The export of coat protein from enteroaggregative *Escherichia coli* by a specific ATP-binding cassette transporter system

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Summary

Enteroaggregative *Escherichia coli* (EAEC) is an emerging enteric pathogen characterized by aggregative adherence (AA) to cultured human mucosal epithelium cells. We have recently characterized a 10.2 kDa protein, called dispersin, which is exported from the bacteria and which promotes dispersal of EAEC across the intestinal mucosa. Here, we present evidence that dispersin is exported by a putative ABC transporter complex which is encoded by a genetic locus of the EAEC virulence plasmid pAA2. We demonstrate that the locus comprises a cluster of five genes (designated *aatPABCD*), including homologs of an inner-membrane permease (*AatP*), an ATP-binding cassette protein (*AatC*) and the outer membrane protein *TolC* (*AatA*). We show that, like *TolC*, *AatA* localizes to the outer membrane independently of its ABC partner. Dispersin appears to require the *Aat* complex for outer membrane translocation but not for secretion across the inner membrane. We also show that, like the dispersin gene, transcription of the *aat* cluster is dependent on AggR, a regulator of virulence genes in EAEC. We propose that the *aat* cluster encodes a specialized ABC transporter which plays a role in the pathogenesis of EAEC by transporting dispersin out of bacterial cell.
Introduction

Enteropathogenic Escherichia coli (EAEC) is an emerging enteric pathogen associated with endemic and epidemic diarrheal illness in both developing and industrialized countries (1,2,3). In addition, EAEC induces intestinal inflammation, which can precipitate growth failure even in the absence of diarrheal symptoms (4). The pathogenesis of EAEC infection is thought to involve the adherence of the bacterium to the intestinal mucosa, possibly in both the small and large intestines, followed by secretion of one or more enterotoxins (5-8). Adherence of EAEC to the mucosa is characterized by the presence of a thick, aggregating biofilm (2, 9), which may promote persistent infection.

The defining feature of EAEC is its characteristic aggregative adherence (AA) phenotype in the standard HEp-2 adherence assay (10). In this in vitro assay, EAEC strains adhere to the epithelial cell surface, to the glass substratum, and to each other in a distinctive stacked-brick formation. We have described the related adhesins Aggregative Adherence Fimbriae I and II (AAF/I and AAF/II), which are encoded on a large virulence plasmid called pAA (11, 12). The proven human pathogenic strain 042 requires AAF/II fimbrial antigen for adherence to the colonic mucosa, suggesting that this adhesin is a virulence factor for human infection (12). An AAF/III adhesin has recently been described (13).

Whereas most EAEC strains lack a recognizable AAF adhesin (7,13), the majority carry the ca. 100 kb pAA plasmid which harbors several conserved genetic loci. Most prominent among the plasmid-encoded factors is a transcriptional activator of the AraC class designated AggR (14). AggR is required for expression of both AAF/I and AAF/II, but it is also present in a large percentage of EAEC strains that do not express any identified AAF (7).

Recently, we characterized a novel AggR-dependent gene lying immediately...
upstream of $aggR$ in EAEC strain 042 (15). This novel gene encodes a secreted 10.2 kD protein, designated Aap (anti-aggregation protein), which appears to form a protein capsule on the bacterial cell surface and which promotes dispersal of EAEC on the intestinal mucosa. In light of this property, Aap has been given the more descriptive name dispersin. Initial studies revealed that the translocation of dispersin to the surface of EAEC does not require the secretion apparatus required to construct the AAF fimbriae; however, the mechanism by which dispersin is translocated remained initially unclear. The current work provides the first insight into the mechanism of dispersin secretion.

In addition to the putative virulence factors AAF, dispersin, Pet, EAST1, and AggR, the pAA plasmid also harbors sequences homologous to an empirically derived EAEC probe (16). In the original report, the EAEC probe was found to be 89% sensitive and 99% specific for EAEC detection, and subsequent data suggest that the probe may constitute a marker for pathogenic EAEC strains (17, 18; M. Cohen, and J. Nataro, unpublished). Whereas the sequence of the probe has been reported (19), no protein product or putative function was suggested for the corresponding genes. We show here that the pAA2 region corresponding to the AA probe encodes a putative ABC transporter apparatus, which is co-regulated with, and required for, efficient translocation of the dispersin protein.
Experimental Procedures

Bacterial strains, plasmids, and growth condition.

Strains and plasmids used in molecular studies are listed in Table 1. Strain 042 was isolated in 1983 from a child with diarrhea in the course of an epidemiological study in Lima, Peru (20); this strain has been shown to cause diarrhea in adult volunteers (21). Previously described EAEC strains used in PCR analysis (Table 2) were from collections of the Center for Vaccine Development and were originally described in reference (7). All E. coli strains were grown aerobically at 37°C in Luria-Bertani (LB) medium unless otherwise stated. All strains were stored at −70°C in Trypticase soy broth with 15% glycerol. AggR-inducing conditions comprised growth at 37°C without shaking in Dulbecco’s Minimal Essential Medium (Gibco/BRL) with 0.45% glucose (DMEM high glucose). Antibiotics were added at the following concentrations where appropriate: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; nalidixic acid, 50 µg/ml.

Molecular cloning and sequencing procedures

Plasmid DNA purification, restriction, ligation, transformation, and agarose gel electrophoresis were performed by standard methods (22). Plasmid DNA was introduced into E. coli DH5α, DH5αλpir, and S17-1λpir by heat shock transformation of competent cells according to the method of Hanahan (23), or into 042 by electroporation using the Gene Pulser II system (Bio-Rad, Hercules, CA).

The sequence of relevant regions of plasmid pAA2 was determined in laboratories at both the University of Maryland School of Medicine and the University of Wisconsin. Sequences from the two facilities were compared and discrepancies reconciled by
sequencing of selected library templates. At the University of Maryland, sequences were
determined from a random pBluescript library, as reported previously (24). DNA
sequence determination was performed in the University of Maryland School of
Medicine Department of Microbiology & Immunology Biopolymer Facility on an
Applied Biosystems model 373A sequencer.

For sequencing at the University of Wisconsin, plasmid pAA2 was propagated in
host strain HB101, and DNA isolated using the Large Construct Kit (Qiagen). Purified
plasmid DNA (5ug) was randomly sheared by nebulization as described (25), fragments
were size-fractionated by agarose gel electrophoresis, then end-repaired and cloned into
M13 Janus (26). Templates for sequencing were prepared by PCR followed by treatment
with Exonuclease I and Shrimp alkaline phosphatase (USB Corporation, Cleveland OH).
Sequencing was performed with dye-terminator chemistry (Applied Biosystems Prism
reagents) and data were collected on ABI 377 or 3700 automated sequencers. Sequence
reads were assembled by Seqman II (DNASTAR) software. Nucleotide sequence analysis
was performed using programs available through the National Center for Biotechnology
Information (www.ncbi.nlm.nih.gov) and the ExPASy server of the Swiss Institute of
Bioinformatics (www.expasy.ch).

The sequence of the Aat cluster has been submitted to Genbank under accession
number AY351860.

Polymerase chain reaction and Reverse transcriptase-PCR

Amplifications were performed with 500 ng of purified genomic DNA as
template in a 50 µl reaction mixture containing 2.5 U of Taq DNA polymerase, 0.5 µM
each primer, 0.2 mM each deoxynucleoside triphosphate, 2 mM MgCl₂ and 5 µl of the
manufacturer’s buffer (Invitrogen, Carlsbad, CA). Amplification reactions were
performed in an MJ Minicycler for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 1 min per kb, concluding with extension at 72°C for 10 min unless otherwise stated. The primer sequences used for PCR are shown in Table 3.

RT-PCR was performed as previously described (27). Total RNA was extracted with an RNeasy mini kit (Qiagen Inc., Valencia, CA) from LB cultures shaking to mid-log phase; preparations were treated with RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN) to eliminate contaminating DNA. The absence of contaminating genomic DNA in RNA preparations was verified by performing PCR for the chromosomal chloramphenicol acetyltranspherase (cat) gene of strain 042. To synthesize cDNA, total RNA (2 µg) was subjected to reverse transcriptase (RT) reactions using Thermoscript RT (Invitrogen) and gene-specific reverse primers according to the manufacturer’s instructions. Primers used for RT-PCR are shown in Table 3. Amplification reactions were performed in an MJ Minicycler for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 1 min per kb, concluding with extension at 72°C for 10 min. The products were separated by 0.7 - 1.0% agarose gels, stained with ethidium bromide and visualized with UV transillumination.

**AatA expression and purification**

The *aatA* gene product was expressed by cloning a 1064-bp fragment generated by PCR into the BamHI and EcoRI sites of the expression vector pET21a(+) (Novagen, Madison, WI). The sequences of *aatA*-F and *aatA*-R primers are shown in Table 3. The protein was thereby expressed as a fusion with a 6-Histidine tag and a T7-tag at the carboxy terminus. Protein expression was achieved by incubating an LB culture of the construct at 37°C with shaking to an OD₆₀₀ of 0.5 to 0.6; expression was then induced with 0.4 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma
Chemical Co., St. Louis, MO.) for 3 h. The fusion protein was purified under denaturing conditions by passage through a Talon metal affinity resin according to manufacturer’s protocols (Clontech, Palo Alto, CA). The column eluate was dialyzed overnight against PBS containing 8 M urea (pH 7.4). The fusion protein was separated by SDS-PAGE and detected by staining with Coomassie brilliant blue R250 (Sigma). To confirm the identity of the protein, the band was excised from an SDS-PAGE gel and analyzed by mass spectrometry at the Protein and Nucleic Acid Research Facility, Stanford University School of Medicine, Palo Alto, CA.

**Generation of AatA-specific antibodies**

Rabbit antiserum specific for AatA was raised by subcutaneous injection of AatA preparations in Freund’s adjuvant as described (28). Affinity purification was performed to concentrate and purify AatA-specific antibodies. The 6His-tag AatA fusion protein (10 mg) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to manufacturer’s protocols, and hyperimmune rabbit antiserum was absorbed by mixing with 6His-AatA protein-coupled Sepharose for 2 h at room temperature. After washing, bound antibodies were eluted with 0.2 M glycine (pH 1.85), and immediately neutralized with 1 M Tris-HCl (pH 8.5). The eluate was dialyzed against PBS, concentrated using a Vivaspin 20, 50,000 MWCO (Vivascience, Hannover, Germany), and used for Western immunoblotting.

**Preparation and analysis of cellular fractions.**

To prepare culture supernatant fractions, strains were grown overnight or for 6 h at 37°C in 5 ml of DMEM 0.45% glucose (high glucose DMEM). After centrifugation at
13,000 x g for 5 min, proteins in the supernatant was precipitated with trichloroacetic acid (TCA) as follows. One-fourth volume of TCA containing 0.4% (wt/vol) deoxycholate was added to culture supernatants and incubated on ice for 30 min. The pellet was centrifuged at 14,000 x g for 15 min, and washed with acetone for 15 min at room temperature. The pellet was collected by centrifugation at 14,000 x g for 15 min, dried, and suspended in 50 µl of Laemmli sample buffer. To remove the Aap protein from the bacterial cell surface, 0.1% Triton X-100 was added to the medium as described (15).

Outer membrane proteins were extracted from cultures grown overnight at 37ºC in 20 ml of high-glucose DMEM. Bacteria were harvested by centrifugation at 6,000 x g for 10 min at 4 ºC and resuspended in 3.0 ml of 10 mM Tris, pH 8.0. The cells were lysed with a French pressure cell and centrifuged for 30 min at 13,000 x g at 4ºC. The pellet was resuspended in 240 µl of 10 mM Tris, pH 8.0, 60 µl of 10% Triton X-100 and 1.5 µl of 1M MgCl₂ (29) or 35 µl of distilled H₂O and 265 µl of 20% Sarkosyl (30). The pellet was incubated at room temperature for 20 min, and resuspended in 50 µl of Laemmli sample buffer.

Periplasmic proteins were extracted from cultures grown overnight at 37ºC in 5 ml of high-glucose DMEM. Bacteria were harvested by centrifugation at 3,800 x g for 10 min and resuspended in 500 µl of lysis buffer (50 mM Tris, pH 8.0, 3 mM EDTA, and 1% Triton X-100) (31). The suspension was kept on ice for 30 min, and centrifuged for 15 min at 3,800 x g. The supernatant was collected and precipitated with TCA as described above. The pellet was resuspended in 100 µl of Laemmli sample buffer for separate analysis.

One-dimensional SDS-PAGE was performed on 10 - 15% (wt/vol) acrylamide separating gels and 4.0% (wt/vol) acrylamide stacking gels according to standard protocols (32). Proteins were detected by staining with Coomassie brilliant blue or the Silver Stain Kit from Bio-Rad Laboratories (Hercules, CA).
For immunoblot analyses, protein samples were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore) using standard protocols. For detection of AatA and Aap, anti-AatA or anti-Aap antiserum (15) were used at dilutions of 1:1,000 and 1:8,000, respectively; antibody binding was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:40,000 and visualized using a chemilluminescence ECL kit (Amersham).

Mutagenesis and complementation.

Insertional mutants of aat genes were constructed by single-crossover insertion of plasmid pJP5603 as previously described (24). Briefly, an internal portion of the target gene was generated by PCR using primers described in Table 3, and the product was cloned into XbaI and EcoRI sites of the π–dependent suicide vector pJP5603 (33). The resulting plasmids (pINTP, pINTA, pINTB, pINTC or pINTD) were propagated in E. coli DH5α λpir prior to transformation into the donor E. coli strain in S17-1 λpir. The mutant strain was then obtained by conjugal mating between the wild type parent strain 042 (which is nalidixic acid resistant) and S17-1λpir(pINT). Transconjugants were selected on LB agar supplemented with kanamycin and nalidixic acid. This process resulted in merodiploid integration of pINT into the homologous site in the aat gene. Integration of pJP5603 constructs resulted in duplication of predicted codons 64-230 of aatP, 100-272 of aatA, 58-204 of aatB, 36-136 of aatC, and 73-239 of aatD.

To complement aat mutants, the aat cluster was amplified by PCR using primers listed in Table 3 and cloned into the single copy vector pZC320. Amplifications were performed with 500 ng of purified genomic DNA as templates in a 50 µl reaction mixture containing 2.5 U of Platinum Pfx DNA polymerase, 0.5 µM each primer, 0.3 mM each deoxynucleoside triphosphate, 2 mM MgCl₂ and 5 µl of the manufacturer’s buffer.
(Invitrogen, Carlsbad, CA). Amplification reactions were performed in an MJ Minicycler for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 54°C for 40 s, and 68°C for 6.5 min, concluding with extension at 68°C for 10 min. The PCR products were cloned into the BamHI and NotI sites of the vector pZC320.

Electron microscopy

Scanning electron microscopy of 042 and its mutants in aap and aat was performed as previously described (15) at the Electron Microscopy Facility, Johns Hopkins University School of Medicine.

Fold recognition and comparative modeling

The amino acid sequence of aatA was submitted to the FUGUE server (http://www-cryst.bioc.cam.ac.uk/fugue/) (34) and searched against the HOMSTRAD database (http://www-cryst.bioc.cam.ac.uk/homstrad) (35), with default parameters. The structure-aided sequence alignment of AatA and TolC was generated with FUGUE and formatted with JOY (36). This alignment was used, along with the atomic coordinates of TolC (PDB code 1EK9), to build a model of AatA using MODELLER (37).

Other programs and databases used for sequence analyses of domain structures, secretion signals and transmembrane regions included SIGNALP (38); ProDom (39); TMPRED (40); SOSUI (41); TOPPRED (42); PSORT (43) and PFAM (44).
Results

Sequence determination and characteristics of open reading frames

The sequence of the pAA2 plasmid from EAEC strain 042 was determined by shotgun sequencing of a pBluescript library. Discrepancies were resolved by directed sequencing of pBluescript clones encompassing the desired region. The complete sequence of plasmid pAA2 will be presented elsewhere. We were especially interested in examining the region corresponding to the AA probe (the insert of plasmid pCVD432), since this cryptic DNA fragment has been shown to be associated with pathogenic EAEC strains and to correlate with the presence of plausible virulence factors. The AA probe sequence has been reported previously (19), and we have noted that it contains one potential open reading frame, but that the predicted polypeptide fragment exhibited no homology to known sequences in any database.

The pAA2 region flanking the AA probe comprises a cluster of five open reading frames (ORFs) in the same orientation and very closely spaced (Fig. 1 and Genbank accession AY351860). Flanking these ORFs at distances of over 500 bp on each side are remnants of insertion sequences (IS), suggesting that the five ORFs comprise a discrete gene cluster. Two of the ORFs exhibit significant identity to bacterial ABC transporter-related proteins (see below), and the cluster has therefore been designated Aat (enteroaggregative ABC transporter). Each gene of the predicted aat gene cluster has a low G+C content; GeneQuest analysis yielded an average G+C of 31.6% for the region encompassing aatP to aatD, ranging from 29.6% (aatA) to 34.3% (aatB). G+C content rises to levels more typical of E. coli immediately upstream of aatP and downstream of aatD. Each gene in the aat cluster exhibits a codon bias characteristic of genes acquired by horizontal transfer from other species, as compiled by Borodovsky (45).
The predicted ORF1 (aatP) product (spanning nucleotides 1425 to 2555 in Fig. 1) is 377 amino acids in length (42.7 kDa), initiating at ATG. The AatP protein bears significant identity with the family of ABC transporter permeases, inner membrane proteins which anchor the ATPases and which participate in the secretion process. The closest AatP homolog is the putative ABC permease protein TTE0258 of *Thermoanaerobacter tengcongensis* (46), with which AatP displays 20% identity and 38% similarity over its entire length (E value = 1 x 10⁻¹⁰). Notably, the permeases of *E. coli* ABC transporters involved in *uptake* have a highly conserved amino acid motif, EAA, near the C-terminus of the protein (47). Neither AatP nor any of the other predicted Aat proteins contain this motif. The permease is thereby predicted to be involved in an export process.

The largest predicted product of ORF2, the *aatA* gene, begins from an atypical start (GTG) codon overlapping the *aatP* ORF by three nucleotides. Translation of this ORF would yield a protein of 412 amino acids (48.5 kDa; nucleotides 2552 to 3790 in Fig.1). Neither the nucleotide sequence nor the deduced amino acid sequences of either predicted *aatA* ORF displays significant identity to any other gene or protein in the databases. NCBI conserved domain search produced a significant alignment (E value = 0.009) of AatA residues 214 – 263 with COG1538, including the *E. coli* outer membrane protein TolC. TolC was identified as an unambiguous homolog of AatA by the FUGUE program (34), which compares sequences with experimentally established three-dimensional structures and scores amino acid substitutions according to local structural environments. With an observed FUGUE Z-score of 13 (>99% confidence) and the conservation of amino acid residues that play important structural roles (see below), it is highly likely that AatA and TolC share common ancestry and would adopt similar three dimensional structures (Fig. 2).

TolC is a versatile OM channel/tunnel, which participates in many multi-drug
efflux pumps and Type I protein transport systems (48-51). TolC is a homotrimeric protein with a membrane-embedded β-barrel portion and an elongated periplasmic portion that is predominantly α-helical and forms a cylindrical α-helical assembly (48, 52, 53). The structure-aided sequence alignment of AatA and TolC, with an annotation of key structural features, is shown in Fig. 2A. Although the amino acid sequence identity between TolC and AatA is only 25%, the two proteins share important structural determinants. For instance, the junctions between the β-barrel and α-barrel have prolines (positions 38 and 233 in Fig. 2A) that are conserved among members of the TolC family (54); these residues are strictly required to accommodate the abrupt turn that links the β-barrel and α-barrel. Also conserved are the glycines at positions 143 and 342 (54), which lie in the turns near the closed end of the TolC channel.

Translation of aatA from the atypical GTG start results in a predicted product exhibiting a strongly predicted signal sequence. Such processing would also make AatA similar to TolC, which is synthesized in a precursor form with a leader sequence that is removed during sec-dependent processing en route to the outer membrane. Based on the alignment in Fig. 2A and the structure of TolC, we have built a three dimensional model of the AatA homotrimer by comparative modeling (Fig. 2B). The model predicts similar structural characteristics to the TolC protein, including an acidic patch near the enclosed, periplasmic end of the trimer.

ORF3 (aatB) encodes a predicted protein of 274 amino acids (31.1 kDa; nucleotides 3687 to 4508 in Fig 1). The predicted ORF3 protein was not found to have significant identity to known protein sequences in the databases. SignalP analysis did not predict a signal sequence, but the product was predicted to contain one transmembrane region near the N-terminus.

ORF4 (aatC) encodes a predicted protein of 210 amino acids (23.3 kDa; nucleotides 4501 to 5130). The predicted AatC protein is 42% identical at the amino acid
level over its entire length to ABC transporter ATP-binding protein of *Clostridium acetobutylicum* (Q97M79). ATP binding proteins exhibit four shared motifs: Walker Boxes A and B, the ABC signature, and the histidine motif (55). All four of these motifs are present in the *aatC* product, with an E value of $1.4 \times 10^{-39}$ against the Pfam ABC transporter motif (PF00005). FUGUE predicts an optimal match of AatC with the crystal structure of the ATPase from the *Thermococcus litoralis* MalK sugar transporter (Z score 38.5). The high Z score gives greater than 99% confidence in the structural similarity of these two proteins.

ORF5 (*aatD*; nucleotides 5142 to 6356) encodes a predicted protein of 405 amino acids (47.2 kDa). The predicted AatD protein is 23% identical over 185 amino acids to a cryptic acid-inducible protein of *Borrelia burgdorferi* (O51253) (E = 0.056). TMPred and Psort analyses suggested the presence of five membrane spanning helices near the N-terminus of the protein (within the first 156 amino acids) yielding a predicted inner membrane N-terminus-outside topology.

*Prevalence of the aat cluster among EAEC.*

To assess the conservation of the *aat* cluster among a collection of EAEC isolates, we performed PCR to detect *aatA* and *aatBCD* in 31 well characterized EAEC strains isolated from children in epidemiologic studies throughout the world (Table 2). The prevalence of the *aatA* gene and *aatBCD* gene cluster was 71.0% (22 of 31) and 74.2% (23 of 31), respectively; *aatA* was uniformly accompanied by *aatBCD* except in one strain. In addition, we found that 19 of 22 *aat*-positive strains were *aggR*-positive, while four of nine *aat*-negative strains were *aggR*-negative (Table 2). The presence of *aggR* by PCR correlated significantly with that of the *aat* cluster (p=0.016).
Transcriptional dependence on AggR.

The correlated occurrence of \textit{aat} and \textit{aggR} in EAEC strains suggested that \textit{aat} transcription could be dependent on the AggR transcriptional activator. Using RT-PCR, we observed an \textit{aatA} transcript in wild type EAEC strain 042 when grown in \textit{aggR}-inducing conditions, but not in an 042\textit{aggR} mutant (Fig. 3), or in either strain in the \textit{aggR}-repressing medium LB without added glucose (not shown). To further substantiate a requirement for AggR in \textit{aatA} transcription, we complemented 042\textit{aggR} in \textit{trans} with \textit{aggR} cloned under control of the arabinose promoter in plasmid pBAD30 (Fig. 3, lanes 4 and 5). \textit{aatA} transcription was observed in 042\textit{aggR}(pBAD\textit{aggR}) when cells were grown in the presence of arabinose (\textit{ara}-inducing conditions) but not glucose (\textit{ara}-repressing conditions). \textit{aatA} transcription was not observed in the negative control strain 042\textit{aggR}(pBAD30) (Fig. 3, lane 3). Using similar methods, AggR was also shown to be essential for transcription of \textit{aatP}, \textit{aatB}, \textit{aatC}, and \textit{aatD} (data not shown).

Localization of the AatA protein

The common co-inheritance of the Aat cluster, coupled with the apparent dependence of all the genes on AggR for expression, suggested that the Aat products act in concert, presumably as an ABC transporter complex. Whether dedicated for import or export, ABC transporters typically comprise three to five gene products, including one or two inner membrane proteins (including the permease noted above), in addition to the ATP binding protein (Reviewed in ref. 56). ABC importers characteristically also feature a periplasmic carrier protein, with or without an accompanying outer membrane protein partner.

Our initial approach to characterizing the role of Aat was to assess the effects of mutations in individual \textit{aat} genes and to localize the AatA product, which was predicted
to reside in the outer membrane. Individual single-crossover insertion mutants were constructed in all five \textit{aat} genes. RT-PCR for \textit{aatA}, \textit{aatC}, and \textit{aatD} transcripts in the respective mutants confirmed that the constructs did not synthesize the respective mRNA transcripts (not shown).

The AatA protein was expressed and purified with a C-terminal 6-His tag, and this protein was used to generate highly specific polyclonal AatA antiserum. Use of this antiserum confirms the absence of the AatA product in the 042\textit{aatA} mutant (Fig. 4A). Western immunoblots were prepared from whole cell, outer membrane, periplasmic, and supernatant fractions of strain 042 grown under \textit{aggR}\textsuperscript{-}-inducing conditions. AatA was observed in the outer membrane but not in the periplasmic (Fig. 4A) or supernatant fractions (not shown). These observations are consistent with our prediction that AatA is a TolC homolog.

Our analyses did not reveal whether AatA reached the outer membrane by virtue of the Sec apparatus or whether it constituted the substrate of the Aat system itself. As shown in Fig. 4B, AatA was observed in the outer membranes of both 042\textit{aatC} and 042\textit{aatD} mutants, suggesting that AatA localization was independent of Aat ATPase activity. As noted above, TolC arrives at the outer membrane through a Sec pathway, and it seems likely that AatA follows the same mechanism.

\textit{The aat cluster promotes translocation of dispersin}

In view of the homology of AatA and TolC, we suspect that Aat may serve as an exporter. To assess a possible role for Aat in protein export, culture supernatants of 042 and its \textit{aat} mutants were concentrated with trichloroacetic acid (TCA) and separated using SDS-PAGE. We observed no differences in the protein profiles of 042 and \textit{aat} mutants grown under \textit{aggR}\textsuperscript{-}-repressing conditions (L-broth). However, when cultivated
under *aggR*-inducing condition (high-glucose DMEM), a ca. 10 kDa species was observed in wild type 042 supernatants but not in supernatants of the *aat* mutants (Fig. 5).

We previously described a protein capsule on the surface of most EAEC strains that is composed of a protein called Aap, or dispersin (15). Aap is a 10.2 kDa species abundantly secreted to the supernatant under *aggR*-inducing conditions; the protein remains associated with the bacterial cell surface but can be removed upon extraction with 0.1% Triton X-100 detergent. Triton X-100 was therefore added to 0.1% in DMEM-glucose cultures of 042 and its *aat* mutants, the supernatants were separated on SDS-PAGE and analyzed by immunoblotting using anti-Aap polyclonal antiserum (Fig. 6). These experiments confirmed that the 10 kDa species was indeed the dispersin protein Aap. Aap secretion was diminished to a similar extent upon interruption of *aatP*, *aatA*, *aatB*, *aatC* or *aatD* (Fig. 6B). As a control for possible effects of the integration of plasmid pJP5603 during mutagenesis, we included in our analyses 042*pet*, which harbors pJP5603 integrated into the gene encoding the autotransporter toxin Pet (ca. 10 kb downstream of the *aat* locus in plasmid pAA2). 042*pet* exhibited similar abundance of Aap in supernatants as wild type 042 (Fig. 6B).

To verify that the *aat* locus is required for the efficient translocation of Aap, we complemented 042*aatA* with various constructs (Fig. 7). Fragments of the *aatPABCD* locus were cloned into the single copy number mini-F vector pZC320 or the expression vector pSE380 (See Fig. 1 and Table 1). When the entire *aatPABCD* locus (construct pJNW) was introduced into an 042*aatA* mutant, the Aap protein was detected in supernatants at approximately wild type levels (Fig. 7B, lane 3). The *aat* subclone in pZC320 which did not include the *aatP* upstream region (pJNAD) was unable to complement the *aatA* mutant (not shown). Construct pJNPA, comprising only the *aatPA* genes in pZC320 (Fig. 7B, lane 4), was able to restore essentially wild type levels of Aap secretion, suggesting that some transcription of downstream genes could occur after
disruption of \textit{aatA}. Construct pJNW was able to complement the \textit{aatC} and \textit{aatD} mutants for Aap secretion (Fig 7C). The \textit{aatD} clone was able to complement \textit{042aatD} but not \textit{042aatC} to restore Aap secretion (Fig. 7D).

We also utilized complementation analysis to address the likely start site of \textit{aatA} translation (Fig. 8). For these experiments, the \textit{aatA} gene was cloned into expression vector pBAD30 to comprise either the upstream GUG or downstream AUG start sites. As shown in Fig. 8, in the presence of arabinose, only the clone including the putative GUG start site was able to complement \textit{042aatA} to restore Aap secretion. These results suggest that the predicted leader sequence of AatA, (encoded via translation at the GUG site) is probably required for the outer membrane localization of the protein and is necessary for Aap secretion.

**Localization of dispersin in aat mutants**

The \textit{aap} gene product has a strongly predicted signal sequence of 21 amino acids. We have previously verified that the N-terminal amino acid sequence of the mature Aap protein corresponds to amino acid residue 22 of the \textit{aap} gene, precisely as predicted by \textit{in silico} analyses (15). We have therefore assumed that Aap enters the periplasm via the Sec apparatus. However, our data raise the possibility that the Aat cluster may mediate coordinated inner membrane secretion and outer membrane translocation of Aap, which is the typical case for proteins secreted by an ABC transporter (eg. \textit{E. coli} hemolysin and colicin).

To assess this possibility, osmotic shock was performed on \textit{042} and its \textit{aat} mutants to extract periplasmic contents, and these preparations were immunoblotted for Aap (Fig. 9). Under \textit{aggR}-inducing conditions, the level of Aap in the periplasm of the \textit{aat} mutants was higher than in the wild type strain, while Aap was decreased at the cell
surface. Taken together, our data suggest that secretion of Aap into the periplasm is independent of the *aat* cluster, but that outer membrane translocation requires multiple proteins of the Aat apparatus.

Phenotype of the *aat* mutants

We have previously shown that *aap* mutants exhibit increased aggregation, mediated at least in part by an abnormal morphology of AAF (15). In such mutants, the AAF filaments were observed to lie along the surface of the bacterial cell, as opposed to the splaying of fimbriae in wild type EAEC. Scanning EM of 042, 042*aap* and 042*aatA* revealed an identical fimbrial morphology in 042*aap* and 042*aatA* mutants (Fig. 10), clearly distinguishable from the wild type. These data are consistent with our observations that the *aat* cluster is required for function of the Aap coat.
Discussion

In this study, we have characterized the plasmid locus corresponding to the previously cryptic AA probe, long used in the clinical detection of EAEC strains. Our data suggest that the locus encodes an ABC transporter cluster comprising five genes. Moreover, we herein report that at least one function of this gene cluster is to translocate the protein capsule of EAEC to the surface of the bacterium.

Evidence in support of this conclusion includes the following: 1) Interruption of \(aatP\), \(aatA\), \(aatB\), \(aatC\) or \(aatD\) reproducibly diminishes Aap export to the cell surface; this effect was complemented by components of the Aat cluster provided in trans. 2) The Aat cluster is found to segregate closely with the \(aap\) gene, despite an unlinked genetic structure. 3) \(aat\) and \(aap\) genes are co-regulated by the AggR transcriptional activator.

Although it is not yet possible to describe fully the mechanism by which the Aat apparatus facilitates Aap translocation, our experimental data coupled with observations made for other ABC systems allow us to draw several inferences. We had initially hypothesized that the Aat apparatus may serve to secrete AatA and/or Aap across the inner membrane, where the two proteins could interact to mediate the latter’s translocation. However, our data instead suggest that Aap reaches the periplasm independent of any of the Aat proteins. We observe that AatA is located in the outer membrane, and its insertion in this membrane requires neither AatC nor AatD. AatA is likely to share common structural features and functional properties with the outer membrane channel protein, TolC (Fig. 2B). With its location in the outer membrane, and its proposed channel-like structure, AatA could act directly as a pore for Aap translocation.

TolC and its homologs function to export large toxins through a “Type I” transport mechanism, and smaller proteins (e.g. the \(E. coli\) heat-stable toxins) from the
periplasm (48). In transporting proteins that do not have periplasmic species, TolC forms a transient assembly with an inner membrane ABC protein and with a periplasmic partner (the class known as “membrane fusion proteins” [50, 51, 54]). Notably, none of the aat components encodes a membrane fusion protein homolog. Our data indicate that dispersin translocation may proceed via a periplasmic intermediate. It therefore seems likely that the mechanism of AatA-mediated transport of dispersin may be more consistent with secretion of the heat-stable toxins.

We summarize a model for dispersin transport in Figure 11. As in all TolC-mediated secretion, the AatA channel would require ATP-dependent activation, presumably provided by its interaction with the AatC ATPase homolog. (48). The AatD and AatB proteins have no apparent ABC function, but one or both may act as adapters, transducing the ATP-dependent signal to the AatA pore. Despite a clear contribution of Aat to dispersin translocation through the outer membrane, we were often able to observe some residual dispersin translocation in the absence of a fully functional Aat complex. This observation was made in both 042aat mutants as well as in K12 not carrying aat genes. This could be due to an alternative general translocation mechanism or to the substitution of another TolC homolog – or TolC itself - in dispersin translocation.

Our data also highlight an emerging central role of the AggR activator in EAEC pathogenesis. The list of factors shown by our laboratory to be under AggR control now includes the AAF adhesions (AAF/I and AAF/II)(14, 24), AggR itself (R. Marques and J. Nataro, unpublished), Aap (15) and Aat. In addition, we have recently discovered an EAEC-specific chromosomal island, multiple genes of which are under AggR control and which is co-inherited with the aforementioned plasmid-borne genes (E. Dudley and J. Nataro, unpublished). Thus, EAEC appears to be similar to other enteric pathogens in which plasmid and chromosomal genes are coordinately controlled to execute complex virulence functions. The central role of AggR is a target of ongoing research efforts.
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Figure legends

Figure 1. Map of the aat cluster. The sequence of the 7-kb fragment of the pAA plasmid from EAEC strain 042 reveals several ORFs greater than 500 bp (indicated). Below the map, the locations of the inserts of aat cluster clones and the location of the AA probe fragment are indicated.

Figure 2. Structural modelling of AatA. (A) The sequence alignment of AatA and TolC, based on the TolC crystal structure. The structural environments of TolC are taken from the crystal structure and those of AatA are predicted from the model shown in (B). The consensus secondary structure is shown below the alignment: α, α-helical positions; 3, 3_10-helical positions; β for β-sheet positions. Shown above the alignment are positions based on AatA numbering of the predicted mature AatA sequence, where insertions with respect to AatA are indicated as ABC, etc. Note that the site of signal peptide cleavage in AatA has not been determined experimentally, but is predicted by one algorithm to be upstream of the lysine residue at position 1 in the figure. Residue environments are indicated by JOY formatting (36) as follows: red, α helices; blue, β-strands; maroon, 3_10 helices; upper-case letters, solvent inaccessible; lower-case letter, solvent accessible; bold type, hydrogen bonds to main chain amides; underlining, hydrogen bonds to main chain carbonyls; tilde (~), hydrogen bonds to other side chain/heteroatoms; italic, positive main chain torsion angle φ. (B) The modeled three-dimensional structure of the homotrimeric AatA based on the crystal structure of TolC. The structure is color-coded to indicate the three subunits. The figure was prepared using MOLSCRIPT (57) and Raster3D (58).
Figure 3. AggR dependence of the aatA gene. pBAD30 containing the aggR gene was introduced into 042aggR and the bacteria were propagated in ara-inducing (0.1% arabinose) or repressing (0.1% glucose) conditions. RNA was extracted and RT-PCR was performed for the ca. 1 kb aatA transcript as described in Experimental Procedures. Products of amplification were separated on a 1% agarose gel. M, marker; Lane 1, 042; Lane 2, 042aggR; Lane 3, 042aggR(pBAD30); Lane 4, 042aggR(pBADaggR) with 0.1% arabinose; Lane 5, 042aggR(pBADaggR) with 0.1% glucose. Cultures were grown to late log-phase in LB media with additional sugars as indicated.

Figure 4. AatA localizes to the outer membrane independently of other aat genes. The AatA protein was localized by Western immunoblot in various fractions of 042 (Panel A) and of outer membrane fractions alone from 042aatC and 042aatD mutants (Panel B). Immunoblot analysis was performed on strains grown in aggR-inducing conditions, comprising high-glucose DMEM for 6 hours. Samples were separated using 10% SDS-PAGE. Western immunoblots were performed with specific polyclonal antibodies against the AatA protein.

Figure 5. SDS-PAGE analysis of Triton X-100-treated culture supernatants from 042 and various mutants. Bacteria were cultured in high-glucose DMEM overnight to induce aggR expression. 0.1% Triton X-100 was added to the culture, bacteria were pelleted by centrifugation and the supernatant was filter-sterilized. The supernatant was treated with one-fourth volume TCA, the precipitate was collected by centrifugation, separated by 15% SDS-PAGE and stained with Coomassie blue. Arrow indicates ca. 10 kDa species.

Figure 6. Role of aat genes in dispersin secretion. (A) Western immunoblot analysis for Aap dispersin in the culture media and the pellet of 042 and 042aatA. Each strain was
cultured in high-glucose DMEM for 6 hours with and without 0.1% Triton X-100, which has been shown to remove the dispersin capsule from strain 042 (15). The supernatant was precipitated with one-fourth volume TCA and the precipitate separated using 15% SDS-PAGE. Western immunoblot was performed by standard methods using anti-Aap polyclonal antibodies. (B) Immunoblot for Aap in 042 and its mutants grown in high glucose DMEM with addition of Triton X-100.

Figure 7. Complementation analysis of 042aat mutants. All experiments are Western immunoblots from high glucose DMEM cultures. Fractions are prepared as described in Experimental Procedures. Inserts of aat clones are indicated in Fig. 1 and Table 1. (A) Western immunoblot for AatA protein. Lane 1 (both panels), 042; Lane 2, 042aatA; Lane 3, 042aatA harboring complete aat cluster clone pJNW, in single copy vector pZC320. (B) Western immunoblot for Aap to demonstrate complementation of aatA mutant. Lane 1, 042; Lane 2, 042aatA; Lane 3, 042aatA(pJNW); Lane 4, 042aatA(pJNPA). Plasmid pJNPA comprises only aatP and aatA genes. (C) Western immunoblot for Aap to demonstrate complementation of aatC and aatD mutants. Lane 1, 042; Lane 2, 042aatC; lane 3, 042aatC(pJNW); lane 4, 042aatD, lane 5, 042aatD(pJNW). (D) Western immunoblot for Aap to demonstrate complementation of aatD mutant. Lane 1, 042; lane 2, 042aatC; lane 3, 042aatC(pSE380aatD); lane 4, 042aatD; lane 5, 042aatD(pSE380aatD). Cultures were grown in high glucose DMEM induced with IPTG as described in Experimental Procedures.

Figure 8. Identification of the likely aatA translational start site by complementation analysis. The predicted aatA gene was cloned downstream of the ara promoter in pBAD30, using primers described in Table 1. pBADaatA-gug initiates from the GUG alternative initiation codon at nucleotide 2594; pBADaatA-aug initiates from the first
methionine of the predicted ORF at nucleotide 2723. Cultures were grown in M9 medium with 0.3% glucose or arabinose. Bacteria were harvested in late log phase and supernatants or whole cell lysates prepared as described in Experimental Procedures. (A) Western immunoblot for Aap in Triton X-100-treated supernatants. (B) Immunoblot to detect Aap in periplasmic fractions of bacteria examined in panel A. (C) Immunoblot to detect the AatA protein in whole cell extracts of cultures examined in panel A. Relevant molecular mass standards are indicated for each blot.

Figure 9. Localization of Aap in aat mutants. Each strain was cultured in high-glucose DMEM with 0.1% Triton X-100 for 6 hours. For supernatant and pellet fractions, bacteria were pelleted by centrifugation and the supernatant proteins precipitated with TCA. TCA precipitates and lysed whole cells were separated using 15% SDS-PAGE and immunoblotted for Aap. Periplasmic fractions were extracted using EDTA-Triton as described in Experimental Procedures.

Figure 10. Scanning electron micrographs of 042 (A), 042aap (B), and 042aatA (C). Bacteria were cultured in high glucose DMEM overnight, then bacteria were pelleted by centrifugation. Pellets were resuspended in one-tenth volume of PBS, applied to silica studs, and stained for scanning electron microscopy. Arrows represent AAF/II fimbriae, which splay out from the bacterium in wild type 042, but which typically lie along the bacterial cell surface in 042aap and 042aatA. Bars 1 µm.

Figure 11. A working model for dispersin secretion via the Aat transporter in EAEC 042. The dispersin protein is secreted into the periplasm in an Aat-independent fashion, most likely via the Sec system; a typical signal sequence is removed in this process. The Aat cluster acts to promote the translocation of the mature dispersin protein across the outer
membrane, where dispersin binds non-covalently to the bacterial cell surface. AatA localizes to the outer membrane, where it may serve as the pore through which dispersin is translocated. The remainder of the Aat complex is shown localized to the inner membrane based on *in silico* analyses, including homology to other ABC transporter systems. Roles for AatP as a permease and AatC as the ATPase can be inferred based on sequence homology; roles for AatB and AatD are not clear, though both AatD and AatB have regions strongly predicted to span the membrane where they may interact with the ATPase/permease complex or mediate interactions with AatA. A, AatA; B, AatB; C, AatC; D, AatD; P, AatP.
Table 1. Bacterial strains and plasmids used in this study.

| Strains | Designation | Characteristics | Reference/source |
|---------|-------------|-----------------|------------------|
| 042     | Wild-type EAEC prototype strain  | 20 |
| 042aatP | 042 harboring pJP5603 integrated into the \textit{aatP} gene. Km$^R$ | This work |
| 042aatA | 042 harboring pJP5603 integrated into the \textit{aatA} gene. Km$^R$ | This work |
| 042aatB | 042 harboring pJP5603 integrated into the \textit{aatB} gene. Km$^R$ | This work |
| 042aatC | 042 harboring pJP5603 integrated into the \textit{aatC} gene. Km$^R$ | This work |
| 042aatD | 042 harboring pJP5603 integrated into the \textit{aatD} gene. Km$^R$ | This work |
| DH5α λpir | K12 \textit{E. coli} lysogenized for the \textit{pir} gene, which permits replication of pJP5603 | 24 |
| S17-1 λpir | Conjugative K12 lysogenized for \textit{pir}. Tet$^R$, Km$^R$ | 24 |
| 042pet | 042 harboring pJP5603 integrated into the \textit{pet} gene. Km$^R$ | 8 |
| 042aggR | 042 carrying TnphoA inserted into the \textit{aggR} gene. Km$^R$ | 27 |
| 042aggR(pBADaggR) | 042aggR carrying the \textit{aggR} gene cloned into pBAD30 to permit expression of AggR in the presence of arabinose | 15 |
| 042aggR(pBAD30) | 042aggR carrying pBAD30 used as a control for 042aggR(pBADaggR) | 15 |

| Plasmids | Designation | Characteristics | Reference/source |
|----------|-------------|-----------------|------------------|
| pET21a(+) | T7 promoter-driven expression vector. Ap$^R$ | Novagen |
| pJP5603 | 3.1-kb R6K suicide plasmid. Km$^R$ | 33 |
| pSE380 | 4.1 kb expression vector; \textit{trc} promoter, \textit{lacIq} Ap$^R$ | Invitrogen |
| PZC320 | 7.5-kb single copy vector. Ap$^R$ | 34 |
| pAatA | 1065-bp fragment of the \textit{aatA} gene cloned into multiple cloning site of pET21a(+) to provide IPTG-inducible expression of the AatA protein as a 6-His fusion, Ap$^R$ | This work |
| pINTP | 517-bp internal fragment of the \textit{aatP} gene in pJP5603 | This work |
| pINTA | 520-bp internal fragment of the \textit{aatA} gene in pJP5603 | This work |
| pINTB | 440-bp internal fragment of the \textit{aatA} gene in pJP5603 | This work |
| pINTC | 300-bp internal fragment of the \textit{aatC} gene in pJP5603 | This work |
| pINTD | 500-bp internal fragment of the \textit{aatD} gene in pJP5603 | This work |
| pJNAD | 4.5-kb fragment encoding \textit{aatA}, \textit{aatB}, \textit{aatC}, and \textit{aatD} cloned into pZC320. Ap$^R$. | This work |
| pJNW | 6.5-kb fragment encoding the complete \textit{aat} cluster cloned into pZC320. Ap$^R$ | This work |
| pJNPA | 3.9-kb fragment encoding \textit{aatP} and \textit{aatA} cloned into pZC320. Ap$^R$ | This work |
|        |                                                                 |      |
|--------|----------------------------------------------------------------|------|
| pBAD30 | High copy number expression vector permitting expression of     | 59   |
|        | foreign genes under control of the arabinose operon promoter.  |      |
|        | Ap^R                                                           |      |
| pBADaggR | *AggR* cloned into multiple cloning site of pBAD30 to permit   | 15   |
|        | expression of AggR in the presence of arabinose. Ap^R         |      |
Table 2. Distribution of the \textit{aat} cluster and \textit{aggR} among EAEC isolates.

| No. | Strain       | \textit{aatA} | \textit{aatBCD} | \textit{aggR} |
|-----|--------------|---------------|----------------|--------------|
| 1   | 042 (Peru)   | +             | +              | +            |
| 2   | 17-2 (Chile) | +             | +              | +            |
| 3   | Brazil 236   | +             | +              | +            |
| 4   | Mexico 60A   | +             | +              | +            |
| 5   | Peru 11145-1 | +             | +              | +            |
| 6   | Peru 11194-2 | +             | +              | +            |
| 7   | Peru 11232-1 | +             | +              | +            |
| 8   | Peru 1132-1  | +             | +              | -            |
| 9   | Peru 1146-2  | +             | +              | -            |
| 10  | Peru 1177-1  | +             | +              | +            |
| 11  | Peru 1192-1  | +             | +              | +            |
| 12  | Peru 133     | +             | +              | +            |
| 13  | Phil DS244-R3| +             | +              | +            |
| 14  | Phil DS61-R2 | +             | +              | -            |
| 15  | Phil DS67-R2 | +             | +              | +            |
| 16  | Thai 103-1-1 | +             | +              | +            |
| 17  | Thai 144-1-1 | +             | +              | +            |
| 18  | Thai 199-1-4 | +             | +              | +            |
| 19  | Thai 253-1-1 | +             | +              | +            |
| 20  | Thai 309-1-1 | +             | +              | +            |
| 21  | Thai 44-1-1  | +             | +              | +            |
| 22  | Thai 6-1-1   | +             | +              | +            |
| 23  | Peru 11223-1 | -             | +              | +            |
| 24  | Peru 1111-1  | -             | -              | +            |
| 25  | Peru 11191-1 | -             | -              | +            |
| 26  | Peru 1172-2  | -             | -              | -            |
| 27  | Phil DS65-R3 | -             | -              | -            |
| 28  | Thai 435-1-1 | -             | -              | -            |
| 29  | Thai 501-1-1 | -             | -              | +            |
| 30  | Japan 101-1  | -             | -              | -            |
| 31  | Serbia 1096  | -             | -              | -            |
Table 3. Primers used in this study.

| Application | Primer name | Gene(s) amplified | Map coordinates | Sequence (5' to 3') | RE* |
|-------------|-------------|-------------------|----------------|---------------------|-----|
| PCR         | aatA-F      | aatA              | 2723-2744      | acggatccatgttacgatataaatag | BamHI |
|             | aatA-R      | aatA              | 3766-3787      | acgaattccatttccctgtaattaggaatg | EcoRI |
|             | aatPint-F   | aatP              | 1623-1642      | actctagatagcacttaaaccgttagg | XbaI |
|             | aatPint-R   | aatP              | 2120-2139      | acgaattcctggctttatgtggataggg | EcoRI |
|             | aatAint-F   | aatA              | 2893-2910      | actctagatgaaatgttgagagagatgag | XbaI |
|             | aatAint-R   | aatA              | 3395-3412      | acgaattccagacgacagactacactg | EcoRI |
|             | aatBint-F   | aatB              | 3861-3880      | acctctagattggacagcatgattagag | XbaI |
|             | aatBint-R   | aatB              | 4281-4300      | acgaattctttcgttataactgcagcgg | EcoRI |
|             | aatCint-F   | aatC              | 4610-4630      | acctctagattggacagactcaactgg | XbaI |
|             | aatCint-R   | aatC              | 4889-4909      | acgaattccggagaagatgatacatta | EcoRI |
|             | aatDint-F   | aatD              | 5361-5380      | acctctagattggacagactcttaagg | XbaI |
|             | aatDint-R   | aatD              | 5841-5860      | acgaattctccatattgtacagggagagagatgag | EcoRI |
|             | aatW-F      | aat cluster       | 001-022        | acggatccagagctgggaggggtaggg | BamHI |
|             | aatW-R      | aat cluster       | 6446-6465      | acgccggctcagctttgtgcgaacgc | NcoI |
|             | aat-PA-R    | aatP and aatA     | 3864-3886      | acgccggctcagctttgtgcgaacgc | NcoI |
|             | aatD-F      | aatD              | 5142-5158      | acggatccatgaaattctctttgtttgtttttg | BamHI |
|             | aatD-R      | aatD              | 6330-6356      | acctctagatcatctctctgtaataaagggtctc | XbaI |
|             | aatAgtg-F   | aatA              | 2552-2576      | acgaattctgctagcatactctttacttcc | EcoRI |
|             | aatAatg-F   | aatA              | 2723-2744      | acgaattctgcttaccagatataaatatag | ScaI |
|             | aatA(BAD)-R | aatA              | 3766-3787      | acctctagatcctttctgtctctgtaattag | XbaI |
| RT-PCR      | aatP-F      | aatP              | 1432-1457      | ctttgcaatattatcttaaatagggg | BamHI |
|             | aatP-R      | aatP              | 2522-2548      | atctctttttattcgtttacaggg | BamHI |
|             | aatA-F      | aatA              | 2723-2744      | atgttaccagatataaatatag | BamHI |
|             | aatA-R      | aatA              | 3766-3787      | cattcccctgtattggaatg | BamHI |
|             | aatB-F      | aatB              | 3687-3709      | atgaattccagagaattgttccag | BamHI |
|             | aatB-R      | aatB              | 4483-4508      | ctataaacatcattataatactaatcacc | EcoRI |
|             | aatC-F      | aatC              | 4501-4523      | atgtagatggtaaaatacaataaaacat | EcoRI |
|             | aatC-R      | aatC              | 5108-5130      | ctatgatattagattgtg | EcoRI |
|             | aatD-F      | aatD              | 5142-5166      | atgaattccgtattgtagtaattg | EcoRI |
|             | aatD-R      | aatD              | 6328-6356      | ctatctgttgtaataaaaggtctc | EcoRI |
|             | aatPmid-F   | aatP              | 2004-2023      | ctcgataacagagtcaatgc | EcoRI |
|             | CAT-F       | cat               | NA             | tcaacctgtggacactttcatgtg | EcoRI |
|             | CAT-R       | cat               | NA             | tcaacctatcgactgtg | EcoRI |

*Restriction endonuclease cleavage site introduced; shown as underlined sequence in primer. # Correspond to coordinates in Genbank accession number AY351860.
Figure 1
Figure 2
Figure 2B
Figure 4

A

| AatA-nulls | Whole cell | Periplasm | Outer membrane |
|------------|------------|-----------|---------------|
| 042 | aatA | 042 | aatA | 042 | aatA | 042 | aatA |

B

| 042 | aatA | 042 | aatC | 042 | aatD |

Figure 5

| Marker | 042 | 042 aatA | 042 aatC | 042 aatD | 042 pet |
|--------|-----|----------|----------|----------|---------|

17kD
9kD
Figure 6

A

|       | Supernatant |         | Pellet |         |
|-------|-------------|---------|--------|---------|
| 0.1% Triton | 042   | 042aatA | 042   | 042aatA |
| -     | +          | -       | +      | -       |

B

|          | 042WT | 042aatP | 042aatA | 042aatC | 042aatD | 042ppt |
|----------|-------|---------|---------|---------|---------|--------|
| 042      |       |         |         |         |         |        |
|          |       |         |         |         |         |        |
|          |       |         |         |         |         |        |
|          |       |         |         |         |         |        |
|          |       |         |         |         |         |        |
|          |       |         |         |         |         |        |
|          |       |         |         |         |         |        |
|          |       |         |         |         |         |        |
Figure 7

A

| Whole cells | Outer membrane |
|-------------|----------------|
| 1           | 1              |
| 2           | 2              |
| 3           | 3              |

B

| 1 | 2 | 3 | 4 |
|---|---|---|---|

C

| 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|

D

| 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|
Figure 8

Figure 9
Figure 10
Figure 11
The export of coat protein from enteroaggregative escherichia coli by a specific ATP-binding cassette transporter system

Junichiro Nishi, Jalaluddin Sheikh, Kenji Mizuguchi, Ben Luisi, Valerie Burland, Adam Boutin, Debra J. Rose, Frederick R. Blattner and James P. Nataro

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