JOURNAL OF BACTERIOLOGY, Mar. 2006, p. 1798–1807 Vol. 188, No. 5
0021-9193/06/$08.00+0 doi:10.1128/JB.188.5.1798–1807.2006

Glycosylation of the Self-Recognizing *Escherichia coli* Ag43 Autotransporter Protein

Orla Sherlock,[1] Ulrich Dobrindt,[2] Jeppe B. Jensen,[3] Rebecca Munk Vejborg,[1] and Per Klemm[1]*

Centre for Biomedical Microbiology, BioCentrum-DTU, Technical University of Denmark, Lyngby, Denmark[1]; Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring Würzburg, Germany[2]; and Department of Life Sciences, Aalborg University, Aalborg, Denmark[3]

Received 11 October 2005/Accepted 10 December 2005

Glycosylation is a common modulation of protein function in eukaryotes and is biologically important. However, in bacteria protein glycosylation is rare, and relatively few bacterial glycoproteins are known. In *Escherichia coli* only two glycoproteins have been described to date. Here we introduce a novel member to this exclusive group, namely, antigen 43 (Ag43), a self-recognizing autotransporter protein. By mass spectrometry Ag43 was demonstrated to be glycosylated by addition of heptose residues at several positions in the passenger domain. Glycosylation of Ag43 by the action of the Aah and TibC glycosyltransferases was observed in laboratory strains. Importantly, Ag43 was also found to be glycosylated in a wild-type strain, suggesting that Ag43-glycosylation may be a widespread phenomenon. Glycosylation of Ag43 does not seem to interfere with its self-associating properties. However, the glycosylated form of Ag43 enhances bacterial binding to human cell lines, whereas the nonglycosylated version of Ag43 does not to confer this property.

Glycosylation is an important covalent modification of proteins. In eukaryotes a plethora of glycoproteins are known, and generally addition of sugar moieties is used for modulation of protein function. Until a few decades ago, the accepted dogma was that protein glycosylation simply did not take place outside the eukaryotic kingdom. This view has dramatically changed, and it is now a well-established fact that protein glycosylation does take place in prokaryotes, particularly in cell surface-associated and secreted molecules (reviewed in reference 6). In gram-negative bacteria only a few glycoproteins are known, and these are generally either bona fide virulence factors or suspected virulence factors, e.g., type 4 pilin of *Neisseria* species and *Pseudomonas aeruginosa* and flagellin of *Campylobacter* species (41, 44, 45). In *Escherichia coli* only two glycoproteins have been characterized so far, both of which are putative virulence factors. These are the AIDA-I and TibA adhesins associated with diarrhea-causing *E. coli* strains. Both proteins are glycosylated by addition of heptose residues, a task performed by specific heptosyl transferases (5, 27). The precursor substrate, ADP-glycero-manno-heptopyranose, is recruited from the lipopolysaccharide (LPS) biosynthetic pathway (5). By virtue of their glycoprotein status, AIDA-I and TibA are adhesins and target selected mammalian cells; in contradistinction, the nonglycosylated forms are unable to bind to such cells (5, 28).

An essential step in the pathogenesis of disease-causing *E. coli* is the initial recognition of and attachment to host tissue surfaces. Without this pivotal initial step, subsequent tissue colonization, cell invasion, or biofilm formation simply will not occur (26). Bacterial attachment is generally provided by specific protein adhesins that protrude from the surface. Usually the receptor target is a specific molecular motif on the surface of epithelial cells, for example, a specific saccharide. Antigen 43 (Ag43) is exceptional in being a self-recognizing adhesin (17, 22, 23). Ag43 is a surface protein that confers bacterial cell-cell aggregation, which can be visualized macroscopically as flocculation and settling of cells from static liquid suspensions; hence, the name *flu* was originally coined for the corresponding genetic locus by Diderichsen (11). In an independent study, a major *E. coli* outer membrane antigen was investigated because of its aggregative properties and was termed antigen 43 (32). Later, Ag43 was identified as the product of the *flu* gene (17, 20). Ag43 is a member of the autotransporter protein family. This family is now the largest group of exported proteins in gram-negative bacteria and encompasses many virulence factors. Autotransporters are characterized by the fact that the proteins contain all information required for traversion of the bacterial membrane system and final routing to the bacterial cell surface (reviewed in reference 19). Ag43 is present in ~50,000 copies per cell (33). It is produced as a precursor of 1,039 amino acids, which subsequently undergoes extended posttranslational modifications. It is processed by removal of a signal peptide and further processed, presumably by autocatalytic action, into a C-terminal translocator domain and an N-terminal passenger domain, each constituting about half of the protein. The translocator moiety forms a β-barrel porin in the outer membrane and via this the passenger moiety gains access to the surface (20, 21, 24). The passenger domain remains attached to the cell surface via interaction with the translocator domain, but it can be detached by brief heat treatment (20). Apart from autoaggregation, Ag43 has been found to induce a frizzy colony morphology (18). Ag43-mediated cell aggregation takes place via an intercellular Ag43-to-Ag43 handshake mechanism. The interaction involves the N-terminal one-third of the passenger domain, and ionic interactions seem to be involved (24). Like its distant relative pertactin of *Bordetella pertussis*, the Ag43 passenger domain has been pre-

* Corresponding author. Mailing address: Centre for Biomedical Microbiology, BioCentrum-DTU, Bldg. 301, Technical University of Denmark, DK-2800 Lyngby, Denmark. Phone: 45 45 25 25 06. Fax: 45 45 93 28 09. E-mail: pkl@biocentrum.dtu.dk.

TABLE 1. Bacterial strains

E. coli strain	Relevant characteristics	Reference or source
536	Uropathogenic strain (06:K15:H31)	8
536DM	536 Δ $orf52_{III}$ Δ$orf47_{V}$	3
H10407	Enterotoxigenic strain	27
2787	Enteropathogenic strain (O126:H27)	4
MG1655	K-12 reference strain	2
MS427	MG1655 Δ*flu*	22
LH56	MS427, pACYC184	38
LH57	MS427, pKKJ128	This study
OS64	MS427, pKKJ128 + pOS28	This study
OS101	MS427, pOS37	38
OS111	MS427, pKKJ128 + pOS38	This study
OS116	MG1655, pOS37	This study
OS123	MG1655, pPKL331	This study
OS135	MG1655, pRMV5	This study
RMV135	UTI536, pKKJ143	This study
RMV136	UTI536 DM, pAGN3	This study
RMV137	UTI536 DM, pAGN5	This study
RMV165	UTI536 DM, pOS29	This study

dicted to fold as an extended β-helix (24). Expression of Ag43 dramatically enhances biofilm formation in bacteria (10, 22, 23, 37). Conversely, lesions in the *flu* gene causing abolishment of Ag43 expression in many cases result in cells with a very limited ability to form a biofilm (23, 37). Ag43 is found in most *E. coli* strains, and, interestingly, it is expressed by many pathogenic strains. Also, many strains possess duplex or multiple copies of the gene, as seems to be the case in many enteropathogenic and enterohemorrhagic strains (24, 35, 43). Ag43 exhibits ~25% sequence identity to the AIDA-I and TibA glycoproteins. With this background, we have probed this interesting protein for possible glycosylation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains, plasmids, and primers used in this study are described in Tables 1, 2, and 3 respectively. Cells were grown at 37°C on solid or in liquid Luria-Bertani (LB) medium (30) supplemented with the appropriate antibiotics unless otherwise stated. Genes of interest were cloned by PCR amplification using the primers listed in Table 3. Primers contained restriction enzyme sites which were subsequently used for cloning in pBAD*myc*-HisA, pACYC184, or pMW119 vectors; for example, the *aah* gene was cloned by PCR amplification (primers 460 and 461) from *E. coli* 2787 and subsequently cloned in the pBAD vector, resulting in plasmid pOS28. Details of construct preparation are in Table 2.

DNA manipulations and genetic techniques. Isolation of plasmid DNA was carried out using the QIAprep Spin Miniprep kit (QIAGEN). Restriction endonucleases were used according to the manufacturer's specifications (Biolabs). Chromosomal DNA purification was done using the GenomicPrep Cell and Tissue DNA isolation kit (Amersham Pharmacia Biotech Inc.). All PCRs were performed with the Expand high-fidelity polymerase system (Roche) essentially as previously described (40). The primers used are listed in Table 3. Amplified products were sequenced to ensure fidelity of the PCR (MWG Biotech, Germany).

Electrophoresis, Western blotting, and detection of glycoproteins. Electrophoresis of proteins was performed under denaturing conditions using a previously described method (40). Cultures grown overnight and of equal optical density were harvested by centrifugation at 10,000 × *g* for 2 min. Passenger domains were released and partly purified as previously described (24). Samples were prepared for electrophoresis by resuspension in 25 μl of 5× sodium dodecyl sulfate (SDS) treatment buffer and 25 μl water. Samples were then boiled at 95°C for 3 min, and 10-μl aliquots were loaded on SDS-polyacrylamide gels.

For Western blotting, samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride microporous membrane filters as described previously (40). Serum raised against the passenger domain of Ag43 was used as primary serum, and the secondary serum was a peroxidase-conjugated anti-rabbit immunoglobulin. Tetramethyl benzidine was used as the substrate.

For glycoprotein staining, samples were electrophoresed and transferred to nitrocellulose filters as described above. Staining was carried out by using method B of the Roche Molecular Biochemicals digoxigenin glycan detection kit according to the manufacturers' instructions.

Colony morphology and cell aggregation. The colony morphology and cell aggregation phenotype was assayed by employing a Carl Zeiss Axioplan epifluorescence microscope, and digital images were captured with a 12-bit cooled slow-scan charge-coupled-device camera (KAF 1400 chip; Photometrics, Tucson, Arizona) controlled by PMIS software (Photometrics).

Mass spectroscopy. Trypsin-digested Ag43, coexpressed with heptosyl transferase Aah or TibC or from the UTI536 strain, was analyzed on a matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometer (ReflexIII; Bruker, Bremen, Germany). Analysis was carried out essentially as previously described (42). The matrix used in these experiments was 2,5-dihydroxybenzoic acid (Sigma-Aldrich). The apparatus was calibrated using a standard mix containing angiotensin II, bombesin, adrenocorticotropic hormone amino acids 18 to 39, and somatostatin, with molecular masses (monoisotopic) of 1,046.5423 Da, 1,619.8229 Da, 2,465.7027 Da, and 3,147.4640 Da, respectively.

Ag43 preparations were subjected to SDS-polyacrylamide gel electrophoresis on an 8% gel, and protein bands were cut out and subjected to tryptic digestion overnight. The resulting peptides were extracted from the gel slabs and concentrated using PorosBeads. The peptides were eluted from the beads with 25% acetonitrile followed by 75% acetonitrile, mixed with 2,5-dihydroxybenzoic acid matrix, and applied to the sample plate (anchor chip). Spectra were obtained by collecting an average of 300 shots. Spectra were analyzed and peaks annotated in the program M/Z, freeware edition, from Proteometrics, LCC. The resulting masses were compared to a theoretical digest performed with the online program ProteinProspector found at http://prospector.ucsf.edu/. The program GPMAW 6.21 from Light House Data was used to compare the theoretical and measured masses and for identification of glycosylated peptides.

TABLE 2. Plasmids used in this study

Plasmid	Relevant genotype or phenotype	Reference or source
pKKJ128	*flu* gene from *E. coli* MG1655 in pACYC184	22
pKKJ143	*flu* gene from *E. coli* MG1655 in pBAD*myc*-HisA	21
pMW119	Low-copy-number cloning vector	Nippon Gene
pOS28	*aah* gene amplified with primers 460 and 461 and cloned in pBAD*myc*-HisA as an XhoI-KpnI fragment	This study
pOS29	*aidA* gene amplified with primers 466 and 467 and cloned in pBAD*myc*-HisA as an XhoI-KpnI fragment	This study
pOS38	*tibC* gene from *E. coli* H10407 on pBAD*myc*-HisA	39
pOS37	*aah* gene on pACYC184	38
pPKL331	*aah* gene amplified with primers 537 and 461 and cloned on plasmid pMW119 as a BamHI-KpnI fragment	This study
pAGN3	$orf52_{III}$ from *E. coli* strain 536 amplified with primers AgIIIforward and AgIIIreverse and cloned on plasmid pGEM T-Easy	This study
pAGN5	$orf47_{V}$ from *E. coli* strain 536 amplified with primers AgVforward and AgVreverse and cloned on plasmid pGEM T-Easy	This study
pRMV5	*tibC* gene amplified with primers 573 and 579 and cloned on pACYC184 as a BamHI-XmaIII fragment	This study

TABLE 3. Primers used in this study

Primer	Sequence
460	5′-CGCGCTCGAGATAATAAGGAATGACTTTCTTATCACCA-3′
461	5′-CGCGCGGGTACCTTAAATGATCCTGTCATGA-3′
466	5′-CGCGCTCGAGATAATAAAGGATCATTTAATGAATAAGGCCTAC-3′
467	5′-CGCGCGGGTACCTCAGGAGCAAGAGTAATCCGCC-3′
537	5′-CGCGGGATCCATAATAAGGAATGACTTTCTTATCACCA-3′
541	5′-CGCGCGGCGGCCGTTAAATGATCCTGTCATGA-3′
573	5′-CGCGGGATCCATAATAAGGATCATTTAATGTCAACGCTGAAG-3′
579	5′-CGCGCCCGGCCGTTCATTGCTTACTCCTT-3′
AgIIIforward	5′-TGCTCTAGACTGAGTCTGCTCACAAAAG-3′
AgIIIreverse	5′-CCCAAGCTTGGGCTGATGGGGGAAATGAATG-3′
AgVforward	5′-TGCTCTAGATGCCTGATTACATTGCGTTA-3′
AgVreverse	5′-CCCAAGCTTCAGGTTCAAAATTCCAGCAC-3′

Cell binding assay. HEp-2 epithelial cells were maintained in RPMI 1640 medium (29) containing 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine, and 100 μg ml^{-1} gentamicin. The cell lines were grown at 37°C in a 6% CO_2 atmosphere. For measurement of attachment to cells by recombinant *E. coli*, the cells were maintained and monolayers were prepared in RPMI medium. To measure adherence of *E. coli* to cell monolayers, bacterial cells were grown to mid-exponential phase or overnight in LB broth at 37°C. Cells were washed in phosphate-buffered saline, resuspended in RPMI medium containing 0.5% (wt/vol) methyl-α-D-manno-pyranoside, and incubated for 10 min at room temperature. Approximately 1×10^7 CFU was added to the monolayers and incubated for 3 hours at 37°C in a 6% CO_2 atmosphere. Following incubation, the monolayers were extensively washed with phosphate-buffered saline, fixed with ice-cold 70% methanol, and stained with 10% Giemsa stain. Cells were examined by phase-contrast microscopy for observation of adhered cells.

RESULTS

The passenger domain of Ag43 can be glycosylated by heptosyl transferases. The AIDA-I and TibA autotransporters of diarrheagenic *E. coli* strains are so far the only glycoproteins known to exist in *E. coli*. The AIDA-I and TibA adhesins are glycosylated by the Aah and TibC heptosyl transferases, respectively (5, 28). In order to probe whether the Aah and TibC heptosyl transferases would accept Ag43 as substrate, we made a series of constructs on compatible plasmid vectors that contained the relevant genes, i.e., *flu*, *aah*, and *tibC*, as well as appropriate controls (Table 1). Plasmids were expressed in a *flu* mutant derivative of *E. coli* K-12, i.e., MS427 (22). To determine if the Ag43 protein was glycosylated when coexpressed with Aah or TibC protein, the Ag43 passenger domains from the relevant strains were isolated and subjected to SDS gel electrophoresis, Western blotting with antiserum raised against the passenger domain of Ag43, and glycoprotein detection using the Roche glycan detection kit. The Western blot of the passenger domains revealed bands with apparent molecular masses of ~50 kDa, as expected (Fig. 1A). Strain MS427, like other *E. coli* K-12 strains, is unable to glycosylate proteins, and consequently the passenger domain of Ag43 does not react in a glycoprotein blot when expressed in this strain background (LH57) (Fig. 1B). Meanwhile, when Ag43 was coexpressed with the Aah heptyltransferase (OS64), a prominent signal was seen in the glycoprotein blot (Fig. 1B). Also, the combination of Ag43 plus TibC (OS111) yielded a good response in this assay (Fig. 1B). Taken together the data indicate that the passenger domain of Ag43 can be glycosylated by the two heptosyl transferases, Aah and TibC, whose natural targets, AIDA-I and TibC, respectively, are only distantly related to Ag43.

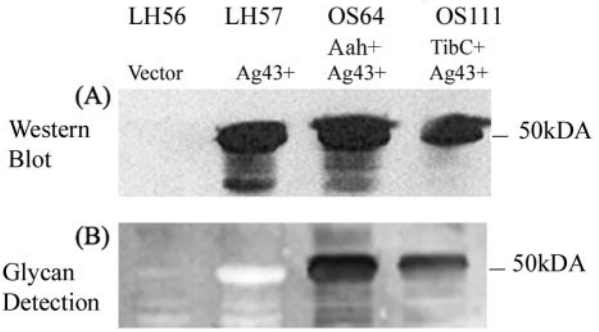


FIG. 1. Glycosylation of the Ag43 passenger domain by the Aah and TibC heptosyl transferases. (A) Western blotting, with antiserum against the Ag43 passenger domain, of α domains liberated from *E. coli* MS427 hosts by brief heat treatment. (B) Glycan detection. Lanes: 1, LH56 (vector control); 2, LH57 ($Ag43^+$); 3, OS64 (Aah^+ $Ag43^+$); 4, OS111 ($TibC^+$ $Ag43^+$).

In wild-type strains, the Ag43 and the TibC proteins are expressed from genes located on the chromosome and the Aah protein is expressed from a gene located on large low-copy-number plasmids (4, 11, 14). In order to eliminate the possibility that the observed glycosylation of Ag43 was an artifact due to high-copy plasmids, we investigated the phenomenon in a low-copy-number scenario (Fig. 2). The results suggested that glycosylation of Ag43 is qualitatively independent of the copy number of the implicated genes.

Glycosylation of Ag43 does not affect self-recognition. Addition of sugars is known often to affect the properties of a protein. As mentioned above, an important property of Ag43 is self-recognition; both receptor-target and receptor recognition are provided by peptide segments in the passenger domain (24). We speculated on whether addition of sugar moieties would block Ag43 self-recognition by shielding sectors of the protein involved in this faculty. In order to test this, we in-

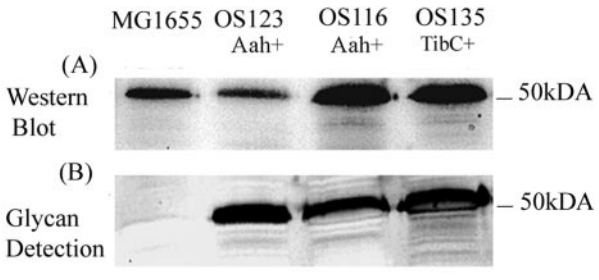


FIG. 2. Glycosylation of Ag43 is qualitatively unaffected by copy number. The product of the chromosomally located *flu* gene in strain MG1655 is glycosylated by heptosyl transferases encoded by low- or high-copy-number plasmids. (A) Western blotting of passenger domains liberated from *E. coli* host cells by brief heat treatment and detected with antiserum raised against the Ag43 passenger domain. (B) Glycan detection. Lanes: 1, MG1655; 2, OS123 (Aah^+) on low-copy plasmid; 3, OS116 (Aah^+) on medium-copy plasmid; 4, OS135 ($TibC^+$) on medium-copy plasmid.

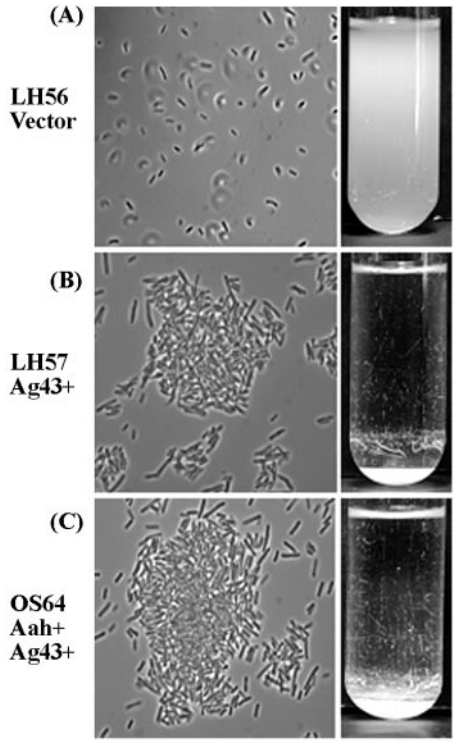


FIG. 3. Glycosylation of Ag43 does not affect Ag43-Ag43 interaction and the associated cell aggregation. (A) LH56 (vector control); (B) LH57 ($Ag43^+$); (C) OS64 (Aah^+ $Ag43^+$).

spected the ability of *E. coli* cells expressing Ag43 and glycosylated Ag43 to aggregate and settle from liquid suspensions. The results show that strains expressing glycosylated Ag43 aggregated and settled in the same way as cells expressing unmodified Ag43 (Fig. 3). Another characteristic phenotype associated with Ag43 expression is a frizzy colony type (18); similarly, glycosylation did not interfere with this phenotype (data not shown). Taken together, the data indicate that the important self-recognition ability of Ag43 is not affected by sugar addition, suggesting that the peptide segments involved in Ag43-Ag43 interaction are not glycosylated.

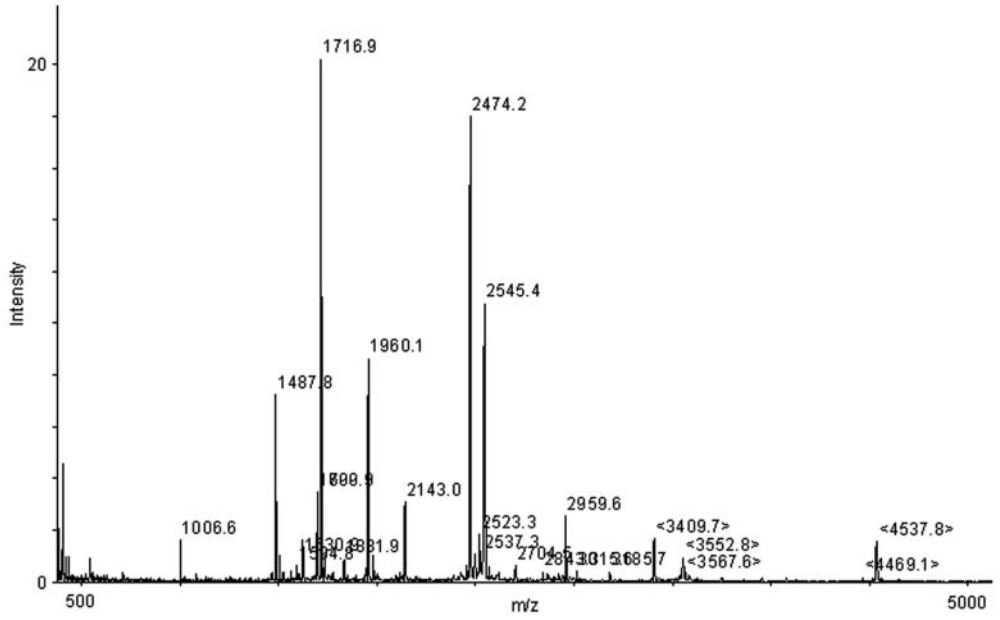
Mass spectrometric analysis of glycosylated segments in the Ag43 passenger domain: glycosylated positions and nature of the sugar side chains. To corroborate the glycoprotein blotting results, the glycosylated Ag43 passenger domain was purified from strain OS64, digested with trypsin, and subjected to MALDI-TOF mass spectrometry (Fig. 4). Twenty-four individual peptides were identified, corresponding to 68% coverage of the passenger domain. Eleven of these were observed in glycosylated forms (Fig. 4 and 5). The Aah and TibC glycosyl transferases have been suggested to confer O glycosylation (27). No consensus sequence(s) for bacterial glycoproteins has yet been identified, but at least it is known that serine and threonine are required (6, 36). Incorporating this into the analysis of Ag43, a picture with potential glycosylated serine and threonine side chains emerges (Fig. 5). The analysis also revealed that the added sugar moieties were heptoses. Taken together, the mass spectrometric analysis indicates that the Ag43 passenger can be decorated by added sugars. Glycosylation of the protein with heptosyl residues takes place at several different positions, presumably involving S and T side chains.

Glycosylated wild-type variant of Ag43. Having demonstrated that the passenger domain of Ag43 can exist as a glycoprotein, we proceeded to probe for the existence of naturally occurring glycosylated forms of Ag43. To this end, a number of wild-type uropathogenic *E. coli* strains were screened for the presence of surface-located glycoproteins that behaved like Ag43, i.e., proteins that could be released from the bacteria by brief heat treatment, were recognized by anti-Ag43 serum, and, importantly, reacted in the glycoprotein assay. In several cases proteins that fulfilled the criteria of our initial screening process were observed (data not shown). We decided to further investigate one of these candidate strains, namely, the uropathogenic *E. coli* strain 536 (UTI536). This strain has been extensively characterized (8, 12). It can express two variants of Ag43, viz., Ag43-III and Ag43-V, encoded by ORF52 on pathogenicity island III_{536} and ORF47 on pathogenicity island V_{536}, respectively (3). Both of these proteins are expressed in a phase-variable manner; however, the Ag43-III variant is better expressed and was chosen for this study. Western blotting with antiserum raised against the passenger domain of Ag43 showed clear signals of this protein in the UTI536 strain (Fig. 6A). Moreover, a clear signal originating from the glycosylated Ag43 passenger domain was observed in the glycoprotein stain (Fig. 6B). Importantly, these signals are missing in heat extracts from a double knockout mutant of UTI536, i.e., strain 536 $\Delta ORF47_V$ $\Delta ORF52_{III}$ (UTI536 DM). Meanwhile, reintroduction of plasmids carrying *flu* genes corresponding to Ag43-III and Ag43-K12 into UTI536 DM resulted in reappearance of signals (Fig. 6). In effect, this makes Ag43 the third known glycoprotein of *E. coli*. Mass spectroscopy of the Ag43 passenger domain isolated from a UTI536 background corroborated the glycoprotein blotting data and identified the presence of heptosyl side chains. However, contamination with small amounts of capsule material hampered this analysis (data not shown).

As shown in this work, Ag43 can be glycosylated by the heptosyl transferases Aah and TibC, belonging to the AIDA and TibC systems, respectively. We investigated whether the glycosyl transferase of UTI536, capable of glycosylating Ag43, would accept AIDA-I as a target. To this end a plasmid, pOS29, carrying the *aidA* gene (but not *aah*) was introduced into UTI536 DM. When heat-released protein from this strain was subjected to glycan blotting, a signal could be seen. Also, the apparent molecular weight of AIDA-I is known to change considerably when it is glycosylated, which is exactly what we observed (data not shown). Taken together, the results indicate that the unknown glycosyl transferase of UTI536, like its functional homologues, Aah and TibC, accepts a spectrum of proteins as targets for glycosylation.

Glycosylated Ag43 mediates adhesion of *E. coli* to HEp-2 epithelial cells. The nonglycosylated form of Ag43 from *E. coli* K-12 promotes efficient bacterial aggregation, but it does not mediate binding to human cell line cells (Fig. 7). We speculated on whether glycosylation of Ag43 by heptosyl transferases would change this. Strain 536 is capable of expressing numerous adhesins and cannot be used directly as a background strain for this purpose, and the glycosylase modifying Ag43 in UTI536 is unknown. Thus, for the purpose of these studies, strain OS64, which in addition to Ag43 also produces the Aah heptosyl transferase,

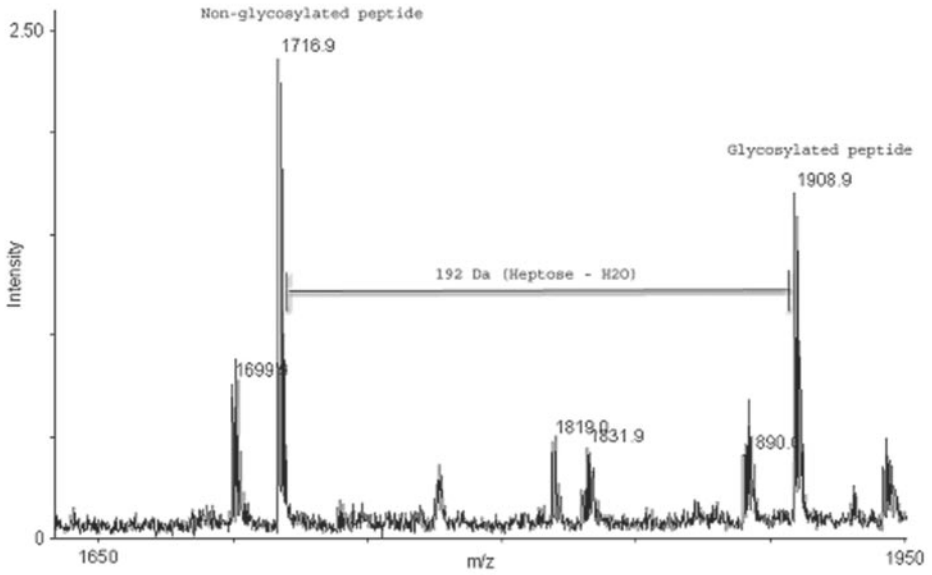
A)



Measured	Theoretical	ppm	Sequence
1006.5537	1006.5573	4	LGAFSVVEGK
1487.8106	1487.8069	3	LQVDAGGTATNVTLK
1594.8311	1594.8288	2	SGSGTLTVSNTTLTQK
1630.9177	1630.9016	10	GTALKLTGSTVLNGAID
1699.8883	1699.8979	6	QGGALVTSTAATVTGINR
1700.8796	1700.9295	30	GRLQVDAGGTATNVTLK
1716.9037	1716.9244	12	QGGALVTSTAATVTGINR
1831.8978	1831.9038	3	EGDALLQGGSLTGNGSVEK
1960.0569	1960.0715	7	GGTLAGTTTLNNGAILTLSGK
2143.0289	2143.0743	21	VNPGGSVSDTVISAGGGQSLQGR
2474.2039	2474.1799	10	GSSFTLNAGDTATDTTVNGGLFTAR
2523.2845	2523.2916	3	ADNVVLENGGRLDVLTGHTATNTR
2537.3497	2537.2266	49	NGGTATTVSMGNGGVLLADSGAAVSGTR
2545.3529	2545.3109	17	AVNLNEGTLTLNDSTVTTDVIAQR
2704.4847	2704.4634	8	QGGALVTSTA ATVTGINRLGAFSVVEGK
2843.3165	2843.3634	16	AVNTTLNGGEQWMHEGAIATGTVINDK
2959.5577	2959.4972	20	TVNNDTLTIREGDALLQGGSLTGNGSVEK
3015.6124	3015.5962	5	AVNLNEGTLTLNDSTVTTDVIAQRGTALK
3185.6978	3185.713	5	LQVDAGGTATNVTLKQGGALVTSTAATVTGINR
<3409.7072>	<3409.7091>	1	EGDALLQGGSLTGNGSVEKSGSGTLTVSNTTLTQK
<3552.8051>	<3552.864>	17	ADNVVLENGGRLDVLTGHTATNTRVDDGGTLDVR
<3566.4837>	<3566.8638>	107	VDDGGTLDVRNGGTATTVSMGNGGVLLADSGAAVSGTR
<4469.0668>	<4468.8848>	41	SDGKAFSIGGGQADALMLEKGSSFTLNAGDTATDTTVNGGLFTAR
<4537.7811>	<4537.9674>	41	TVNNDTLTIREGDALLQGGSLTGNGSVEKSGSGTLTVSNTTLTQK

FIG. 4. MALDI-TOF mass spectroscopy. (A) Trypsin-digested Ag43 passenger domain isolated from a host strain unable to perform glycosylation. (B) Representative enlarged segment of spectrum of trypsin-digested Ag43 passenger domain isolated from a host strain capable of performing protein glycosylation. The indicated peaks correspond to the same peptide with and without heptose. The mass difference represents heptose minus water. Values in angle brackets indicate average masses; all other masses are represented as monoisotopic. Masses are presented as M + H. The asterisk indicates three heptose residues.

B)



Theoretical non-gly	Theoretical gly	Measured	ppm	Sequence
1716.9244	1908.9244	1908.9499	13	QGGALVTSTAATVTGINR
2143.0743	2335.0743	2335.1992	53	VNPGGSVSDTVISAGGGQSLQGR
2426.2276	2618.2276	2618.3776	57	LDVLTGHTATNTRVDDGGTLDVR
2704.4634	2896.4634	2896.6043	49	QGGALVTSTAATVTGINRLGAFSVVEGK
<3187.6003>	<3379.6003>	<3379.5918>	3	LQVDAGGTATNVTLKQGGALVTSTAATVTGINR
<3954.259>	<4146.2590>	<4146.5534>	71	VDDGGTLDVRNGGTATTVSMGNGGVLLADSGAAVSGTRSDGK
<2961.2327>	*<3537.2327>	<3537.1205>	32	TVNNDTLTIREGDALLQGGSLTGNGSVEK

FIG. 4—*Continued.*

was employed. In contrast to the control strains that produced either Ag43 or Aah, OS64 was demonstrated to bind to cells of the human cell line HEp-2 (Fig. 7). This suggests that glycosylation of Ag43 can modify its binding characteristics to target to human cells.

DISCUSSION

E. coli is a versatile pathogen capable of causing a range of diseases, such as diarrhea, bladder and kidney infections, pneumonia, and meningitis. The versatility of *E. coli* stems from the fact that different strains have acquired different sets of virulence genes through acquisition of pathogenicity islands, plasmids, etc. Different sets of virulence-related genes permit infection and survival in different host tissues. In addition a small group of virulence factors seem to be able to fulfill more than one function; e.g., flagella can in some cases mediate not only motility but also adhesion (16).

Ag43 seems to be such a multipurpose virulence factor. Ag43 confers efficient bacterial aggregation via intercellular self-recognition. It has become clear that the ability to form aggregates seems to be a common trait among many bacterial pathogens. Such aggregates are known to be able to resist various host defenses, e.g., complement attack and phagocytosis, more efficiently than solitary bacteria (7, 15, 31). Bacteria expressing the Ag43 aggregating phenotype may exist as tight communities of cells encompassing all of the beneficial aspects of this type of existence. In this respect it is interesting to speculate that the autoaggregating function of Ag43 may be a tool used to aid survival of the organism on route to a mammalian host. Aggregation may also assist transfer of bacteria across the gastric barrier on the way to the intestines. It is noteworthy that the ability to aggregate greatly enhances the infectivity of *Vibrio cholerae* (47). These observations lend strong support to the notion that aggregation is an important virulence mechanism. In contrast to aggregation systems based on polymeric structures that reach far out from the bacterial surface, such as fimbriae and curli, Ag43 is anchored directly to the outer membrane. Thus, Ag43-mediated aggregation results in a more intimate cell-cell contact than is seen with other systems. Recently, Ag43 was shown to be expressed by uropathogenic *E. coli* in vivo during formation of intracellular bacterial aggregates or pods in bladder cells (1).

(A)

(Peptide 1. (2143.0743 MI) VNPGGSVSDTVISAGGGQSLQGR
Glycosylated with one heptose.

Peptide 2. (3631.9192 AVG) GWQVVKPGTVATDTVVNTGAEGGPDAENGDTGQFVR
Glycosylated with one heptose, also found with no glycosylation.

Peptide 3. (4175.7593 AVG) LQVDAGGTATNVTLKQGGALVTSTAATVTGINRLGAFSVVEGK
Glycosylated with two heptoses or a di-heptose.

Peptide 4. (3187.6003 AVG) LQVDAGGTATNVTLKQGGALVTSTAATVTGINR
Glycosylated with one heptose, also found with no glycosylation. This peptide is the left part of peptide 3.

Peptide 5. (1716.9244 MI) QGGALVTSTAATVTGINR
Glycosylated with one heptose, also found with no glycosylation. This peptide is the middle of peptide 3.

Peptide 6. (2704.4634 MI) QGGALVTSTAATVTGINRLGAFSVVEGK
Glycosylated with one heptose, also found with no glycosylation. This peptide is the right part of peptide 3.

Peptide 7. (3954.259 AVG) VDDGGTLDVRNGGTATTVSMGNGGVLLADSGAAVSGTRSDGK
Glycosylated with one heptose, also found with no glycosylation.

Peptide 8. (2426.2276 MI) LDVLTGHTATNTRVDDGGTLDVR
Glycosylated with one heptose. This peptide is the left part of peptide 7.

Peptide 9. (2521.256 MI) NGGTATTVSMGNGGVLLADSGAAVSGTR
Glycosylated with one heptose. This peptide is the middle part of peptide 7.

Peptide 10. (3409.69 AVG) EGDALLQGGSLTGNGSVEKSGSGTLTVSNTTLTQK
Glycosylated with one heptose, also found with no glycosylation.

Peptide 11. (2961.2327 AVG) TVNNDTLTIREGDALLQGGSLTGNGSVEK
Glycosylated with tree heptoses or a tri-heptose. This peptide is the left part of peptide 10.

(B)

ADIVVHPGETVNGGTLANHDNQIVFGTTNGMTISTGLEYGPDNEANTGGQWVQDGGTANKTTVTS
GGLQRVNPGGSVSDTVISAGGGQSLQGRAVNTTLNGGEQWMHEGAIATGTVINDKGWQVVKPGT
VATDTVVNTGAEGGPDAENGDTGQFVRGDAVRTTINKNGRQIVRAEGTANTTVVYAGGDQTVHG
HALDTTLNGGYQYVHNGGTASDTVVNSDGWQIVKNGGVAGNTTVNQKGRLQVDAGGTATNVTL
KQGGALVTSTAATVTGINRLGAFSVVEGKADNVVLENGGRLDVLTGHTATNTRVDDGGTLDVRN
GGTATTVSMGNGGVLLADSGAAVSGTRSDGKAFSIGGGQADALMLEKGSSFTLNAGDTATDTTVN
GGLFTARGGTLAGTTTLNNGAILTLSGKTVNNDTLTIREGDALLQGGSLTGNGSVEKSGSGTLTVSN
TTLTQKAVNLNEGTLTLNDSTVTTDVIAQRGTALKLTGSTVLNGAID

(C)

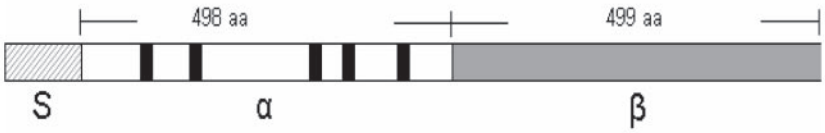


FIG. 5. Summary of identified tryptic peptides from the Ag43 passenger domain found to be glycosylated with heptose residues by MALDI-TOF mass spectrometry. Potential O-glycosylated amino acids are indicated in blue. (A) Identified glycosylated peptides. Molecular masses (in daltons) are indicated. MI and AVG, monoisotopic and average masses, respectively. Glycosylation status is indicated. Note that several of the peptides are overlapping due to partial tryptic digestion. (B) Primary structure of the passenger domain with glycosylated peptides indicated. (C) Overall picture of Ag43 glycosylation. Glycosylated segments are indicated as black bars. S, signal peptide; α, passenger domain; β, transmembrane domain; aa, amino acids.

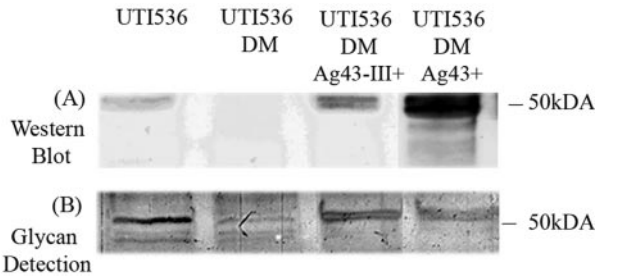


FIG. 6. Ag43-variants from *E. coli* UTI536 are glycosylated. (A) Western blotting of Ag43 passenger domains liberated from *E. coli* host cells by brief heat treatment and detected with anti Ag43 passenger domain serum. (B) Glycan detection. Lanes: 1, UTI536; 2, UTI536 DM; 3, UTI536 DM (Ag43-III$^+$); 4, UTI536 DM (Ag43-K12$^+$). The latter detection analysis was somewhat hampered by the heavy encapsulation of UTI536, which accounts for the capsule material observed in the double mutant.

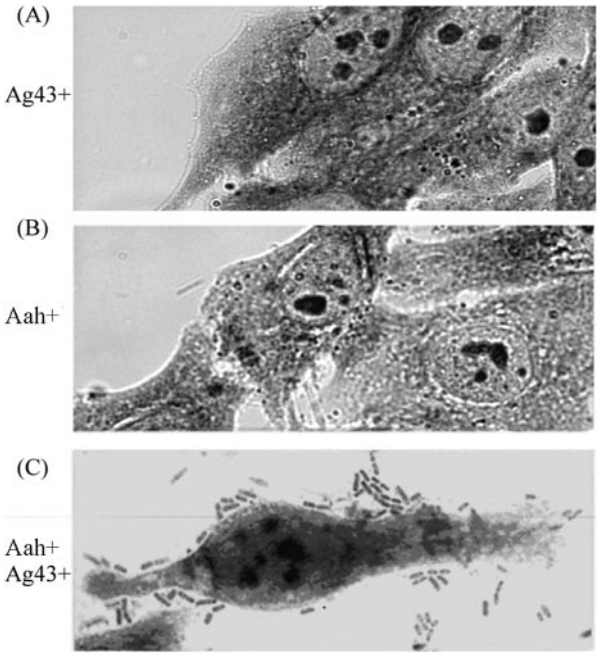


FIG. 7. Glycosylated Ag43 promotes adhesion to HEp-2 cells. Interaction of HEp-2 epithelial cell line cells with *E. coli* strains expressing nonglycosylated Ag43 (LH57, Ag43$^+$) (A), Aah heptosyl transferase (OS101, Aah$^+$) (B), or glycosylated Ag43 (OS64, Aah$^+$ Ag43$^+$) (C) is shown.

Another important phenotype associated with Ag43 is biofilm formation. Expression of Ag43 greatly enhances bacterial biofilm not only in *E. coli* but also in other gram-negative bacteria (22, 23, 24). The ability to form biofilms is a trait closely associated with bacterial persistence and virulence, and many persistent and chronic bacterial infections, including periodontitis, otitis media, biliary tract infections, and endocarditis, are now believed to be linked to the formation of biofilms (9, 13).

The observations presented in this study suggest that Ag43 can exist in a glycosylated version and that this form enhances bacterial attachment to human cells. In effect, in addition to its other virulence-related traits, the glycosylated form of Ag43 seems to be an adhesin that can mediate binding to human cells. Specific attachment to a host tissue is of paramount importance in bacterial pathogenesis, and adherence and subsequent colonization of a host tissue are generally considered to be key events in bacterial pathogenesis (reviewed in references 25 and 26). Specific adhesion provides bacteria with both the ability to select a target surface (tissue tropism) and the ability to resist removal in an environment subjected to hydrodynamic shear forces such as the urinary tract. This third novel virulence-related phenotype of Ag43 truly makes it a "molecular Swiss army knife." It is not clear whether addition of sugar residues to Ag43 somehow makes it able to bind to a molecular motif present on the surface of HEp-2 cells or whether the added sugar residues make it a target for lectins present on surface of the HEp-2 cells. In this context, we can add that addition of heptose monosaccharide to the buffer does not seem to affect Ag43-mediated cell attachment.

Both the Aah and TibC heptyltransferases readily accepted Ag43 as a substrate. These heptyltransferases exhibit significant homology to the WaaQ (formerly RfaQ) heptyltransferase involved in the biosynthesis of the *E. coli* LPS (46). For example, TibC exhibits 30% identity to WaaQ over a stretch of 180 amino acids (27). Like WaaQ, both Aah and TibC employ ADP-glycero-manno-heptopyranose as a precursor substrate (5). WaaQ appears to be a HepIII transferase that transfers HepIII to HepII in the LPS core (46). The nature of the glucosyl transferase that confers glycosylation of Ag43 in strain 536 is not clear. It would appear conceivable that there are variants of WaaQ that instead of or in addition to performing O glycosylation of another heptose sugar can do this to threonine or serine residues in selected proteins. In both the AIDA and TibA systems, the gene encoding the heptosyl transferase is found immediately adjacent to the autotransporter-encoding gene. This is not the case in the UTI536 strain, where investigation of the partially resolved genome sequence indicates that no heptosyl transferase is located anywhere near the *flu* genes.

Whereas the distant relatives of Ag43, viz., AIDA-I and TibA, are restricted to limited subsets of diarrheagenic strains, Ag43 is widespread among *E. coli* strains. Indeed, a survey among enteropathogenic and uropathogenic strains showed that 77% and 60%, respectively, of these were capable of Ag43 expression. Furthermore, the presence of more than one copy of the *flu* gene seems to be the rule rather than the exception (24, 34, 35, 43). We have recently characterized Ag43 variants that are unable to self-associate, and it seems conceivable that such proteins can be glycosylated, act as adhesins, and contribute to the pathogenicity and environmental fitness of the strains in question.

The region encompassing the N-terminal ~150 amino acid residues of the Ag43 passenger domain has been implicated in Ag43-Ag43 self-recognition, and the first 47 amino acid residues were found to be especially important (24). This region harbors peptide segments that provide the interaction required for self-association to take place. Decoration of the Ag43 passenger domain by addition of sugars apparently does not block this interaction, as shown in this work. Glycosylation of the passenger domain takes place at several different positions. In this context, it is noteworthy that none of the Ag43-derived

tryptic peptides that were found to be glycosylated originated from the N-terminal 70-amino-acid-residue segment and only one glycosylated peptide originated from region consisting of the first 120 amino acid residues (Fig. 4). It is interesting to speculate that the N-terminal region of the protein is kept free of glycosylation in order not to interfere with the self-recognition mechanism of Ag43.

A novel picture of the Ag43 autotransporter is emerging. Indeed, this molecule seems to be a highly versatile virulence factor fulfilling multiple potential roles in bacterial pathogenesis: (i) it is capable of mediating bacterial aggregation via intercellular self-recognition, (ii) it is a highly efficient initiator of biofilm formation, and (iii) it can exist as a glycoprotein and as such demonstrates an adhesive phenotype with affinity for human cells.

ACKNOWLEDGMENTS

We thank Birthe Jul Jorgensen (Technical University of Denmark) and Barbara Plaschke (Universität Würzburg) for expert technical assistance. We are grateful to Annika Lehmann (Universität Würzburg) for the construction of pAGN3 and pAGN5.

This work was supported by grants from the Danish Technical Research Council (26-02-0183) and the German Research Foundation (DFG, SFB479 TP A1).

REFERENCES

1. **Anderson, G. G., J. J. Palermo, J. D. Schilling, R. Roth, J. Heuser, and S. J. Hultgren.** 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science **301:**105–107.
2. **Bachmann, B.** 1996. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 2460–2488. *In* F. C. Neidhardt, R. Curtis III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.). *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
3. **Beloin, C., K. Michaelis, K. Lindner, P. Landini, J. Hacker, J.-M. Ghigo, and U. Dobrindt.** 2006. The transcriptional antiterminator RfaH represses biofilm formation in *Escherichia coli*. J. Bacteriol. **188:**1316–1331.
4. **Benz, I., and M. A. Schmidt.** 1989. Cloning and expression of an adhesin (AIDA-I) involved in diffuse adherence of enteropathogenic *Escherichia coli*. Infect. Immun. **57:**1506–1511.
5. **Benz, I., and M. A. Schmidt.** 2001. Glycosylation with heptose residues mediated by the *aah* gene product is essential for adherence of the AIDA adhesin. Mol. Microbiol. **40:**1403–1413.
6. **Benz, I., and M. A. Schmidt.** 2002. Never say never again: protein glycosylation in pathogenic bacteria. Mol. Microbiol. **45:**267–276.
7. **Berge, A., B.-M. Kihlberg, A. G. Sjöholm, and L. Björck.** 1997. Streptococcal protein H forms soluble complement activating complexes with IgG, but inhibits complement activation by IgG-coated targets. J. Biol. Chem. **272:**20774.
8. **Berger, H., J. Hacker, A. Juarez, C. Hughes, and W. Goebel.** 1982. Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in *Escherichia coli*. J. Bacteriol. **152:**1241–1247.
9. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Biofilms: a common cause of persistent infections. Science **284:**2137–2142.
10. **Danese, P. N., L. A. Pratt, S. L. Dove, and R. Kolter.** 2000. The outer membrane protein, Antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. Mol. Microbiol. **37:**424–432.
11. **Diderichsen, B.** 1980. *flu*, a metastable gene controlling surface properties of *Escherichia coli*. J. Bacteriol. **141:**858–867.
12. **Dobrindt, U., G. Blum-Oehler, G. Nagy, G. Schneider, A. Johann, G. Gottschalk, and J. Hacker.** 2002. Genetic structure and distribution of four pathogenicity islands [PAI I(536) to PAI IV(536)] of uropathogenic *Escherichia coli* strain 536. Infect. Immun. **70:**6365–6372.
13. **Donlan, R. M., and J. W. Costerton.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. **15:**167–193.
14. **Elsinghorst, E. A., and D. J. Kopecko.** 1992. Molecular cloning of epithelial cell invasion determinants from enterotoxigenic *Escherichia coli*. Infect. Immun. **60:**2409–2417.
15. **Frick, I.-M., M. Mörgelin, and L. Björck.** 2000. Virulent aggregates of *Streptococcus pyogenes* are generated by homophilic protein-protein interactions. Mol. Microbiol. **37:**1232–1247.
16. **Giron, J. A., A. G. Torres, and J. B. Kaper.** 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Mol. Microbiol. **44:**361–379.
17. **Hasman, H., T. Chakraborty, and P. Klemm.** 1999. Antigen-43-mediated autoaggregation of *Escherichia coli* is blocked by fimbriation. J. Bacteriol. **181:**4834–4841.
18. **Hasman, H., M. A. Schembri, and P. Klemm.** 2000. Antigen 43 and type 1 fimbriae determine colony morphology of *Escherichia coli* K-12. J. Bacteriol. **182:**1089–1095.
19. **Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez, and D. A. Aldeen.** 2004. Type V protein secretion pathway: the autotransporter story. Microbiol. Mol. Biol. Rev. **68:**692–744.
20. **Henderson, I. R., and P. Owen.** 1999. The major phase-variable outer membrane protein of *Escherichia coli* structurally resembles the immunoglobulin A1 protease class of exported protein and is regulated by a novel mechanism involving Dam and OxyR. J. Bacteriol. **181:**2132–2141.
21. **Kjaergaard, K., H. Hasman, M. A. Schembri, and P. Klemm.** 2002. Antigen 43-mediated autotransporter display, a versatile bacterial cell surface presentation system. J. Bacteriol. **184:**4197–4204.
22. **Kjaergaard, K., M. A. Schembri, H. Hasman, and P. Klemm.** 2000. Antigen 43 from *Escherichia coli* induces inter- and intraspecies cell aggregation and changes in colony morphology of *Pseudomonas fluorescens*. J. Bacteriol. **182:**4789–4796.
23. **Kjaergaard, K., M. A. Schembri, C. Ramos, S. Molin, and P. Klemm.** 2000. Antigen 43 facilitates formation of multispecies biofilms. Environ. Microbiol. **2:**695–702.
24. **Klemm, P., L. Hjerrild, M. Gjermansen, and M. A. Schembri.** 2004. Structure-function analysis of the self-recognizing Antigen 43 autotransporter protein from *Escherichia coli*. Mol. Microbiol. **51:**283–296.
25. **Klemm, P., and M. A. Schembri.** 2000. Bacterial adhesins: structure and function. Int. J. Med. Microbiol. **290:**27–35.
26. **Klemm, P., and M. A. Schembri.** 2004. Type 1 fimbriae, curli and antigen 43, adhesion, colonization and biofilm formation. Chapter 8.3.2.6. *In* R. Curtis III (editor in chief), *EcoSal—Escherichia coli and Salmonella*: cellular and molecular biology. [Online.] http://www.ecosal.org. ASM Press, Washington, D.C.
27. **Lindenthal, C., and E. A. Elsinghorst.** 1999. Identification of a glycoprotein produced by enterotoxigenic *Escherichia coli*. Infect. Immun. **67:**4084–4091.
28. **Lindenthal, C., and E. A. Elsinghorst.** 2001. Enterotoxigenic *Escherichia coli* TibA glycoprotein adheres to human intestine epithelial cells. Infect. Immun. **69:**52–57.
29. **Moore, G. E., R. E. Gerner, and H. A. Franklin.** 1967. Culture of normal human leucocytes. JAMA. **199:**519–524.
30. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
31. **Ochiai, K., T. Kurita-Ochiai, Y. Kamino, and T. Ikeda.** 1993. Effect of co-aggregation on the pathogenicity of oral bacteria. J. Med. Microbiol. **39:**183–190.
32. **Owen, P.** 1983. Antigens of the *Escherichia coli* cell envelope, p. 347–373. *In* O. J. Bjerrum (ed.), Electroimmununochemical analysis of membrane proteins. Elsevier Science Publishing, Inc., Amsterdam, The Netherlands.
33. **Owen, P.** 1992. The gram-negative outer membrane: structure, biochemistry and vaccine potential. Biochem. Soc. Trans. **20:**1–6.
34. **Owen, P., M. Meehan, H. de Loughry-Doherty, and I. Henderson.** 1996. Phase-variable outer membrane proteins in *Escherichia coli*. FEMS Immunol. Med. Microbiol. **16:**63–76.
35. **Roche, A. J., J. P. McFadden, and P. Owen.** 2001. Antigen 43, the major phase-variable protein of the *Escherichia coli* outer membrane, can exist as a family of proteins encoded by multiple alleles. Microbiology **147:**161–169.
36. **Schäffer, C., M. Graninger, and P. Messner.** 2001. Prokaryotic glycosylation. Proteomics **1:**248–261.
37. **Schembri, M. A., K. Kjaergaard, and P. Klemm.** 2003. Global gene expression in *Escherichia coli* biofilms. Mol. Microbiol. **48:**253–267.
38. **Sherlock, O., M. A. Schembri, A. Reisner, and P. Klemm.** 2004. Novel roles for the AIDA adhesin from diarrheagenic *Escherichia coli*: aggregation and biofilm formation. J. Bacteriol. **186:**8058–8065.
39. **Sherlock, O., R. M. Vejborg, and P. Klemm.** 2005. The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* is self recognizing and induces bacterial aggregation and biofilm formation. Infect. Immun. **73:**1954–1963.
40. **Stentebjerg-Olesen, B., L. Pallesen, L. B. Jensen, G. Christiansen, and P. Klemm.** 1997. Authentic display of a cholera toxin epitope by chimeric type 1 fimbriae: effects of insert position and host background. Microbiology **143:**2027–2038.
41. **Thibault, P., S. M. Logan, J. F. Kelly, J. R. Brisson, C. P. Ewing, T. J. Trust, and P. Guerry.** 2001. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. J. Biol. Chem. **276:**34862–34870.
42. **Thiede, B., W. Höenwarter, A. Krah, J. Mattow, M. Schmid, F. Schmidt, and P. R. Jungblut.** 2001. Peptide mass fingerprinting. Methods **35:**237–247.
43. **Torres, A. G., N. T. Perna, V. Burland, A. Ruknudin, F. R. Blattner, and J. B. Kaper.** 2002. Characterization of Cah, a calcium-binding and heat-

extractable autotransporter of enterohaemorrhagic *E. coli*. Mol. Microbiol. **45:**951–966.

44. **Virji, M.** 1997. Post-translational modifications of meningococcal pili, identification of common substituents: glycans and α-glycerophosphate—a review. Gene **192:**141–147.
45. **Virji, M., J. R. Saunders, G. Sims, K. Makepeace, D. Maskell, and D. J. P. Ferguson.** 1993. Pilus-faciliated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in amino acid sequence and the glycosylation status of pilin. Mol. Microbiol. **10:**1013–1028.
46. **Yethon, J. A., D. E. Hendrichs, M. A. Monteiro, M. B. Perry, and C. Whitfields.** 1998. Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. J. Biol. Chem. **273:**26310–26316.
47. **Zhu, J., and J. J. Mekalanos.** 2001. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. Dev. Cell **5:**647–656.