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Differentiation of F18ab$^+$ from F18ac$^+$ *Escherichia coli* by Single-Strand Conformational Polymorphism Analysis of the Major Fimbrial Subunit Gene (*fedA*)

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Toxin-producing *Escherichia coli* expressing F18 fimbriae colonizes the small intestines of weaned pigs and causes diarrhea, edema disease, or both. The F18 family is composed of two antigenic variants, F18ab and F18ac. Because many strains do not express F18 fimbriae in vitro, identification and differentiation of these two variants are difficult. Single-strand conformational polymorphism (SSCP) analysis is a rapid method for identifying genetic mutations and polymorphisms. The F18 major fimbrial subunit genes (*fedA*) of 138 strains were amplified by PCR, and genetic differences were detected by SSCP analysis. The SSCP analysis of the *fedA* gene differentiated F18ab$^+$ strains from F18ac$^+$ strains. Most strains classified as F18ab$^+$ by SSCP analysis contained Shiga toxin 2e and enterotoxin genes. Most strains classified as F18ac$^+$ by SSCP analysis contained only enterotoxin genes. The SSCP analysis was a useful method for predicting the antigenicity of F18$^+$ *E. coli* and could also be used for analysis of other virulence genes in *E. coli* and other pathogenic bacteria.

Enterotoxigenic *Escherichia coli* (ETEC) and *E. coli* organisms that produce Shiga toxin 2e (STEC) colonize the porcine small intestine and cause diarrhea and edema disease, respectively. The fimbrial adhesins of K99, F41, K88, and 987P fimbriae mediate adherence and promote ETEC colonization of the neonatal pig's small intestine. Of these four fimbriae, only K88 is frequently detected in ETEC isolated from both weaned and neonatal pigs (24). The F18 fimbria mediates colonization of both ETEC and STEC in weaned, but not neonatal, pigs. The F18 fimbrial family is composed of two antigenic variants, F18ab and F18ac, and has been previously referred to as F107, 2134P, Av24, and 8813 (1–4, 11, 14, 15, 19, 20, 25).

Differentiation of strains expressing F18ab from those expressing F18ac may be important in development and selection of effective vaccines for ETEC and STEC infections in weaned pigs. Differentiation of strains producing F18ab and F18ac is also important because of a correlation between the type of toxin produced and clinical sequelae in infected swine. F18ab$^+$ strains are generally STEC and are associated with edema disease, while F18ac$^+$ strains are generally ETEC and are associated with diarrhea (4, 15, 26). Monospecific polyclonal antisera and the monoclonal antibody 6C7/C1, which is specific for F18ac$^+$ strains, can differentiate between these two antigenic variants (3, 4, 15, 19). However, serologic differentiation is not always possible because many strains do not express F18 when cultured in vitro under standard culture conditions (1, 8, 25, 26). The gene encoding the major fimbrial subunit of F18 (*fedA*) in both F18ab$^+$ and F18ac$^+$ strains has been sequenced, and differences have been found (9). A PCR-restriction fragment length polymorphism (RFLP) test consisting of amplification of the *fedA* gene followed by digestion with the restriction enzyme *Ngo*MI has been used to differentiate F18ab$^+$ from F18ac$^+$ strains (9, 15, 19). This PCR-RFLP test avoided the problems of serologic differentiation, which requires in vitro pilus expression, and was based upon the DNA sequences of seven different *fedA* genes, *fedA* and *fedA.1* to *fedA.6* (9). These seven different sequences were determined by sequencing of the *fedA* genes of only 10 unique strains (9), demonstrating that the *fedA* gene is highly polymorphic.

Single-strand conformational polymorphism (SSCP) analysis can rapidly identify polymorphisms in a gene and is useful when a large number of samples are being analyzed. Single-strand DNA migrates according to size and shape in a nondenaturing gel. The shape is dependent upon folding due to intermolecular interactions which are DNA sequence dependent (5). The major objective of this study was to determine if SSCP analysis of the *fedA* gene could differentiate F18ab$^+$ from F18ac$^+$ strains.

MATERIALS AND METHODS

Bacterial strains and SSCP analysis. Identification of *fedA*$^+$ strains in the *E. coli* collection at the National Animal Disease Center was done by colony blot hybridization with a 510-bp DNA fragment bearing *fedA* (7). The presence of genes encoding Shiga toxin 2e (Stx2e) and enterotoxins (LT, STa, and STb) was determined by colony blot hybridization (13, 18). A total of 138 unique *fedA*$^+$ strains were analyzed for polymorphisms in *fedA* by SSCP analysis as previously described (16). Bacterial DNA was isolated by boiling approximately 10^6 CFU in 30 μl of water for 5 min. The *fedA* gene was amplified by PCR with *fedA*-specific primers (sense strand, 5′-GTGAAAAGACTAGTGTTTATTTC-3′, and antisense strand, 5′-CTTGTAAGTAACCGCGTAAGC-3′) as previously described (7). The 10-μl reaction mixture contained a 1.0 μM concentration of each primer, a 70.0 μM concentration of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.1 μl of [α-^{32}P]dATP (10 μCi/μl), 2 U of *Taq* polymerase (Promega, Madison, Wis.), and 5 μl of bacterial DNA. A solution containing 5 μl of an amplified product, 1 μl of a 0.1% sodium dodecyl sulfate–0.1 M EDTA solution, and 5 μl of a stop solution (95% formamide; United States Biochemicals, Cleveland, Ohio) was denatured by being heated to 95°C for 5 min. Three microliters of the denatured solution was loaded on a nondenaturing gel and subjected to electrophoresis at 5 W overnight at room temperature. SSCPs were detected by autoradiography of the nondenaturing gel.

Nomenclature for the *fedA* genes was in accordance with that of Imberechts et al. (9). A list of fimbrial subunit genes identified follows, with each gene followed by the *E. coli* strain used for determining its DNA and deduced amino acid sequence, as well as the strain's phenotype: *fedA*, strain 107/86, serogroup O139,

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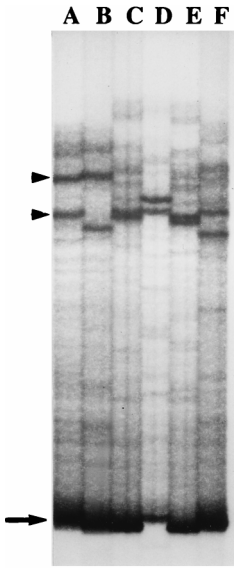

FIG. 1. Autoradiograph showing results of an SSCP analysis of the following *fedA* genes on a nondenaturing gel: *fedA* (lane A), *fedA.1* (lane B), *fedA.2* (lane C), *fedA.3* (lane D), *fedA.7* (lane E), and *fedA.8* (lane F). The top two bands are denatured single-stranded DNA, and the bottom band is nondenatured double-stranded DNA. The denatured single-stranded DNA and nondenatured double-stranded DNA in lane A are shown by arrowheads and an arrow, respectively.

Stx2e$^+$ F18ab$^+$ (9, 15, 25); *fedA.1*, strain S221/90, serogroup O138, Stx2e$^+$ F18ab$^+$ (9, 25); *fedA.2*, strain 2134, serogroup O157, STa$^+$ STb$^+$ F18ac$^+$ (9, 14, 15, 25); *fedA.3*, strain 8199, serogroup O141, STa$^+$ STb$^+$ F18ac$^+$ (9, 25); *fedA.7*, strain 2680, serogroup unknown, LT$^+$ STb$^+$ F18ac$^+$ (this study) (generously provided by Bela Nagy, Budapest, Hungary); and *fedA.8*, strain 2415, serogroup O149, STa$^+$ STb$^+$ (this study) (generously provided by Bela Nagy).

DNA sequencing. The *fedA* gene was amplified by PCR as described above except that no radioactive nucleotides were included and the concentration for each deoxynucleoside triphosphate was increased to 200 μM. The amplified gene was cloned into the PCRII vector and transformed into a recipient strain according to the instructions of the manufacturer of the kit used in the procedure (TA cloning kit; Invitrogen Co., San Diego, Calif.). Three clones for each *fedA* gene were sequenced with the T7 Sequenase kit (version 2.0; United States Biochemicals) in both directions to ensure that the DNA sequence contained no PCR-generated artifacts. The LASERGENE DNA program (DNASTAR Inc., Madison, Wis.) was used to predict antigenicity of the major fimbrial subunit on the basis of hydrophilicity, surface probability, flexibility, and secondary structure by the method of Jameson and Wolf (10).

Immunoassay. Bacterial strains were grown overnight on Trypticase soy agar at 37°C in 5% CO_2. Expression of F18ac fimbriae was detected by a colony blot immunoassay with the monoclonal antibody 6C7/C1, as previously described (3).

RESULTS

SSCP and analysis of deduced amino acids. The SSCP analysis of 138 *fedA*$^+$ strains identified 19 different *fedA* genes. The *fedA* gene migrated as three bands corresponding to single-stranded DNA molecules (Fig. 1, upper two bands) and nondenatured double-stranded DNA (Fig. 1, lower band). The SSCP analysis determined that the major fimbrial subunit gene in 125 of the 138 strains was one of the following *fedA* genes: *fedA*, *fedA.1*, *fedA.2*, *fedA.3*, *fedA.7*, and *fedA.8* (Fig. 1). Each of the *fedA* genes of the remaining 13 strains had a unique migration pattern and the DNA sequences were not determined.

The deduced amino acid sequences of the six most common *fedA* genes in this study are shown in Fig. 2. Strains used for determining *fedA* and *fedA.1* sequences were F18ab$^+$ and did not react with the F18ac-specific monoclonal antibody 6C7/C1. Strains used for determining the DNA sequences of *fedA.2*,

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fedA     MKRLVFISFVALSMTAGSAMAQQGDVKFFGNVSATTCNLTPQISG    45
fedA.1   ----------------------------------------
fedA.2   ------------------------------S-------------
fedA.3   ------------------------------S-------------
fedA.7   ------------------------------S-------------
fedA.8   ------------------------------S---I----------

fedA     TVGDTIQLGTVAPSGTGSEIPFALKASSNVGGCASLSTKTADITW    90
fedA.1   ---------------A-R--------------------------
fedA.2   -----------T-N--------------AT-------N-------
fedA.3   -----------T-N-I------------TT-------N-------
fedA.7   -----------T-N----A---------TA------AN-------
fedA.8   -----------T-N----A---------TA-------N-------

fedA     SGQLTEKGFANQGGVANDSYVALKTVNGKTQ GQEVKASNSTVSF   135
fedA.1   ------------------------------ -------------
fedA.2   ----------------R--------------PA------------
fedA.3   ----------------R--------------PA----------N-
fedA.7   ------------------------------RPE------------
fedA.8   ------------------------------RPE------------

fedA     DASKATTEGFKFTAQLKGGQTPGDFQGAAAYAVTYK            171
fedA.1   -----------------------------------
fedA.2   -----------------------------------
fedA.3   -----------------------------------
fedA.7   -----------------------------------
fedA.8   -----------------------------------
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FIG. 2. Deduced amino acid sequences (single-letter designation) of the major fimbrial subunit genes *fedA* and *fedA.1*, *-2*, *-3*, *-7*, and *-8*. Amino acid differences are shown, and identical amino acids are represented as dashes. The putative leader peptide is underlined and the predicted antigenic regions are double underlined.

TABLE 1. Genotypes and phenotypes of *fedA*$^+$ *E. coli*

fedA gene	No. of strains that were:					
	Identified	Reactive with 6C7/C1[a]	ETEC[b]	STEC[c]	STEC/ETEC[d]	Toxin negative
fedA	15	0	3	4	8	0
fedA.1	56	0	6	4	43	3
fedA.2	22	16	18	0	3	1
fedA.3	14	13	13	0	1	0
fedA.7	16	12	13	1	2	0
fedA.8	2	0	2	0	0	0
fedA.—[e]	13	9	9	0	4	0

[a] F18ac-specific monoclonal antibody.
[b] ETEC strains were STa, STb, or LT positive.
[c] STEC strains were Stx2e positive.
[d] STEC and ETEC strains were Stx2e and STa, STb, or LT positive.
[e] Unique *fedA* genes were identified by SSCP analysis for 13 strains, 11 of which were designated F18ac$^+$ and 2 of which were designated F18ab$^+$.

fedA.3, and *fedA.7* were F18ac$^+$ and reacted with 6C7/C1. Neither of the two strains containing the *fedA.8* gene reacted with 6C7/C1 (Table 1). However, these strains were designated F18ac$^+$, as *fedA.8* had a higher deduced amino acid homology with *fedA* genes found in F18ac$^+$ strains than with those found in F18ab$^+$ strains (Fig. 2).

There was a high overall homology at the deduced amino acid level for all six *fedA* genes. The deduced amino acid sequences of *fedA.1*, *-2*, *-3*, *-7*, and *-8* were, respectively, 99, 95, 94, 94, and 94% identical to the deduced amino acid sequence of *fedA*. All six had an identical putative signal sequence encoding 21 amino acids. The two *fedA* genes found in F18ab$^+$ strains differed by only two amino acids, while there were more differences among the four *fedA* genes found in F18ac$^+$ strains (Fig. 2). Five amino acids that differentiated *fedA* and *fedA.1* (F18ab$^+$ strains) from *fedA.2*, *-3*, *-7*, and *-8* (F18ac$^+$ strains) were identified. These five amino acids were at positions 31, 57, 59, 83, and 122 (Fig. 2).

Predicted antigenicity. Computer analysis of the deduced amino acid sequences of all six *fedA* genes identified the same two regions as highly antigenic. One of the regions was homologous in all six (Fig. 2, amino acids 151 to 160). The other region predicted to be antigenic in all six had a variable amino acid composition (amino acids 116 to 130 [Fig. 2]). The major difference in this region was that *fedA* genes in F18ac$^+$ strains encoded an additional amino acid (proline) not encoded by *fedA* genes found in F18ab$^+$ strains.

Classification of unique *fedA* genes. The SSCP analysis demonstrated that the *fedA* genes of F18ab$^+$ strains migrate differently than the *fedA* genes of F18ac$^+$ strains. The migration of the uppermost band was slower for *fedA* and *-1* (F18ab$^+$ strains) than it was for *fedA.2*, *-3*, *-7*, and *-8* (F18ac$^+$ strains) (Fig. 1). This difference in migration was used to predict the antigenicity of the 13 strains with unique *fedA* genes that migrated differently during SSCP analysis than did *fedA* and *fedA.1*, *-2*, *-3*, *-7*, and *-8*. Nine of 11 strains with unique *fedA* genes classified as F18ac$^+$ by this criterion reacted with monoclonal antibody 6C7/C1. Neither of the two strains with unique *fedA* genes classified as F18ab$^+$ by this criterion reacted with the F18ac-specific monoclonal antibody.

Toxin profile and antigenicity. None of the 73 strains identified as F18ab$^+$ by SSCP analysis reacted with monoclonal antibody 6C7/C1 (Table 1). Sixty-one of 73 strains (84%) contained the Stx2e gene, while 60 of 73 strains (82%) contained one or more enterotoxin (LT, STa, or STb) genes. Three strains were nontoxigenic.

Fifty of the 65 strains (77%) designated F18ac$^+$ by SSCP analysis reacted with monoclonal antibody 6C7/C1 (Table 1). Only 8 of the 65 strains (12%) contained the Stx2e gene, while 63 of 65 (97%) were probe positive for one or more of the enterotoxin (LT, STa, or STb) genes. One strain classified as F18ac$^+$ was nontoxigenic.

DISCUSSION

Identification of and differentiation between F18ab$^+$ and F18ac$^+$ strains by serologic techniques are not always possible, because some strains do not express fimbriae when cultured in vitro under standard culture conditions (1, 8, 25, 26). Recently, Wittig et al. reported that in vitro fimbria expression is possible with nonconventional culture techniques (25, 26). Microaerobic culture is required, and some strains require agar containing alizarin yellow and eosin for fimbria expression, which varies from colony to colony for some strains (25).

The DNA sequences of the major fimbrial subunit genes *fedA* and *fedA.1* to *-6* were used by Imberechts et al. (9) to develop a PCR-RFLP test that differentiates F18ab$^+$ from F18ac$^+$ strains. *fedA* and *fedA.1*, present in F18ab$^+$ strains, do not contain a proline-encoding triplet (CCG) and are resistant to digestion with restriction enzyme *Ngo*MI, which recognizes GCCGGC. *fedA.2* to *-6*, present in F18ac$^+$ strains, contain a proline-encoding triplet and are digested by the restriction enzyme *Ngo*MI (9). *fedA.7* and *-8* also contained this proline-encoding triplet and were classified as F18ac$^+$ in the present study. However, *fedA.7* and *-8* would not be digested by *Ngo*MI because of differences in the base pairs adjacent to the proline-encoding triplet. Strains with these *fedA* genes would be misclassified as F18ab$^+$ by the PCR-RFLP test proposed by Imberechts et al. (9). The accuracy of this PCR-RFLP test when used to classify strains with *fedA* genes of undetermined sequence identified in the present study or strains with *fedA* genes not yet identified is unknown.

Others have recently used SSCP analysis to detect genetic polymorphisms in bacterial, viral, and protozoal genes (6, 12, 22, 23). In RFLP analysis, genetic differences in only the recognition site of the restriction enzyme are recognized, while SSCP analysis can detect differences anywhere in the amplified gene. The sensitivity of SSCP analysis to DNA differences is inversely correlated with the size of the amplified product and can be affected by the conditions used with the nondenaturing gel, such as temperature of electrophoresis. It has been estimated that SSCP analysis can detect >80% of single base substitutions in amplicons of 400 bp (5). We analyzed an approximately 500-bp amplicon because it contained the entire open reading frame of the major fimbrial subunit gene. Samples were analyzed on a nondenaturing gel at room temperature as previously described (16). While not all single base substitutions may have been identified in an amplicon of this size under the conditions we used, SSCP analysis differentiated between *fedA* genes that differed by only two bases (*fedA* versus *fedA.1*, *fedA.2* versus *fedA.3*, and *fedA.7* versus *fedA.8*).

SSCP analysis differentiated F18ab$^+$ strains from F18ac$^+$ strains and identified 19 variations of the *fedA* gene. Most, but not all, of the strains classified as F18ac$^+$ by SSCP analysis reacted with monoclonal antibody 6C7/C1, while none of the strains classified as F18ab$^+$ reacted with 6C7/C1. This confirms the specificity of 6C7/C1 for F18ac$^+$ *E. coli* (4, 15) and demonstrates the usefulness of SSCP analysis in differentiating strains that do not express F18 in vitro.

Sixty-one of 73 strains designated F18ab$^+$ by SSCP analysis contained the Stx2e gene (STEC) and could cause edema disease. A number of these strains also contained one or more

enterotoxin genes and could also cause diarrhea. Diarrhea is occasionally a component of edema disease in field outbreaks and may be caused by strains producing both Stx2e and enterotoxins (17). Only 8 of 65 strains classified as F18ac$^+$ by SSCP analysis were STEC, while more than 90% were ETEC. This confirms that F18ab$^+$ strains are commonly STEC, while F18ac$^+$ strains are frequently ETEC (4, 15, 26).

We were able to classify the 13 strains with unique *fedA* genes as either F18ab$^+$ or F18ac$^+$ by SSCP analysis. The upper single-stranded DNA molecules of *fedA* genes from strains classified as F18ac$^+$ migrated farther in the nondenaturing gel than did those from strains classified as F18ab$^+$. It is possible that the increased migration of one of the single-stranded *fedA* DNA molecules in F18ac$^+$ strains is due to the proline-encoding triplet (CCG) or its complementary sequence, which is absent in *fedA* genes of F18ab$^+$ strains. This additional triplet could significantly modify folding of the single-stranded DNA, resulting in increased mobility on the nondenaturing gel.

The major difference between *fedA* genes of F18ab$^+$ and F18ac$^+$ strains was an additional proline-encoding triplet in the *fedA* genes of F18ac$^+$ strains, and this proline was in a region predicted by this study to be antigenic. This proline may affect antigenicity as a part of the epitope or may indirectly affect antigenicity by modifying secondary structure (9). Recently, it has been shown that inoculation with either F18ab$^+$ or F18ac$^+$ strains reduces shedding in swine that are subsequently challenged with either the homologous or heterologous F18 antigenic variant (21). Future studies should be directed at determining which amino acids compose the shared epitope(s) of F18ab and F18ac fimbriae. Sequencing several *fedA* genes increases the likelihood of identifying this shared epitope(s) and should lead to vaccines that prevent disease caused by either F18ab$^+$ or F18ac$^+$ *E. coli.*

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