Research Note

Staphylococcal Enterotoxin D Production by Staphylococcus aureus FRI 100

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ABSTRACT

Staphylococcus aureus FRI 100 is commonly used as a control strain for staphylococcal enterotoxin A (SEA) assays. When FRI 100 was used in PCR-based enterotoxin detection methods, the strain gave a positive result for both SEA and staphylococcal enterotoxin D (SED). Production of SED was confirmed by testing concentrated and unconcentrated culture supernatants with the TECRA staphylococcal enterotoxin visual immunoassay. SED was detected after 24 h of growth in Trypticase soy broth. Primers were created to amplify the entire sed gene by PCR for subsequent sequencing. The sequenced gene showed high similarity to a previously sequenced sed gene. The SED-like gene in FRI 100 exhibited four point mutations and two deletions. Changes in the FRI 100 open reading frame altered the primary structure of the SED-like protein, allowing for coding of only the first 150 amino acids followed by a stop codon. Because the SED active site is at the proximal end, where there was no change in DNA sequence, we conclude FRI 100 produces a variant form of SED. It is necessary to note that, when using FRI 100 as an SEA control strain, it does produce a variant of the SED protein, which exhibits immunological activity, and the sed-like gene is detected by commonly used PCR primers. This phenomenon may be an important general consideration when using PCR to characterize strains of toxin-producing S. aureus. S. aureus enterotoxin–positive PCR results should be confirmed by immunological techniques.

Staphylococcal enterotoxins (SEs) are low-molecular-weight extracellular proteins produced during the exponential and stationary phases of growth by some strains of Staphylococcus aureus. These toxins can cause illness in humans when consumed at a dose as low as 100 ng (3). Enterotoxin poisoning is characterized by gastroenteritis occurring 2 to 4 h after ingestion and lasting for about 24 h. According to the Centers for Disease Control and Prevention (7), there were 189 S. aureus related foodborne outbreaks between the years 1990 and 2003. The majority of the SE illnesses were traced to meats and prepared foods. A great deal of research has been conducted on staphylococcal enterotoxins, and currently 18 different SE genes have been characterized by PCR (11, 15, 17, 19, 24).

S. aureus strain FRI 100 is commonly used as a control in assays for staphylococcal enterotoxin A (SEA) production. FRI 100 was originally isolated in 1932 from a chocolate éclair that caused foodborne illness (12). Strain FRI 100 produces SEA in large quantities and was found to produce SEA with an immunodiffusion assay (9, 13). While characterizing strains of S. aureus isolated from mastitic cows for the presence of enterotoxin genes, we noted an amplicon produced from FRI 100 when staphylococcal enterotoxin D (SED) detection primers were employed. The purpose of the present study was to determine if S. aureus FRI 100 contains the gene coding for SED or SED-like protein (16) and to determine if FRI 100 produces SED by an enzyme-linked immunosorbent assay (ELISA) method.

MATERIALS AND METHODS

Bacterial strains. Strains of S. aureus FRI 100 and FRI 472 were acquired from Dr. Amy Wong at the Food Research Institute (University of Wisconsin, Madison). FRI 472 was used as a positive SED control. S. aureus strain ATCC 51651 (American Type Culture Collection, Manassas, Va.), a toxic shock syndrome toxin-producing (TSST-1) strain, was used as the SED negative control.

PCR amplification of the sed gene. S. aureus strains were grown on Baird-Parker agar (Difco, Becton Dickinson, Sparks, Md.) for 12 to 16 h. A single colony was picked and transferred to a sterile PCR tube. Cells were lysed according to the microwave method of Bollet et al. (6) as modified by Kullen et al. (14). A negative control sample without added DNA was also analyzed. Primers used to amplify the sed gene are shown in Table 1. Primers F-243, F-174, F-111, F-34, and R807 were selected by using the sed sequence published by Bayles and Iandolo (4) and Primer3 software (18). The PCR amplification mixture (50 μl) consisted of 10X reaction buffer, 400 μM dNTP mixture, 3 mM MgCl2, 1 μM of each forward and reverse primer, and 1 U Taq polymerase. All PCR reagents were obtained from Promega (Madison, Wis.), and primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). PCR conditions consisted of denaturation at 95°C for 1 min, annealing at 56°C for 2 min, and elongation at 72°C for 2 min carried out in a Mircrocer system personal (Eppendorf, Hamburg, Germany). For amplicon sizes less than 1,000 bp, the elongation step was decreased to 1 min. PCR products were electrophoresed through a 1% agarose (Promega) gel at 90 V. The gel
was stained with ethidium bromide (Promega) and visualized under UV illumination with an Alpha Imager (Alpha Innotech, San Leandro, Calif.). A 100-bp DNA ladder (Promega) was used to determine amplicon size of the PCR products.

**DNA extraction from agarose gel and sequencing.** DNA was extracted from agarose slices with a QIAquick Gel Extraction Kit (QIAGen, Valencia, Calif.). PCR products were electrophoresed through a 1.5% agarose gel at 80 V and stained with ethidium bromide (Promega). Bands were excised from the agarose and placed in 1.0-mL sterile Eppendorf tubes. After DNA extraction according to QIAGen manufacturer’s instructions, samples were frozen at −20°C until sequenced at the Nucleic Acid Facility (The Pennsylvania State University, University Park, Pa.). Forward and reverse sequences were generated by using cycle sequencing and primers F-174, F-111, and R807. The sequence obtained was compared with the known sed sequence published by Bayles and Iandolo (4) and ending 53 bp downstream of the protein-coding region as seen in Figure 1.

**ELISA test for enterotoxin production.** To assay production of SED, strains were grown for 24 h in Trypticase soy broth (TSB; Difco, Becton Dickinson) and analyzed for toxin production with the TECRA SIDVIA72 kit (International Bioproducts, Bothell, Wash.) according to the manufacturer’s instructions. Following incubation, the absorbance was read with a microtiter plate reader (Biotek Instruments, Inc., Winooski, VT) at dual wavelengths of 405 and 490 nm, blanked with the negative control. FRI 100 and FRI 472 were first grown in TSB for 24 h at 37°C and then cells were spun out at 3,700 × g for 1 min in an effort to concentrate the culture. FRI 100 supernatant was concentrated 6.4-fold, and FRI 472 was concentrated 5.6-fold with Centricon-10 concentrator kits (Amicon, Inc., Beverly, Mass.). Centricon tubes were spun at 3,700 × g for 30-min intervals, with 1 to 2 mL of supernatant added at each interval. The TECRA analysis was performed again with concentrated toxin.

**RESULTS**

**sed gene amplification.** Using primers cited by Johnson et al. (11) and Monday and Bohach (17), PCR analysis revealed the presence of the proximal and distal portions, respectively, of the gene encoding SED in FRI 100 (data not shown). Both primers were designed to detect sed but actually only amplify a small fragment of the gene. New primers generated with Primer3 (18) allowed amplification of the entire sed gene in FRI 100 beginning 111 bp upstream of the protein-coding region and ending 53 bp downstream of the protein-coding region as seen in Figure 1.

**SED detection by TECRA.** TECRA analysis revealed strain FRI 100 produced both SEA and SED. SED was detected with lower reactivity than the positive SED control strain, FRI 472. Following TECRA analysis, the color intensity matched between standards #2 and #3 on the scoring sheet included in the TECRA kit, indicating a borderline positive result for SED. From initial analysis, SED absorbance for FRI 100 was 0.101 compared with 1.820 for FRI 472. When the FRI 100 culture supernatant was concentrated, the absorbance increased from 0.128 to 0.150 and the color intensity matched standard no. 3 on the scoring sheet, indicating a positive SED result. Although there appears to be weak immunological activity, the identity of the protein causing this reaction was not directly confirmed.

**Sequencing of the sed gene in FRI 100.** The enterotoxin-like gene in FRI 100 contained 798 bp, and, based on BLAST (2) comparison, it was 98% homologous to the sequence published by Bayles and Iandolo (4). ClustalW (8) analysis revealed four point mutations and two deletions in the FRI 100 sequence. When compared with the sequence of Bayles and Iandolo (4), the point mutations resulted in changes of amino acid 88 from phenylalanine to cysteine, amino acid 114 from proline to alanine, amino acid 128 from arginine to lysine, and amino acid 149 from lysine to asparagine. A single-base deletion occurred at bp 317, resulting in a frame shift and changes in subsequent amino acids coded by the gene. Following the first deletion, the sequence coded for six additional amino acids followed by a stop codon, as seen in Figure 2.

**TABLE 1. Primers used to detect the sed gene in FRI 100**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F41b</td>
<td>CTAGTTTGGTAAATATCTCCT</td>
<td>317</td>
<td>11</td>
</tr>
<tr>
<td>R338b</td>
<td>TAATGCTATATCTTATAGGG</td>
<td>384</td>
<td>17</td>
</tr>
<tr>
<td>F368b</td>
<td>GTGGTGAATTAGTAAAGCTGC</td>
<td>1,136</td>
<td>This study</td>
</tr>
<tr>
<td>R733b</td>
<td>ATATGAAAGGTGCTCTCTGTTG</td>
<td>1,001</td>
<td>This study</td>
</tr>
<tr>
<td>F-243</td>
<td>TGGCGGCTAGTCTCCTGAATA</td>
<td>938</td>
<td>This study</td>
</tr>
<tr>
<td>F-174</td>
<td>TGGCGGCTAGTCTCCTGAATA</td>
<td>861</td>
<td>This study</td>
</tr>
<tr>
<td>F-111</td>
<td>TGAATGTATTGTCTACATAATCGGTG</td>
<td>1,136</td>
<td>This study</td>
</tr>
<tr>
<td>F-34b</td>
<td>AAAGGTGCTCAATTATTGAAA</td>
<td>1,001</td>
<td>This study</td>
</tr>
<tr>
<td>R807</td>
<td>AGCATCTTTTTGAAATGCGTTT</td>
<td>938</td>
<td>This study</td>
</tr>
</tbody>
</table>

a F, forward; R, reverse; and gene location relative to the protein coding region.
b Primer location shown in Figure 2.

**FIGURE 1. Results of PCR analysis indicating the presence of the entire SED protein encoding region. Lane 1, FRI 100; lane 2, FRI 472; lane 3, ATCC 51651 (negative control); and lane 4, no template control. a, b, and c are PCR products produced using primers F-174, F-111, and F-34, respectively, with R807.**
FIGURE 2. DNA and amino acid sequence of the sed gene in FRI 100. Bold amino acids from sequence of Bayles and Iandolo (4). Underlined are primers in sequence with labels above. *, deletion; ***, stop codon.

DISCUSSION

The common SEA-producing strain, FRI 100, contains an open reading frame, which is detectable by PCR and appears to encode a shortened form of the SED protein. SED was also detected in FRI 100 by PCR when Tsen et al. (22) were characterizing SEA strains of S. aureus from around the world. Although they reported a positive PCR result, these authors did not verify that the SED protein was produced by FRI 100. Changes in the sed gene found in FRI 100 allowed for translation of only the first 150 of the 258 amino acids in the native protein (4). The first substitution observed at base 265 resulted in a change from Phe to Cys and is considered significant because of the potential change in secondary and tertiary structure resulting from addition of a sulfur group to the FRI 100 protein (23). The second point mutation of C to G at base 340, which resulted in a change from Pro to Ala, is a common variant of the protein according to Al-Daccak et al. (1). The modification of Arg to Lys (bp 384) is likely insignificant because these two amino acids have similar structure (10). The last point mutation, Lys to Asp, changed the amino acid from positively charged to neutral, which may affect the protein structure. Following a single base deletion at base 449, the subsequent amino acids encoded are different from the native SED protein. The presence of SED protein, however, was not tested directly by physical methods. Another explanation for the positive TECRA result includes minor cross-reactions with other SEs or related proteins. In conclusion, when FRI 100 is used as an SEA control strain, it is necessary to note that the strain does produce an SED-like protein, which is slightly immunologically active and is detected by common PCR primers targeting the sed gene. Issues may arise when using FRI 100 to quantify general toxin production or when using it in other assays because the SE genes and proteins have high sequence homology (5). This phenomenon can be an important consideration when using PCR to characterize strains of toxin-producing S. aureus.

REFERENCES


