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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 enterotoxigenic 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 fimbriae mediate 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 (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 *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 l of water for 5 min. The *fedA* gene was amplified by PCR with *fedA*-specific primers (sense strand, 5'-GTATGACTATGTTAATTC-3' and anti- sense strand, 5'-CTGAAAGATCGGCTGTAACG-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-32P]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. 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.

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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 pCR II 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).

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 non-denatured 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,
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, 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 genes found 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.4 and -I (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 monoclonoal 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 monoclonoal 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 nontoxicogenic.

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 nontoxicogenic.

**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 fimbriae 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 NgoMI, which recognizes GCCGGC. fedA.2 to -6, present in F18ac⁻ strains, contain a proline-encoding triplet and are digested by the restriction enzyme NgoMI (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 NgoMI 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 monoclonoal 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 inclusion of 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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