Characterization of the AggR Regulon in Enteroaggregative *Escherichia coli*

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AggR is a transcriptional regulator of enteroaggregative *Escherichia coli* (EAEC) and has been proposed as the defining factor for typical EAEC strains. Expression of multiple putative virulence factors, including the aggregative adherence fimbriae (AAF), dispersin, the dispersin translocator Aat, and the Aai type VI secretion system, have been found to be regulated by AggR. Here, we confirm the existence of at least 44 AggR-regulated genes using DNA microarray and real-time quantitative reverse transcription-PCR (qRT-PCR); these genes include chromosomal and plasmid-borne loci and 19 previously unsuspected genes. Two previously uncharacterized virulence plasmid-encoded open reading frames (ORFs) (designated ORF3 and ORF4) exhibit significant identity with isoprenoid biosynthesis genes of *Bacteria* and *Archaea*. The predicted ORF4 product is closely related to isopentenyl isomerase (IDI) enzymes, whereas the predicted product of the adjacent ORF3 exhibits an aspartate-rich region that is common among *trans*-isoprenyl phosphate synthases. We show that mutations in these ORFs confer changes in bacterial surface properties. AggR coordinately controls expression of a large number of EAEC genes.

Enterocolic *Escherichia coli* (EAEC) is increasingly recognized as a cause of diarrhea in adults and children in both industrialized and nonindustrialized countries (1–5). EAEC may be the most common cause of acute diarrheal illness among all age groups in the United States (2), and the recent deadly outbreak of Shiga toxin-encoding EAEC in Europe suggests that it may become a cause of significant morbidity and mortality (6, 7). EAEC may be the second most common cause of traveler's diarrhea (8, 9).

EAEC pathogenomic studies have focused on the regulator called AggR. AggR is a member of the AraC/XylS family of bacterial transcriptional activators (10, 11), exhibiting the greatest levels of amino acid identity with the CfaD (68%), Rns (66%), and CsrV (62%) regulators of enterotoxigenic *E. coli* (ETEC) (11). Multiple epidemiologic studies suggest that strains expressing AggR are more likely to cause diarrheal disease than those without it, leading us to propose the term “typical EAEC” to describe strains harboring the as-yet-incompletely characterized AggR regulon (8, 12, 13).

A number of AggR-regulated genes have been described previously in archetype EAEC strain 042. The genes encoding aggregative adherence fimbriae (AAF) were the first found to be regulated by AggR (11), followed by *aap* (encoding the dispersin surface protein) (14) and the Aat secretion system, which is required for transport of dispersin to the bacterial surface (15). AggR also activates expression of the Aai type VI secretion system (T6SS) in 042 (16), though the role of Aai in EAEC virulence remains unknown.

Here we provide additional characterization of the AggR regulon. We show that AggR activates expression of at least 44 genes, including 19 previously unsuspected genes. Sixteen out of 44 AggR-regulated genes encode hypothetical proteins, some of which are promising candidates for further characterization.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Bacterial strains used in this study are shown in Table 1. Strains 042, EAEC 042aggR, and EAEC 042aggR(pBADaggR) were previously described by our laboratory (14, 17, 18). Bacterial cultures were routinely propagated in Luria broth (LB) (American Bioanalytical, Natick, MA) from a fresh agar plate and grown overnight at 37°C. When needed, kanamycin (kan) was added to a final concentration of 50 µg ml⁻¹. For the microarray study, bacterial strains were cultured in Dulbecco's modified Eagle's medium with 0.4% glucose (DMEM high glucose) (Gibco, Grand Island, NY). Transcription of *aggR* was determined from bacterial broth cultures in DMEM high glucose, DMEM with 0.1% glucose (DMEM low glucose), DMEM low glucose with 0.4% maltose, DMEM low glucose with 3% bile, and DMEM low glucose with 45 mM NaHCO₃. Transcription of *ntrC* and *aap* was determined in DMEM high glucose with 0.2% ammonium sulfate.

The mutants described in this study were generated by using the λ red recombinase system (19) as previously described.

RNA isolation. To generate total RNA, 042, 042aggR, and 042aggR(pBADaggR) strains were grown overnight in LB. Duplicate subcultures were generated by diluting the samples 1:100 into 50 ml of DMEM high glucose. The strains were incubated with shaking at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.8. RNA was extracted using TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Contaminating DNA was removed using an RNase-free DNase

Received 9 July 2012 Returned for modification 30 July 2012

Accepted 15 October 2012

Published ahead of print 22 October 2012

Editor: B. A. McCormick

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.00676-12>.

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doi:10.1128/IAI.00676-12

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
Strains		
<i>E. coli</i> HS	Commensal <i>E. coli</i> strain	25
EAEC 042	Prototype EAEC strain	17
EAEC 042 <i>aggR</i>	042 derivative carrying <i>aggR</i> deletion	14, 18
EAEC 042 <i>aggR</i> (pBAD <i>aggR</i>)	042 <i>aggR</i> complemented in <i>trans</i> by pBAD <i>aggR</i>	14, 18
EAEC 042 <i>orf3</i>	042 derivative carrying <i>orf3</i> deletion	This study
EAEC 042 <i>orf3</i> (pBAD <i>orf3</i>)	042 <i>orf3</i> complemented in <i>trans</i> by pBAD <i>orf3</i>	This study
EAEC 042 <i>orf4</i>	042 derivative carrying <i>orf4</i> deletion	This study
EAEC 042 <i>orf4</i> (pBAD <i>orf4</i>)	042 <i>orf4</i> complemented in <i>trans</i> by pBAD <i>orf4</i>	This study
EAEC 042 <i>orf3-4</i>	042 derivative carrying <i>orf3</i> and <i>orf4</i> deletions	This study
EAEC 042 <i>orf3-4</i> (pZC320 <i>orf3-4</i>)	042 <i>orf3-4</i> complemented in <i>trans</i> by <i>orf3</i> and <i>orf4</i>	This study
Plasmids		
pBAD30	Plasmid used to <i>trans</i> complement	Lab collection
pBAD <i>aggR</i>	<i>Trans</i> -complemented plasmid for <i>aggR</i>	Lab collection
pBAD <i>orf3</i>	<i>Trans</i> -complemented plasmid for <i>orf3</i>	This study
pBAD <i>orf4</i>	<i>Trans</i> -complemented plasmid for <i>orf4</i>	This study
pZC320 <i>orf3-4</i>	<i>Trans</i> -complemented plasmid for <i>orf3</i> and <i>orf4</i>	This study

set (Qiagen, MD), and total RNA was quantified using a NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA) spectrophotometer.

Construction of an oligonucleotide microarray. The EAEC microarray was synthesized by NimbleGen (Madison, WI) and included 19- to 24mer oligonucleotide probes for each of 4,899 open reading frames (ORFs) predicted from the 042 chromosome sequence (deposited by the Sanger Center in GenBank under accession number [FN554766](#)) and 160 ORFs of plasmid pAA2 (GenBank accession number [FN554767](#)). Each probe was present in duplicate on the chip. RNA samples of EAEC 042 and 042*aggR* were prepared and shipped to NimbleGen to perform array hybridization experiments using proprietary protocols. The normalized array data received from NimbleGen were analyzed using the Significance Analysis of Microarrays program (SAM) at <http://www-stat.stanford.edu/~tibs/SAM/> (20). The complete data set was visualized using Arraystar software.

qRT-PCR. mRNA transcripts were quantitated by reverse transcription followed by quantitative real-time PCR (qRT-PCR). Cultures of 042, 042*aggR*, 042*aggR*(pBAD30), and 042*aggR*(pBAD*aggR*) were grown similarly in LB overnight. The overnight cultures were diluted 1:100 in 5.0 ml of DMEM high glucose. The strains were grown to an OD₆₀₀ of approximately 0.8. *aggR* expression was induced in the strains by adding arabinose (to a 2% final concentration) and incubating for 1 h at 37°C. RNA was extracted as described above, and qRT-PCR was performed by using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The primer sets used in this study are reported in Table S1 in the supplemental material. Reactions were done in duplicate using two different RNA samples for each strain. The expression level for each queried gene was normalized to the constitutively expressed *cat* gene as previously described (21).

SEM. We performed scanning electron microscopy (SEM) as previously described (18). Strains were grown in L broth overnight with shaking at 37°C. Static cultures were grown in DMEM in 24-well culture dishes with glass coverslips for 6 h to form a biofilm. Samples were incubated with 2% glutaraldehyde (Electron Microscopy Sciences Inc., Fort Washington, PA) in CaCo buffer (0.1 M CaCo, 3 mM CaCl₂) (Electron Microscopy Sciences Inc.) for 1 h at room temperature. Fixed samples were prepared and examined by the Electron Microscopy facility at the University of Maryland (EM-UMB).

TEM. Strains were grown in LB overnight with shaking at 37°C. The bacterial culture was diluted 1:100 in 5.0 ml of DMEM high glucose. The strains were grown to an OD₆₀₀ of approximately 0.8. One milliliter of bacterial broth (OD₆₀₀, 0.8) was pelleted by centrifugation, washed in 1 ml

of phosphate-buffered saline (PBS), and incubated overnight at 4°C in 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), 2.5% glutaraldehyde, and 0.1 M CaCo buffer. The samples were washed in 1 ml of sterile water and suspended in 500 µl of water. Ten-microliter bacterial samples were adsorbed onto a Formvar mesh grid for 1 min. The grids were then negatively stained with 2% uranyl acetate (Electron Microscopy Sciences Inc., Fort Washington, PA) for 5 s. The sample was blotted dry and viewed on a Jeol JEM1230 transmission electron microscope (TEM) (80 kV) at the Advanced Microscopy Laboratory at the University of Virginia (AML-UVA).

Cationic antimicrobial peptide susceptibility. Strains were grown in LB overnight with shaking at 37°C. The overnight bacterial culture was diluted 1:100 in DMEM high glucose and grown to an OD₆₀₀ of 0.8. One milliliter of bacterial culture was pelleted, washed in PBS, and resuspended in 1 ml of 2-µg/ml defensin-1 (HNP-1) (Sigma-Aldrich, St. Louis, MO), freshly prepared in 10 mM potassium phosphate buffer with 0.1% tryptic soy broth. The samples were incubated for 1 h at 37°C and then serially diluted in PBS and plated to determine the percent survival. For the polymyxin B assay, the bacteria were grown as described above. One milliliter of bacterial culture was pelleted, washed, and incubated with 0.3 µg/ml polymyxin B (Sigma-Aldrich, St. Louis, MO) in PBS for 1 h, and surviving bacteria were enumerated by serial plating.

Lipid A analysis. Lipid A from each strain was extracted using an ammonium hydroxide/isobutyric acid method (22) and subjected to negative-ion matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis. Lyophilized pellets from 5 ml of O/N cultures grown in DMEM high glucose were resuspended in 400 µl isobutyric acid/1 M ammonium hydroxide (5:3 [vol/vol]) and incubated at 100°C for 2 h, with occasional vortexing. Samples were cooled on ice for 5 min and centrifuged (2,000 × g) for 15 min at room temperature. The resulting supernatant was transferred to a new tube, diluted with an equal volume of water, and lyophilized. The dried lipid A was resuspended in 400 µl methanol and centrifuged as described above twice. The lipid A pellet was solubilized in 200 µl chloroform/methanol/water (3:1.5:0.25 [vol/vol/vol]) and spotted (1 µl) directly onto the MALDI sample plate, followed by 1 µl of 20-mg/ml 5-chloro-2-mercapto-benzothiazole (CMBT) MALDI matrix dissolved in chloroform/methanol (1:1 [vol/vol]). All MALDI-TOF experiments were performed using a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Incorporated); each spectrum comprised an average of 300 shots. A tuning mix (Agilent) was used to calibrate the MALDI-TOF.

FIG 1 Microarray analysis of the AggR regulon. RNA samples of 042 (black bars) and 042 Δ aggR (open bars) were hybridized in duplicate to an EAC oligonucleotide microarray (NimbleGen, Inc.). (A) *aggR* transcription was evaluated in DMEM supplemented with glucose, bicarbonate, maltose, or bile salts. (B) Transcript levels for *aap* and *aggR* were determined by RT-PCR in 042, 042 Δ aggR, 042 Δ aggR(pBAD30), and 042 Δ aggR(pBADaggR), grown as described in the text.

RESULTS

Microarray transcriptome analysis to characterize the AggR regulon. To identify mRNAs whose abundance was affected by the presence of AggR, we compared transcriptomes of log-phase bacterial cultures of 042 and those of its isogenic *aggR* mutant, cultivated under *aggR*-inducing conditions (Fig. 1A). mRNA was prepared from bacterial cultures in DMEM high glucose, reverse transcribed, and hybridized to an oligonucleotide array constructed from the whole genome of strain 042. Results were compared using the Arraystar program.

The large majority of genes on the microarray were unchanged in their expression level between the wild type and the *aggR* mu-

tant. No genes in 042 Δ aggR were expressed more than 2 standard deviations below their expression levels in 042. In contrast, SAM analysis yielded 179 genes with expression levels at least 2-fold higher in the wild type than in its *aggR* mutant (data not shown).

Fifty of the 179 putative AggR-upregulated genes were found to be part of the NtrC regulon, though upregulation of these genes was observed in only one replicate (23). NtrB/C comprises a two-

TABLE 2 AggR-dependent genes confirmed by qPCR

Gene type and ORF name	Fold change ^a	Function and gene name
Type VI secretion system		
ECO42_4562	8.169065932	Putative type VI secretion protein, <i>aaiA</i>
ECO42_4563	8.039148147	Putative type VI secretion protein, <i>aaiB</i>
ECO42_4564	6.785280803	Putative type VI secretion protein, <i>aaiC</i>
ECO42_4565	8.022616485	Putative type VI secretion protein, <i>aaiD</i>
ECO42_4566	8.261976862	Putative type VI secretion protein, <i>aaiE</i>
ECO42_4568	7.847518419	Putative type VI secretion protein, <i>aaiF</i>
ECO42_4569	7.969137365	Putative type VI secretion protein, <i>aaiG</i>
ECO42_4570	6.894393014	Putative type VI secretion protein, <i>aaiH</i>
ECO42_4571	5.890466358	Putative type VI secretion protein, <i>aaiI</i>
ECO42_4572	7.114036791	Putative type VI secretion protein, <i>aaiJ</i>
ECO42_4573	7.285659665	Putative type VI secretion protein, <i>aaiK</i>
ECO42_4574	8.613568278	Putative type VI secretion protein, <i>aaiL</i>
ECO42_4574A	ND	Putative type VI secretion protein
ECO42_4575	7.523960434	Putative type VI secretion protein, <i>aaiM</i>
ECO42_4576	8.146141969	Putative type VI secretion protein, <i>aaiN</i>
ECO42_4577	7.866923361	Putative type VI secretion protein, <i>aaiO</i>
ECO42_4579B	ND	Conserved hypothetical protein
ECO42_4580	5.579747087	Conserved hypothetical protein, <i>aaiR</i>
ECO42_4581	5.587540357	Conserved hypothetical protein, <i>aaiS</i>
ECO42_4582	6.5817138	Hypothetical protein, <i>aaiT</i>
ECO42_4583	5.480597346	Hypothetical protein, <i>aaiU</i>
Dispersin and Aat secretion system		
ECO42_pAA007	2.528355119	Permease protein, <i>aatP</i>
ECO42_pAA008	13.37722907	Outer membrane protein, <i>aatA</i>
ECO42_pAA009	14.69941902	Conserved hypothetical protein, <i>aatB</i>
ECO42_pAA010	7.410381723	ABC transporter, ATP-binding protein, <i>aatC</i>
ECO42_pAA011	5.651047838	Putative membrane protein, <i>aatD</i>
ECO42_pAA055	276.7756858	Dispersin, <i>aap</i>
Fimbrial genes		
ECO42_pAA030	15.73008247	Fimbrial adhesin, <i>aafB</i>
ECO42_pAA031	14.59676314	Aggregative adherence fimbria II usher protein, <i>aafC</i>
ECO42_pAA046	210.1889912	Chaperone protein, <i>aafD</i>
ECO42_pAA048	26.49096839	Major fimbrial subunit of aggregative adherence fimbria II, <i>aafA</i>
Genes encoding hypothetical proteins		
ECO42_3181	2.282558933	Putative transcriptional regulator
ECO42_3182	5.529536167	ParB-like nuclease
ECO42_3183	2.919604813	Conserved hypothetical protein
ECO42_3184	30.58693943	Conserved hypothetical protein
ECO42_3187	3.197781365	Putative helicase
ECO42_4006	8.192256093	Putative exported protein
ECO42_pAA003	29.2147097	Hypothetical protein (ORF3)
ECO42_pAA004	18.19583241	Putative isopentenyl-diphosphate (ORF4)
ECO42_pAA005	3.316512405	Hypothetical protein
ECO42_pAA005A	ND	Conserved hypothetical protein
ECO42_pAA020	ND	Conserved hypothetical protein
ECO42_pAA047	ND	Hypothetical protein
ECO42_pAA056	ND	Hypothetical protein
ECO42_pAA060	7.615732952	Conserved hypothetical protein
Genes encoding regulatory proteins		
ECO42_pAA052	30.97680088	<i>aggR</i>
Other genes		
ECO42_pAA021	3.670469957	Polysaccharide deacetylase, <i>shf</i>

^a Fold change by microarray between 042 and 042 Δ aggR. ND, not detected by microarray.

FIG 2 AggR-regulated genes located in the chromosome. (A) Transcriptional levels of AggR-regulated genes were determined by qRT-PCR in 042 (black bars), 042aggR (open bars), and 042aggR(pBAD30aggR) (gray bars). (B) Graphic representation of the chromosomal genes regulated by AggR.

component regulatory system that activates genes responsible for nitrogen scavenging and utilization of alternative nitrogen sources. To test possible dependence of the NtrC regulon on AggR, transcription of *ntrC* was evaluated by qRT-PCR in DMEM supplemented with exogenous nitrogen sources or not supplemented; these experiments failed to confirm any variation in *ntrC* expression between 042 and 042aggR (see Fig. S1 in the supplemental material). In addition, supplementation of the medium with nitrogen reduced the level of *ntrC*-derived mRNA but not that of the AggR-dependent gene *aap* (see Fig. S1 in the supplemental material). These data suggest that expression of the NtrC regulon is independent of AggR.

We sought to confirm AggR-dependent expression for all ORFs with signals at least 3-fold higher in the wild type than in the *aggR* mutant strain. Genes which had lower-level induction but which were part of multigene clusters potentially under AggR control were also included. Using this selection criterion, 74 genes were considered candidates for AggR-controlled expression (Table 2; see also Table S2 in the supplemental material). The nomenclature used by NCBI (<http://www.ncbi.nlm.nih.gov>) to describe genomic sequences in 042 (GenBank accession number [FN554766](#)) or genes in plasmid pAA2 (GenBank accession number [FN554767](#)) was applied in this study. Puta-

tive gene assignments and homologies for AggR-regulated genes are listed in Table 2.

Forty-two out of the 74 candidate AggR-regulated genes suggested by the microarray were consistently confirmed as more abundantly expressed in 042 than in 042aggR and also in 042aggR(pBADaggR) in the presence of arabinose than in the absence of arabinose. Thirty-two genes were not confirmed (see Table S2 in the supplemental material). Complementation of *aggR* was demonstrated with 042aggR(pBADaggR) but not with the vector alone [042aggR(pBAD30)] (Fig. 1B).

Chromosomal AggR-regulated regions. Of the 44 genes found to be under AggR control, 23 were localized to the bacterial chromosome. These were mapped to two distantly located gene clusters (ECO42_3181 to ECO42_3187 and ECO42_4562 to ECO42_4583) and one isolated ORF (ECO42_4006). In the region from 3181 to 3187, the ORFs ECO42_3182 and ECO42_3184 were 2.9- and 21-fold upregulated, respectively, in the wild-type 042 strain compared to the 042aggR strain (Fig. 2) by real-time qPCR. ECO42_3187 expression was 2.7-fold higher in 042aggR(pBADaggR) in the presence of arabinose than in the *aggR* mutant (Fig. 2).

By BLAST analysis, ECO42_3182 was suggested to encode a ParB-like nuclease, reported to be highly conserved among *E. coli*, *Shigella* spp., and *Klebsiella pneumoniae*. ECO42_3184 is a hypo-

FIG 3 AggR-regulated genes located in pAA2. (A) Transcriptional levels of plasmid-borne AggR-regulated genes were quantitated by qRT-PCR in 042 (black bars), 042aggR (open bars), and 042aggR(pBAD30aggR) (gray bars). (B) Graphic representation of the genes in the pAA2 plasmid.

thetical gene identified in EAEC and avian pathogenic *E. coli* strains. The sequence analysis revealed no signal peptide sequence or other motifs required for secretion, suggesting that ECO42_3184 most likely encodes a cytoplasmic protein.

ECO42_3187 exhibits conserved domains characteristic of the UvrD helicase family, present in *E. coli*, *Vibrio* spp., *Salmonella* spp., and *Citrobacter rodentium*. ECO42_4006, expressed 4.3-fold more abundantly in the wild-type strain than in the 042aggR mutant strain (Fig. 2), is also known as *yicS* and encodes a highly conserved hypothetical protein in *E. coli*, *Salmonella* spp., *Shigella* spp., and *Citrobacter rodentium*.

In agreement with previous findings in our laboratory (16), we

found that 20 genes carried in a pathogenicity island (designated PAI-1) inserted adjacent to *pheU* were expressed at least 4-fold more abundantly in the wild-type strain than in 042aggR (Fig. 2). In the microarray analysis, this region was upregulated 6- to 8-fold in the wild-type strain than in the *aggR* mutant (Table 2). This region includes the Aai type VI secretion system (ECO42_4562 to ECO42_4577), followed by four hypothetical proteins (ECO42_4580 to ECO42_4583). The AggR-regulated T6SS of EAEC secretes the product of ECO42_4564 (AaiC), which was also confirmed to be regulated by AggR in this work (Fig. 2).

The region from ECO42_4580 to ECO42_4583 is also carried in the PAI-1 region and was expressed 4-fold more abundantly in

A. ORF3

gi 322369459	-----MTDASTESREELVMQTVEQRRELVN--AAIEEELPIVEPERLYEASRYLLDAG	51
gi 169235957	-----MSQEAREAVLA AVRERRRQQVN--DAIDDDLPVAEERLYEASRYLLEAG	48
gi 16329282	-----MVAQQTRTDFDLAQYLQVKKGVVE--AALDSSLAIRPEKIYEAMRYSLLAG	50
gi 284924584	MKVVSDFSNDMHKRRYFNDTMDIINYNRKTIRYTDIIFYFRTLKEIRLKYPFKELESFLD	60
	: . : : . : . * . .	
gi 322369459	GKRLRPVLLLLVADALADVEPMSEAYRKFPALDGTELDVMAAAVSIEVIQSFTLIHDDI-	110
gi 169235957	GKRLRPVLLLLTAEALADTS-DTPPYREFPDCIGGHVDVMSAAVSIEVIQSFTLIHDDI-	106
gi 16329282	GKRLRPILCITACELCGGDE-----ALALPTCALEMIHTMSLIHDDLP	94
gi 284924584	PLNIYFFYYFMPFLFISDLHDKQGYIERIR----YVGIISTLIARRAILTDLFVDEQY-	114
	: . * : . . : : : . * : : : :	
gi 322369459	-MDDDDLRRGVPSPVHRAYDTETAILAGDTLYSKAFEIMLETD--ATPDRCVTALDVLAST	167
gi 169235957	-MDDDDLRRGSPAVHREYDTETAILAGDTLYSKAFEIMLETG--APPERGLRAMRTLAE	163
gi 16329282	SMNDNDFRRGKPTNHKVYGEDIAILAGDGLLAYAFEYVVVTHTPQADPQALLQVIARLGR	154
gi 284924584	---EQLISRNMGDLKKEYLGYLLQIIDVEINNIASKFIEPG-----SFYAFYNR	161
	: : : * . : : * : . : * : : : : : :	
gi 322369459	C--TRICEGQALDVAFEERPDVLPDEYLEMIENKTA VLYGAAAKVPVALLGADEETTEAL	225
gi 169235957	C--THICEGQAMDIDFETRGDVVTDDEYLEMVELKTA VLYAAAATLPLGLLSADEQETVEAL	221
gi 16329282	VGAAGLVGGQVLDLDESGRDTITPETLTFIHHTKTGALLEASVLTGAILAGATGEQQQRL	214
gi 284924584	R-FNEYVNTMLMEKDISPLIGMDEKAFKKYAVGKSLHLLVSDMVLYIIK--KQELTHQF	218
	: : . . : . * : . : : : * . :	
gi 322369459	YRYGIDVGRAFIQDDVDLDTVPSDKLGKQRGSDLVESKQTIITLHARSQGVDDVDSLVT	285
gi 169235957	YNYGLRVGGAQFIQDDVDLDTVPSAELGKQRGSDLVEDKTTITLHAHGHVDDVDDLDA	281
gi 16329282	ARYAQNIGLAFQVVDLIDITATQEEELGKTAGKDVKAQKATYPSLLG-----L	262
gi 284924584	DNMMKSFIMYLTQLQDDVDLIMEDIQKQPSHFYPWISDGKIIESFDEN-VEKAVLLKFYL	277
	. . : : **:** : . : :	
gi 322369459	DDVDGVTAEAEIDEAVQRLEAAGSIEYATEKAQELVQRGKDHLSVLPDNESRYLLEQIADY	345
gi 169235957	EDPADVTGAEEAAVQAALQDAGSIQYASDLAEDVEEGKAHLEVLPNEARHRLLEQIADY	341
gi 16329282	EASRAQAQSLIDQAI VALEPFG-----PSAEPLQAI AEY	296
gi 284924584	GGGIERCEDLINKYVEDIITAVKKTNHPLKSWLEVIERM SLKTKDKLDSFKA VRDELHFF	337
	: : : : : : : :	
gi 322369459	LIERGY 351	
gi 169235957	LIERGY 347	
gi 16329282	IVARKY 302	
gi 284924584	LSQQG- 342	
	: :	

B. ORF4

gi 16761820	MTEHHVLLDEQDKPSGTLEKYAAHTLNTPLHLAFSCWLFNEDGQLLVTRRSLSKKAWPG	60
gi 283788453	MTGEHVILLDAQDTPSGTLEKYAAHSTHTPLHFAFSCWLFNEDGQLLVTRRSLMKKAWPG	60
gi 284922837	MQTEHVILLNAQGVPTGTLEKYAAHTADTLHLAFSSWLFNAKGQLLVTRRSLSKKAWPG	60
gi 157162349	MQTEHVILLNAQGVPTGTLEKYAAHTADTLHLAFSSWLFNAKGQLLVTRRSLSKKAWPG	60
gi 284924585	--MENVILIDHND CETGTAEKLYTHKKG-ILHRAVS VYICNSDGKLL LQQRALGKYHSPG	57
	::: :. :* ** :. ** *. *::* *.*: :*: * **	
gi 16761820	VWTVSVCVGHPPQGETTEEAII RRCRFELGVEITDLPVYPHFSYRATDPNGIVENEVCVPV	120
gi 283788453	VWTVSVCVGHPPQGETTEEAII RRCRFELGVEITDLPVYPHFSYRATDPNGIVENEVCVPV	120
gi 284922837	VWTVSVCVGHPPQLGESNEDAVIRRCRFELGVEITAPEVPYDPDFRYRATDPNGIVENEVCVPV	120
gi 157162349	VCTNSVCVGHPPQLGESNEDAVIRRCRYELGVEITPESYIYDPDFRYRATDPNGIVENEVCVPV	120
gi 284924585	LWSNTSCTHPFPGESNL SAANRLREEMGIECP--LSKLLKIYVNVYVGDLTEHEIAHI	115
	: ::* ** ** . * ** * *: * . . : * . . . :*: :. :	
gi 16761820	FAARATSVLQVNSEEVMDYQWSEFKSVQWSSLATPWAFSPWMVMQASDEQ-ARKRLNLNYC	179
gi 283788453	YAAQVSVLQVNSEEVMEYQWSELQSVFAADATPWAFSPWMVMQASNSA-ARALLRAYC	179
gi 284922837	FAARTTSALQINDDEVMDYQWCDLADVLHGIDATPWAFSPWMVMQATNRE-ARKRLSAFT	179
gi 157162349	FAARTTSALQINDDEVMDYQWCDLADVLHGIDATPWAFSPWMVMQAAANSE-ARKLLSAFA	179
gi 284924585	FYGISDDEPVLNSLEAMS YKYVSLTELSSEIKFNNDAFSRWFVYCFPIKNAFLNESNYT	175
	: . . :*. *.*: :. :. : . *** *. * . *	
gi 16761820	QR-- 181	
gi 283788453	QR-- 181	
gi 284922837	QLK- 182	
gi 157162349	QHN- 182	
gi 284924585	NLLI 179	

FIG 4 Homologs for ORF3 and ORF4. (A) ECO42_pAA003 (GenBank accession number gil284924584) and homologs in *Archaea* (*Halobacterium salinarum* R1, GenBank accession number gil169235957; *Haladaptatus paucihalophilus* DX253, GenBank accession number gil322369459) and *Cyanobacteria* (*Synechocystis* sp. PCC 6803, GenBank accession number gil16329282) were aligned. (B) ECO42_pAA004 (GenBank accession number gil284924585) showed homology to isopentenyl-diphosphate delta isomerase (IDI) of *Salmonella enterica* serovar Typhi strain CT18 (GenBank accession number gil16761820), *Citrobacter rodentium* ICC168 (GenBank accession number gil283788453), *E. coli* HS (GenBank accession number gil157162349), and 042 (GenBank accession number gil284922837).

TABLE 3 Prevalence of selected AggR-regulated genes among diarrheagenic *E. coli* strains

Gene	Frequency of gene (% of strains positive) in:	
	EAEC	Non-EAEC diarrheagenic <i>E. coli</i> ^a
<i>aggR</i>	32 (89)	0 (0)
ORF3	27 (75)	0 (0)
ORF60	27(75)	7 (17)
ECO_3184	3 (11)	4 (12.5)

^a A total of 41 strains representing enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), ETEC, enteroinvasive *E. coli* (EIEC), and diffusely adhering *E. coli* (DAEC) from collections of the authors.

042 than in 042*aggR* (Fig. 2) by qRT-PCR. The region contains four hypothetical proteins that are conserved within the genomes of other pathogenic *E. coli* strains, including the O104:H4 Shiga toxin-encoding EAEC strain C227-11, implicated in the German outbreak of 2011 (7).
ECO42_4581 and ECO42_4582 proteins exhibited low identity (31 to 35%) to other hypothetical proteins in *Yersinia* spp. An ECO42_4580 homolog is also present in *C. rodentium*, with an identity of 78%. None of the translated sequences revealed significant similarity to any characterized protein in the databases.

Plasmid pAA2-associated AggR-regulated regions. The pAA2 virulence plasmid harbors a large array of putative virulence genes, accompanied by an apparently complete conjugation apparatus and plasmid replication machinery (GenBank accession number [FN554767](#)). None of the genes bearing significant similarity to known conjugation and maintenance functions exhibited differential expression in the wild type versus the *aggR* mutant.
In contrast, the large pAA2 region distinct from the conjugation apparatus has previously been shown to harbor over 20 genes with putative virulence functions. A large number of these genes were found here to exhibit reduced expression in the *aggR* mutant by microarray. Expression of each of these ORFs (designated pAA003 to pAA061) was analyzed by qRT-PCR. All of these genes, with the exception of pAA015, pAA016, *ccdA*, *ccdB*, *pet*, pAA041, pAA042, and pAA061, were found to be AggR upregulated. In total, the region spanning from ECO42_pAA003 to ECO42_pAA060 comprised 22 AggR-regulated genes, among which were the *aat*-encoded secretion system (*aatP*, *aatA*, *aatB*, *aatC*, and *aatD*), the AAF/II biogenesis genes (*aafC*, *aafB*, *aafD*, and *aafA*), and 7 genes encoding hypothetical products (Fig. 3).

FIG 5 SEM of EAEC 042 and 042orf3-4 biofilms. Strains 042 (A and B) and 042orf3-4 (C and D) were visualized by SEM; samples were visualized at a magnification of $\times 4,000$ (A and C), $\times 20,000$ (B), or $\times 30,000$ (D).

FIG 6 ORF3 and ORF4 mutants are more susceptible to defensin and polymyxin B treatment. Strains were treated with polymyxin B (panel A), defensin-1 (panel B, open bars), or PBS (panel B, black bars) for 1 h. Cultures were serially diluted in PBS, and viable counts were determined by plating. Percent survival of the original inoculum was recorded after 24 h. The difference between groups was significant ($P < 0.001$; Kruskal-Wallis).

The ORFs ECO42_pAA003 to ECO42_pAA005A were upregulated 8- to 64-fold in wild-type 042 compared to the 042aggR mutant (Fig. 3). Sequence analysis revealed that ECO42_pAA003 (GenBank accession number gil284924584) shared 13 to 14% sequence identity with the superfamily of *trans*-isoprenyl diphosphate synthases in *Archaea* (*Halobacterium salinarum* R1, GenBank accession number gil169235957; *Haladaptatus paucihalophilus* DX253, GenBank accession number gil322369459) and *Cyanobacteria* (*Synechocystis* sp. PCC 6803, GenBank accession number gil16329282) (Fig. 4).

ECO42_pAA005, predicted to encode a protein of only 50 amino acids, displayed 53% identity to ECO42_pAA003 in a region of 36 residues, though the functions of these ORFs remain cryptic.

ECO42_pAA004 exhibited significant homology to a gene encoding a putative isopentenyl diphosphate isomerase (IDI). A second IDI-encoding ORF was found in the chromosome of 042 (GenBank accession number gil284922837); the predicted product of this gene was virtually identical to the IDI found in all published *E. coli* strains and chromosomes as well as those of most related species of *Enterobacteriaceae*, and this chromosomal locus presumably confers the basic metabolic function of IDI enzymes (24). This chromosomal IDI was not under AggR con-

trol. Alignment of the IDI-encoding genes from *Salmonella enterica* (GenBank accession number gil16761820), *C. rodentium* (GenBank accession number gil283788453), and *E. coli* commensal strain HS (GenBank accession number gil157162349) (25) is shown in Fig. 4. Adjacent to ORF3 and ORF4, ECO42_pAA005A is predicted to encode a hypothetical protein also present in *E. coli* O104:H4 (90% identical); its function remains unknown.

In agreement with previous findings reported by our lab, *aap* (ECO42_pAA055) and the *aat* secretion system were shown in the microarray analysis (ECO42_pAA007 to ECO42_pAA011) to be upregulated by AggR (14, 15). The *aat* genes were upregulated >8-fold in 042 (Fig. 3); *aap*, encoding the dispersin surface protein, was remarkably >10³-fold upregulated in the EAEC 042 strain versus 042aggR (Fig. 3).

In the region spanning ECO42_pAA015 to ECO42_pAA023, only ECO42_pAA020 and ECO42_pAA021 (carrying the previously identified *shf* gene) were AggR regulated (2- to 4-fold). The effect was less pronounced for pAA022 (Fig. 3). ECO42_pAA020 encodes a hypothetical protein highly conserved in *E. coli*, *Salmonella* spp., and *Shigella* spp.

AggR induced expression of AAF/II biogenesis genes (*aafD* and *aafA*) as previously suggested by our group (26); AggR-dependent transcription had not previously been demonstrated for the unlinked *aafB* and *aafC* genes. ECO42_pAA047, *aafD*, and *aggR* itself (previously described) (27) showed high levels of differential expression (at 10²- to 10³-fold) (Fig. 3).

ECO42_pAA047, ECO42_pAA056, and ECO42_pAA060 are predicted to encode small hypothetical proteins. Interestingly, close but cryptic homologs of ECO42_pAA060 were identified in ETEC, *Haemophilus* spp., *Aggregatibacter* spp., *Actinobacillus* spp., *Yersinia* spp., and *Mannheimia haemolytica*. ECO42_pAA047, ECO42_pAA056, and ECO42_pAA060 functions remain cryptic.

Frequency of ORF3, ORF60, and ECO42_3184 among a collection of EAEC strains. We reasoned that if ORF3, ORF60, and ECO42_3184 were relevant to EAEC pathogenesis, they would be found commonly within a collection of EAEC strains and they would segregate with the *aggR* gene and with each other. Using PCR analysis for primers derived from the coding regions of ORF3 and ORF60, we detected products of the predicted sizes in the majority of EAEC strains from a well-characterized strain library consisting of isolates from geographically distinct regions (28). In contrast, the chromosomal gene ECO42_3184 was detected in a minority of EAEC strains (Table 3). ECO42_3184 and ORF60 were also found in 12.5 to 17% of the non-EAEC strains, whereas ORF3 was found exclusively in EAEC strains (Table 3).

Mutations in ORF3 and ORF4 result in altered surface properties. Our data suggested that ORF3 and ORF4 were coexpressed under AggR control in a large percentage of EAEC strains. The similarity of ORF3 and ORF4 to isoprenoid synthesis enzymes suggested to us that these genes may encode proteins with roles in bacterial cell surface modification. A deletion mutation was constructed in each ORF individually, and a mutation spanning both genes was constructed. The pattern of 042 biofilm formation was dramatically different in the ORF3 and ORF4 mutants, with bacteria adhering in a sheet conformation with no individual bacteria observed (Fig. 5). Aggregated 042 bacteria possess clear surface boundaries, while outer membranes (OMs) of the double mutant appear partially fused during bacterial apposition (Fig. 5). In agreement with the SEM findings, we also observed structural

FIG 7 Mutations in ORF4 result in altered surface properties. Transmission electron microscopy of negatively stained samples was performed for 042 (A to C), 042orf4 (D to F), and 042orf4(pBADorf4) (G to I). The samples were stained with 2% uranyl acetate and examined on a Jeol JEM1230 transmission electron microscope (80 kV) at the Advanced Microscopy Laboratory at the University of Virginia (AML-UVA). Samples were visualized at a magnification of $\times 5,000$ (A, D, and G), $\times 10,000$ (B, E, and H), or $\times 25,000$ (C, F, and I).

differences in the bacterial surface of the ORF4 mutant and ORF4-complemented in *trans* strains by TEM (see Fig. 7).

To assess surface integrity, strains were evaluated for resistance to defensins and polymyxin B. We found that deletion of ORF3, ORF4, or both renders the strain more susceptible to killing by polymyxin B than the wild-type parent (Fig. 6A). To ascertain susceptibility to defensins, the strains were incubated with defensin-1 for 1 h and surviving bacteria were enumerated by serial plating. 042 ORF3 and ORF4 strains were more susceptible to defensin-1 killing than was the wild-type strain (Fig. 6B).

We did not observe differences in lipid A structure in an *orf3-4* mutant (see Fig. S2 in the supplemental material). Importantly, growth curves of each of the mutants were indistinguishable from those of parent strain 042 under AggR-induced (DMEM) or AggR-repressed (LB) conditions (data not shown).

DISCUSSION

Using microarray and qRT-PCR approaches, we have confirmed at least 44 AggR-regulated genes in the genome of EAEC strain 042. Twenty-five out of the 44 genes were previously known to be

so regulated, identified as part of the Aai T6SS (16 genes), the dispersin secretion system (5 genes), and the AAF/II fimbrial biogenesis system (4 genes) (Fig. 2 and 3). Sixteen out of the 44 genes are predicted to encode hypothetical proteins, and only 5 of these genes showed homology to other genes encoding known bacterial proteins, suggesting new virulence-related functions.

The predicted products of two newly identified AggR-dependent genes, ORF3 and ORF4, were found to bear significant similarity to enzymes in the isoprenoid synthesis pathway (Fig. 4), and each was found to be both functional and commonly carried by EAEC strains. In the isoprenoid synthesis pathway, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the building blocks for biosynthesis of isoprenoid compounds (29, 30). Conversion of DMAPP to IPP is catalyzed by IPP isomerase (IDI) (29, 30). In the same pathway, linear isoprenoids are produced by isoprenyl diphosphate synthases that catalyze consecutive condensations of IPP with DMAPP on growing prenyl chains (31). The synthases can be classified as *cis*- and *trans*-isoprenyl pyrophosphate synthase according to the stereochemical nature of their products, and unlike the *trans*-type enzymes, *cis*-prenyltransferases lack the DDxxD motifs (32–35). The ORF3 sequence described here contains a single DDxxD motif which is commonly present twice in *trans*-isoprenyl diphosphate synthases and in the closest homologues, identified in *Archaea* and *Cyanobacteria* (Fig. 4). The predicted protein sequence for ORF4 showed high similarity to the canonical IDIs and contains all of the active site residues necessary for isomerase activity (Fig. 4). Interestingly, we found in the 042 chromosome evidence of the common *E. coli* IDI. We postulate, therefore, that 042 controls IDI expression via two systems, one tightly regulated by AggR (ORF4) and the other presumably under the control system employed by all *E. coli* strains. When the AggR regulon is activated, the ORF4 product hypothetically provides supplemental IDI activity to meet the demand of the concurrently expressed ORF3 isoprenyl synthase.

The biofilm phenotype of 042 Δ orf3-4 was strikingly different from that of its parent, with the cells adhering in a solid-sheet configuration (Fig. 5). Taken together, the findings substantiate the expression of ORF3 and ORF4 products. In addition, we show that 042orf3, 042orf4, and 042 Δ orf3-4 strains are more susceptible to polymyxin and defensin treatment (Fig. 6), compatible with surface modification or perturbation. We recognize that increased susceptibility to polymyxin and defensins may be due to altered membrane permeability, general debility, and/or accumulation of toxic compounds within the bacterium, although the general growth curves of the mutants were indistinguishable from those of the wild type under AggR-inducing conditions. Under TEM, we observed that 042orf4 releases abundant small vesicles (Fig. 7D to 7F); the production of OM vesicles has been identified as a bacterial stress response (36, 37). Under ORF3-expressing conditions, deletion of ORF4 may affect negatively the stores or ratio of DMAPP and IPP.

Our inference that newly detected ORF3 and ORF4 are somehow important to EAEC is supported by the finding of their high frequency and coinheritance with each other and with *aggR* in a collection of EAEC strains from around the world. Molecular epidemiologic and pathogenicity studies will be required to understand their concerted action in the context of virulence.

In conclusion, this work further extends our knowledge of genes under the control of the EAEC master virulence regulator AggR, though additional genes may yet be identified. The func-

tions of many genes described here are largely enigmatic. Possession of the *aggR* gene has been frequently associated with EAEC illness in epidemiologic studies, and thus, identifying the genes controlled by AggR is crucial to our understanding of EAEC pathogenesis.

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