuscript and Anh-Linh Bui and Diane Capps for technical assistance. We thank Dr. John E. Shively (Beckman Research Institute City of Hope, Duarte, CA) for generously providing the construct for CEA N-A3 expression. We also thank David J. Evans (Institute of Virology, University of Glasgow) for kindly providing the constructs for DAF expression.

REFERENCES

1. Garcia, M. I., Gounon, P., Courcoux, P., Labigne, A., and Le Bouguenec, C. (1996) Mol. Microbiol. 19, 683–693
2. Foxman, B., Zhang, L., Tallman, P., Palin, K., Rode, C., Bloch, C., Gillespie, B., and Marrs, C. F. (1995) J. Infect. Dis. 172, 1536–1541
3. Nowicki, B., Labigne, A., Moseley, S., Hull, R., Hull, S., and Moulds, J. (1990) Infect. Immun. 58, 279–281
4. Servin, A. L. (2005) Clini. Microbiol. Rev. 18, 264–292
5. Nowicki, B., Moulds, J., Hull, R., and Hull, S. (1988) Infect. Immun. 56,
Dr Adhesins Bind Independently to DAF and N-domain of CEA

1057–1060

6. Guignot, J., Peiffer, I., Bernet-Camard, M. F., Lublin, D. M., Carnoy, C., Moseley, S. L., and Servin, A. L. (2000) Infect. Immun. 68, 3554–3563

7. Medof, M. E., Walter, E. L, Rutgers, J. L., Knowles, D. M., and Nussenzweig, V. (1987) J. Exp. Med. 165, 848–864

8. Hammarstrom, S. (1999) Semin. Cancer Biol. 9, 67–81

9. Berger, C. N., Billker, O., Meyer, T. F., Servin, A. L., and Kansau, I. (2004) J. Exp. Med. 190, 331–340

10. Obrink, B. (1997) Curr. Opin. Cell Biol. 9, 616–626

11. Prall, F., Nollau, P., Neumaier, M., Haubeck, H. D., Drzeniek, Z., Helmchen, U., Loning, T., and Wagener, C. (1996) Mol. Microbiol. 24, 2485–2493

12. Kodera, Y., Isobe, K., Yamauchi, M., Satta, T., Hasegawa, T., Oikawa, S., Kondo, K., Akiyama, S., Itoh, K., Nakashima, I., and Takagi, H. (1993) Br. J. Cancer 68, 130–136

13. Metze, D., Bhardwaj, R., Amann, U., Eades-Perner, A. M., Neumaier, M., Wagener, C., Jantschek, P., Gruent, F., and Lugert, T. A. (1996) J. Invest. Dermatol. 106, 64–69

14. Leusch, H. G., Drzeniek, Z., Markos-Pusztai, Z., and Wagener, C. (1991) Infect. Immun. 59, 2051–2057

15. Sauter, S. L., Rutherfurd, S. M., Wagener, C., Shively, J. E., and Hefta, S. A. (1991) Infect. Immun. 59, 2485–2493

16. Sauter, S. L., Rutherfurd, S. M., Wagener, C., Shively, J. E., and Hefta, S. A. (1993) J. Biol. Chem. 268, 15510–15516

17. Virji, M., Watt, S. M., Barker, S., Makepeace, K., and Doyonnas, R. (1996) Mol Microbiol. 22, 929–939

18. Virji, M., Evans, D., Hadfield, A., Gruent, F., Teixeira, A. M., and Watt, S. M. (1999) Mol Microbiol. 34, 538–551

19. Virji, M., Evans, D., Griffith, J., Hill, D., Serino, L., Hadfield, A., and Watt, S. M. (2000) Mol Microbiol. 36, 784–795

20. Bos, M. P., Kuroki, M., Krop-Watorek, A., Hogan, D., and Belland, R. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9584–9589

21. Tollem, M., Aho, E., and Virji, M. (2001) Cell Microbiol. 3, 33–44

22. Hill, D. J., and Virji, M. (2003) Mol Microbiol. 48, 117–129

23. Hill, D. J., Tollem, M. A., Evans, D. J., Vilhulas, S., Van Alphen, L., and Virji, M. (2001) Mol Microbiol. 39, 850–862

24. Gray-Owen, S. D. (2003) Scand. J. Infect. Dis. 35, 614–618

25. Maniatis, T. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

26. You, Y. H., Hefta, L. J., Yazaki, P. I., Wu, A. M., and Shively, J. E. (1998) Anticancer Res. 18, 3193–3201

27. Hellwig, S., Robin, F., Drossard, J., Raven, N. P., Vaquero-Martin, C., Shively, J. E., and Fischer, R. (1999) Biotechnol. Appl. Biochem. 30, 267–275

28. Van Loy, C. P., Sokurenko, E. V., Samudrala, R., and Moseley, S. L. (2002) Mol Microbiol. 45, 439–452

29. Powell, R. M., Ward, T., Evans, D. J., and Almond, J. W. (1997) J. Virol. 71, 9306–9312

30. Myszka, D. G. (1999) J. Mol. Recognit. 12, 279–284

31. Anderson, K. L., Billington, J., Pettigrew, D., Cota, E., Simpson, P., Roversi, P., Chen, H. A., Urvil, P., du Merle, L., Barlow, P. N., Medof, M. E., Smith, R. A., Nowicki, B., Le Bouguenec, C., Lea, S. M., and Matthews, S. (2004) Mol. Cell 15, 647–657

32. Carnoy, C., and Moseley, S. L. (1997) Mol. Microbiol. 23, 365–379

33. Pettigrew, D., Anderson, K. L., Billington, J., Cota, E., Simpson, P., Urvil, P., Rabuzin, F., Roversi, P., Nowicki, B., du Merle, L., Le Bouguenec, C., Matthews, S., and Lea, S. M. (2004) J. Biol. Chem. 279, 46851–46857

34. Tan, K., Zelus, B. D., Meijers, R., Liu, J. H., Bergelson, J. M., Duke, N., Zhang, R., Joachimiak, A., Holmes, K. V., and Wang, J. H. (2002) EMBO J. 21, 2076–2086

35. Boehm, M. K., Mayans, M. O., Thornton, J. D., Begent, R. H., Keep, P. A., and Perkins, S. J. (1996) J. Mol. Biol. 259, 718–736

36. Bos, M. P., Hogan, D., and Belland, R. J. (1999) J. Exp. Med. 190, 331–340

37. Hefta, L. J., Chen, F. S., Ronk, M., Sauter, S. L., Sarin, V., Oikawa, S., Nakazato, H., Hefta, S., and Shively, J. E. (1992) Cancer Res. 52, 5647–5655

38. Hunter, I., Sawa, H., Edlund, M., and Obrink, B. (1996) Biochem. J. 320, 847–853

39. Krop-Watorek, A., Oikawa, S., Ohnami, Y., and Nakazato, H. (1998) Biochem. Biophys. Res. Commun. 242, 79–83

40. Talieri, M., Saragovi, U., Fuchs, A., Makkerb, J., Mort, J., and Stanners, C. P. (2000) J. Biol. Chem. 275, 26955–26943

41. Markel, G., Gruda, R., Achdout, H., Katz, G., Nechama, M., Blumberg, R. S., Kammerer, R., Zimmermann, W., and Mandelboim, O. (2004) J. Immunol. 173, 3732–3739

42. Watt, S. M., Teixeira, A. M., Zhou, G. Q., Doyonnas, R., Zhang, Y., Gruent, F., Blumberg, R. S., Kuroki, M., Skubitz, K. M., and Bates, P. A. (2001) Blood 98, 1469–1479

43. Kansau, I., Berger, C., Hospital, M., Amsellem, R., Nicolas, V., Servin, A. L., and Bernet-Camard, M. F. (2004) Infect. Immun. 72, 3733–3742

44. Das, M., Hart-Van Tassell, A., Urvil, P. T., Lea, S., Pettigrew, D., Anderson, K. L., Samet, A., Kur, J., Matthews, S., Nowicki, S., Popov, V., Goluszko, P., and Nowicki, B. (2005) Infect. Immun. 73, 6119–6126

45. Jouve, M., Garcia, M. I., Courcoux, P., Labigne, A., Gounon, P., and Le Bouguenec, C. (1997) Infect. Immun. 65, 4082–4089

46. Plancon, L., Du Merle, L., Le Friec, S., Gounon, P., Jouve, M., Guignon, J., Servin, A., and Le Bouguenec, C. (2003) Cell Microbiol. 5, 681–693

47. Selvarangan, R., Goluszko, P., Popov, V., Singhal, J., Pham, T., Lublin, D. M., Nowicki, S., and Nowicki, B. (2000) Infect. Immun. 68, 1391–1399

48. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell Biol. 1, 31–39

49. McCaw, S. E., Liao, E. H., and Gray-Owen, S. D. (2004) Infect. Immun. 72, 2742–2752