Characterization of Saa, a Novel Autoagglutinating Adhesin Produced by Locus of Enterocyte Effacement-Negative Shiga-Toxigenic *Escherichia coli* Strains That Are Virulent for Humans

ADRIENNE W. PATON, POTJANEE SRIMANOTE, MATTHEW C. WOODROW, AND JAMES C. PATON*

Department of Molecular Biosciences, Adelaide University, Adelaide, South Australia 5005, Australia

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The capacity of Shiga toxigenic *Escherichia coli* (STEC) to adhere to the intestinal mucosa undoubtedly contributes to pathogenesis of human disease. The majority of STEC strains isolated from severe cases produce attaching and effacing lesions on the intestinal mucosa, a property mediated by the locus of enterocyte effacement (LEE) pathogenicity island. This element is not essential for pathogenesis, as some cases of severe disease, including hemolytic uremic syndrome (HUS), are caused by LEE-negative STEC strains, but the mechanism whereby these adhere to the intestinal mucosa is not understood. We have isolated a gene from the megaplasmid of a LEE-negative O113:H21 STEC strain (98NK2) responsible for an outbreak of HUS, which encodes an auto-agglutinating adhesin designated Saa (STEC autoagglutinating adhesin). Introduction of *saa* cloned in pBC results in a 9.7-fold increase in adherence of *E. coli* JM109 to HEp-2 cells and a semilocalized adherence pattern. Mutagenesis of *saa* in 98NK2, or curing the wild-type strain of its megaplasmid, resulted in a significant reduction in adherence. Homologues of *saa* were found in several unrelated LEE-negative STEC serotypes, including O48:H21 (strain 94CR) and O91:H21 (strain B2F1), which were also isolated from patients with HUS. Saa exhibits a low degree of similarity (25% amino acid [aa] identity) with YadA of *Yersinia enterocolitica* and Eib, a recently described phage-encoded immunoglobulin binding protein from *E. coli*. Saa produced by 98NK2 is 516 aa long and includes four copies of a 37-aa direct repeat sequence. Interestingly, Saa produced by other STEC strains ranges in size from 460 to 534 aa as a consequence of variation in the number of repeats and/or other insertions or deletions immediately proximal to the repeat domain.

Shiga toxigenic *Escherichia coli* (STEC) is an important cause of gastrointestinal disease in humans, particularly since such infections may result in life-threatening sequelae, such as the hemolytic uremic syndrome (HUS) (13, 20, 28). It has been recognized for a number of years that STEC strains causing human disease may belong to a very broad range of O serogroups. However, a subset of these (notably O157 and O111) appear to be responsible for the majority of serious cases (those complicated by HUS) (13, 28). These STEC have the capacity to produce attaching and effacing (A/E) lesions on intestinal mucosa, a property mediated by the locus of enterocyte effacement (LEE) pathogenicity island. The presence of LEE is not essential for pathogenesis, as a minority of sporadic cases of HUS are caused by LEE-negative STEC strains. These cases are frequently reported to be at the severe extreme of the clinical spectrum. Nevertheless, until 1998, virtually all recorded outbreaks of STEC disease complicated by HUS had been caused by LEE-positive STEC belonging to serogroups O157 or O111 (28). The only exception to this was an outbreak of HUS in a German child care center caused by a Shiga toxigenic strain of *Citrobacter freundii* (39). In April 1998, there was a small outbreak involving three cases of HUS in South Australia, Australia. Culture, PCR, and serological analyses indicated that a LEE-negative O113:H21 STEC strain was responsible (26). This is one of the more common LEE-negative STEC serotypes associated with human disease (13, 28).

Indeed, O113:H21 was among the first STEC serotypes to be causally linked to cases of HUS by Karmali et al. (14).

Although Stx is a *sine qua non* of virulence, adherence to the intestinal epithelium and colonization of the gut by the STEC is also an important component of the pathogenic process. The mechanism whereby LEE-positive strains adhere intimately and generate A/E lesions has been the subject of intensive study in recent years (for review, see reference 20). A/E lesions are characterized phenotypically by localized destruction of the apical microvilli of intestinal epithelial cells resulting from rearrangement of the cytoskeleton and intimate attachment of bacteria to the enterocyte surface. All of the genes required for this are located on LEE, which encodes a type III secretion system, several secreted effector proteins (Esp), the outer membrane protein intimin, and a receptor (Tir) which is translocated into the host cell membrane. Ebel et al. (8) have shown that during lesion formation, initial binding of STEC to host cells is mediated by filamentous structures, of which EspA is a major component. EspD is essential for the formation of these EspA filaments (17), which in turn are required for the translocation of EspB and Tir into epithelial cells and for the activation of epithelial signal transduction (15, 16, 36). It has been proposed that while EspA appendages mediate the initial interaction of STEC with host cells, this is later replaced by the intimate attachment mediated by intimin and Tir (8).

Two other recent studies have described additional putative adhesins from LEE-positive STEC. Tarr et al. (35) have described a chromosomally encoded 67-kDa homologue of *Vibrio cholerae* IrgA, termed Iha, which mediated adherence of O157:H7 STEC to HeLa cells. Also, Nicholls et al. (21) have described a very large (365-kDa) adhesin, termed Efa1, which

* Corresponding author. Mailing address: Department of Molecular Biosciences, Adelaide University, Adelaide, S.A. 5005, Australia. Phone: 61-8-83035929. Fax: 61-8-83033262. E-mail: james.paton@adelaide.edu.au.

mediated adherence to CHO (Chinese hamster ovary) cells. However, although adherence phenotypes have been examined for several LEE-negative STEC strains (5, 7, 31), virtually nothing is known of the actual mechanisms involved. In a previous study, we showed that the capacity of the O113:H21 STEC responsible for the South Australian HUS outbreak to adhere to Henle 407 and HEp-2 cells was similar to that of LEE-positive STEC reference strains (26). In the present study, we describe a novel autoagglutinating adhesin isolated from the outbreak strain, the first adhesin to be characterized from a LEE-negative STEC.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. The O113:H21 STEC strain 98NK2 was isolated from a patient with HUS at the Women's and Children's Hospital, South Australia, Australia, as previously described (26). All other STEC strains used in this study, as well as the O111 enteropathogenic *E. coli* (EPEC) strain 87A, were also isolated at the Women's and Children's Hospital and have been described previously (27), except for the O157:H7 strain EDL933 (provided by R. Robins-Browne), the O91:H21 strain B2F1 (provided by A. Melton-Celsa), and the O113 strains 3848 and 1183 (provided by J. Bennett). *E. coli* K-12 strains DH1 and JM109 have been described previously (10, 41). The cosmid vector pHC79 has also been described previously (11). The phagemids pBluescript SK (encoding ampicillin resistance) and pBC SK (encoding chloramphenicol resistance) were obtained from Stratagene, La Jolla, Calif. All cells of *E. coli* strains were routinely grown in Luria-Bertani (LB) medium (19) with or without 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.). Where appropriate, ampicillin or chloramphenicol was added to growth media at concentrations of 50 and 40 µg/ml, respectively.

HEp-2 adherence assay. HEp-2 adherence assays were carried out essentially as described previously for Henle 407 cells (27). Briefly, *E. coli* cells were grown overnight at 37°C in LB broth and diluted to a density of 10⁴ CFU/ml (confirmed by viable count) in Dulbecco's modified Eagle's medium (DMEM), buffered with 20 mM HEPES and supplemented with 10% fetal calf serum and 2 mM L-glutamine. Washed HEp-2 monolayers in 24-well tissue culture plates were then infected with 1-ml aliquots of bacterial suspension. After incubation at 37°C for 3 h, the culture medium was removed and the monolayers were washed four times with phosphate-buffered saline (PBS) to remove nonadherent bacteria. The cell monolayers were then detached from the plate by treatment with 100 µl of 0.25% trypsin–0.02% EDTA. Cells were then lysed by addition of 400 µl of 0.025% Triton X-100, and 50-µl aliquots (and serial 10-fold dilutions thereof) were plated on LB agar to determine the total number of adherent bacteria per well. Assays were carried out in quadruplicate, and results were expressed as the mean (± standard error) CFU/well. The significance of differences in adherence was analyzed using Student's unpaired *t* test (two-tailed).

For Giemsa staining, HEp-2 cells were grown on 1.0-cm-diameter coverslips in 24-well tissue culture trays and were used when approximately 50% confluent. Wells were infected with approximately 10⁶ CFU of bacteria suspended in 1 ml of tissue culture medium and incubated at 37°C for 3 h. Monolayers were then washed four times in PBS, fixed with 70% methanol, and stained with Giemsa stain. After washing, the coverslips were mounted on glass slides and examined by light microscopy.

DNA manipulations. Routine DNA manipulations (restriction digestion, agarose gel electrophoresis, ligation, transformation, Southern hybridization analysis, etc.) were carried out essentially as described by Maniatis et al. (19).

Construction of cosmid gene bank. Construction of the cosmid gene bank of 98NK2 DNA has been described previously (24). Briefly, high-molecular-weight chromosomal DNA was extracted as described previously (25) from STEC 98NK2 and was digested partially with *Sau3A1* so as to optimize the yield of fragments in the size range of 35 to 40 kb. This DNA was ligated with a fivefold molar excess of pHC79 DNA, which had been digested with *Bam*HI. Ligated DNA was packaged into lambda heads by using a Packagene kit (Promega Biotec, Madison, Wis.) and was transfected into *E. coli* DH1 cells which had been grown in LB broth–2% maltose. The cells were then plated onto LB agar supplemented with ampicillin, and after incubation, clones were stored in LB broth–ampicillin–15% glycerol in microtiter plates at –70°C.

DNA sequencing. For DNA sequencing, plasmid DNA template was purified using a QIAprep Spin miniprep kit (Qiagen, Hilden, Germany); alternatively, PCR products were purified using the Bresapin PCR purification kit (Bresagen, Adelaide, Australia). The sequences of both strands were then determined using dye-

terminator chemistry and either universal M13 sequencing primers or custom-made oligonucleotide primers on an Applied Biosystems model 377 automated DNA sequencer. The sequences were analysed using DNASIS and PROSIS Version 7.0 software (Hitachi Software Engineering, South San Francisco, Calif.).

Isolation of megaplasmid-cured derivative of 98NK2. 98NK2 was cured of its megaplasmid by direct electrotransformation with a temperature-sensitive suicide plasmid pCACTUS, which encodes chloramphenicol resistance and carries *sacB* (40). Transformants were selected by plating on minimal agar containing 25 µg of chloramphenicol/ml at 30°C and were subcultured three times in LB broth containing 25 µg of chloramphenicol/ml at 30°C. Serial dilutions were then plated on LB agar and incubated at 42°C, and single colonies were then replated on LB agar supplemented with 6% sucrose and incubated at 42°C. Loss of the megaplasmid from the 98NK2 derivative (designated 98NK2-Cu) was confirmed by the absence of a product after PCR using *ehx4*-specific primers (23) and the absence of high-molecular-weight plasmid DNA in ethidium bromide-stained agarose gels of genomic DNA extracts; loss of pCACTUS was also confirmed by the absence of a product after PCR using pCACTUS-specific primers.

PCR amplification of *saa*. The complete *saa* genes from various STEC strains were amplified by PCR using an Expand High Fidelity PCR kit (Roche Molecular Diagnostics) and primers SaaF (5'-CCTCATCTTCTGCAAATACC-3') and SaaR (5'-GTTGTCCTGCAGATTTTACCATCCAATGGACATG-3'). These primers directed amplification of the region from nucleotides (nt) 1107 to 2795 of the insert of pJCP562, which incorporates the complete *saa* open reading frame as well as upstream sequences containing the putative promoter and ribosome binding site. When required, PCR products were purified and blunt cloned into the *Sma*I site of pBluescript SK.

Construction of *saa*-negative derivative of 98NK2. To generate a *saa*-negative derivative of 98NK2, the 3,758-bp 98NK2 DNA insert was excised from pJCP562 with *Pst*I, cloned into pCACTUS, and electroporated into *E. coli* DH5α cells. Transformants were selected by plating on LB agar containing 25 µg of chloramphenicol/ml at 30°C. Inverse PCR was then used to generate a deletion derivative of this construct lacking 1,402 bp from the *saa* open reading frame (equivalent to nt 1298 to 2699 of the insert of pJCP562). The primers used were 5'-CCGTAGATCTGCTGCATGAGTACTCGTTGCC-3', which incorporates a *Bgl*II site (underlined), and 5'-TCCCGTAGATCTAACACCGTCGTCAGACTAAACACC-3', which also contains a *Bgl*II site (underlined). PCR was performed using the Expand long template PCR system (Roche) and plasmid DNA template; the product was digested with *Bgl*II, recircularized with T4 ligase, and electroporated into *E. coli* DH5α cells. The plasmid containing the deleted *saa* operon was then extracted and linearized with *Bgl*II, end-filled with Klenow fragment, and blunt-ligated with a 1,252-bp *Hind*III fragment from pUC4K (Roche), which contains a functional kanamycin resistance gene. Sequence analysis of the resultant construct (designated pΔ*saa*::kan) confirmed deletion of the major proportion of *saa* and insertion of the kanamycin cartridge. This construct was then electroporated into 98NK2 cells, and cells were plated on LB agar supplemented with 25 µg of chloramphenicol/ml and 50 µg of kanamycin/ml at 30°C. A randomly selected transformant was grown in LB broth–kanamycin–chloramphenicol at 30°C overnight, and serial dilutions in PBS were plated on LB agar–kanamycin and grown at 42°C (which is nonpermissive for pCACTUS derivatives) to select for cointegrants. Ten randomly selected colonies were then grown overnight at 37°C in LB broth–kanamycin, and serial dilutions were grown at 30°C on LB agar without NaCl, supplemented with 6% sucrose and kanamycin, in order to isolate recombinants lacking pCACTUS-derived *sacB*.

Purification of Saa and preparation of antiserum. Purification of Saa was carried out using a QIAexpress kit (Qiagen). In order to construct an N-terminal His₆-Saa fusion protein, the region of the insert of pJCP562 encoding amino acids 29 to 516 was amplified by high-fidelity PCR using primers SaaH6F (5'-CATAATGGATCCACTTATGAAAGCCTTGAAAAAGGG-3') and SaaR. The two primers incorporate *Bam*HI and *Pst*I sites near their respective termini, enabling the resultant product to be cloned between the *Bam*HI and *Pst*I sites of pQE30 (Qiagen). Correct insertion of the *saa* fragment was confirmed by sequence analysis. The recombinant plasmid was then transformed into the expression host *E. coli* SG13009[pREP4]. For purification of His₆-Saa, transformants were grown in 1 liter of LB broth supplemented with 50 µg of ampicillin/ml, and when the culture reached an *A*₆₀₀ of 0.5, the culture was induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated for a further 3 h. Cells were harvested by centrifugation, resuspended in 24 ml of buffer A (6 M guanidine-HCl, 0.1 M NaH₂PO₄, 10 mM Tris, pH 8.0), and stirred at room temperature for 1 h. Cell debris was removed by centrifugation at 10,000 × *g* for 25 min at 4°C. The supernatant was then loaded (at a rate of 15 ml/h) onto a 2-ml column of Ni-nitrilotriacetic acid resin (ProBond; Invitrogen), which had been pre-equilibrated with 20 ml of buffer A supplemented with 0.5 M NaCl and 15 mM imidazole. The column was then washed with 40 ml of buffer A, followed by

FIG. 1. Adherence of *E. coli* strains to HEp-2 cells (Giemsa stain). (A) STEC strain 98NK2; (B) *E. coli* DH1:pHC79; (C) *E. coli* DH1:pJCP561; (D) *E. coli* JM109:pJCP562.

20 ml of buffer B (8 M urea, 0.1 M NaH_2PO_4 , 10 mM Tris, pH 8.0) and then 16 ml of buffer C (8 M urea, 0.1 M NaH_2PO_4 , 10 mM Tris, pH 6.3), which was supplemented with 0.25 M NaCl and 5 mM imidazole. Saa was then eluted with a 30-ml gradient of 0 to 500 mM imidazole in buffer C; 3-ml fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Peak fractions were pooled, and the denatured Saa protein was refolded by dialysis against 100 ml of buffer B to which 1 liter of PBS was added dropwise at a rate of 60 ml/h. This was followed by dialysis against two changes of PBS. The total yield of purified Saa was approximately 5 mg, which was >95% pure, as judged by SDS-PAGE after staining with Coomassie brilliant blue R250.

Six BALB/c mice were then immunized by intraperitoneal injection of three 10- μg doses of purified Saa in 0.2 ml of PBS containing 5 μg of alum adjuvant (Imjectalum; Pierce, Rockford, Ill.) at 2-week intervals. Mice were exsanguinated by cardiac puncture 1 week after the third immunization, and pooled immune serum was stored in aliquots at -15°C .

Western blot analysis. Crude lysates of STEC or other *E. coli* strains were separated by SDS-PAGE as described by Laemmli (18), and antigens were electrophoretically transferred onto nitrocellulose filters as described by Towbin et al. (38). Filters were probed with mouse anti-Saa antibody (used at a dilution of 1:5,000), followed by goat anti-mouse immunoglobulin G (IgG) conjugated to alkaline phosphatase (Bio-Rad Laboratories). Labeled bands were visualized using a chromogenic nitroblue tetrazolium/X-phosphate substrate system (Roche Molecular Diagnostics).

Immunogold electron microscopy. *E. coli* cells suspended in PBS were applied to poly-L-lysine-coated grids for 5 min, washed twice in PBS-bovine serum albumin (BSA), and reacted with polyclonal anti-Saa antibody diluted 1:20 in PBS-BSA for 15 min. Grids were then washed twice with PBS-BSA and reacted with protein G-20-nm gold conjugate (ICN Biomedicals, Aurora, Ohio) diluted 1:40

in PBS-BSA for 15 min. The grids were then washed twice in PBS and examined without further staining using a Philips CM100 electron microscope at 80 kV.

Nucleotide sequence accession number. The DNA sequence described in this paper has been deposited with GenBank under accession number AF325220.

RESULTS

Adherence phenotype of O113:H21 STEC strain 98NK2 and isolation of adherent clones. As a first step, the adherence properties of 98NK2 to HEp-2 cells were examined using a quantitative adherence assay and Giemsa staining as described in Materials and Methods. Total adherence after 3 h of incubation was (mean $\pm$ standard error) $1.16 (\pm 0.13) \times 10^5$ CFU per well, and Giemsa staining indicated large clusters of adherent bacteria on the epithelial cell surface (Fig. 1A). We describe this adherence pattern as “semilocalized” to distinguish it from the “localized” adherence phenotype associated with LEE-positive enteropathogenic *E. coli* (EPEC) strains, which is characterized by tightly bound bacterial microcolonies on the cell surface. Macroscopically, 98NK2 cells autoagglutinated when grown at 37°C for 16 h in DMEM, a property coincidentally also observed for the EPEC strain 87A.

To eliminate the possibility that the adherence mechanism of 98NK2 was related to that for EPEC strains, restricted genomic DNA from 98NK2 was subjected to low-stringency

FIG. 2. Map of the 98NK2 DNA insert of pJCP562 showing the location of the *saa* open reading frame (thick arrow) and its putative promoter (*p*) and transcription termination site (*t*). The regions with DNA sequence homology with portions of IS3 elements and pO157 (*P*) are shown beneath the map.

Southern hybridization analysis using a digoxigenin-labeled probe specific for *bfpA*, which encodes the EPEC bundle-forming pilus (32, 33). A strong hybridization signal was obtained for digests of DNA from EPEC strain 87A, but no probe-reactive fragments were detected for 98NK2 (result not shown). 98NK2 DNA did, however, react with probes specific for the *irgA*-related gene *iha*, and a library of 98NK2 DNA constructed in *E. coli* DH1 cells by using cosmid pHCT9 (see Materials and Methods) was screened for clones reacting with this probe. One of these (designated pJCP561) was selected for further analysis.

The adherence of *E. coli* DH1:pJCP561 to HEP-2 cells in a standard 3-h assay was (mean $\pm$ standard error) $6.9 (\pm 0.89) \times 10^4$ CFU per well: this was significantly greater than that for DH1:pHCT9, which was $1.15 (\pm 0.14) \times 10^4$ CFU per well ($P < 0.001$). Furthermore, examination of Giemsa-stained infected HEP-2 cells indicated that pJCP561 but not pHCT9 conferred a semilocalized adherence phenotype upon DH1 similar to that seen for 98NK2 (Fig. 1B and C). A series of random subclones of pJCP561 were then constructed in pBC. One of these (designated pJCP562) contained a 3.8-kb *PstI* fragment and conferred semilocalized adherence upon *E. coli* JM109, as judged by Giemsa staining (Fig. 1D). In the quantitative HEP-2 adherence assay, JM109:pJCP562 exhibited a total adherence of $5.58 (\pm 0.26) \times 10^4$ CFU per well. This was significantly greater than that for JM109:pBC, which was $5.75 (\pm 0.63) \times 10^3$ CFU per well ($P < 0.001$). Both DH1:pJCP561 and JM109:pJCP562, but not JM109:pBC, autoagglutinated when grown in DMEM.

Sequence analysis of pJCP562. Sequence analysis indicated that the 98NK2 DNA insert in pJCP562 was 3,758 nt long and did not contain the *irgA/iha*-related gene. It contained only one intact open reading frame (nt 1230 to 2780) sufficient to encode a 516-aa protein. As shown in Fig. 2, the insert was flanked by two oppositely oriented incomplete copies of an IS3 element (nt 1 to 885 and 3183 to 3758); indeed, the last 576 nt of the insert is 99% identical to an inverted repeat of the first 576 nt. A 140-bp region from nt 890 to 1030 was 87% identical to nt 22485 to 22627 (a noncoding region) of pO157, the large virulence plasmid of O157:H7 STEC strain EDL933 (2), but the remainder of the pJCP562 insert had no homology with any pO157 sequences. The open reading frame which we have designated *saa* (STEC autoagglutinating adhesin) is preceded by a strong ribosome binding site (the sequence of nt 1216 to 1223 is AGGAAGAA). The NNPP program (29) (available at <http://dot.imgen.bcm.tmc.edu>) predicted that within the entire plus strand of the insert of pJCP562, the region most likely to be a promoter sequence was immediately upstream of the ri-

bosome binding site for *saa* (nt 1156 to 1201); transcription was predicted to start at T₁₁₉₆. Immediately downstream of *saa* (nt 2980 to 3003) is a potential stem-loop element ($\Delta G = -15.2$ kcal/mol) which may function as a transcription terminator.

BLASTP analysis (1) indicated that the first 155 aa (30%) of the 516-aa Saa protein had no similarities with any known protein. However, a low but significant degree of sequence similarity was detected between the distal 70% of Saa and YadA, a plasmid-encoded outer membrane protein of *Yersinia enterocolitica* implicated in epithelial cell adherence and invasion (34), as well as a recently described family of phage-encoded immunoglobulin-binding proteins (Eib) of *E. coli* (30). Amino acids 155 to 516 of Saa exhibited 24% identity and 40% similarity with aa 53 to 455 of YadA, albeit with 13% gaps; aa 187 to 500 of Saa exhibited 27% identity and 42% similarity to EibD, with 22% gaps (slightly lesser similarities were seen between Saa and EibC or EibE). The N terminus of Saa comprises charged residues followed by a hydrophobic domain typical of signal peptides, and the program SignalP V1.1 (22) predicted a signal peptidase cleavage site between two alanine residues (A₂₂ and A₂₃). Interestingly, the C-terminal half of Saa comprises four complete copies and one partial copy of a 37-aa repeated element (LQHEATARIEGDAQTLKSANTYTNKQVNALEQNTNQQ) (covering the region from aa 266 to 429). There is absolute sequence identity between the various copies of the repeat unit at both the DNA and amino acid levels. This region of Saa is strongly helical and has a high probability of forming coiled-coil structures, as predicted by the program COILS (available at <http://www.ch.embnet.org>) with a 14-aa window setting. The latter half of each 37-aa repeat unit and a stretch of 15 residues (aa 430 to 444) immediately following the repeat domain had the highest coiled-coil potential. The extreme C-terminal portion of Saa is also hydrophobic and so may represent a membrane anchorage domain.

Presence of *saa* in other STEC strains. Southern hybridization analysis was used to determine the presence of *saa*-related genes in other *E. coli* strains, including 32 STEC isolates which had previously been tested for presence of the LEE pathogenicity island and the plasmid-carried enterohemolysin gene *ehxA* (also referred to as EHEC-*hlyA*) (27) (Table 1). No hybridization signal was obtained at either high or low stringency with the *E. coli* K-12 strain C600 or C600 lysogenized with the Stx2-converting bacteriophage 933W. Neither was any signal obtained with the EPEC strain 87A or with any of the LEE-positive STEC strains tested, including representatives belonging to serotypes O157:H7, O157:H⁻, O111:H⁻, and O26. However, high-stringency *saa* homologues were present in 19 of the 26 LEE-negative STEC strains. All of these were

TABLE 1. Presence of *saa* homologues in various *E. coli* strains

<i>E. coli</i> strain	Serotype	Source ^a	Presence of <i>saa</i> ^b	Presence of LEE ^c	Presence of <i>ehxA</i> ^c
98NK2	O113:H21	HUS	+	—	+
C600			—	—	—
C600:933W			—	—	—
EPEC 87A	O111	D	—	+	—
EDL933	O157:H7	BD	—	+	+
96GR1	O157:H ⁺	HUS	—	+	+
95ZG1	O26	BD	—	+	+
96RO1	O111:H ⁺	HUS	—	+	+
95NR1	O111:H ⁺	HUS	—	+	+
99BG1	O111:H ⁺	BD	—	+	+
97MW1	O113:H21	BD MHA/T	+	—	+
3848	O113:H21	HUS	+	—	+
1183	O113:H21	HUS	+	—	+
MW10	O113:H21	Food	+	—	+
94CR	O48:H21	HUS	+	—	+
99AM1	O23	BD	+	—	+
MW5	Ont ^d	Food	+	—	+
00MC2	O6	BD	+	—	+
B2F1	O91:H21	HUS	+	—	+
MW13	O98	Food	+	—	+
MW2	Ont	Food	+	—	+
MW9	OR ^d	Food	+	—	+
MW14	ND ^d	Food	+	—	+
MW8	Ont	Food	+	—	+
MW6	OR	Food	+	—	+
MW3	O82:H8	Food	+	—	+
MW7	Ont:H11	Food	+	—	+
95HE4	O91	D	+	—	+
MW15	O141	Food	—	—	—
MW4	Ont	Food	—	—	—
95MV1	OR:H9	HUS	—	—	—
MW11	ND	Food	—	—	—
95AS1	O128	D	—	—	—
031	OX3:H21	SIDS	—	—	—
MW12	O159	Food	—	—	—

^a STEC strains were isolated either from foods or from the feces of patients with uncomplicated diarrhea (D), bloody diarrhea (BD), microangiopathic hemolytic anemia and thrombocytopenia (MHA/T), hemolytic uremic syndrome (HUS), or sudden infant death syndrome (SIDS).

^b Determined by Southern hybridization analysis using a digoxigenin-labeled PCR product corresponding to the complete *saa* open reading frame as probe.

^c Determined by PCR for *eae* and *ehxA* (23, 27).

^d Ont, O non-typable; OR, O rough; ND, serotype not determined.

also positive for *ehxA*. Homologues of *saa* were not present in any of the seven LEE-negative STEC strains which were negative for *ehxA*. Thus, it appears that *saa* is only present in LEE-negative STEC strains that also carry the plasmid-carried *ehxA*, implying that *saa* is located on the respective large virulence plasmid. This was confirmed by PCR analysis of DNA extracted from 98NK2 and a derivative (designated 98NK2-Cu) which had been cured of its megaplasmid (see Materials and Methods), using *saa*- and *ehxA*-specific primers. PCR was also used to test additional LEE-positive STEC isolates (three O157, five O111, and three O26 strains), all of which were positive for *ehxA* but negative for *saa* (results not shown).

As a further test of the contribution of *saa* to adherence of STEC, in vitro adherence of 98NK2 to HEp-2 cells was compared with that of 98NK2-Cu; total adherence after a 3-h incubation was (mean $\pm$ standard error) $9.87 (\pm 0.82) \times 10^4$ and $4.66 (\pm 0.47) \times 10^4$ CFU/well, respectively ($P < 0.01$). Geimsa staining also indicated that 98NK2-Cu displayed a more diffuse adherence pattern (results not shown).

Size heterogeneity of *saa*. The complete *saa* genes were amplified by PCR from genomic DNA extracted from STEC

strains 94CR, MW10, MW5, 97MW1, and B2F1, using primers SaaF and SaaR, as described in Materials and Methods. Interestingly, the various PCR products differed in length. Those obtained using 94CR and 97MW1 DNA templates were slightly larger (approximately 1,720 bp) than the 98NK2 PCR product (1,689 bp), whereas the PCR products from the MW10, MW5, and B2F1 templates were smaller (approximately 1,630, 1,500, and 1,500 bp, respectively) (results not shown). This was confirmed by direct sequence analysis of the various amplicons, which demonstrated that the products of the *saa* genes from 94CR and 97MW1 were 534 aa, compared with 516 aa for 98NK2. On the other hand, *saa* from MW10 encoded a 497-aa product, while *saa* from MW5 and B2F1 encoded 460-aa products. The alignment of these sequences (Fig. 3) indicates that the size differences result from variations in the number of 37-aa repeat units, as well as deletions and insertions immediately upstream of this domain. However, apart from these differences, the Saa proteins were highly conserved amongst the various strains.

The in vitro adherence of these *saa*-positive STEC strains was then compared using the quantitative HEp-2 assay (Fig. 4). The three strains whose Saa proteins contained four copies of the repeat unit (98NK2, 94CR, and 97MW1) did not differ significantly in adherence. However, MW5 and B2F1, whose Saa proteins contain only two copies of the repeat unit, exhibited significantly reduced adherence (41 and 27%, respectively) relative to that for 98NK2 (P values of <0.01 and <0.001 , respectively). Adherence of MW10, whose Saa protein contains three copies of the repeat unit, exhibited intermediate adherence (73% of that for 98NK2).

Expression of *saa*. The weaker adherence of MW10, MW5, and B2F1 could be directly attributable to the shorter predicted Saa proteins or, alternatively, to poor expression of *saa* in these strains. To examine this, Saa from 98NK2 was purified as a His₆-tagged fusion protein and was used to raise a polyclonal mouse antiserum as described in Materials and Methods. This antiserum was used for Western immunoblot analysis of lysates from various wild-type STEC strains grown in LB broth at 37°C (Fig. 5). Immunoreactive bands of the expected size (approximately 54 to 56 kDa) were seen in lysates of the fully adherent STEC strains 98NK2, 97MW1, and 94CR. Lysates of the STEC strains with shorter *saa* genes also contained immunoreactive species, the apparent sizes of which (approximately 50 kDa for MW10 and 45 to 47 kDa for MW5 and B2F1) were essentially consistent with their respective gene sequences. However, there was a slight difference between the apparent SDS-PAGE mobility of Saa from B2F1 and MW5, even though their deduced amino acid sequences are the same length. There are a total of nine predicted amino acid differences between the two proteins. Three of these are in the signal peptide and one is at position +1 of the mature protein, raising the possibility of defective cleavage by the signal peptidase. However, the SignalP program predicts that the signal peptide of Saa from B2F1 will still be cleaved at the same position, albeit with a lower probability score. Two of the five other amino acid differences are K/Q substitutions; thus, the resultant difference in net charge could affect the SDS-PAGE mobility of the respective proteins. The overall level of the expression of *saa* cannot account for the difference in adherence between the various STEC strains, as the intensity of the

FIG. 5. Western blot analysis of STEC strains. STEC strains were grown in LB broth at 37°C, and lysates were separated by SDS-PAGE, blotted, and probed with anti-Saa antibody as described in Materials and Methods. The mobilities of protein size markers (in kilodaltons) are also shown.

rivatives carrying PCR-amplified *saa* genes from 98NK2, MW10, or MW5 cloned in pBluescript (designated pJCP563, pJCP564, and pJCP565, respectively) (Fig. 7A). In all three clones, the *saa* gene was oriented in the opposite direction of the vector *lac* promoter. The lysate of JM109:pJCP563 contained a strongly immunoreactive band which comigrated with Saa purified

from 98NK2. JM109:pJCP564 and JM109:pJCP565 expressed slightly smaller immunoreactive proteins, as predicted from their respective DNA sequences. The JM109:pJCP563 and JM109:pJCP564 lysates both contained smaller immunoreactive species which may reflect proteolytic degradation of the overexpressed proteins. The JM109:pJCP563 lysate also contained a small amount of a much larger immunoreactive species. Separate Western blot analysis indicated that JM109:pJCP563 lysates which had not been boiled prior to SDS-PAGE contained much larger amounts of high-molecular-weight anti-Saa-reactive material, with an apparent size of >180 kDa (Fig 7B). Boiled lysates contained relatively more Saa monomer (56 kDa), as well as traces of an immunoreactive species with a size of 112 kDa, which might be a Saa dimer. Interestingly, all the *saa*-expressing clones exhibited a semilocalized adherence pattern on HEp-2 cells, as judged by Giemsa staining (Fig. 8), and (unlike JM109:pBluescript) autoagglutinated when grown in DMEM.

Effect of Saa on serum resistance. Given that the two proteins most closely related to Saa (YadA and Eib) both confer serum resistance, aliquots of 98NK2, 98NK2-Cu, JM109:pJCP562, and JM109:pBC were tested for susceptibility to the bactericidal effects of fresh human serum (or heat-inactivated serum) as described by El Tahir et al. (9). However, no differ-

FIG. 6. Electron micrographs of STEC strains after staining with anti-Saa and gold-labeled (20 nm) protein M conjugate. Bar, 0.5 μm.

FIG. 7. Western blot analysis of *E. coli* JM109 expressing cloned *saa* genes. (A) Lysates of JM109 carrying the indicated plasmids, or purified Saa, were separated by SDS-PAGE, blotted, and probed with anti-Saa antibody as described in Materials and Methods. The mobilities of protein size markers (in kilodaltons) are also shown. (B) A fresh lysate of *E. coli* JM109:pJCP563 was subjected to SDS-PAGE with or without boiling, blotted, and probed with anti-Saa antibody. The apparent size of immunoreactive species (arrows) was calculated with reference to the mobility of size markers.

ences in resistance were observed between the Saa-expressing strains and their respective Saa-negative controls (results not presented).

Construction and analysis of *saa*-negative 98NK2. A derivative of 98NK2 in which a 1,402-bp internal portion of *saa* was deleted and replaced with a kanamycin resistance cartridge was constructed by allelic exchange using the suicide vector pCACTUS, as described in Materials and Methods. Mutagenesis of the *saa* operon in the mutant (designated 98NK2-S) was confirmed by PCR amplification using primers SaaF and SaaR. These primers direct amplification of a 1,689-bp fragment from 98NK2. However, a single 1,538-bp PCR product was obtained using 98NK2-S DNA as template. Sequence analysis confirmed that this fragment comprised the 1,252-bp kanamycin cartridge flanked by 190- and 96-bp fragments derived from the 5' and 3' termini of the *saa* operon. Absence of any 1,689-bp PCR product indicated that all copies of the megaplasmid in 98NK2-S contained the mutagenized *saa* operon. Retention of the megaplasmid was also confirmed by PCR using *ehxA*-specific primers and by agarose gel electrophoresis of extracted plasmid DNA (results not presented). Western blot analysis using polyclonal anti-Saa antibody was also used to confirm that 98NK2-S did not produce the Saa protein (Fig. 9A).

The adherence phenotypes of 98NK2 and 98NK2-S were then compared. In the quantitative HEP-2 assay, total adherence after a 3-h incubation was (mean $\pm$ standard error) $3.07 (\pm 0.12) \times 10^5$ and $1.48 (\pm 0.05) \times 10^5$ CFU/well, respectively ($P < 0.001$). Giemsa staining also indicated that 98NK2-S

FIG. 8. Adherence of *E. coli* JM109 expressing cloned *saa* genes to HEp-2 cells (Giemsa stain). (A) *E. coli* JM109:pBluescript; (B) JM109:pJCP563; (C) JM109:pJCP564; (D) JM109:pJCP565.

FIG. 9. Analysis of *saa*-negative derivative of 98NK2. (A) Western blot analysis of 98NK2 and 98NK2-S lysates using polyclonal anti-Saa antibody. (B) Adherence of 98NK2 and 98NK2-S to HEp-2 cells (Giemsa stain).

displayed a diffuse, rather than the semilocalized, adherence pattern (Fig. 9B).

Effect of anti-Saa antibody or exogenous purified Saa on adherence to HEp-2 cells. To examine the capacity of antibodies to Saa to inhibit adherence of STEC, HEp-2 monolayers were infected with STEC strain 94CR in the presence or absence of anti-Saa serum diluted 1:100 or 1:400. In the absence of antibody, total adherence after a 3-h incubation was (mean $\pm$ standard error) $1.89 (\pm 0.26) \times 10^5$ CFU/well. In the presence of anti-Saa antibody, adherence was reduced in a dose-dependent fashion ($6.97 [\pm 0.98] \times 10^4$ CFU/well and $1.41 [\pm 0.17] \times 10^5$ CFU/well for 1:100 and 1:400 dilutions, respectively). For the 1:100 serum dilution, the reduction in adherence was statistically significant ($P < 0.01$).

The effect of exogenous purified Saa on STEC adherence was also investigated. HEp-2 monolayers were preincubated with or without Saa at concentrations of 5 or 25 $\mu\text{g/ml}$ for 30 min and then infected with 98NK2. After a 3-h incubation, total adherence was (mean $\pm$ standard error) $1.97 (\pm 0.17) \times 10^5$ CFU/well in the absence of Saa, compared with $3.52 (\pm 0.21) \times 10^5$ and $4.38 (\pm 0.25) \times 10^5$ CFU/well in the presence of 5 and 25 $\mu\text{g/ml}$ of Saa, respectively. At both concentrations, the stimulation of adherence by Saa was statistically significant (P values of <0.01 and <0.001 , respectively).

DISCUSSION

Although a wide variety of STEC strains are capable of causing severe human gastrointestinal disease and life-threatening systemic complications, such as HUS, research into pathogenic mechanisms has been focused largely upon STEC belonging to serogroup O157 (particularly O157:H7). This has been prompted by the fact that strains belonging to this sero-

group are the most frequent causes of STEC disease in humans in many parts of the world. However, this apparent predominance is due at least in part to the fact that O157 strains are much easier to detect than all other STEC strains (they are unable to ferment sorbitol). STEC belonging to serogroup O157 and a limited range of other groups, including O111 and O26, carry the LEE pathogenicity island, which encodes the capacity to adhere intimately to enterocytes. Although LEE is unquestionably an important accessory virulence factor for these strains, it is not essential for STEC virulence, and many strains lacking LEE are capable of causing life-threatening disease in humans. A substantial body of information has been accumulated on the complex molecular interactions between LEE-positive STEC and the intestinal epithelium. However, virtually nothing is known of adherence mechanisms of LEE-negative STEC, in spite of the fact that they are capable of highly efficient colonization of the human gut (28). Dytoc et al. (7) examined the in vitro and in vivo adherence phenotype of a different O113:H21 STEC strain, also isolated from a patient with HUS. This strain adhered diffusely to HEp-2 cells and caused microvillus effacement of rabbit caecum epithelial cells, but without generating the cytoskeletal rearrangements associated with LEE-positive strains. The presence of pili on the surface of this strain was also demonstrated by transmission electron microscopy, but the role of these in adherence was not investigated.

In the present study, we have described a gene we have designated *saa*, which encodes a novel autoagglutinating adhesin unique to LEE-negative STEC. The *saa* gene was isolated from cosmid pJCP561, which also contained a homologue of *iha* which has been reported by Tarr et al. (35) to facilitate adherence of *E. coli* O157:H7. *E. coli* DH1:pJCP561 had six-fold-higher adherence to HEp-2 cells relative to that of DH1:pHC79 and exhibited a semilocalized adherence phenotype. *E. coli* JM109 carrying a subclone of this cosmid in pBC (pJCP562) was 9.7 times more adherent than JM109:pBC. However, this subclone did not contain the *iha*-related gene, and the only intact open reading frame in the 3.8-kb 98NK2 DNA insert in pJCP562 was *saa*. JM109:pJCP562 also exhibited a semilocalized adherence phenotype indistinguishable from that of DH1:pJCP561. High-stringency homologues of *saa* were found in a number of unrelated STEC strains, all of which were LEE negative. All of these strains were positive for the STEC-associated enterohemolysin gene *ehxA*. All *saa*-negative, LEE-negative STEC cells were also negative for *ehxA*, suggesting that *saa* might also be plasmid carried, a fact confirmed by the absence of *saa* in a derivative of 98NK2 which had been cured of its large virulence plasmid. Interestingly, the vast majority of LEE-positive STEC strains also carry large *ehxA*-carrying plasmids, but the absence of *saa* from these strains indicates that there are important differences between the plasmids of these two STEC classes.

Saa appears to be located in the outer membrane and is exposed on the cell surface, as judged by immunogold electron microscopy. It exhibits a low degree of similarity (24 to 27% identity) with two other outer membrane proteins, notably YadA of *Y. enterocolitica* and EibD from *E. coli*. YadA is an important virulence factor in *Y. enterocolitica*, contributing to autoagglutination, serum resistance, complement inactivation, epithelial cell adherence, and resistance to phagocytosis and

binding to a variety of extracellular matrix proteins (3, 9, 34). Like Saa, YadA is expressed at 37°C, but not at 25°C, and is encoded on a large, virulence-related plasmid (4). EibD is a member of a family of four related proteins encoded by a prophage in *E. coli* strain ECOR-9, responsible for nonimmune IgG binding and serum resistance (30). However, in the present study, we found no evidence that Saa contributes to serum resistance either in 98NK2 or when expressed by *E. coli* K-12 clones.

Saa exhibits interesting structural features, in particular, the presence of four copies of a 37-aa repeat unit in the C-terminal region, which contains a motif predicted to form coiled coils, as does a region immediately downstream of the repeat domain. Such coiled-coil motifs are frequently implicated in protein-protein interactions. SDS-PAGE analysis of unboiled extracts suggests that Saa, which lacks cysteines, is capable of forming multimers with masses of >180 kDa. Interestingly, YadA of *Y. enterocolitica* also lacks cysteines and contains a region with a high probability of forming coiled coils. Recent structural analysis of YadA (12) suggests that the protein is oligomeric (probably trimeric or tetrameric) with a tripartite "lollipop" organization comprising an N-terminal oval head (responsible for functions such as adherence and autoagglutination) followed by a coiled-coil stalk and a C-terminal membrane anchor. EspA, which is produced by LEE-positive STEC and EPEC strains, also has a coiled-coil domain, and this region is essential for assembly of the EspA filaments which mediate initial attachment of these bacteria to epithelial cells and deliver other secreted effector proteins into the host cell (6).

Interestingly, *saa* genes from diverse STEC strains exhibited marked variation in size, due largely to deletion of one or two of the direct repeat units, such that the encoded Saa proteins ranged from 460 to 534 aa in length. Wild-type STEC strains MW10, MW5, and B2F1, which carried the shorter *saa* genes, exhibited lower adherence to HEp-2 cells. Western blot analysis using a Saa-specific polyclonal antiserum confirmed that these strains produce smaller Saa proteins, as predicted. When the shorter *saa* genes from these strains were cloned into *E. coli* JM109 using pBluescript, Saa was produced and the recombinant bacteria exhibited a semilocalized adherence phenotype, indicating that these gene products are still functional, at least when overexpressed from a high-copy-number plasmid. Unfortunately, however, these clones grew poorly in vitro with respect to the parental strain, preventing direct quantitative examination of the effect of the number of repeat units on adherence in an otherwise isogenic background.

Three further pieces of evidence support the hypothesis that Saa functions as an adhesin in LEE-negative STEC. Firstly, both the plasmid-cured derivative 98NK2-Cu and the defined *saa*-negative mutant 98NK2-S exhibited significantly reduced adherence relative to 98NK2. Adherence was reduced to a similar extent in both derivatives (47 and 48% of the wild-type level, respectively). It should be emphasized, however, that in neither case was adherence abolished, which indicates that additional adherence mechanisms also operate.

Secondly, a polyclonal antiserum raised against purified Saa from 98NK2 significantly inhibited in vitro adherence of the *saa*-positive STEC 94CR in a dose-dependent fashion. This strain was chosen to confirm that anti-Saa antibody was capable of interfering with adherence of heterologous STEC sero-

types, an important consideration if Saa is to be examined as a potential vaccine antigen for LEE-negative STEC. The reduction in adherence only reached statistical significance at the lowest serum dilution tested (1:100), which may imply poor antibody avidity. When overexpressed as a His₆-tagged fusion protein, Saa was insoluble, presumably due to formation of inclusion bodies. It was purified after solubilization with 6 M guanidine-HCl, and after Ni-nitrilotriacetic acid chromatography, the purified protein was refolded by dialysis against decreasing concentrations of urea. This resulted in a soluble antigen, which elicited antibodies capable of reacting with (denatured) Saa on Western blots after separation by SDS-PAGE. However, there is no guarantee that the final conformation accurately reflects that of the native protein, and important conformational epitopes may have been disrupted.

Finally, exogenous purified Saa significantly enhanced in vitro adherence of 98NK2 to HEp-2 cells. Purified exogenous protein might have been expected to compete with the endogenous adhesin for available binding sites on the epithelial cell surface, thereby reducing adherence. However, given that Saa contains predicted coiled-coil domains, it is also plausible that exogenous protein might be capable of directly interacting with surface-localized Saa and enhance interactions either with the epithelial surface or with other bacterial cells.

An understanding of the adherence mechanisms of STEC may be crucial to the development of effective anti-STECC vaccines. Vaccines based on inactivated Shiga toxins are likely to prevent the systemic complications, but they will not prevent transmission of STEC from person to person. For this reason, vaccines targeting products of the LEE locus are being developed to block colonization of either humans or animal reservoirs by LEE-positive STEC strains. However, in spite of the long-recognized capacity of LEE-negative STEC strains to cause life-threatening human disease, there have been very few studies of adherence mechanisms. Saa is the first adhesin to be characterized for this important class of human pathogens, and it warrants further investigation as a candidate vaccine antigen.

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