Characterization and Functional Analysis of AatB, a Novel Autotransporter Adhesin and Virulence Factor of Avian Pathogenic *Escherichia coli*

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Autotransporter (AT) proteins constitute a large family of extracellular proteins that contribute to bacterial virulence. A novel AT adhesin gene, *aatB*, was identified in avian pathogenic *Escherichia coli* (APEC) DE205B via genomic analyses. The open reading frame of *aatB* was 1,017 bp, encoding a putative 36.3-kDa protein which contained structural motifs characteristic for AT proteins: a signal peptide, a passenger domain, and a translocator domain. The predicted three-dimensional structure of AatB consisted of two distinct domains, the C-terminal β -barrel translocator domain and an N-terminal passenger domain. The prevalence analyses of *aatB* in APEC indicated that *aatB* was detected in 26.4% (72/273) of APEC strains and was strongly associated with phylogenetic groups D and B2. Quantitative real-time reverse transcription-PCR analyses revealed that AatB expression was increased during infection *in vitro* and *in vivo*. Moreover, AatB could elicit antibodies in infected ducks, suggesting that AatB is involved in APEC pathogenicity. Thus, APEC DE205B strains with a mutated *aatB* gene and mutated strains complemented with the *aatB* gene were constructed. Inactivation of *aatB* resulted in a reduced capacity to adhere to DF-1 cells, defective virulence capacity *in vivo*, and decreased colonization capacity in lung during systemic infection compared with the capacities of the wild-type strain. Furthermore, these capacities were restored in the complementation strains. These results indicated that AatB makes a significant contribution to APEC virulence through bacterial adherence to host tissues *in vivo* and *in vitro*. In addition, biofilm formation assays with strain AAEC189 expressing AatB indicated that AatB mediates biofilm formation.

Several common strategies are used by pathogenic organisms to sustain themselves and overcome host barriers, such as the firm adhesion of the microorganism to host cells (1). Pathogenic bacteria use surface structures, such as cell adhesin molecules, to adhere to host cells, and this adhesion helps bacteria overcome host barriers. Distinct bacterial adhesins have been identified, including fimbrial and nonfimbrial adhesins (1, 2). The autotransporter (AT) adhesins secreted through type V transport pathways are nonfimbrial adhesins, which are present in a number of Gram-negative pathogenic bacteria (3–6). In principal, all members of the AT family share conserved structural features: a secretion signal peptide in the N terminus, a conserved C-terminal translocation domain inserting into the outer membrane of the bacterial cell, and a variable internal functional passenger domain, which is translocated to the bacterial surface (3–5). The trimeric autotransporters (TAAs) represent a subfamily of AT proteins and are defined by the presence of a very short C-terminal translocator domain that forms highly stable trimers in the outer membrane (7–9). All TAA proteins identified to date display bacterial adherence, adhesion, and aggregation activities to mediate bacterial interactions with either host cells or extracellular matrix (ECM) proteins. The conventional ATs are involved in the adhesion, cytotoxicity, and lipase or protease activities of bacteria (4, 7, 8, 10–15).

There are many ATs and TAAs that have been identified in different Gram-negative bacterial pathogens, and these play different roles in the pathogenicity of bacteria (14, 16–21). Three AT proteins in avian pathogenic *Escherichia coli* (APEC), including Tsh (temperature-sensitive hemagglutinin), Vat (vacuolating autotransporter toxin), and AatA (APEC autotransporter adhesin),

were found to play roles in APEC pathogenicity (22–26). The YadA adhesin from *Yersinia enterocolitica* is the best-characterized TAA protein and mediates adherence and serum resistance (27, 28). TAAs Hia and Hsf were identified in *Haemophilus influenzae* and mediate adherence to host cells (29, 30). *Neisseria meningitidis* TAAs include NadA, which mediates the invasion of epithelial cells, and NhhA, which mediates adhesion to human epithelial cells and ECM components (11, 31). Saa is a TAA adhesin involved in the adherence and aggregation of Shiga toxin-producing *Escherichia coli* (STEC) (32). A new member of the TAA family, UpaG, has been identified in uropathogenic *E. coli* (UPEC) and promotes cellular aggregation and biofilm formation in strain CFT073 (33). SadA, a TAA from *Salmonella enterica* serovar Typhimurium, promotes biofilm formation and provides limited protection against infection (10).

Systemic infections caused by APEC are economically devastating to poultry industries. APEC enters and colonizes the avian respiratory tract by inhalation of fecal dust, leading to localized infections such as airsacculitis and pneumonia. In certain cases,

Received 30 January 2013 Returned for modification 11 March 2013

Accepted 21 April 2013

Published ahead of print 29 April 2013

Editor: S. M. Payne

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doi:10.1128/IAI.00102-13

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
DE205B	O2:K1 Nal ^r	38–40
DE205BΔ <i>aatB</i>	DE205B Δ <i>aatB</i>	This study
DE205BPΔ <i>aatB</i>	DE205B Δ <i>aatB</i> with plasmid pUC18	This study
DE205BCΔ <i>aatB</i>	DE205B Δ <i>aatB</i> with plasmid pUC18- <i>aatB</i>	This study
AAEC189	MG1655 Δ <i>fim</i>	43
AAEC189P	AAEC189 with plasmid pUC18	38
AAEC189P- <i>aatB</i>	AAEC189P with plasmid pUC18- <i>aatB</i>	This study
DH5α	F [−] Δ(<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K [−] m _K ⁺) <i>phoA supE44</i> λ [−]	Tiagen
BL21(DE3)	F [−] <i>ompT hsdS_B</i> (r _B [−] m _B [−]) <i>gal dcm</i> (DE3)	Tiagen
Plasmids		
pET28a (+)	Kan, F1 origin, His tag	Novagen
pET28a- <i>aatB</i>	pET28a (+) carrying <i>aatB</i> gene	This study
pMD18-T vector	Amp <i>lacZ</i>	TaKaRa
pUC18	Amp <i>lacZ</i>	TaKaRa
pUC18- <i>aatB</i>	pUC18 carrying <i>aatB</i> ORF and its putative promoter	This study
pKD46	Amp; expresses bacteriophage λ red recombinase	48
pKD4	<i>kan</i> gene, template plasmid	48
pCP20	Cm Amp, yeast Flp recombinase gene, FLP	48

APEC can spread into various internal organs, typically causing pericarditis, perihepatitis, peritonitis, salpingitis, and other extraintestinal diseases (34, 35). Although research on APEC has increased greatly over the years, the molecular basis of APEC pathogenesis is not yet fully understood. However, the discovery and characterization of ATs provide new insight not only into the diverse functions of these proteins but also into the various modes of pathogen-host interactions (36). In the present study, a novel AT adhesin gene, *aatB*, was identified in APEC DE205B by whole-genome sequence analysis. The novel AT adhesin gene was named *aatB* (APEC autotransporter adhesin B) to differentiate it from another AT gene, *aatA* (22, 23). The distribution and function of the novel AT adhesin AatB were also examined to elucidate the precise role of AatB in APEC pathogenicity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. The APEC strain DE205B was isolated from the brain of a duck with septicemia and neurological symptoms and belonged to the phylogenetic *E. coli* reference (ECOR) group B2, as determined by phylogenetic analysis using multiplex PCR (37, 38). This strain harbored the virulence-associated genes *tsh*, *mat*, *fyuA*, *irp2*, *iucD*, *iutA*, *iss*, *aatA*, *aatB*, *vat*, *malX*, *fimC*, *ompA*, *ibeA*, *ibeB*, *yijp*, *gimB*, and *aslA* but was negative for *papC* and *hlyA* by PCR analysis (38–40). Strain DE205B was used for infection studies, mutant construction, and functional assays.

To determine the distribution of the putative APEC adhesin gene *aatB*, a strain collection (*n* = 273) available at the College of Veterinary Medicine, Nanjing Agricultural University (Nanjing, China), was screened. The ECOR groups of single isolates were determined using triplex PCR, as described previously (23, 37). *E. coli* strain DH5α was used for cloning procedures, BL21(DE3) cells were used for protein expression (41, 42), and the *fim*-negative *E. coli* strain AAEC189 (43) was used in biofilm formation and aggregation experiments. All *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C with aeration. When necessary, the LB medium was supplemented with appropriate antibiotics unless otherwise specified: ampicillin (Amp; 100 μg ml^{−1}) and kanamycin (Kan; 50 μg ml^{−1}).

DNA and genetic manipulations. DNA manipulations and transformations were performed using standard methods. All restriction enzymes were purchased from TaKaRa (Dalian, China). Plasmid DNA was isolated using a High Pure plasmid miniprep kit (Invitrogen, Shanghai, China). PCR products and DNA extracted from agarose gels were purified using an agarose gel DNA fragment recovery kit (TaKaRa) according to the manufacturer’s guidelines. For *aatB* sequencing and expression, TaKaRa PrimeSTAR HS DNA polymerase was used for PCR, whereas 2× PCR premix (Tiagen Biotech, Beijing, China) was used in screening assays according to the manufacturer’s instructions. The descriptions and sequences of the primers used in this study are provided in Table 2. The prevalence of the *aatB* gene was assessed by PCR using primers *aatB*-F and *aatB*-R.

DNA and protein sequence analysis. DNA sequences were analyzed using DNASTar Lasergene 7 software and the online Basic Local Alignment Search Tool (BLAST) program (BLASTN and BLASTX) from the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/>). Promoter prediction analyses were carried out with prediction program tools available at http://www.fruitfly.org/seq_tools/promoter. Signal peptide prediction analyses were carried out with the SignalP, version 4.0, server (<http://www.cbs.dtu.dk/services/SignalP/>). For phylogenetic analyses of AT proteins, ClustalW analyses were performed using Molecular Evolutionary Genetic Analysis (MEGA), version 4.0, software (<http://www.megasoftware.net/mega4/mega.html>). Phylogenetic neighbor-joining trees were obtained using complete protein sequences of autotransporter proteins. The I-TASSER online server was used to predict the three-dimensional (3D) structure of the amino acid sequences (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) (44–46).

Expression of AatB, antibody production, and immunoblotting. The *aatB* open reading frame (ORF), except for the sequence encoding the signal peptide (1 to 69 bp), was amplified with primers ZGXX1F and ZGXX2R with the addition of EcoRI and XhoI recognition sites (Table 2). The obtained PCR fragments were subcloned into the pMD18-T vector for sequencing. The *aatB* fragment was then digested with EcoRI and XhoI, followed by ligation into EcoRI/XhoI-digested pET28a(+) vectors (Novagen, Madison, WI). The resulting plasmid, pET28a-*aatB*, was transformed into competent *E. coli* BL21(DE3) cells, and the AatB protein was expressed by isopropyl-beta-D-thiogalactopyranoside (IPTG) induction at a final concentration of 1 mM. Protein purification was performed

TABLE 2 Primers used in this study

Primer	Sequence (5' to 3') ^a	Target gene or region
aatB-F	ATGAAACGTAAACGTAGCTTTACTGG	<i>aatB</i>
aatB-R	TTAGAACGAATATCCTACGCCGACAC	<i>aatB</i>
ZGXX1F	CCGGAATTCGGTGATACCATTAAGCGGC	<i>aatB</i>
ZGXX2R	CCGCTCGAGGAACGAATATCCTACGCCG	<i>aatB</i>
ZGXX3F	TTATTTGTTTCTTATAGAAACATCAATGCGACTTATTTGAACAAGAGA	pKD4
	AAATGATGTAGGCTGGAGCTGCTTCGA	
ZGXX4R	AAGAGGTGAAACCCGGCAAGCTTCCTTGCTCTTATTTATATTGAGGATT	pKD4
	GAAATACATATGAATATCCTCCTTAG	
ZGXX5F	CCGGAATTCCTTAGCACACATCAGGAACCG	Upstream region of <i>aatB</i>
ZGXX6R	CGCGGATCCGAAGTGGGTGAACCTACAGCC	Downstream region of <i>aatB</i>
k1	CAGTCATAGCCGAATAGCCT	pKD4
k2	CGGTGCCCTGAATGAAGTGC	pKD4
aatBup-F	CCAGTAATCAGGTTGGTCCAGGTAA	Upstream region of <i>aatB</i>
aatBdown-R	CAGCGTGAACCGAGGAGGCTAATAA	Downstream region of <i>aatB</i>
dnaE RT-F	ATGTCGGAGGCGTAAGGCT	<i>dnaE</i>
dnaE RT-R	TCCAGGGCGTCAGTAAACAA	<i>dnaE</i>
aatB RT-F	ACTGACGCCAGCAAACGCA	<i>aatB</i>
aatB RT-R	CAGTCGCGGCGAGCGTTTTT	<i>aatB</i>

^a Restriction sites are underlined.

using a HisTrap high-performance column (GE Healthcare, Shanghai, China) according to the manufacturer's guidelines. The final protein concentration was determined by the Bradford method using a SmartSpec 3000 spectrophotometer (Bio-Rad, Shanghai, China) (47). Expression of the fusion protein was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Polyclonal anti-AatB antiserum was raised in New Zealand White rabbits by subcutaneous immunization, as described previously (23, 38, 39). The immunization dose was 300 µg of purified AatB protein mixed with an equal volume of Montanide ISA 206 adjuvant (SEPPIC, Lyon, France). Immunizations were repeated three times at 2-week intervals. At 10 days after the final immunization, serum samples were collected.

The production of convalescent-phase and immunized anti-DE205B antiserum was performed. Ducks were infected intramuscularly with APEC DE205B twice. At 10 days postinfection, serum was collected from the survivor.

For immunoblot analysis, protein samples were loaded into 12% SDS gels and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) using a semidry blotting apparatus (TE77; Amersham Pharmacia Biotech), as described previously (23, 38, 39). Anti-AatB and anti-DE205B immune sera were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG or anti-duck IgG was used as the secondary antibody, and 3,3'-diaminobenzidine (DAB) was used as the substrate. For the detection of AatB expression in each strain, enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) was used.

Construction of an *aatB* deletion mutant. The *aatB* gene deletion mutant was constructed according to the methods of Datsenko and Wanner (48). The *aatB* gene was replaced with a kanamycin resistance cassette, which was PCR amplified from plasmid pKD4 using primers ZGXX3F and ZGXX4R (Table 2). The PCR products were purified and transformed by electroporation into strain DE205B containing the bacteriophage lambda red recombinase expression plasmid pKD46. After electroporation, samples were incubated at 37°C for 1 h in superoptimal broth with catabolite repression (SOC) broth and plated on LB agar with Kan to select for *aatB* mutants. Mutants were confirmed by PCR and sequenced using primers k1 and k2 in combination with primers aatBup-F and aatBdown-R, whose sequences flanked the *aatB* region. The Kan resistance cassette was cured by transforming the pCP20 plasmid into the mutant and selecting for a Kan-sensitive mutant strain, which was finally designated strain DE205BΔ*aatB*.

Complementation of the *aatB* mutant and fimbria-negative *E. coli* strain AAEC189. For complementation studies, the *aatB* operon, including its putative promoters, was amplified and subcloned into the pUC18 vector using the primer pair ZGXX5F and ZGXX6R with restriction enzyme recognition sites EcoRI and BamHI, as described previously (23, 38, 39). The colonies were tested for the presence of *aatB*. The mutant strain DE205BΔ*aatB* was transformed with the control vector pUC18 and the recombinant plasmid pUC18-*aatB*, and the resulting strains were designated DE205BpΔ*aatB* and DE205BCΔ*aatB*, respectively. For complementation of the *aatB* gene in fimbria-negative *E. coli* strain AAEC189, plasmids pUC18 and pUC18-*aatB* were transformed into AAEC189, generating strains AAEC189P (AAEC189/pUC18) and AAEC189P-*aatB* (AAEC189/pUC18-*aatB*), respectively.

Biofilm formation assay. Biofilm formation assays were performed in 96-well plates using a previously described method (38). Briefly, strains were grown to the stationary phase in LB medium at 37°C and then diluted 1/100 in LB medium supplemented with 5 g liter⁻¹ of glucose. Aliquots of 200 µl for each dilution were dispensed per well into a microtiter plate. Negative-control wells contained uninoculated medium. Plates were cultured aerobically without shaking at 37°C for 24 h. The medium of the plates was then poured off and the plates were washed twice with sterile phosphate-buffered saline (PBS). Microplates were then stained with 200 µl of 1% (wt/vol) crystal violet for 30 min, washed four times with PBS to remove unbound dye, and air dried for 1 h. After drying, adherent cells were resuspended with 200 µl of an 80:20 (vol/vol) solution of ethanol and acetone. The absorbance was measured at 595 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad microplate reader, model 550). All tests were carried out three times.

DF-1 cell adhesion assays. The adhesion capacities of wild-type strain DE205B, mutant strain DE205BΔ*aatB*, and complementation strain DE205BCΔ*aatB* were studied with cultured chicken embryo fibroblasts, as previously described (23, 39). Briefly, semiconfluent monolayers of DF-1 cells were washed and incubated with Dulbecco's modified Eagle's medium (DMEM) without fetal bovine serum containing bacteria at a multiplicity of infection of 100. Infected monolayers were incubated for 2 h at 37°C under a 5% CO₂ atmosphere to allow cell adhesion. Then, DF-1 cells were washed three times with PBS following treatment with 1% Triton X-100, and the number of adherent bacteria was determined on LB agar plates in a dilution series. Negative-control wells containing only DF-1 cells were used in all experiments. The assay was performed three times in triplicate. Results are expressed as percent adhesion compared to that of parent strain DE205B.

The adherence inhibition experiments were done as described previously (23). Briefly, bacteria were pretreated with anti-AatB serum before infection of DF-1 cells. Adherence assays were carried out as described above. The assay was performed three times in duplicate.

Animal experiments. The virulence of each strain was evaluated in duck and mouse models, as described previously (38, 39). The 50% lethal dose (LD₅₀) was ascertained to determine the differences in virulence between the parental, mutant, and complementation strains. Strains were grown to the exponential phase and collected, washed twice in PBS, and then adjusted to the appropriate doses. Seven-day-old ducks were inoculated intratracheally with 0.2 ml of each bacterial suspension containing different numbers of CFU. The numbers of bacterial CFU contained in the injected inoculum were confirmed by plating on LB agar. Negative controls were injected with PBS. Eight animals were used per dose. Mortality was monitored until 7 days postinfection. The experiment was repeated three times. The LD₅₀ of each strain was also tested with 8-week-old imprinting control region (ICR) mice. LD₅₀ results were calculated using the Spearman-Kärber method (49).

Animal experiments were also carried out to determine the colonization capabilities during systemic infections, as described previously (38, 39, 50). Briefly, groups of six 7-day-old ducks were infected intratracheally with a bacterial suspension containing 10⁸ CFU. At 24 h postinfection, ducks were euthanized and dissected. Bacteria were isolated from the lungs and brains as follows: organ samples were weighed, suspended in PBS (1 ml/g), homogenized, appropriately diluted, and plated on LB agar containing nalidixic acid to determine the number of bacteria colonizing the internal organs of the ducks during systemic infection. Animal experiments were also conducted with mice. Groups of 8-week-old ICR mice were infected intraperitoneally with a bacterial suspension containing 10⁷ CFU. At 18 h postinfection, mice were euthanized and dissected and bacteria were isolated as described above.

All animal experimental protocols were approved by the Laboratory Animal Monitoring Committee of Jiangsu Province, China.

qRT-PCR. The levels of *aatB* expression *in vitro* and *in vivo* were studied by quantitative real-time reverse transcription-PCR (qRT-PCR). The RNA was isolated from logarithmic-phase bacteria *in vitro* using an E.Z.N.A. bacterial RNA kit (Omega Bio-Tek, Beijing, China) according to the manufacturer's instructions. Total RNA was isolated from the infected cells and duck tissues using the TRIzol reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Contaminating DNA was removed from the samples with RNase-free DNase I (TaKaRa). cDNA synthesis was performed using a PrimeScript RT reagent kit (TaKaRa) according to the manufacturer's protocol. qRT-PCR was performed to determine the levels of *aatB* transcription using SYBR *Premix Ex Taq* DNA polymerase (TaKaRa) and gene-specific primers (Table 2). The relative gene expression was normalized to the expression of the housekeeping gene *dnfE* via the $\Delta\Delta C_T$ method (where C_T is the threshold cycle) (51). The PCR efficiency (>90%) for each of the genes was verified via standard dilution curves.

Statistical analyses. Statistical analyses for *in vitro* and *in vivo* experiments were carried out using the GraphPad software package (GraphPad Software, La Jolla, CA). One-way analysis of variance (ANOVA) was used for analysis of the adhesion assay data *in vitro*, and two-way ANOVA was performed on the qRT-PCR results. The animal infection study analysis was performed using the nonparametric Mann-Whitney U test. The mean values are shown in the figures. Statistical significance was established at a *P* value of <0.05.

Nucleotide sequence accession number. The sequence of the novel putative adhesin/invasin gene *aatB* was submitted to GenBank and may be found under accession number JX402062.

RESULTS

Characterization of novel putative autotransporter adhesin AatB. Analysis of the genome sequence of APEC DE205B revealed the novel putative adhesin/invasin gene *aatB* (GenBank accession

no. JX402062), which was flanked by genes encoding hypothetical proteins. The ORF of *aatB* was 1,017 bp, which encodes a hypothetical 36.3-kDa protein consisting of 338 amino acid (aa) residues. The sequence analysis revealed that *aatB* was likely a single gene locus. Promoter prediction analysis with 200 bp upstream of the ATG sequence showed three possible transcriptional start sites at positions −193 (*P* = 0.97), −161 (*P* = 0.97), and −128 (*P* = 0.96) relative to the ATG of the *aatB* ORF in DE205B.

Amino acid sequence analysis of potential AatB proteins revealed that AatB comprised a signal peptide (1 to 23 aa) at the N terminus and a YadA-like C-terminal region, which were the outer membrane proteins and β domain of ATs. Moreover, the putative passenger domain of AatB shared some amino acid sequence identity with the domain of TAA Hia of *H. influenzae* (Fig. 1A). Moreover, BLASTP analysis indicated that the AatB protein was homologous to an AT adhesin or an adhesin/invasin-link protein. Thus, we can assume that the *aatB* gene likely encoded a novel AT adhesin.

The 3D structure of AatB was predicted using the I-TASSER online server (44–46). As shown in Fig. 1B, the predicted structure of AatB consisted of two distinct domains, the C-terminal β -barrel translocator domain and an N-terminal passenger domain. The β -barrel domain has 4 antiparallel β strands, which was in accordance with other TAAs, such as YadA, Hia, and Saa (8, 27, 30, 32). Like other TAAs, the β domain is assembled from three subunits that each contributes 4 β strands (8, 27, 30, 32). Although the overall structure of the β -barrel domain of AatB is similar to that of other TAAs, there were differences in the characterization of the hydrophilic core. The length and diameter of the hydrophilic core of AatB were about 42 Å and 26 Å, respectively. The entire passenger domain was well ordered and displayed the characteristic alpha-beta-alpha fold. This passenger domain structure might contribute to the interactions (both electrostatic and hydrogen bond interactions) with the barrel domain (Fig. 1B). The membrane anchor was the only homologous domain throughout the TAAs, and the structural predictions of AatB showed strong similarity to those of the TAAs that have been characterized, such as YadA, Hia, and Saa (8, 27, 30, 32). According to these data, AatB looks more like a TAA rather than a classical AT.

To determine the relation of AatB to other autotransporter family members, ClustalW analyses were performed with 28 protein sequences from previously known adhesins of the autotransporter family, including proteins from *E. coli*, *S. Typhimurium*, *N. meningitidis*, *H. influenzae*, *Haemophilus ducreyi*, *Y. enterocolitica*, *Moraxella catarrhalis*, *Helicobacter pylori*, *Xylella fastidiosa*, *Bordetella pertussis*, and the newly identified APEC AT adhesin AatB. As shown in the phylogenetic (neighbor-joining) tree, AatB was clustered within a group together with the TAAs Saa, Hia, and Hsf, which mediate adherence to host cells (Fig. 2) (29, 30, 32).

Prevalence of the *aatB* gene in APEC. A collection of APEC strains was screened to determine the presence of the *aatB* gene via PCR. As shown in Table 3, 26.4% (72/273) APEC isolates were positive for *aatB*. For all strains examined in this study, our results were compared with the phylogenetic ECOR group data. The *aatB* gene was detected in 3.9% (5/128) of phylogenetic ECOR group A members, 10.9% (6/55) of group B1 members, 69.2% (18/26) of group B2 members, and 67.2% (43/64) of group D members. Furthermore, of the isolates positive for *aatB*, 5 belonged to ECOR group A (6.9%), 6 belonged to group B1 (8.3%), 18 belonged to group B2 (25%), and 43 belonged to group D (59.7%). Thus,

FIG 1 *In silico* analysis of AT adhesin AatB. (A) Schematic illustration of the domain in AatB. The signal peptide, passenger domain, and translocator domain are indicated. The passenger domains are similar to those of TAA Hia and pfam11932. The translocator domain is similar to the YadA superfamily protein. (B) Predicted 3D structure of autotransporter adhesin AatB. (a) Backbone representation viewed from the side, with the protein colored by a gradient from blue at the N terminus to yellow at the C terminus. The translocator and passenger domains are indicated. The hydrophilic core of the translocator domain is approximately 42 Å long and has a diameter of approximately 26 Å. (b) Surface view of AatB with the translocator and passenger domains.

more than 60% of the APEC strains belonging to phylogenetic groups B2 and D were *aatB* positive. Moreover, phylogenetic ECOR group D was the predominant group for isolates harboring *aatB*.

Expression and purification of adhesin AatB from strain DE205B. The recombinant vector pET28a-*aatB* was successfully expressed in *E. coli* BL21(DE3) under the control of the IPTG-inducible T7 promoter. The molecular mass of the fusion protein AatB was 38.4 kDa (Fig. 3A). The purified AatB protein concentration was 0.6 mg ml⁻¹.

Deletion of *aatB* does not affect growth kinetics of APEC strain DE205B. A clean *aatB* isogenic mutant strain, DE205BΔ*aatB*, was generated using the methods described previously (48). For genetic complementation, the ORF and putative promoter of *aatB* from DE205B were amplified and cloned into plasmid pUC18. The resulting vector, pUC18-*aatB*, was transformed into mutant strain DE205BΔ*aatB*, yielding complementation strain DE205BCΔ*aatB*. No significant differences in generation times or final optical densities were observed between any of the three strains during growth in LB medium (data not shown).

AatB was expressed and triggers antibody production in duck. The expression of *aatB* in the wild-type, mutant, and complementation strains was compared by SDS-PAGE. No differences

in protein patterns between the wild-type and mutant strains were detected (data not shown). Thus, immunoblotting was performed using our anti-AatB serum for detecting *aatB* expression in each strain. As shown in Fig. 3B, incubation with anti-AatB led to the detection of protein bands of the expected size for AatB in the total lysate extract of wild-type strain DE205B and complementation strain DE205BCΔ*aatB* but not in the total lysate extract of mutant strains DE205BΔ*aatB* and DE205BPΔ*aatB*. This result indicated that AatB was expressed in LB medium under laboratory conditions. Furthermore, this result also verified the construction of the *aatB* mutant strain.

To determine whether *aatB* was expressed *in vivo* and triggered antibody production during infection, convalescent-phase and immunized anti-DE205B serum was raised. Then, purified AatB protein was transferred to a polyvinylidene fluoride membrane and anti-DE205B serum was used as the primary antibody. As shown in Fig. 3C, incubation with anti-DE205B led to the detection of bands of purified AatB protein, indicating that AatB elicited an antibody response in the host.

AatB expression was increased during infection *in vitro* and *in vivo*. To determine the level of *aatB* expression during infection *in vitro* and *in vivo*, qRT-PCR was performed. RNA was isolated from DF-1 cells and control DMEM infected by DE205B. Then, cDNA synthesis and qRT-PCR were carried out. Our analysis re-

FIG 2 Phylogenetic tree of autotransporter adhesins, including AatB. The phylogenetic trees were calculated with the neighbor-joining algorithm on the basis of a ClustalW multiple alignment of sequences of 28 proteins from known autotransporter adhesins family, including AatB. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Protein sequences were obtained from the NCBI database. Symbols indicate the species: *, *Escherichia coli*; Δ, *Haemophilus influenzae*; #, *Neisseria meningitidis*; +, *Yersinia enterocolitica*; °, *Haemophilus ducreyi*; §, *Moraxella catarrhalis*; &, *Helicobacter pylori*; ✕, *Xylella fastidiosa*; \$, *Salmonella Typhimurium*; and \$, *Bordetella pertussis*.

vealed that significantly increased *aatB* transcription (3.8-fold) was observed during APEC infection compared to that observed in DMEM ($P < 0.01$) (Fig. 4).

On the other hand, the level of *aatB* expression in strain DE205B from lung tissue of ducks was analyzed by qRT-PCR. The results showed that the level of *aatB* expression was significantly increased by 7.42-fold *in vivo* ($P < 0.01$) (Fig. 4). These results indicated that AatB expression is increased during infection *in vitro* and *in vivo*, suggesting that AatB might be involved in the infection and pathogenicity of APEC DE205B.

AatB contributes to biofilm formation of *E. coli*. AT proteins participate in bacterial adherence, aggregation, and biofilm formation (4, 7, 8). Thus, the roles of AatB in cell aggregation and biofilm formation were determined. The capacity of mutant strain DE205BΔ*aatB* to produce biofilm was not statistically significantly different from that of wild-type DE205B and complementation strain DE205BCΔ*aatB* (data not shown). Thus, the complementation of *aatB* in *E. coli* strain AAEC189 was performed and resulted in a significantly ($P < 0.05$) higher level of biofilm

formation compared with that for the control strain AAEC189P (Fig. 5). These results indicated that AatB contributes to the biofilm formation of *E. coli*. However, no differences in aggregation between wild-type strain DE205B and mutant strain DE205BΔ*aatB* were detected. Furthermore, the aggregation capacity of AAEC189P was similar to that of AAEC189P-*aatB*, indicating that AatB has no role in cell aggregation of *E. coli*.

AatB facilitates APEC adhesion to DF-1 cells. The role of AatB in the adhesion to and invasion of APEC in avian cell lines was determined. DF-1 cells were infected with strains DE205B, DE205BΔ*aatB*, and DE205BCΔ*aatB* and the complementation strain DE205BCΔ*aatB*. The number of bacteria adhering to and invading the DF-1 cells was estimated after washing and plating the bacteria for determination of viable titers. However, the invasion capacity of mutant strain DE205BΔ*aatB* was similar to that of wild-type strain DE205B and complementation strain DE205BCΔ*aatB*, indicating that AatB had no role in APEC invasion of DF-1 cells (data not shown). As shown in Fig. 6A, there was a significant reduction of 53% in the adherence of mutant strain DE205BΔ*aatB* compared with that of parental strain DE205B ($P < 0.01$). Furthermore, the adhesion capacity was restored in complementation strain DE205BCΔ*aatB*, the level of which was statistically significantly increased compared to that for strain DE205BΔ*aatB* ($P < 0.01$). These results suggested that AatB enhanced the capacity of APEC to adhere to DF-1 cells.

In a complementary adhesion inhibition approach, DF-1 cells were infected with specific anti-AatB antibody-pretreated bacteria. Bacteria pretreated with preimmune serum served as a control. The anti-AatB antibody could significantly inhibit the adhesion of DE205B compared to the adhesion for the negative control ($P < 0.01$) (Fig. 6B). In conclusion, we can assume that AatB is involved in APEC adhesion to DF-1 cells.

***aatB* deletion attenuates APEC strain DE205B virulence *in vivo*.** To determine the effect of AatB on APEC DE205B virulence, the LD₅₀ values of the parental, mutant, and complementation strains were evaluated in the duck and mouse models. Animals were infected with different strains, and animal mortality was observed for 7 days postchallenge. As shown in Table 4, the LD₅₀ values were 4.7×10^6 CFU/duck with mutant strain DE205BΔ*aatB* and 4.7×10^5 CFU/duck with wild-type strain DE205B, whereas the LD₅₀ of complementation strain DE205BCΔ*aatB* was similar to that of the wild-type strain (6.3×10^5 CFU/duck). Similar results were also observed in the mouse model (Table 4). Together, these results indicated that AatB is an important virulence factor in the pathogenicity of APEC strain DE205B.

AatB is involved in colonization of the lung *in vivo*. To determine the effect of AatB *in vivo*, systemic infection experiments were performed by infecting ducks intratracheally and isolating

TABLE 3 Distribution of *aatB* among 273 APEC strains

ECOR group	Total no. of strains per group	No. of strains positive for <i>aatB</i>	%
A	128	5	3.9
B1	55	6	10.9
B2	26	18	69.2
D	64	43	67.2
Summary	273	72	26.4

FIG 3 (A) Purification of AatB expressed in *E. coli* BL21(DE3). The whole ORF of *aatB* was cloned into pET28a(+), leading to the expression of the 38.4-kDa fusion protein AatB. BL21(DE3) cells were incubated in LB medium at 37°C with (lane 2) or without (lane 3) addition of IPTG, which induces the expression of AatB. Proteins from the total bacteria extracts (lanes 2 and 3) and the elution of the purified AatB (lane 1) were separated on an SDS-polyacrylamide gel and stained with Coomassie. Lane M, protein marker. (B) Immunoblotting analysis of total cell lysates prepared from different *E. coli* strains using anti-AatB antibody. Expression of AatB was detected in wild-type strain DE205B and complementation strain DE205BC Δ *aatB* but not mutant strain DE205B Δ *aatB* or DE205BP Δ *aatB*. Lane M, prestained protein marker; lane 1, DE205B (*aatB* positive); lane 2, DE205B Δ *aatB* (*aatB* negative); lane 3, DE205BC Δ *aatB* (*aatB* positive); lane 4, DE205BP Δ *aatB* (*aatB* negative). (C) Immunoblotting analysis of the purified AatB protein using anti-DE205B antibody. Incubation with anti-DE205B led to the detection of protein bands of the expected size for the purified AatB protein. Lane M, prestained protein marker; lane 1, anti-DE205B.

bacteria from the lungs and brains at 24 h postinfection. All 3 strains were able to colonize and invade deeper tissues and caused systemic infections. However, the colonization capacity in the lungs of mutant strain DE205B Δ *aatB* was significantly reduced compared to that of wild-type strain DE205B ($P < 0.01$). Furthermore, the colonization capacity of DE205BC Δ *aatB* in lung tissues

was fully restored and was significantly greater than that of mutant strain DE205B Δ *aatB* ($P < 0.01$) (Fig. 7A). As shown in Fig. 7B, although the amounts of mutant bacteria recovered in the brain were reduced, the difference was not statistically significant com-

FIG 4 Quantification of *aatB* expression during infection *in vitro* and *in vivo*. The levels of *aatB* expression in DE205B during infection *in vitro* (DF-1 cells) and *in vivo* (lungs of duck) were analyzed by qRT-PCR. RNA was isolated from infected DF-1 cells or lungs of duck. cDNA synthesis and qRT-PCR were carried out, and data were normalized to those for the housekeeping gene *dnaE*. The results showed that levels of *aatB* expression were significantly increased by 3.8-fold and 7.42-fold during APEC infection *in vitro* and *in vivo*, respectively (**, $P < 0.01$). Statistical significance analysis was performed using two-way ANOVA.

FIG 5 AatB contributes to biofilm formation of *E. coli*. Biofilm formation by *E. coli* strains AAEC189P (*aatB* negative) and AAEC189P-*aatB* (*aatB* positive) was studied. All strains were cultured in LB medium supplemented with 5 g liter⁻¹ glucose. Biofilm formation was examined in polystyrene microtiter plates. All experiments were repeated at least three times. The columns represent the mean $\pm$ standard deviation of the data. The expression of *aatB* led to significantly increased biofilm formation for AAEC189P-*aatB* compared with that for control strain AAEC189P (*, $P < 0.05$). Statistical significance analysis was performed using one-way ANOVA. OD595nm, optical density at 595 nm.

FIG 6 AatB plays a role in adhesion of APEC to DF-1 cells. (A) Adhesion of each strain to DF-1 cells was examined. The values represent the average data of three independent experiments. The standard deviations are expressed as lines above the bars. The difference between DE205B and mutant strain DE205BΔaatB was statistically significant with a *P* value of <0.01 (**). Furthermore, the adhesion capacity was restored in complementation strain DE205BCΔaatB, which was statistically significantly different from that for DE205BΔaatB (**, *P* < 0.01). One-way ANOVA was performed for the statistical significance analysis. (B) The anti-AatB antibody inhibits the capacity of DE205B to adhere to DF-1 cells. DE205B was incubated with preimmune serum (control) and anti-AatB antibody. Then, bacteria from each experiment were used to infect DF-1 cells. The anti-AatB antibody significantly reduced APEC DE205B invasion into DF-1 cells compared to the level for the negative control (**, *P* < 0.01).

pared to those for wild-type strain DE205B and complementation strain DE205BCΔaatB (*P* > 0.05). Systemic infection experiments were also tested with mice, with similar results (Fig. 7C and D). Statistical analysis was performed using the nonparametric Mann-Whitney U test. These results indicated that the AT AatB is involved in the process of adhesion to lung tissue *in vivo*.

DISCUSSION

AT proteins constitute a large family of extracellular proteins that contribute to bacterial virulence. APEC harbored 3 ATs, including AatA, Tsh, and Vat, which were involved in APEC pathogenicity (22–25). In the present study, the novel AT adhesin AatB was identified through genomic sequence analyses. Compared to the sequences of other ATs, AatB of APEC DE205B contained all structural motifs characteristic for members of the AT protein family: a signal peptide at the N terminus, which can be recognized by the Sec secretion machinery; a passenger domain; and a C-terminal translocator domain. Furthermore, the predicted 3D struc-

ture of AatB consisted of two distinct domains, the C-terminal β-barrel translocator domain and an N-terminal passenger domain. The obvious difference between the conventional AT and TAA subfamily is the C-terminal translocator domain. Conventional ATs are monomeric proteins and form their C-terminal translocator domain with 12 transmembrane β strands from their C-terminal 250 to 300 aa residues. However, the translocator domain of TAAs contains 60 to 70 aa and forms trimers in the outer membrane (28, 52, 53). The amino acid sequence and structural predictions of AatB showed strong similarity to those of other TAAs, such as Saa, YadA, Hia, and Hsf (8, 27, 30, 32), indicating that AatB might be a novel TAA.

The prevalence of ATs has been determined previously and showed different phylogenetic distributions (54). APEC aatA was detected in *E. coli* strains belonging to phylogenetic groups D and B2 (22, 23). The distribution of vat showed a significant association with isolates assigned to phylogenetic group B2 (25). However, tsh was located on virulence plasmids, which led to a wider

TABLE 4 Calculations of LD₅₀ for different strains

Challenge dose (no. of CFU)	No. of dead animals/total no. tested					
	Ducks			Mice		
	DE205B	DE205BΔaatB	DE205BCΔaatB	DE205B	DE205BΔaatB	DE205BCΔaatB
2 × 10 ⁸	8/8	8/8	8/8	10/10	10/10	10/10
2 × 10 ⁷	8/8	6/8	8/8	10/10	9/10	10/10
2 × 10 ⁶	6/8	3/8	7/8	9/10	4/10	7/10
2 × 10 ⁵	3/8	0/8	1/8	2/10	0/10	2/10
2 × 10 ⁴	0/8	0/8	0/8	0/10	0/10	0/10
LD ₅₀ value	4.7 × 10 ⁵	4.7 × 10 ⁶	6.3 × 10 ⁵	5.0 × 10 ⁵	3.2 × 10 ⁶	8.0 × 10 ⁵

FIG 7 Animal systemic infection experiments. Groups of six 7-day-old ducks were intratracheally infected with 10^8 CFU of bacteria. The counts of bacteria isolated from the lungs (A) and brains (B) at 24 h postinoculation were determined by plate counting, as described in Materials and Methods. The amount of bacteria recovered from the lungs was significantly ($P < 0.01$) reduced for mutant strain DE205BΔaatB compared to that for wild-type strain DE205B. The colonization capacity was restored in complementation strain DE205BCΔaatB and was statistically significantly different from that for DE205BΔaatB ($P < 0.01$). Although the amounts of bacteria recovered from the brain were reduced (DE205BΔaatB) and restored (DE205BCΔaatB), the differences were not statistically significant ($P > 0.05$). (C, D) Systemic infection experiments were also performed with mice, except that a dose of 10^7 CFU of bacteria was used, and results similar to those for ducks were also observed. A nonparametric Mann-Whitney U test was carried out for statistical significance analysis. *, $P < 0.05$; **, $P < 0.01$.

distribution among all phylogenetic groups (55). UpaG was the first trimeric AT protein to be characterized in detail from UPEC and was found to be strongly associated with *E. coli* strains from phylogenetic groups B2 and D (33). Thus, the prevalence of novel AT gene *aatB* was determined, and the findings showed that 26.4% of APEC strains harbored the *aatB* gene. These results indicated that more than half of APEC strains belonging to phylogenetic groups B2 and D were *aatB* positive. Furthermore, phylogenetic ECOR group D was the predominant group for isolates harboring *aatB*.

Bacteria modulate their patterns of gene expression by upregulating genes that are specifically required for survival and virulence

during infection. Thus, it is likely that bacterial virulence genes are upregulated during infection or under conditions simulating infection compared to their level of regulation during routine culture (56). AatB expression was detected in wild-type strain DE205B cultured in LB medium, which could elicit antibodies in infected ducks. Moreover, significantly increased levels of *aatB* expression for strain DE205B during infection *in vitro* and *in vivo* were determined. These results suggested that AatB might be involved in APEC pathogenicity.

The ability of bacteria to adhere to a diverse range of surfaces, including different host tissues and abiotic elements, is essential for colonization, survival, and persistence (2, 57). The importance

of adhesion to the lifestyle of bacteria is reflected by the wide range of different adhesins found even in a single species. AT proteins are nonfimbrial adhesins and promote adhesion of bacteria (10, 21, 39). Therefore, we determined whether AatB could mediate adhesion, aggregation, and biofilm formation. The results showed that the adhesion capacity of an *aatB* mutant strain was significantly reduced compared to that of wild-type strain DE205B. Moreover, the adhesion capacity was fully restored in the complementation strain, which indicated that AatB mediates the adhesion of APEC to DF-1 cells. Multiple factors, such as fimbrial adhesins and AatA, contribute to APEC and neonatal meningitis *E. coli* invasion, which might imply that *aatB* mutant strains also maintained their capability to adhere to DF-1 cells.

Residence in a biofilm community offers to bacteria certain advantages, such as the enhanced ability to cause disease, which is detrimental to animal and human public health (58). Except for adherence, AT proteins play roles in cell aggregation and biofilm formation (10, 13, 21, 39). Therefore, whether AatA could mediate aggregation and biofilm formation was determined. The capacity of mutant strain DE205BΔ*aatB* to produce biofilm was not statistically significantly different from that of wild-type strain DE205B and complementation strain DE205BCΔ*aatB* (data not shown), which might be because other surface factors contribute to biofilm formation, since this single deletion in *aatB* should not lead to significantly decreased biofilm formation. Thus, the complementation of *aatB* in *E. coli* strain AAEC189 was performed and resulted in a significantly ($P < 0.05$) higher level of biofilm formation compared with that for control strain AAEC189P (Fig. 5). The results indicated that AatB is involved in biofilm formation of *E. coli*, although the precise function of AatB in this process is still unclear. However, AatB has no role in cell aggregation in *E. coli*.

Since AatB was found to be involved in biofilm formation and adhesion to DF-1 cells, the influence of AatB on APEC pathogenicity was examined. The virulence of the *aatB* mutant was attenuated compared with that of the wild-type strain in the duck and mouse models. Furthermore, virulence was restored for the complementation strain, which indicated that AatB is necessary for the full virulence of APEC strain DE205B. To determine the precise roles of AatB in the pathogenicity of DE205B *in vivo*, animal infection studies were performed, as described previously (38, 40). The results indicated that the loss of *aatB* resulted in a significant reduction in the amount of bacteria recovered in the lungs of ducks compared to the lungs of the wild-type strain. The colonization capacity was fully restored for the complementation strain. Similar results were also observed in the mouse model. This might explain the attenuated virulence of mutant strain DE205BΔ*aatB*. Although the colonization and proliferation capacity of mutant bacteria in brain tissue was reduced compared to that of the wild-type strain, the differences were not statistically significant ($P > 0.05$). These results indicate that AatB is involved in early lung colonization steps during systemic infection. On the basis of the animal infection study and the adherence assay results, it can be concluded that AatB significantly contributes to APEC virulence through bacterial adherence to host tissues *in vivo* and *in vitro*.

In summary, a novel AT adhesin gene, *aatB*, was identified in APEC DE205B and was predominantly associated with phylogenetic groups B2 and D of APEC. The functional analyses showed that AatB is involved in adherence of APEC to host tissues *in vivo* and *in vitro*, which is important for APEC pathogenicity.

ACKNOWLEDGMENTS

This work was supported by the Fund of Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and the National Basic Fund for University, which is supported by Nanjing Agricultural University (KYZ201214).

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