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Detection of a Streptomycin/Spectinomycin Adenylyltransferase Gene (*aadA*) in *Enterococcus faecalis*

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Genes encoding streptomycin/spectinomycin adenylyltransferases [ANT(3″)(9)] have been reported to exist in gram-negative organisms and *Staphylococcus aureus*. During a study of high-level aminoglycoside resistance in enterococci, we encountered an isolate of *Enterococcus faecalis* that was streptomycin resistant but did not appear to contain the 6′-adenylyltransferase gene (*aadE*) when examined by PCR with specific primers. Phosphocellulose paper binding assays indicated the presence of an ANT(3″)(9) enzyme. Streptomycin and spectinomycin MICs of 4,000 and 8,000 μg/ml, respectively, were observed for the isolate. PCR primers corresponding to a highly conserved region of the *aadA* gene were used to amplify a specific 284-bp product. The product hybridized with a digoxigenin-labeled PCR product from *E. coli* C600(pHP45Ω) known to contain the *aadA* gene. The *aadA* gene was transferred via filter matings from the *E. faecalis* donor to *E. faecalis* JH2-2. PCR primers designed for analysis of integrons were used to amplify a 1-kb product containing the *aadA* gene, which was cloned into the vector pCRII and transformed into *Escherichia coli* DH5-α competent cells. D-Rhodamine dye terminator cycle sequencing was used to determine the gene sequence, which was compared to previously reported sequences of *aadA* genes. We found the *aadA* gene in *E. faecalis* to be identical to the *aadA* genes reported by Sundström et al. for *E. coli* plasmid R6-5 (L. Sundström, P. Rådström, G. Swedberg, and O. Sköld, Mol. Gen. Genet. 213:191–201, 1988), by Fling et al. for the *aadA* within transposon Tn*7* (M. E. Fling, J. Kopf, and C. Richards, Nucleic Acids Res. 13:7095–7106, 1985), and by Hollingshead and Vapnek for *E. coli* R538-1 (S. Hollingshead and D. Vapnek, Plasmid 13:17–30, 1985). Previous reports of the presence of the *aadA* gene in enterococci appear to be erroneous and probably describe an *aadE* gene, since the isolates were reported to be susceptible to spectinomycin.

Streptomycin continues to be an important drug for synergistic therapy of serious enterococcal infections (10). Resistance to streptomycin can be mediated by ribosomal mutation or by enzymatic modification of the drug. Ribosomal resistance is usually associated with streptomycin MICs of >32,000 μg/ml (11), while enzyme-mediated resistance produces lower MICs. The *aadA* gene, which encodes resistance to streptomycin and spectinomycin via an adenylyltransferase [ANT(3″)(9)] enzyme, has been reported to exist in gram-negative organisms (3, 6, 15, 16, 33) and *Staphylococcus aureus* (7). Although there have been reports of streptomycin adenylyltransferases in enterococci (6, 8, 18), none of the isolates reported were resistant to spectinomycin, suggesting a mechanism other than one involving the ANT(3″)(9) enzyme. In large surveys of clinical isolates, neither Leclercq et al. nor Ounissi et al. reported the identification of an ANT(3″)(9) in enterococci (20, 25). However, a clinical isolate of *Enterococcus faecalis* exhibiting high-level resistance to streptomycin and spectinomycin (MICs of ≥2,000 μg/ml) was recently recognized in a multilaboratory study of screening methods for detection of high-level aminoglycoside resistance in enterococci (32). The isolate did not exhibit ribosomal resistance since the streptomycin MIC was <32,000 μg/ml, failed to produce an *aadE* (also referred to as the *ant6′* gene)-specific product when tested by PCR, and did not hybridize with an *aadE*-specific gene probe, suggesting an alternate mechanism of streptomycin resistance.

The goal of this study was to characterize the mechanism of streptomycin resistance in this isolate.

MATERIALS AND METHODS

Bacterial isolates. The *E. faecalis* strain used in this study (W4770) was originally isolated from a patient at the University of Wisconsin Hospital, Madison, Wis., and was sent to the Centers for Disease Control and Prevention, where it was confirmed to be *E. faecalis* by the performance of biochemical tests described by Facklam and Collins (12). Strains were grown on Trypticase soy agar with 5% sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, Md.) at 35°C. The control strains used throughout the study were as follows: rifampin-resistant (Rif^r) and fusidic acid-resistant (Fus^r) *E. faecalis* JH2-2 (plasmid free) (17); streptomycin-resistant (Str^r), spectinomycin-resistant (Sp^r), and ampicillin-resistant *Escherichia coli* C600(pHP45Ω) (*aadA*) (26); *E. faecalis* ATCC 49532 [*aac*(6′)-*aph*(2″)] (32), ATCC 49533 (*aadE*) (32), and Str^r (ribosomal) E1-R (11); Sp^r *S. aureus* 8325-4 [*ant*(9′)] (24); *E. coli* C600(pFCT3103) [*ant*(2″)] (34); and *Bacillus subtilis* pUBH$_2$ [*ant*(4′)] (9).

Antimicrobial susceptibility testing. All isolates were tested by the broth microdilution method as described by the National Committee for Clinical Laboratory Standards, using cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) (23). The antimicrobials tested were ampicillin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, penicillin, quinupristin/dalfopristin, rifampin, spectinomycin, streptomycin, teicoplanin, tetracycline, and vancomycin.

AME assays. Lysates of bacterial strains were screened for the presence of aminoglycoside-modifying enzymes (AMEs) by the phosphocellulose binding assay as described by Cooksey et al. (4). The only modification of their procedure was that the wash buffer (10 mM Tris, 1 mM EDTA; pH 7.4) and the suspension buffer (10 mM Tris, 10 mM $MgCl_2$, 10 mM dithiothreitol; pH 6.8) used for the enterococci were those described by Chen and Williams (2).

PCR amplification of resistance genes. The oligonucleotide primers chosen for amplification of the 6′-streptomycin adenylyltransferase (*aadE*) and the 3″-

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TABLE 1. PCR amplification of aminoglycoside resistance genes

Gene(s)	Primer pair (5′→3′)[a]	Product size (bp)	Reference
aadE	(1268) ACT GGC TTA ATC AAT TTG GG (1845) GCC TTT CCG CCA CCT CAC CG	597	13
aadA	(564)[b] TGA TTT GCT GGT TAC GGT GAC (829)[b] CGC TAT GTT CTC TTG CTT TTG	284	16
aac(6′)-*aph*(2″)	(348) TGA TGA TTT TCC TTT GAT GT (1723) CAA TCT TTA TAA GTC CTT TT	1,395	14

[a] The numbers in parentheses denote the positions of the initial nucleotides in the primer sequences.

[b] Since the *aadA* gene sequences for several different organisms are published, the nucleotide number varies and the primers were selected from within a universally conserved sequence.

adenylyltransferase (*aadA*) genes (Table 1) were selected from published sequences (13, 16) with the assistance of OLIGO software, version 4.0 (National Biosciences, Hamel, Maine). The PCR mixtures and conditions for amplification of the *aac*(6′)-*aph*(2″) and the *aadE* genes have been described previously (32).

For amplification of the *aadA* gene, four or five bacterial colonies from an overnight plate were suspended in 100 μl of a chilled reaction mixture containing 10 mM Tris–2.0 mM $MgCl_2$–50 mM KCl (pH 8.3), 0.2 mM deoxynucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), 0.5 μM each primer, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). A model 9600 DNA thermocycler (Perkin-Elmer Cetus, Emeryville, Calif.) was programmed as follows: an initial denaturation for 10 min at 95°C; 30 cycles consisting of a 30-s denaturation step at 94°C, a 30-s annealing at 60°C, and a 30-s extension step at 72°C; a 10-min extension step at 72°C; and incubation at 4°C until the analysis was performed.

Resistance gene probes. The probe used for detection of the *aadE* gene was that described by Swenson et al. (32) and consisted of a digoxigenin-labeled 597-bp PCR product from *E. faecalis* JH2-2(pJH-1) (17). For detection of the *aadA* gene, a 284-bp PCR product from *E. coli* plasmid pHP45Ω (26) was labeled with digoxigenin by using a Genius kit (Boehringer Mannheim), and hybridization was performed under stringent conditions at 68°C according to the manufacturer's recommendations (1).

Transfer of resistance genes. Filter matings were performed by using *E. faecalis* W4770 as the donor and *E. faecalis* JH2-2 (17) as the recipient, as previously described (35). Overnight Trypticase soy broth (TSB) cultures of Str^r, Sp^r *E. faecalis* W4770 (donor) and Rif^r, Fus^r *E. faecalis* JH2-2 (recipient) were mixed in a 20:1 ratio and filtered through a 0.22-μm-pore-size Nalgene filter (Nalgene Nunc International, Rochester, N.Y.). The membrane was placed on a brain heart infusion agar (BBL) plate and incubated for 24 h at 37°C. The growth was resuspended in 5 ml of TSB, and transconjugants were selected on brain heart infusion agar containing 50 μg of rifampin, 20 μg of fusidic acid, 50 μg of streptomycin, and 50 μg of spectinomycin/ml.

The transconjugants were examined for the presence of the resistance gene by PCR with specific *aadA* gene primers. Expression of the gene was demonstrated by determination of streptomycin, spectinomycin, and tetracycline MICs by the broth microdilution method (23).

Sequencing of the resistance gene. Using the oligonucleotide primers designed by Lévesque et al. for PCR analysis of integrons (21, 22), we generated a PCR product of approximately 1 kb, using cesium chloride-purified plasmid DNA as the template (27). The 100-μl reaction mixture included 1× PCR buffer II; 2 mM $MgCl_2$; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 10 μl of each primer (2.5 pmol/ml stock); and 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, Calif.). The thermocycler was programmed as follows: an initial denaturation for 12 min at 95°C; 30 cycles consisting of a 30-s denaturation step at 94°C, a 30-s annealing at 55°C, and a 30-s extension step at 72°C; a 10-min extension step at 72°C; and incubation at 4°C until the analysis was performed. The products were purified on QIAquick PCR spin columns (Qiagen, Inc., Chatsworth, Calif.), and the DNA was eluted from the columns with 10 mM Tris, pH 9.0.

The integron PCR DNA was ligated into the pCRII vector (TA Cloning Kit; Invitrogen, Carlsbad, Calif.) and transformed into *E. coli* Library Efficiency DH5-α competent cells (Gibco/BRL Life Technologies, Inc., Gaithersburg, Md.). The DH5-α competent cells are Str^s, allowing streptomycin to be used for selection of the transformants; the competent cells normally supplied with the TA Cloning Kit are resistant. The transformants were inoculated into 5 ml of TSB containing 100 μg of ampicillin/ml and incubated at 37°C with shaking for 6 h. The plasmid DNA was purified by using a Qiagen plasmid minikit and then digested with *Eco*RI (Gibco/BRL Life Technologies, Inc.) to check for insertions into the vector DNA. The purified transformant plasmid DNA from multiple clones with an insert of approximately 1 kb was used as the template for the sequencing reaction.

The samples for sequencing were prepared in a 10-μl volume by using an ABI PRISM dRhodamine terminator cycle sequencing kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer Applied Biosystems), and 3.2 pmol of stocks of both the forward and reverse integron and *aadA* gene PCR primers. The thermocycler was preheated to 96°C, the program was aborted, and the samples were added; then the thermocycler was programmed for 25 cycles of a 10-s denaturing step at 96°C, a 5-s annealing step at 50°C, a 4-min extension step at 60°C, and a holding step at 4°C. The dye terminators were removed from the samples by passing the samples through CentriSep columns (Princeton Separations, Adelphia, N.J.), and the samples were dried in a SpeedVac vacuum centrifuge (Savant Instruments, Inc., Holbrook, N.J.) without heat. The samples were electrophoresed in a 5% Gene Page-Plus acrylamide gel with 6 M urea in 1× Tris-borate-EDTA buffer (Ultrapure grade TBE; AMRESCO, Solon, Ohio) on an ABI PRISM model 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

Nucleotide sequence accession number. The *E. faecalis aadA* gene sequence has been deposited in the GenBank database under accession no. AF052459.

RESULTS

Antimicrobial susceptibility testing. *E. faecalis* W4770 was resistant to streptomycin and spectinomycin (MICs, 4,000 and 8,000 μg/ml, respectively), as well as to tetracycline (MIC, >16 μg/ml). The isolate was susceptible to ampicillin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, penicillin, quinupristin/dalfopristin, rifampin, teicoplanin, and vancomycin.

AME assays. The isolate and controls were lysed and tested for AME activity. Only the assays for adenylyltransferase and phosphotransferase were performed, since streptomycin and spectinomycin are modified only by those classes of enzymes. W4770 gave a modification pattern consistent with the production of the ANT(3″)(9) enzyme by modifying streptomycin and spectinomycin but not amikacin, butirosin, gentamicin, kanamycin, neomycin, paromomycin, or tobramycin, as did *E. coli* C600(pPHP45Ω), which is known to produce ANT(3″)(9) enzyme. No phosphotransferase activity was demonstrated except in the control isolate *E. faecalis* ATCC 49532.

PCR amplification of resistance genes and specific gene probing. The specific 284-bp *aadA* gene product was amplified from both plasmid and total DNA obtained from W4770, and the specificity of the PCR product was confirmed with the digoxigenin-labeled product from *E. coli* C600(pHP45Ω). Control strains containing the *aadA* gene were positive by PCR for the 284-bp product; no product was demonstrated with strains containing other AME genes [*aadE*, *ant(2″)*, *ant(4″)*, *ant(9′)*, *aac(6′),* or *aph(2″)*]. No gene amplification was seen in the isolate with the *aadE*- and *aph(2″)-aac(6′)*-specific primers.

Transfer of resistance genes. A resistance plasmid (pNCC801) of approximately 80 kb was transferred by conjugation from strain W4770 to *E. faecalis* JH2-2 at a frequency of $\sim 3 \times 10^{-10}$. The MICs of streptomycin and spectinomycin for the transconjugants were 2,000 and 4,000 μg/ml, respectively; the transconjugants were also resistant to tetracycline. The 284-bp *aadA* gene product was amplified from the transconjugants. The *Bam*HI, *Eco*RI, and *Hin*dIII restriction profiles for pNCC801 and the plasmids present in the JH2-2 transconjugants were identical (data not shown).

Cloning and sequencing of the resistance gene. The 1,009-bp PCR product amplified by integron primers 1 and 2 from *E. faecalis* W4770 was cloned into pCRII. The sequence from the integron containing the *aadA* gene is presented in Fig. 1. Our 1,009-bp sequence is identical to that reported by Sundström et al. for *E. coli* plasmid R6-5 (GenBank accession no. X12870) from bases 1192 to 2201 (30), as well as to bases 5298 to 6306 of the recently assembled sequence of transposon Tn*21* (GenBank accession no. AF071413). The 791-bp enterococcal *aadA* structural gene sequence (bases 107 to 898) was identical to that in plasmid R538-1 in *E. coli* K-12 (bases 399 to 1190 of GenBank accession no. M10241) and was 99.4% homologous

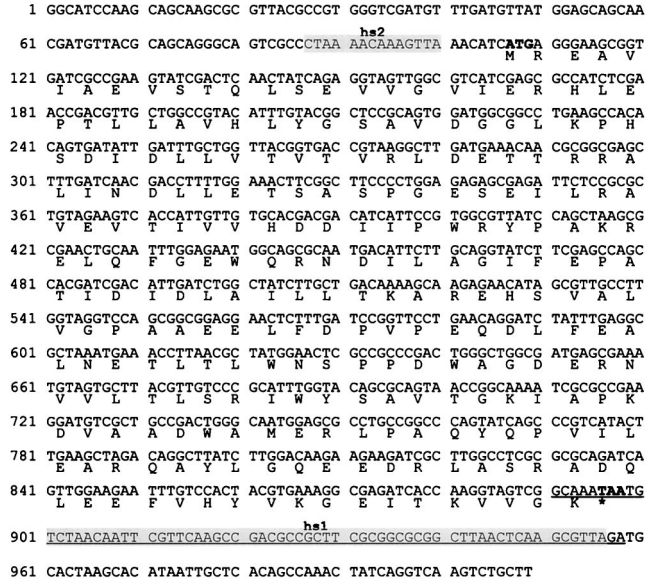

FIG. 1. Nucleotide sequence and predicted amino acid sequence of the plasmid-mediated *aadA-1* gene of *E. faecalis* W4770. The 1,009-bp PCR product was amplified with oligonucleotide primers specific for the integron cassette described by Lévesque et al. (21, 22). The gene insertion sites (GTTA) for the integron, at the 3′ end of each of the two shaded areas which highlight the hs2 (14 bp) and hs1 (55 bp) hot spots; the initiation and stop codons are in boldface type; and the 59-bp element is underlined (28).

to the sequence reported by Fling et al. (15) for Tn*7* (GenBank accession no. X03043, bases 82 to 873), except for the substitutions of an A for a G at position 790 and a C for a T at position 856, neither of which resulted in an amino acid change. However, an AGA insertion in the enterococcal sequence (bases 814 to 816) did result in an additional glutamic acid (E) residue.

When the entire 1,009-bp enterococcal sequence was compared with the sequence of R538-1 determined by Hollingshead and Vapnek (16), there was 99.5% homology as a result of a deletion of a C residue after base 933, the addition of a CGGC sequence (bases 938 to 941), and deletion of an A after base 954. Our sequence of the 1,009-bp amplification product from control organism *E. coli* C600(pHP45Ω) containing the *aadA* gene from R100.1 (26) is identical to that from W4770.

The 59-bp element sequences containing the hs1 hot spot (28), located downstream of the *aadA* gene, were identical in W4770, Tn*7*, and pHP45Ω (Fig. 1). However, the Tn*7* integron sequence upstream of the 5′ insertion site (GTTA) also contains an hs1 hot spot (28). Thus, it differs from the W4770, pHP45Ω, and R538-1 sequences, which are identical to each other and contain hs2 hot spots (28) (Fig. 1).

DISCUSSION

In 1981, Kono et al. reported the presence of ANT(3″) activity in an isolate of *E. faecalis* (18). Similar results were reported by Courvalin et al. in 1978 for the *E. faecalis* transconjugants of three clinical isolates (8), including strains JH2-14 (derived from *Enterococcus faecium* JH-7), JH2-15 (derived from *E. faecalis* JH-1 containing pJH-1), and JH2-12 (derived from *E. faecalis* JH-6). All of the isolates had high streptomycin MICs of >2,000 μg/ml but were susceptible to spectinomycin (MIC, 64 μg/ml). Courvalin et al. (8) attributed the adenylylation of streptomycin to the ANT(6′) (*aadE*) activity in their strains, since they were susceptible to spectinomycin as is typical for *S. aureus* strains carrying this determinant (31).

In 1992, Leclercq et al. noted that the AME ANT(3″)(9) was produced in staphylococci but not in enterococci (20). Our report of the ANT(3″)(9) enzyme in enterococci was not surprising since many other enterococcal genes, e.g., β-lactamase, *ant(4′)*, and *aac(6′)-(aph(2″),* were originally recognized in staphylococci (5, 6, 20, 25, 36). The ANT(9) enzyme, which mediates resistance to spectinomycin only, also appears to have its origins in staphylococci (19, 20).

Integrons are found either as part of transposons or independently on broad-host-range plasmids, but to our knowledge their presence has only been reported in gram-negative organisms (21, 29). Integrons typically have two conserved segments, 5′ and 3′, separated by a variable region which includes integrated antimicrobial resistance genes or cassettes. The 5′ conserved segment contains the integrase gene and, on the opposite strand, a common promoter region, P1-P2, which is directed toward the site of integration. Most genes inserted into integrons lack their own promoters and are expressed by the use of the common promoter region. The 3′ conserved segment contains the genes that determine resistance to quaternary ammonium compounds and sulfonamide, in addition to an open reading frame (21, 29). At the 3′ end of each resistance gene cassette is a short inverted-repeat element called the 59-bp element. The 59-bp element, which can vary to a certain degree in sequence and length, is important in the site-specific recombination event that results in the insertion of individual genes.

The integron sequence containing the *aadA* gene from *E. faecalis* W4770 was located on a plasmid of approximately 80 kb (pNCC801) which was transferable at a low frequency to *E. faecalis* JH2-2. The DNA sequences of the 1,009-bp amplification product from W4770 and the *E. coli* plasmids R6-5 (30) and pHP45Ω (26) were identical. The differences between our sequences and those of Hollingshead and Vapnek (16) for R538-1 were probably due to technical factors relating to earlier sequencing methods, as previously noted by Fling et al. (15). Our *aadA* sequence was preceded by an hs2 hot spot and followed by an hs1 hot spot, which included the characteristic 59-bp element described by Schmidt et al. (28). Our results suggested that the *aadA* gene was the only resistance gene inserted within the integron, since the PCR product was not of sufficient size to include other cassettes. It is unclear whether this integron is part of a larger transposon, but based on the sequence data, the integron does appear to have originated from an enteric organism.

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