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Association of Putative Enteroaggregative *Escherichia coli* Virulence Genes and Biofilm Production in Isolates from Travelers to Developing Countries[▽]

Jamal A. Mohamed,[1,2] David B. Huang,[5] Zhi-Dong Jiang,[3] Herbert L. DuPont,[1,2,3,4] James P. Nataro,[6] Jaime Belkind-Gerson,[7] and Pablo C. Okhuysen[1,2,3,4]*

Division of Infectious Diseases, Department of Internal Medicine,[1] and Center for the Study of Emerging and Reemerging Pathogens,[2] University of Texas Medical School, and Center for Infectious Diseases, School of Public Health,[3] Baylor College of Medicine,[4] Houston, Texas 77030; Boehringer-Ingelheim, Inc., Ridgefield, Connecticut 06877[5]; Center for Vaccine Development, Departments of Pediatrics, Medicine, Microbiology, and Immunology, University of Maryland School of Medicine, Baltimore, Maryland 21201[6]; and National Institute of Public Health, Cuernavaca, Mexico[7]

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Enteroaggregative *Escherichia coli* (EAEC) is an emerging enteric pathogen that causes acute and chronic diarrhea among children, human immunodeficiency virus-infected patients, and travelers to developing regions of the world. The pathogenesis of EAEC strains involves the production of biofilm. In this study, we determined the association between presence of putative EAEC virulence genes and biofilm formation in 57 EAEC isolates (as defined by HEp-2 adherence) from travelers with diarrhea and in 18 EAEC isolates from travelers without diarrhea. Twelve nondiarrheagenic *E. coli* isolates from healthy travelers were used as controls. Biofilm formation was measured by using a microtiter plate assay with the crystal violet staining method, and the presence of the putative EAEC virulence genes *aap*, *aatA*, *aggR*, *astA*, *irp2*, *pet*, *set1A*, and *shf* was determined by PCR. EAEC isolates were more likely to produce biofilm than nondiarrheagenic *E. coli* isolates ($P = 0.027$), and the production of biofilm was associated with the virulence genes *aggR*, *set1A*, *aatA*, and *irp2*, which were found in 16 (40%), 17 (43%), 10 (25%), and 27 (68%) of the biofilm producers versus only 4 (11%), 6 (6%), 2 (6%), and 15 (43%) in non-biofilm producers ($P = 0.008$ for *aggR*, $P = 0.0004$ for *set1A*, $P = 0.029$ for *aatA*, and $P = 0.04$ for *irp2*). Although the proportion of EAEC isolates producing biofilm in patients with diarrhea (51%) was similar to that in patients without diarrhea (61%), biofilm production was related to the carriage of *aggR* ($P = 0.015$), *set1A* ($P = 0.001$), and *aatA* ($P = 0.025$). Since *aggR* is a master regulator of EAEC, the presence of *aap* ($P = 0.004$), *astA* ($P = 0.001$), *irp2* ($P = 0.0006$), *pet* ($P = 0.002$), and *set1A* ($P = 0.014$) in an *aggR* versus an *aggR*-lacking background was investigated and was also found to be associated with biofilm production. This study suggests that biofilm formation is a common phenomenon among EAEC isolates derived from travelers with or without diarrhea and that multiple genes associated with biofilm formation are regulated by *aggR*.

Bacterial diarrhea is one of the most common causes of morbidity and mortality among infants and children of developing nations (4, 21). Diarrheagenic *Escherichia coli* is the most commonly identified pathogen, and at least five distinct pathotypes have been characterized, including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enteroinvasive *E. coli*, and enteroaggregative *E. coli* (EAEC). EAEC is an emerging food-borne pathotype that can cause acute and persistent diarrhea in children, human immunodeficiency virus-infected persons, and international travelers (1, 2, 15, 16, 24, 27). EAEC has been associated with diarrhea in both developing and industrialized countries (13, 24), including the United States (5, 24), and sometimes causes large outbreaks of gastrointestinal illness (14).

Aggregative adherence (AA) to the intestinal mucosa is the first step in EAEC pathogenesis (23). EAEC adheres to the small and large intestinal mucosal surface and stimulates mucus secretion, forming a thick aggregating biofilm (12, 21, 30, 32, 33). After adhesion, multiplication, and colonization on a surface (6), the bacteria surround themselves with exopolymeric substances and recruit more cells to form microcolonies interspersed with fluid-filled channels. Because of the restricted penetration of antimicrobials, decreased growth rate, and the expression of possible resistance genes, colonies in biofilm are not easily eradicated by bactericidal antibiotics (6) and may also be protected from attack by the intestinal immune system, resulting in prolonged infections.

The genetic determinants involved in biofilm production by EAEC have just begun to be understood (8, 18, 30, 34). Several virulence factors such as *aggA*, *aggR*, and *aap* (dispersin) are important in EAEC adherence to the intestinal mucosa and in stimulating biofilm formation (29, 30, 34). In addition, Sheikh et al. (30) have shown that *fis* and *yafK*, through the activation of *aggR*, are also important in biofilm formation. Several other genes, including *aatA*, *pet* (plasmid encoded toxins), ShET1 (*Shigella* enterotoxins), *irp2* (yersiniabactin biosynthesis gene), and *shf* (cryptic open reading frame), are also thought to be involved in EAEC pathogenesis (7, 9, 11, 29, 31). The relationship of known or putative EAEC virulence genes and biofilm formation in isolates derived from subjects with travelers' diarrhea has not been studied.

* Corresponding author. Mailing address: Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical School, 6431 Fannin St., Houston, TX 77030. Phone: (713) 500-6736. Fax: (713) 500-5495. E-mail: Pablo.C.Okhuysen@uth.tmc.edu.

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TABLE 1. Primers used in this study

Gene[a]	Putative function	Primer sequence	Amplicon size (bp)	Accession no. and/or reference	Starting PCR conditions
*aggR**	Transcriptional activator for AAF/I and AAF/II	5-CTAATTGTACAATCGATGTA-3′ 5′-ATGAAGTAATTCTTGAAT-3′	308	Z18751 (7)	1 min at 94°C, 1 min at 42°C, 1 min at 72°C
*aap**	Dispersin	5′-ATGAAAAAAATTAAGTTTGTTATCTT-3′ 5′-TTATTTAACCCATTCGGTTAGAGC-3′	351	Z32523	1 min at 94°C, 1 min at 52°C, 1 min at 72°C
*aatA**	Dispersin transporter protein/CVD432	5′-CTGGCGAAAGACTGTATCAT-3′ 5′-CAATGTATAGAAATCCGCTGTT-3′	630	X81423	1 min at 94°C, 1 min at 55°C, 1 min at 72°C
astA	EAST1 heat-stable toxin	5′-ATGCCATCAACACAGTATAT-3′ 5′-GCGAGTGACGGCTTTGTAGT-3′	110	S81691	1 min at 94°C, 1 min at 55°C, 1 min at 72°C
pet	Plasmid encoded toxin	5′-ACTGGCGGACTCATTGCTGT-3′ 5′-GCGTTTTTCCGTTCCCTATT-3′	832	AFO56581	1 min at 94°C, 1 min at 55°C, 1 min at 72°C
shf	Cryptic open reading frame	5′-ACTTTCTCCCGAGACATTC-3′ 5′-CTTTAGCGGGAGCATTCAT-3′	613	AF134403 (7)	1 min at 94°C, 1 min at 50°C, 1 min at 72°C
irp2†	Yersiniabactin biosynthesis	5′-AAGGATTCGCTGTTACCGGAC-3′ 5′-TCGTCGGGCAGCGTTTCTTCT-3′	264	Schubert et al. (28)	1 min at 94°C, 1 min at 55°C, 1 min at 72°C
set1A†	*Shigella* enterotoxin 1	5′-TCACGCTACCATCAAAGA-3′ 5′-TATCCCCCTTTGGTGGTA-3′	309	AF097644	1 min at 94°C, 1 min at 55°C, 1 min at 72°C

[a] *, Plasmid-borne genes known to be regulated by AggR; †, chromosomal encoded gene.

MATERIALS AND METHODS

Study population and microbiology. Stool samples were collected during the period from 1999 to 2004 from travelers from industrialized countries with diarrhea ($n = 57$) and without diarrhea ($n = 30$) during short-term stays in Mexico (57 isolates), India (10 isolates), and Guatemala (8 isolates) (10) and studied for the presence of enteric pathogens as previously described (19). A total of 75 EAEC isolates (57 from travelers with diarrhea and 18 from travelers without diarrhea) derived from fresh stool culture and stored in peptone stabs were used in the present study. Twelve *E. coli* isolates from subjects without diarrhea that did not demonstrate HEp-2 adherence and did not carry genes for ETEC heat-labile toxin (LT) and/or heat-stable toxin (ST) production were also used in the present study as nondiarrheagenic *E. coli* controls. The study was approved by the University of Texas Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston, and written informed consent was obtained from each subject.

Detection of virulence factors by PCR. *E. coli* isolates were picked from a single colony grown on Luria broth agar plates. EAEC virulence factors were identified by PCR using specific primers (Table 1) as described previously (7, 28), including the putative virulence genes *irp2* of *Yersinia* species (28) and shET1 (*set1A*) of *Shigella* (7), which have been previously identified in some EAEC isolates.

Biofilm assay. To test for biofilm formation, isolates grown overnight in Luria broth with 0.25% glucose at 37°C with agitation were diluted 1:100 in Dulbecco modified Eagle medium plus 0.45% glucose, and 200 μl of the bacterial suspension was inoculated into individual wells of sterile 96-well polystyrene microtiter plates. After incubation at 18 h at 37°C, a modified biofilm assay was carried out according to previously published methods (17), except that bacteria were fixed with 200 μl of Bouin fixative for 15 min at room temperature and rinsed once with phosphate-buffered saline. The fixed bacterial cells were then stained with 0.5% crystal violet for 15 min at room temperature and rinsed thoroughly with distilled water. After air drying, crystal violet was solubilized in 200 μl of ethanol-acetone (80:20 [vol/vol]) for 30 min, and the optical density at 570 nm (OD_{570}) was measured by using a microplate reader (MultiskanSpectrum, Thermo Labsystems, Vantaa, Finland). Each assay was performed in quadruplicate and repeated on at least three different occasions. EAEC strain 042 served as a positive control (30) for the strong biofilm producer, and the nonpathogenic *E. coli* strains HS and *E. coli* DH5α served as negative controls for the non-biofilm producer.

Statistical analysis. Statistical analyses were performed by using the Fisher exact test and chi-square test for categorical data, while the nonparametric Mann-Whitney test, as well as analysis of variance (GraphPad Prism 4) was used for continuous variables. The median OD_{570} and interquartile range values for clinical isolates were calculated by using GraphPad Prism 4 statistical software.

(This study was presented in part at the 106th General Meeting of the American Society for Microbiology, 21 to 25 May 2006, Orlando, FL.)

RESULTS

Biofilm formation by EAEC isolates. The biofilm mean OD_{570} values for the negative controls *E. coli* HS and *E. coli* DH5α were 0.377 ± 0.026 and 0.243 ± 0.029, respectively, whereas for the positive control EAEC 042 the mean OD_{570} value was 3.341 ± 0.074. Based on OD_{570} readings after incubation and crystal violet staining, *E. coli* isolates were deemed biofilm formers if the OD_{570} readings exceeded the mean plus two standard deviations of the *E. coli* HS and DH5α negative control strains (i.e., >0.46). We also arbitrarily classified samples as strong biofilm ($OD_{570} \geq 1$), weak biofilm ($OD_{570} > 0.46 < 1$), and non-biofilm ($OD_{570} \leq 0.46$) producers (Fig. 1). For the entire group of isolates, the OD_{570} readings ranged from 0.16 to 3.36 U (Fig. 1). Forty (53%) of the isolates were classified as biofilm producers with a mean OD of 1.143 ± 0.744. Of the rest of the isolates, 35 (47%) were classified as non-biofilm producers with a mean OD of 0.292 ± 0.075. Based on the definition given above, 40 of the 75 (53%) EAEC isolates were biofilm producers; in contrast, only 2 of the 12 (17%) nondiarrheagenic *E. coli* isolates produced biofilm ($P = 0.027$; chi-squre test), and when they did it was of the weak phenotype (Fig. 1). Among the 75 EAEC isolates, 16 (21%) were strong biofilm (mean OD = 1.852 ± 0.722), 24 (32%) were weak (mean OD = 0.670 ± 0.127), and 35 (47%) were non-biofilm (mean OD = 0.292 ± 0.075) producers (Tables 2 and 3). Among 75 EAEC isolates, 31 (54%) of the 57 isolates from Mexico, 5 (50%) of the 10 isolates from India, and 5

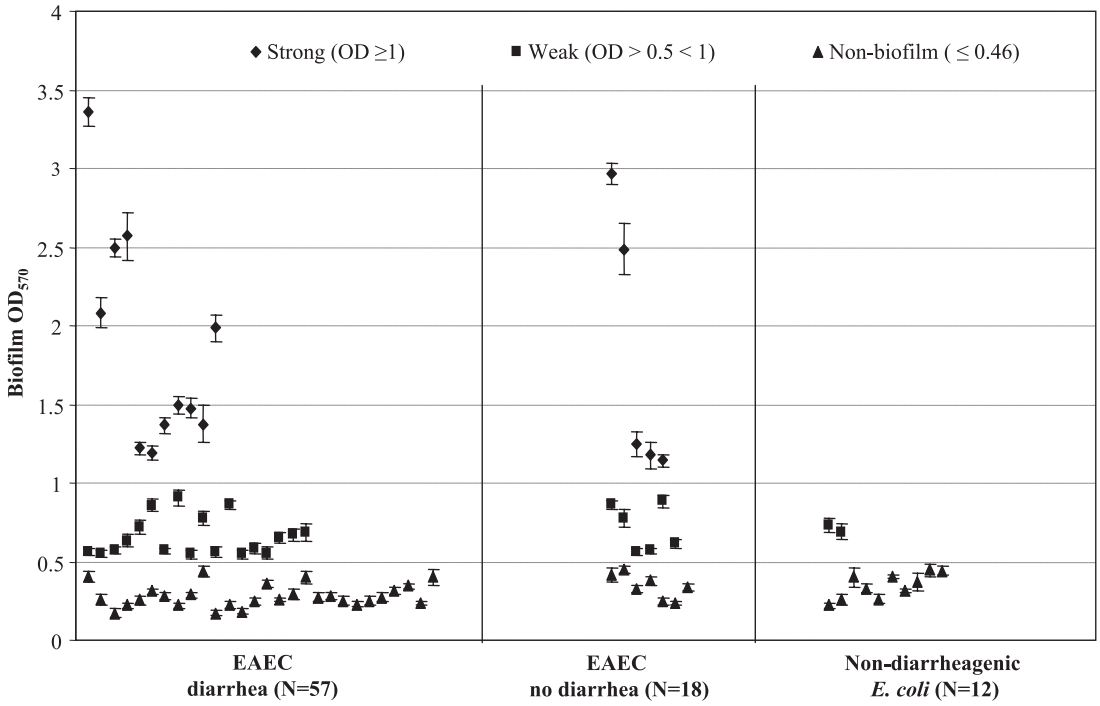

FIG. 1. Biofilm formation by EAEC isolates derived from travelers' with diarrhea or without diarrhea. Biofilm formation on polystyrene surface after 18 h was assessed by crystal violet staining. Each dot indicates the mean values with standard deviations. The biofilm assay was performed three times, with quadruplicates in each assay. Biofilm formation by nondiarrheagenic *E. coli* isolates was also tested. *E coli* isolates were deemed biofilm formers if the OD_{570} readings exceeded the mean plus two standard deviations of the *E. coli* strains HS and DH5α negative control for non-biofilm producers (i.e., >0.46).

(62%) of the 8 isolates from Guatemala produced biofilm (the *P* value was not significant [NS]; Fisher's exact test).

Association between the presence of putative EAEC virulence genes and biofilm formation. We observed an association between the presence of *aggR* and biofilm formation. Of the 75 EAEC isolates, 16 (40%) of 40 the biofilm producers contained *aggR* versus only 4 of 35 (11%) of the non-biofilm producers ($P = 0.008$; Fisher's exact test) (Tables 2 and 3). Among these isolates, *aggR* was detected in 8 of 16 (50%) of the strong biofilm producers and in 8 of 24 (33%) of the weak biofilm producers. We also identified an association with the presence of the *set1A* gene and biofilm production, which was present in 17 of 40 (43%) of biofilm producers versus 2 of 35 (6%) of non-biofilm producers ($P = 0.0004$; Fisher's exact test) (Tables 2 and 3). Of 40 biofilm-positive isolates, 10 (25%) carried the *aatA* gene compared to only 2 of 35 (6%) biofilm-negative isolates ($P = 0.029$; Fisher's exact test) (Tables 2 and 3).

Of 40 biofilm producers, 27 (68%) carried *irp2* versus 15 (43%) of 35 non-biofilm producers ($P = 0.038$; Fisher's exact

TABLE 2. Occurrence of biofilm formation (BF+) and presence of various genes in *E. coli* isolates from U.S. travelers to Mexico, India, and Guatemala in EAEC isolates ($n = 75$)

Gene[a]	Biofilm formation [no. of isolates (%)][b]				*P*[c]
	Strong ($n = 16$)	Weak ($n = 24$)	Non-BF ($n = 35$)	All BF+ ($n = 40$)	
*aggR**	8 (50)	8 (33)	4 (11)	16 (40)	0.008
set1A†	8 (50)	9 (38)	2 (6)	17 (43)	0.0004
*aatA**	6 (38)	4 (17)	2 (6)	10 (25)	0.029
irp2†	9 (56)	18 (75)	15 (43)	27 (68)	0.038
*aap**	6 (38)	7 (29)	7 (20)	13 (33)	0.298
pet	8 (50)	8 (33)	8 (23)	16 (40)	0.14
shf	3 (19)	7 (29)	14 (40)	10 (25)	0.217
astA	12 (75)	14 (58)	25 (71)	26 (65)	0.624

[a] *, plasmid-borne genes known to be regulated by AggR; †, chromosomal encoded gene.
[b] Strong, $OD_{570} \geq 1$; weak, $OD_{570} > 0.46 < 1$; Non-BF, $OD_{570} \leq 0.46$.
[c] *P* values represent biofilm formers versus non-biofilm formers (Fisher's exact test).

TABLE 3. Occurrence of biofilm formation (BF+) and presence of various genes in *E. coli* isolates from U.S. travelers to Mexico, India, and Guatemala in non-EAEC isolates ($n = 12$)

Gene[a]	Biofilm formation [no. of isolates (%)][b]				*P*[c]
	Strong ($n = 0$)	Weak ($n = 2$)	Non-BF ($n = 10$)	All BF+ ($n = 2$)	
*aggR**	0	0	0	0	1.000
set1A†	0	0	4 (40)	0	0.515
*aatA**	0	0	0	0	1.000
irp2†	0	1 (50)	8 (80)	1 (50)	0.455
*aap**	0	0	0	0	1.000
pet	0	0	3 (30)	0	1.000
shf	0	1 (50)	2 (30)	1 (50)	0.455
astA	0	1 (50)	4 (40)	1 (50)	1.000

[a] *, plasmid-borne genes known to be regulated by AggR; †, chromosomal encoded gene.
[b] Strong, $OD_{570} \geq 1$; weak, $OD_{570} > 0.46 < 1$; Non-BF, $OD_{570} \leq 0.46$.
[c] *P* values represent biofilm formers versus non-biofilm formers (Fisher's exact test).

TABLE 4. Incidence of biofilm (BF+) formation and the presence of various genes among EAEC isolates from patients with diarrhea (n = 57)

Gene[a]	Biofilm formation [no. of isolates (%)]		P[b]
	BF+ (n = 29)	BF− (n = 28)	
*aggR**	12 (41)	3 (11)	0.015
set1A†	14 (48)	2 (7)	0.001
*aatA**	8 (27)	1 (4)	0.025
irp2†	19 (65)	11 (39)	0.065
aap†	9 (31)	5 (18)	0.360
pet	12 (41)	5 (18)	0.082
shf	9 (31)	11 (39)	0.585
astA	23 (79)	22 (79)	1.000

[a] *, plasmid-borne genes known to be regulated by AggR; †, chromosomal encoded gene.
[b] P values represent biofilm formers versus non-biofilm formers (Fisher's exact test).

TABLE 5. Incidence of biofilm (BF+) formation and the presence of various genes among EAEC isolates from patients without diarrhea (n = 18)

Gene[a]	Biofilm formation [no. of isolates (%)]		P[b]
	BF+ (n = 11)	BF− (n = 7)	
*aggR**	4 (36)	1 (14)	0.596
set1A†	3 (27)	0	0.245
*aatA**	2 (18)	1 (14)	1.000
irp2†	8 (73)	4 (57)	0.627
aap†	4 (36)	2 (29)	1.000
pet	4 (36)	3 (43)	1.000
shf	1 (9)	3 (43)	0.245
astA	3 (27)	3 (43)	0.627

[a] *, plasmid-borne genes known to be regulated by AggR; †, chromosomal encoded gene.
[b] P values represent biofilm formers versus non-biofilm formers (Fisher's exact test).

test) (Tables 2 and 3). No significant relationship between the presence of other putative virulence factors, including *aap*, *astA*, *pet*,and *shf*, and the biofilm phenotype were identified. Thirteen (13 of 40 or 32%) biofilm-producing EAEC isolates carried four genes (*aggR*, *set1A*, *aatA*, and/or *aap*) simultaneously in contrast to none (0 of 35) of the non-biofilm-producing EAEC isolates (P = 0.0001; Fisher's exact test). Of these 13 biofilm-producing isolates, 10, 2, and 1 isolate were derived from Mexico, India, and Guatemala, respectively (P = NS; Fisher's exact test). Of interest, 10 of the 12 nondiarrheagenic *E. coli* isolates had at least one or more of the genes studied (*set1A*, *irp2*, *pet*, *astA*, and *shf*) (Tables 2 and 3); however, they demonstrated weak or no biofilm formation.

The median biofilm OD value was significantly higher in biofilm producers carrying *aggR* (median OD of 0.72 versus 0.40), *set1A* (median OD of 0.86 versus 0.40), and *aatA* (median OD of 1.376 versus 0.44) than in isolates lacking these genes (P = 0.001 for *aggR*; P = 0.0003 for *set1A*; and P = 0.01 for *aatA*; Mann-Whitney test). In contrast, no significant association between biofilm and carriage of the *aap*, *astA*, *pet*, and *shf* genes were observed.

Association of biofilm and virulence genes with diarrhea. As shown in the Tables 4 and 5, the proportion of EAEC isolates producing biofilm from patients with diarrhea (29 of 57 [51%]) was similar to that of subjects without travelers' diarrhea (11 of 18 [61%]; P = NS; Fisher's exact test), and biofilm production by EAEC isolates from travelers' diarrhea was related to the carriage of virulence genes, particularly *aggR*, *set1A*, and *aatA* (P = 0.015 for *aggR*, P = 0.001 for *set1A*, and P = 0.025 for *aatA*; Fisher's exact test).

Role of AggR regulator and biofilm formation. Since AggR is a master regulator in the pAA plasmid, the relatedness of *aggR* to other genes was studied. Twelve biofilm producers had both *aggR* and *set1A* genes (eight strong, six weak), and none of non-biofilm producers had both these genes together. However, 20 biofilm producers (5 strong, 15 weak) and 34 non-biofilm producers did not possess both genes ($P < 0.0001$; Fisher's exact test). The presence of *aap*, *astA*, *irp2*, *pet*, and *set1A* virulence genes in the *aggR* background for biofilm production showed that the presence of these genes was associated with biofilm as long as *aggR* was also present. The pooled median biofilm ODs of *set1A*+/*aggR*+ versus *set1A*+/*aggR*− (P = 0.014), *aap*+/*aggR*+ versus *aap*+/*aggR*− (P = 0.004), *astA*+/*aggR*+ versus *astA*+/*aggR*− (P = 0.0019), *irp2*+/*aggR*+ versus *irp2*+/*aggR*− (P = 0.0006), and *pet*+/*aggR*+ versus *pet*+/*aggR*− (P = 0.0028) were statistically significant (Mann-Whitney test) (Fig. 2).

DISCUSSION

The objective of this study was to determine the occurrence of various EAEC virulence factors and of biofilm formation among clinical isolates of EAEC collected from travelers from industrialized countries to Mexico, India, and Guatemala during short-terms stays. Of 75 EAEC isolates, 45 (53%) produced biofilm on an abiotic surface, which is lower than the rates reported by others (34) and may be due to our more stringent definition of biofilm formation or to differences in study design. In contrast to the findings of a previous study (34) wherein isolates were initially screened for biofilm and then characterized for aggregative phenotype, we initially screened for the aggregative phenotype and then characterized the isolates for biofilm formation. The frequency of biofilm formation by EAEC versus nondiarrheagenic *E. coli* isolates was significant, confirming that biofilm formation is more common in EAEC isolates than in nondiarrheagenic *E. coli* isolates (30, 34).

Isolates carrying *aggR* alone or in combination with other virulence genes (so-called typical EAEC) were strongly associated with biofilm formation. *aggR* is an EAEC master regulator formerly described as a transcriptional activator for AAF/I expression (19, 20) and later proved also to be required for AAF/II expression (9). Although other investigators have reported that no correlation exists between *aggR* and biofilm formation (26, 30), our findings are consistent with the work of Wakimoto et al. (34). In a related study, EAEC adhesins were found to be allelic in nature, and biofilm formation was shared by all members of the AAF family among Indian isolates (3). Taken together, these results suggest the sequence of factors AggR → AAF allele → biofilm production though other pathways can produce the same phenotype.

We also investigated the relationship of dispersin and its transporter in biofilm formation. Dispersin is a 10-kDa secreted protein coded by the *aap* gene that coats the bacterial

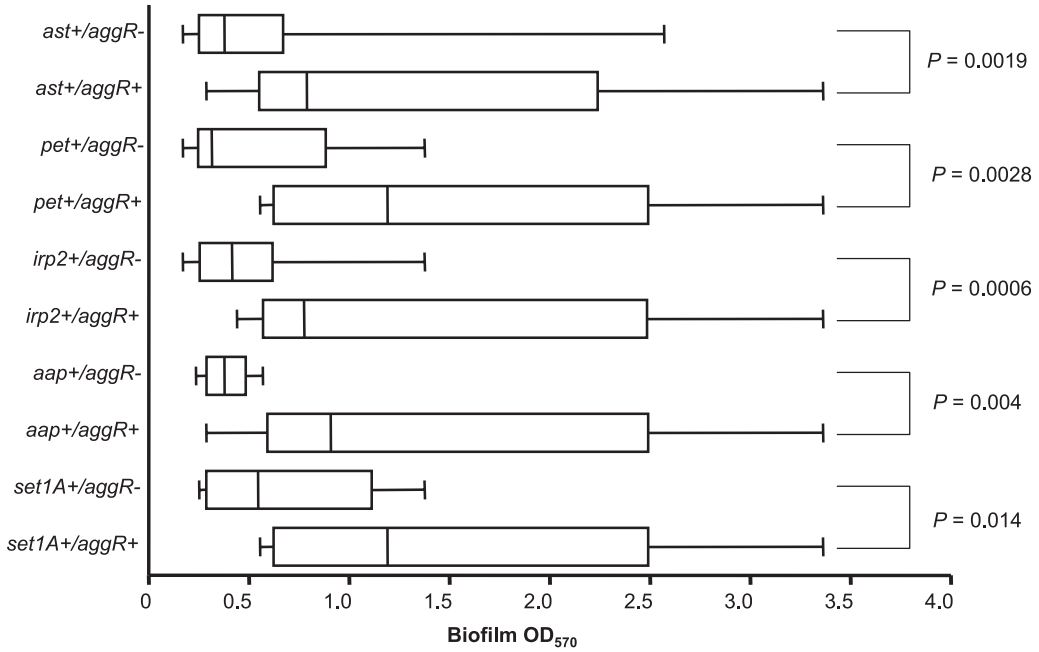

FIG. 2. Biofilm formation by EAEC isolates based on the presence of various genes in *aggR* background. Biofilm formation on a polystyrene surface after 18 h was assessed by crystal violet staining. Values are based on pooled median biofilm OD_{570} values shown by the box-and-whiskers plot which represents an upper quartile, median, and lower quartile.

surface and was selected for study since this protein promotes the dispersal of EAEC on the intestinal mucosa (29). Biofilm formation also correlated with the presence of the *aatA* gene that codes for a transporter protein that is a homolog of *E. coli* outer membrane protein, TolC (22). This was also of interest to us because the transporter facilitates the export of dispersin across the outer membrane in EAEC (29) and is the target for probe CVD 432, which is widely used to identify EAEC by molecular methods.

A novel finding in our study was the fact that the chromosomally located genes, *set1A* (*Shigella* enterotoxin 1) and *irp2* (yersiniabactin biosynthesis gene) were also associated with EAEC biofilm production. *set1A* encodes an oligomeric enterotoxin, ShET1 (7, 11) and it contributes to secretory diarrhea caused by EAEC and *Shigella* infections. EAEC strains carrying *set1A* have been associated with diarrhea in Brazilian children and adult travelers from Spain (25). *irp2* in turn encodes a protein involved in yersiniabactin expression and is designated iron-repressible high-molecular-weight protein 2 (Irp2) (7, 28). *irp2* represents part of an unstable pathogenicity island that was acquired as a result of a horizontal transfer. Since *set1A* is located on the same island as a cluster of AggR-regulated genes, the relationship may be coincidental since it travels together with other putative virulence genes involved in biofilm formation.

In the present study, the proportion of EAEC isolates that were biofilm producers was similar in travelers with diarrhea and in travelers with asymptomatic colonization (2) and may explain in part the ability of EAEC to cause chronic colonization (1).

Since *aggR* is a master regulator that controls both plasmid and chromosomally encoded genes in EAEC, the biofilm formation ability of EAEC isolates carrying *aap*, *astA*, *irp2*, *pet*, and *set1A* with or without an *aggR* background was examined. EAEC isolates possessing the *aap*, *astA*, *irp2*, *pet*, and *set1A* genes in an *aggR* background produced more biofilm than those with an *aggR*-lacking background. This suggests that *aggR* regulates other genes needed for biofilm formation in EAEC. Recently, Nataro and Dudley have shown that AggR not only controls the plasmid-borne AAF fimbriae but also chromosomal genes located on an island located at 94 min of the EAEC chromosome (E. Dudley and J. Nataro, unpublished results). Further, it seems that additional genes are also under the control of *aggR* such as *aatA* (the dispersin transporter) and *aap* (dispersin). Sheikh et al. (30) have proved that the Fis protein contributes to biofilm production through AAF/II biogenesis, apparently by the activation of *aggR* expression.

Nine HEp-2 adherent isolates produced biofilm but did not carry either *aggR*, or *aatA*, or *irp2*, or *set1A* genes, indicating that there are additional factors involved in biofilm production in EAEC. It has recently been shown that atypical EAEC strain C1096 harboring Incl1 plasmid encoding a type IV pilus can contribute to biofilm formation (8). Additional work is needed to characterize HEp-2-adherent, biofilm-forming EAEC strains that are atypical (negative for *aggR*).

In summary, the present study suggests that biofilm formation is a common phenomenon among EAEC isolates derived from travelers with or without diarrhea. In vitro biofilm production from EAEC isolates is associated with *aggR*, and its regulated genes *aatA*, *irp2*, and *set1A*. Multiple EAEC genes located on the plasmid, as well as on its chromosome, are associated with biofilm formation.

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REFERENCES

1. **Adachi, J. A., C. D. Ericsson, Z. D. Jiang, M. W. DuPont, S. R. Pallegar, and H. L. DuPont.** 2002. Natural history of enteroaggregative and enterotoxigenic *Escherichia coli* infection among US travelers to Guadalajara, Mexico. J. Infect. Dis. **185:**1681–1683.
2. **Adachi, J. A., Z. D. Jiang, J. J. Mathewson, M. P. Verenkar, S. Thompson, F. Martinez-Sandoval, R. Steffen, C. D. Ericsson, and H. L. DuPont.** 2001. Enteroaggregative *Escherichia coli* as a major etiologic agent in traveler's diarrhea in 3 regions of the world. Clin. Infect. Dis. **32:**1706–1709.
3. **Bhardwaj, R., S. Majumdar, N. K. Ganguly, N. Taneja, S. Dutta, T. Ramamurthy, and A. Chakraborti.** 2006. Characterization of adhesin variants in Indian isolates of enteroaggregative *Escherichia coli*. FEMS Microbiol. Lett. **258:**274–283.
4. **Clarke, S. C.** 2001. Diarrhoeagenic *Escherichia coli*: an emerging problem? Diagn. Microbiol. Infect. Dis. **41:**93–98.
5. **Cohen, M. B., J. P. Nataro, D. I. Bernstein, J. Hawkins, N. Roberts, and M. A. Staat.** 2005. Prevalence of diarrheagenic *Escherichia coli* in acute childhood enteritis: a prospective controlled study. J. Pediatr. **146:**54–61.
6. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322.
7. **Czeczulin, J. R., T. S. Whittam, I. R. Henderson, F. Navarro-Garcia, and J. P. Nataro.** 1999. Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli*. Infect. Immun. **67:**2692–2699.
8. **Dudley, E. G., C. Abe, J. M. Ghigo, P. Latour-Lambert, J. C. Hormazabal, and J. P. Nataro.** 2006. An IncI1 plasmid contributes to the adherence of the atypical enteroaggregative *Escherichia coli* strain C1096 to cultured cells and abiotic surfaces. Infect. Immun. **74:**2102–2114.
9. **Elias, W. P., Jr., J. R. Czeczulin, I. R. Henderson, L. R. Trabulsi, and J. P. Nataro.** 1999. Organization of biogenesis genes for aggregative adherence fimbria II defines a virulence gene cluster in enteroaggregative *Escherichia coli*. J. Bacteriol. **181:**1779–1785.
10. **Greenberg, D. E., Z. D. Jiang, R. Steffen, M. P. Verenker, and H. L. DuPont.** 2002. Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative *Escherichia coli* pathogenicity. J. Infect. Dis. **185:**944–949.
11. **Henderson, I. R., S. Hicks, F. Navarro-Garcia, W. P. Elias, A. D. Philips, and J. P. Nataro.** 1999. Involvement of the enteroaggregative *Escherichia coli* plasmid-encoded toxin in causing human intestinal damage. Infect. Immun. **67:**5338–5344.
12. **Hicks, S., D. C. Candy, and A. D. Phillips.** 1996. Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. Infect. Immun. **64:**4751–4760.
13. **Huppertz, H. I., S. Rutkowski, S. Aleksic, and H. Karch.** 1997. Acute and chronic diarrhoea and abdominal colic associated with enteroaggregative *Escherichia coli* in young children living in western Europe. Lancet **349:**1660–1662.
14. **Itoh, Y., I. Nagano, M. Kunishima, and T. Ezaki.** 1997. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. J. Clin. Microbiol. **35:**2546–2550.
15. **Jiang, Z. D., B. Lowe, M. P. Verenkar, D. Ashley, R. Steffen, N. Tornieporth, F. von Sonnenburg, P. Waiyaki, and H. L. DuPont.** 2002. Prevalence of enteric pathogens among international travelers with diarrhea acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay). J. Infect. Dis. **185:**497–502.
16. **Mathewson, J. J., P. C. Johnson, H. L. DuPont, D. R. Morgan, S. A. Thornton, L. V. Wood, and C. D. Ericsson.** 1985. A newly recognized cause of travelers' diarrhea: enteroadherent *Escherichia coli*. J. Infect. Dis. **151:**471–475.
17. **Mohamed, J. A., W. Huang, S. R. Nallapareddy, F. Teng, and B. E. Murray.** 2004. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. Infect. Immun. **72:**3658–3663.
18. **Moreira, C. G., S. M. Carneiro, J. P. Nataro, L. R. Trabulsi, and W. P. Elias.** 2003. Role of type I fimbriae in the aggregative adhesion pattern of enteroaggregative *Escherichia coli*. FEMS Microbiol. Lett. **226:**79–85.
19. **Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine.** 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect. Immun. **60:**2297–2304.
20. **Nataro, J. P., J. B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M. M. Levine.** 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediatr. Infect. Dis. J. **6:**829–831.
21. **Nataro, J. P., T. Steiner, and R. L. Guerrant.** 1998. Enteroaggregative *Escherichia coli*. Emerg. Infect. Dis. **4:**251–261.
22. **Nishi, J., J. Sheikh, K. Mizuguchi, B. Luisi, V. Burland, A. Boutin, D. J. Rose, F. R. Blattner, and J. P. Nataro.** 2003. The export of coat protein from enteroaggregative *Escherichia coli* by a specific ATP-binding cassette transporter system. J. Biol. Chem. **278:**45680–45689.
23. **Okeke, I. N., A. Lamikanra, J. Czeczulin, F. Dubovsky, J. B. Kaper, and J. P. Nataro.** 2000. Heterogeneous virulence of enteroaggregative *Escherichia coli* strains isolated from children in Southwest Nigeria. J. Infect. Dis. **181:**252–260.
24. **Okeke, I. N., and J. P. Nataro.** 2001. Enteroaggregative *Escherichia coli*. Lancet Infect. Dis. **1:**304–313.
25. **Piva, I. C., A. L. Pereira, L. R. Ferraz, R. S. Silva, A. C. Vieira, J. E. Blanco, M. Blanco, J. Blanco, and L. G. Giugliano.** 2003. Virulence markers of enteroaggregative *Escherichia coli* isolated from children and adults with diarrhea in Brasilia, Brazil. J. Clin. Microbiol. **41:**1827–1832.
26. **Sarantuya, J., J. Nishi, N. Wakimoto, S. Erdene, J. P. Nataro, J. Sheikh, M. Iwashita, K. Manago, K. Tokuda, M. Yoshinaga, K. Miyata, and Y. Kawano.** 2004. Typical enteroaggregative *Escherichia coli* is the most prevalent pathotype among *E. coli* strains causing diarrhea in Mongolian children. J. Clin. Microbiol. **42:**133–139.
27. **Scaletsky, I. C., S. H. Fabbricotti, S. O. Silva, M. B. Morais, and U. Fagundes-Neto.** 2002. HEp-2-adherent *Escherichia coli* strains associated with acute infantile diarrhea, Sao Paulo, Brazil. Emerg. Infect. Dis. **8:**855–858.
28. **Schubert, S., A. Rakin, H. Karch, E. Carniel, and J. Heesemann.** 1998. Prevalence of the "high-pathogenicity island" of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. Infect. Immun. **66:**480–485.
29. **Sheikh, J., J. R. Czeczulin, S. Harrington, S. Hicks, I. R. Henderson, C. Le Bouguenec, P. Gounon, A. Phillips, and J. P. Nataro.** 2002. A novel dispersin protein in enteroaggregative *Escherichia coli*. J. Clin. Investig. **110:**1329–1337.
30. **Sheikh, J., S. Hicks, M. Dall'Agnol, A. D. Phillips, and J. P. Nataro.** 2001. Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. Mol. Microbiol. **41:**983–997.
31. **Suzart, S., B. E. Guth, M. Z. Pedroso, U. M. Okafor, and T. A. Gomes.** 2001. Diversity of surface structures and virulence genetic markers among enteroaggregative *Escherichia coli* (EAEC) strains with or without the EAEC DNA probe sequence. FEMS Microbiol. Lett. **201:**163–168.
32. **Tzipori, S., J. Montanaro, R. M. Robins-Browne, P. Vial, R. Gibson, and M. M. Levine.** 1992. Studies with enteroaggregative *Escherichia coli* in the gnotobiotic piglet gastroenteritis model. Infect. Immun. **60:**5302–5306.
33. **Vila, J., A. Gene, M. Vargas, J. Gascon, C. Latorre, and M. T. Jimenez de Anta.** 1998. A case-control study of diarrhea in children caused by *Escherichia coli* producing heat-stable enterotoxin (EAST-1). J. Med. Microbiol. **47:**889–891.
34. **Wakimoto, N., J. Nishi, J. Sheikh, J. P. Nataro, J. Sarantuya, M. Iwashita, K. Manago, K. Tokuda, M. Yoshinaga, and Y. Kawano.** 2004. Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*. Am. J. Trop. Med. Hyg. **71:**687–690.