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Cloning and Nucleotide Base Sequence Analysis of a Spectinomycin Adenyltransferase AAD(9) Determinant from *Enterococcus faecalis*

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***Enterococcus faecalis* LDR55, a human clinical isolate, is resistant to tetracycline (Tc^r), erythromycin (Em^r), and high levels (>2,000 μg/ml) of spectinomycin (Sp^r) but not streptomycin. Filter matings between strain LDR55 and *E. faecalis* OG1-RF produced transconjugants with the following resistance phenotypes: Tc^r Em^r Sp^r, Tc^r Em^r, Tc^r Sp^r, and Tc^r only but never Em^r or Sp^r only. The genetic determinant encoding resistance to spectinomycin was cloned in *Streptococcus sanguis* Challis from pDL55, a 26-kb plasmid harbored by a Tc^r Sp^r transconjugant. Subcloning experiments yielded a 1.1-kb *Cla*I-*Nde*I fragment that encoded very high-level Sp^r in *S. sanguis* (10 mg/ml) and *Escherichia coli* (50 mg/ml). Cell extracts of cultures obtained from Sp^r strains expressed adenylating activity for spectinomycin but not for streptomycin, indicating that Sp^r was due to an AAD(9) activity. The nucleotide base sequence of the 1.1-kb *Cla*I-*Nde*I fragment contained a single 750-base open reading frame. The protein predicted from the open reading frame consisted of 250 amino acids and had a calculated size of ~28,000 daltons, similar to the size estimated from maxicell analysis (29,000 daltons). The deduced amino acid sequence of the streptococcal AAD(9) was compared with that of the AAD(9) encoded by staphylococcal transposon Tn*554*. The two proteins shared approximately 39% amino acid identity, which was expanded to 53% when conservative amino acid changes were included. When the streptococcal protein was compared with an AAD(3″)(9) protein of *E. coli*, the degrees of identity were 27 and 47%, on the basis of actual amino acids and conservative replacements, respectively. The cloning and nucleotide base sequence analyses of the spectinomycin AAD(9) determinant from *E. faecalis* that results in high-level Sp^r when transferred to *S. sanguis* or *E. coli* are presented.**

Resistance to high levels (>2,000 μg/ml) of aminoglycoside antibiotics, such as neomycin, kanamycin (Km^r), and streptomycin (Sm^r), is common among clinical isolates of group D streptococci (16). Resistance to gentamicin (Gm^r) has been on the increase (20) since its first report in group D streptococci in 1979 (10). These resistance traits in isolates of *Enterococcus faecalis* usually are carried on plasmids (2), many of which are also transferable by conjugation (2). The genetic determinants encoding Km^r and Sm^r on pJH1, the first conjugative plasmid reported in streptococci (12), have been disseminated widely among enterococcal isolates of human and animal origins (16). These two genes, as well as the gene encoding the bifunctional enzyme AAC(6′)-APH(2″), responsible for the recent emergence of Gm^r in enterococci (6), are also common in *Staphylococcus aureus* (16, 25). Thus, it seems clear that the staphylococci and streptococci share a common pool of genetic determinants encoding antibiotic resistance traits.

Bacterial spectinomycin resistance (Sp^r), although much less common than resistance to other aminoglycoside-aminocyclitol antibiotics, is usually due to an adenylating enzyme, AAD(3″)(9), which modifies spectinomycin at the 9-OH position of the spectinomycin actinamine ring and streptomycin at the 3″-OH position of the streptinomycin glucosamine ring (29). Consequently, any bacterial strain exhibiting high-level Sp^r will also exhibit Sm^r. To date, the AAD(9) activity encoded by the *S. aureus* transposon Tn*554* described by Murphy (21) appears to be the only exception to this rule. During a survey of 26 human and 225 animal isolates of group D streptococci, it was observed that 1 human isolate and 59 animal isolates were resistant to >2,000 μg of spectinomycin per ml. Unexpectedly, the single human isolate and eight of the Sp^r animal isolates were Sm^s, suggesting that Sp^r in these isolates may be due to an AAD(9) activity. Because a gene for this activity has been reported only in *S. aureus* and because genetic determinants for high-level resistance to aminoglycosides are commonly shared by strains of *S. aureus* and group D streptococci, the human Sp^r streptococcal isolate was chosen for further study.

This report describes the cloning, sequencing, and characterization of the gene encoding spectinomycin AAD(9) activity from *E. faecalis* LDR55. It also presents a comparison of the deduced protein product of this gene with the AAD(9) gene product encoded by *S. aureus* as well as with an AAD(3″)(9) protein common among members of the family *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. faecalis* LDR55 was obtained from the collection of L. D. Rollins (23). *E. faecalis* OG1-RF (4) served as the recipient in conjugation experiments. *Streptococcus sanguis* Challis strain DL1 (13) and *Escherichia coli* TB1, an HsdR$^-$ HsdM$^+$ derivative of JM83 (30) obtained from Bethesda Research Laboratories, Gaithersburg, Md., were the respective host strains for cloning procedures with the streptococcal plasmid vectors pDL412 (14), pDL413 (14), and pVA380-1 (17) and the *E. coli* plasmid vector pUC19 (30). *E. coli* JM103 and JM107 served as hosts for M13mp18 and M13mp19 phage vectors (30) and their

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recombinant derivatives. *E. coli* maxicell strain CSR603 (26) was obtained from M. Yeung (University of Texas Health Science Center at San Antonio).

Streptococci and *E. faecalis* were grown routinely in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) or in brain heart infusion broth supplemented with 10 mM D-glucose at 37°C without aeration. *E. coli* strains were grown in LB broth (18) at 37°C with aeration. Solid media contained 1.5% Bacto-Agar (Difco). Strain LDR55 was identified as *E. faecalis* as described by Facklam and Carey (5). Bile-esculin and tellurite agars were from Difco, and carbohydrates were from Sigma Chemical Co. (St. Louis, Mo.).

Biochemical reagents and enzymes. Restriction endonucleases, bacterial alkaline phosphatase, T4 DNA ligase, DNA polymerase I (Klenow fragment), exonuclease III, and deoxyribonucleoside and dideoxyribonucleoside triphosphates were obtained from Bethesda Research Laboratories. [α-^{35}S]dATP (specific activity, >400 Ci/mmol) and [U-^{14}C] ATP (specific activity, 400 to 600 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.).

Conjugation experiments. Streptococcal matings, which included incubation of donor and recipient strains on membrane filters or mixed incubation in broth cultures, were done as described previously (15). Recipient *E. faecalis* OG1-RF was selected on media containing 25 μg each of rifampin and fusidic acid per ml. Transconjugant colonies were selected by the addition of 500 μg of spectinomycin per ml to the above-described media.

Plasmid DNA isolation and molecular cloning. Plasmid DNA was purified from exponential-phase cultures of streptococci as described previously (15) and from *E. coli* cultures by standard procedures (18). Vector and passenger DNAs were prepared for cloning by digestion with appropriate restriction endonucleases, mixing, denaturation, annealing, and ligation with T4 DNA ligase as described by Davis et al. (3). Transformation of *E. coli* was done by the method of Hanahan (7). Competent cultures of *S. sanguis* Challis were prepared as described by Rollins et al. (23) and transformed with ligated DNA mixtures as described previously (14). Antibiotic concentrations for selection of streptococcal transformants were 250 μg of spectinomycin, 1,000 μg of streptomycin, or 500 μg of kanamycin per ml. *E. coli* transformants were selected in the presence of 50 μg each of spectinomycin and ampicillin per ml.

Aminoglycoside-aminocyclitol adenylation assays. A detailed protocol for the aminoglycoside-aminocyclitol adenylation assay (1) was provided by B. Mederski-Samoraj (University of Toronto, Toronto, Ontario, Canada). Cell extracts were prepared as described by Ranhand (22) with minor modifications as described previously (11). Overnight cultures were diluted 1:10 in brain heart infusion broth and incubated at 37°C until the optical density at 660 nm reached 0.5 to 0.6. Washed cells were resuspended in 0.01 culture volume of TMN buffer (10 mM Tris-HCl [pH 7.6], 10 mM Mg acetate, 70 mM NH_4Cl, 1 mM dithiothreitol). Protein concentrations were determined with a microdetermination kit (690-A; Sigma). The 40-μl reaction mixture contained 20 μl of ATP mixture (see below), 10 μl of antibiotic or distilled H_2O, and 10 μl of cell extract or TMN buffer. The ATP mixture contained 0.1 μCi of ^{14}C-ATP and 0.25 mM ATP in 0.5× buffer containing 67 mM Tris-HCl (pH 7.4), 42 mM $MgCl_2$, 0.4 M NH_4Cl, and 1.7 mM dithiothreitol. The concentration of spectinomycin or streptomycin added was varied from 100 to 4,000 μg/ml. A concentration of 200 μg/ml was chosen empirically for standard reactions. Reaction mixtures were incubated at 32°C for 60 min, after which 20-μl aliquots were spotted onto cellulose phosphate papers (Whatman P81). After 15 s, the papers were boiled in distilled H_2O for 3 min and washed three times for 2 min each time in distilled H_2O. The papers were dried and counted in scintillation fluid. An enzyme activity unit was defined as the amount of extract required for the binding of 1 pmol of ATP to the paper, on the basis of 40 cpm/pmol of ATP. Specific activities are reported as picomoles of ATP bound per minute per milligram of protein.

Restriction endonuclease mapping and nucleotide base sequence determinations. Partial restriction endonuclease maps of cloned fragments encoding Sp^r were constructed from single and double enzyme digests of recombinant plasmids. Subclones to be used for nucleotide base sequence determinations were obtained either by cloning specific fragments into M13mp18 and/or M13mp19 or by generating nested deletions with exonuclease III as described by Henikoff (8). Sequence determinations were performed on both DNA strands by the dideoxy chain termination method (28) and [α-^{35}S]dATP labeling. Labeled fragments were separated on 0.2-mm sequencing gels (Macrophor system; LKB Instruments, Gaithersburg, Md.). DNA sequence data were compiled and analyzed with a Beckman Instruments Micro Genie program.

Maxicell analyses. Maxicell lysates were prepared as described by Sancar et al. (27) with the following modifications. pUC19, pDL269 (expressing Sp^r and Ap^r [ampicillin resistance]), and pDL270 (expressing Sp^r Ap^s [ampicillin susceptibility]) were used to transform *E. coli* CSR603 (ATCC 35577; F$^-$ *supE44 lacY1 thr-1 leuB6 proA2 argE3 mcrB galK2 ara-14 xyl-5 mtl-1 gyrA98 rpsL31 recA1 phr-1 uvrA6 thi-1 tsx-33* λ^-) by the method of Hanahan (7) as modified by Maniatis et al. (18). CSR603 and plasmid-containing transformants were grown in M9 media (18) plus 1% Casamino Acids to cell densities of 10^8 CFU/ml (optical density at 660 nm, 0.5) and irradiated with 2.0 J in a UV Stratalinker 1800 (Stratagene, LaJolla, Calif.). The irradiated cells were allowed to recover (1 hr, 37°C, with shaking), after which cycloserine (ICN Biochemicals, Cleveland, Ohio) was added to a final concentration of 100 μg/ml. After overnight incubation, the cells were harvested by centrifugation and washed with 1× M9 salts without sulfate. The cells were resuspended in M9 media without sulfate but supplemented with 2% threonine, 1% leucine, 2% proline, 2% arginine, 0.1% thiamine, and 0.4% glucose and incubated for 1 h at 37°C. ^{35}S-methionine (>1,050 Ci/mmol; NEN DuPont) was added to a final concentration of 5 μCi/ml, and incubation was continued for an additional hour. The cells were harvested by centrifugation and resuspended in 100 μl of 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (18). The cell lysates were boiled for 2 min, and 10- to 50-μl aliquots were loaded onto 12% SDS-PAGE gels. Electrophoresis and autoradiography were done as described previously (27).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported here is M69221.

RESULTS

Characterization of strain LDR55 and its resistance to spectinomycin. A streptococcal isolate obtained in 1982 from the urine of a Washington, D.C., hospital patient with a urinary tract infection was designated strain LDR55. The isolate was classified as *E. faecalis* on the basis of the following criteria: ability to grow in the presence of 6.5%

TABLE 1. Determination of spectinomycin resistance and spectinomycin-specific adenylating activity

Strain tested	MIC (mg/ml) of spectinomycin[a]	Spectinomycin-specific adenylating activity[b]
E. faecalis		
OG1-RF	0.5	0.01
LDR55	5	5
DL186	50	15
S. sanguis		
DL1	0.5	0.01
DL1(pDL265)	25	50
DL1(pDL266)	25	15
DL1(pDL267)	25	15
DL1(pDL273)	25	15
E. coli		
TB1	0.01	0.1
TB1(pDL268)	100	15
TB1(pDL269)	100	100

[a] Determined in LB broth for *E. coli* and in brain heart infusion broth plus glucose for *E. faecalis* and *S. sanguis*.

[b] One enzyme unit equals 1 pmol of ATP bound to paper per min per mg of protein in a standard assay.

NaCl and at 45°C; positive reactions on bile-esculin and tellurite agar plates; acid production from mannitol, lactose, sucrose, sorbitol, melizitose, and glycerol (aerobically); and growth but no acid production in the presence of L-arabinose, sorbose, and glycerol (anaerobically). LDR55 was resistant to erythromycin (25 μg/ml) (Em^r), tetracycline (10 μg/ml) (Tc^r), and spectinomycin (2,000 μg/ml) but not streptomycin (2,000 μg/ml).

The ability of strain LDR55 to transfer its resistance traits to plasmid-free *E. faecalis* OG1-RF was tested in broth and filter matings. Transconjugants were obtained only after overnight filter matings. All transconjugants selected in the presence of erythromycin were also Tc^r and Sp^r. Among transconjugants selected for resistance to tetracycline, 54% were Tc^r only, 23% were Tc^r Em^r, 21% were Tc^r Em^r Sp^r, and 2% were Tc^r Sp^r. All transconjugants selected in the presence of 1,000 μg of spectinomycin per ml were also Tc^r, and 20% of these were also Em^r. No transconjugants that were Em^r or Sp^r only were obtained. Transconjugants which specified Sp^r in addition to other markers did not serve as resistance donors in subsequent mating experiments.

One transconjugant exhibiting an Sp^r Tc^r phenotype, strain DL186, was chosen for further study. Strain DL186 contained a 26-kb plasmid (pDL55) and was 100 times more resistant to spectinomycin than was the recipient strain, OG1-RF (Table 1). It also exhibited a higher level of resistance than did the donor strain, LDR55. Spectinomycin-specific adenylating activity was detected in cell extracts of strain LDR55 and was 1,500 times greater in DL186 than in OG1-RF (Table 1). No adenylating activity was detected in extracts of the transconjugant when streptomycin (400 μg/ml) was used as the substrate. Streptomycin-specific adenylating activity was readily detected when OG1-RF containing plasmid pJH1 (12) was used as a positive control for AAD(6) activity (data not shown).

Cloning of the Sp^r gene from pDL55. A series of cloning and subcloning experiments, involving both streptococcal and *E. coli* host-vector systems, located the *E. faecalis* Sp^r determinant on a 1.1-kb DNA fragment from pDL55. The results of these experiments are summarized in Fig. 1. Plasmid DNA was purified from the pDL55-containing transconjugant that was resistant to spectinomycin and tetracycline. Initially, two derivatives of streptococcal plasmid pVA380-1 (17) were used as vectors to clone the Sp^r determinant from pDL55. pDL55 and Km^r vector pDL413 (14) were digested with *Eco*RI, mixed, treated with DNA ligase, and used to transform *S. sanguis* Challis, with selection for resistance to kanamycin and spectinomycin. Recombinant plasmid pDL265 from an Sp^r Km^r transformant contained pDL413 and a 6.4-kb *Eco*RI fragment from pDL55. A similar experiment involving *Cla*I-digested pDL55 and Sm^r plasmid pDL412 (14) provided an Sp^r Sm^r transformant containing recombinant plasmid pDL266 composed of vector pDL412 and a 4.2-kb *Cla*I fragment from pDL55. Restriction endonuclease maps of recombinant plasmids pDL265 and pDL266 revealed the presence of a common 2.5-kb *Cla*I-*Eco*RI fragment. This fragment was subcloned into *Cla*I- and *Eco*RI-digested pVA380-1 by selection for an Sp^r *S. sanguis* transformant which contained pDL267. The expression of the *E. faecalis* Sp^r gene in *E. coli* was established when the 2.5-kb *Cla*I-*Eco*RI fragment from pDL267 and *Acc*I- and *Eco*RI-digested pUC19 DNA were mixed, treated with DNA ligase, and used to transform *E. coli* TB1, with selection for Sp^r transformants. Recombinant plasmid pDL268 was isolated from one of the *E. coli* transformants, digested with *Nde*I, religated, and used to transform strain TB1, again with selection for Sp^r transformants. This experiment generated recombinant plasmid pDL269, which contained only 1.1 kb of pDL55-derived DNA. Subsequently, *S. sanguis* recombinant plasmid pDL267 was digested with *Nde*I, religated, and used to produce Sp^r transformants of *S. sanguis* Challis. The recombinant plasmid obtained from this experiment, pDL273, also contained only the 1.1-kb *Cla*I-*Nde*I fragment from pDL55.

The MICs of spectinomycin for all of the *S. sanguis* and *E. coli* recombinant clones described above were 25,000 and 100,000 μg/ml, respectively (Table 1). Cell extracts from these clones also expressed specinomycin-specific adenylating activity. Extracts of the *E. coli* clone containing pDL269, as expected, did not have any detectable streptomycin-specific adenylating activity.

Sequencing of the 1.1-kb DNA fragment encoding Sp^r. A partial restriction endonuclease map of the 1.1-kb *Cla*I-*Nde*I fragment was constructed from single and double digests of recombinant plasmids (Fig. 1, bottom). Subclones for sequencing were obtained by cloning specific fragments into M13mp18 and/or M13mp19 and by generating nested deletions with exonuclease III (8). The sequence (Fig. 2) contained an open reading frame (ORF) of 750 bases, sufficient to encode a 250-amino-acid protein with a calculated size of 27,750 daltons. Supportive evidence that the ORF (bases 286 to 1035) encoded Sp^r came from a mutation at the *Kpn*I site (base 555), generated by blunting of the ends produced by this enzyme and by religation. The mutation, in pDL269 (Fig. 1), resulted in the loss of the Sp^r phenotype when the plasmid was reintroduced into *E. coli*. The probable translational start codon, TTG, at base 286, was preceded by a strong ribosome binding site (AGGAGG) which had a calculated free energy of interaction of −20 kcal (ca. −84 kJ) and a spacer of 8 bases and which conformed well to the ribosome binding sites of *Bacillus subtilis* (19). A potential promoter was found at positions 2 to 7 (−35; TCGATT) and 27 to 32 (−10; TATAAT), with a separation of 19 bases. However, the −35 sequence did not match the consensus sequence, TTGACA (24). A second putative promoter was

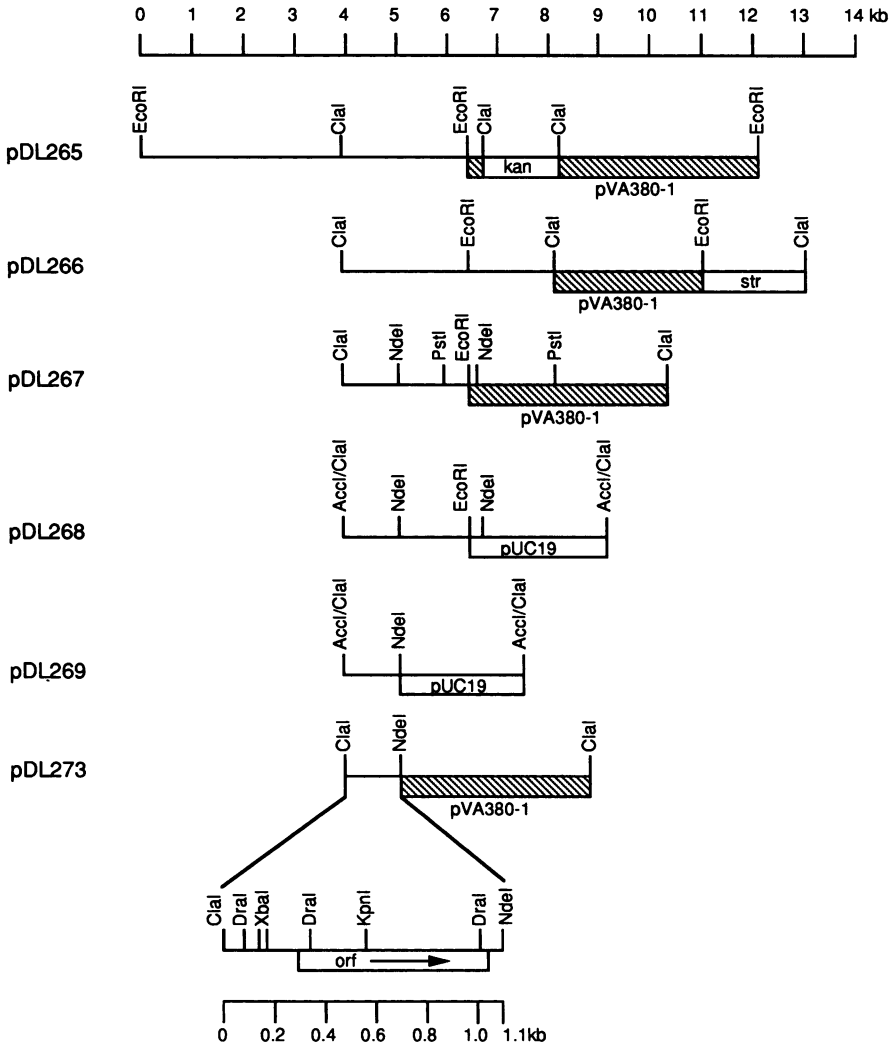

FIG. 1. Cloning and subcloning of the Sp^r determinant from pDL55. All recombinant plasmids were obtained by transformation of *S. sanguis* Challis (pVA380-1 vector) or *E. coli* TB1 (pUC19 vector) with appropriate ligation mixtures, with selection for Sp^r transformants. pDL265, 6.4-kb *Eco*RI fragment from pDL55 cloned into pDL413 (pVA380-1 containing the Km^r determinant of pJH1); pDL266, 4.2-kb *Cla*I fragment from pDL55 cloned into pDL412 (pVA380-1 containing the Sm^r determinant of pJH1); pDL267, 2.5-kb *Cla*I-*Eco*RI fragment from pDL266 subcloned into pVA380-1; pDL268, 2.5-kb *Cla*I-*Eco*RI fragment from pDL267 subcloned into pUC19; pDL269, produced by digestion of pDL268 with *Nde*I, religation, and transformation of strain TBI; pDL273, 1.1-kb *Cla*I-*Nde*I fragment of pDL267 subcloned into pVA380-1. Bottom of figure, expanded restriction endonuclease map of 1.1-kb *Cla*I-*Nde*I fragment from pDL55 encoding Sp^r.

found at positions 111 to 117 (−35; ATGGAAA) and 135 to 140 (−10; TATAAT), with a separation of 17 bases. In this instance, the proposed −35 sequence was very similar to that of the *aac6-aph2* gene (ATGAAAA) from *E. faecalis* (6). A UAA translational termination codon (base 1038) was followed by a palindromic region capable of forming a hairpin structure with a calculated free energy of −14 kcal (ca. −59 kJ). The palindromic region ended with a run of U residues, a feature common to rho-independent terminator sequences (24).

Maxicell analysis of the protein encoded by the Sp^r gene. The size of the protein encoded by the Sp^r gene was determined with the maxicell system (27) as described in Materials and Methods. Three plasmids were transferred to the maxicell strain, CSR603. The original vector, pUC19, was used as a control for the β-lactamase protein. pDL269 encoded both β-lactamase and spectinomycin AAD(9) activities. An Ap^s derivative of pDL269 was constructed by digestion of plasmid DNA with *Ava*II, religation, and transformation of *E. coli*, with selection for Sp^r. Colonies resistant to spectinomycin were tested for susceptibility to ampicillin. An Ap^s transformant contained a plasmid, pDL270, which was identical to pDL269, with the exception of a 222-bp deletion in the Ap^r gene. The proteins encoded by each of these plasmids in strain CSR603 are illustrated in the autoradiograph shown in Fig. 3. The transformant harboring the original vector, pUC19, produced a single protein band (lane 2) that migrated at a rate equivalent to that of a protein of approximately 30 kDa. The only protein expected from this plasmid was β-lactamase, with a predicted size of 31.5 kDa. A double protein band was produced by the transformant carrying pDL269 (lane 3) in the same region of the gel as the band produced by the pUC19-containing transformant. Strain CSR603 harboring pDL270 produced a protein band

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   1 ATCGATTTTC GTTCGTGAAT ACATGTTATA ATAACTATAA CTAATAACGT
                                 ==== ==
  51 AACGTGACTG GCAAGAGATA TTTTTAAAAC AATGAATAGG TTTACACTTA
 101 CTTTAGTTTT ATGGAAATGA AAGATCATAT CATATATAAT CTAGAATAAA
                                      ======      ----
 151 ATTAACTAAA ATAATTATTA TCTAGATAAA AAATTTAGAA GCCAATGAAA
     --    ----  --
 201 TCTATAAATA AACTAAATTA AGTTTATTTA ATTAACAACT ATGGATATAA
                      RBS                            ---
 251 AATAGGTACT AATCAAAATA GTGAGGAGGA TATATTTGAA TACATACGAA
     ---
 301 CAAATTAATA AAGTGAAAAA AATACTTCGG AAACATTTAA AAAATAACCT
 351 TATTGGTACT TACATGTTTG GATCAGGAGT TGAGAGTGGA CTAAAACCAA
 401 ATAGTGATCT TGACTTTTTA GTCGTCGTAT CTGAACCATT GACAGATCAA
 451 AGTAAAGAAA TACTTATACA AAAAATTAGA CCTATTTCAA AAAAAATAGG
 501 AGATAAAAGC AACTTACGAT ATATTGAATT AACAATTATT ATTCAGCAAG
 551 AAATGGTACC GTGGAATCAT CCTCCCAAAC AAGAATTTAT TTATGGAGAA
 601 TGGTTACAAG AGCTTTATGA ACAAGGATAC ATTCCTCAGA AGGAATTAAA
 651 TTCAGATTTA ACCATAATGC TTTACCAAGC AAAACGAAAA AATAAAAGAA
 701 TATACGGAAA TTATGACTTA GAGGAATTAC TACCTGATAT TCCATTTTCT
 751 GATGTGAGAA GAGCCATTAT GGATTCGTCA GAGGAATTAA TAGATAATTA
 801 TCAGGATGAT GAAACCAACT CTATATTAAC TTTATGCCGT ATGATTTTAA

 851 CTATGGACAC GGGTAAAATC ATACCAAAAG ATATTGCGGG AAATGCAGTG
 901 GCTGAATCTT CTCCATTAGA ACATAGGGAG AGAATTTTGT TAGCAGTTCG
 951 TAGTTATCTT GGAGAGAATA TTGAATGGAC TAATGAAAAT GTAAATTTAA
1001 CTATAAACTA TTTAAATAAC AGATTAAAAA AATTATAAAA AAATTGAAAA
                                                 ----------
1051 AATGGTGGAA ACACTTTTTT CAATTTTTTT GTTTTATTAT TTAATATTTG
     ------>  <----------------------
1101 GGAAATATTC ATTCTAATTG GTAATCAGAT TTTAGAAAAC AATAAACCCT
1151 TGCATATG
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FIG. 2. Nucleotide base sequence of 1.1-kb *ClaI-NdeI* fragment from pDL55 encoding Sp^r. Boldface letters indicate the ORF. RBS, ribosome binding site. Putative promoters, −10 and −35, are represented by single and double underlinings, respectively. A palindrome and a putative transcriptional terminator are indicated by arrows.

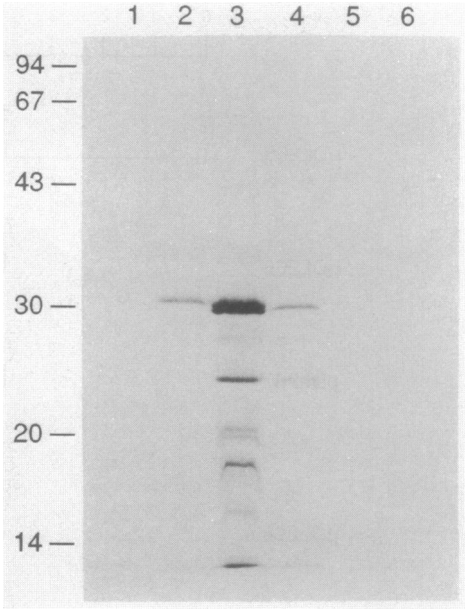

FIG. 3. Autoradiogram identifying the protein responsible for Sp^r. Total proteins produced by *E. coli* CSR603, with and without vector or recombinant plasmid DNA, were separated on an SDS-PAGE slab gel and stained with Coomassie brilliant blue R250. After drying of the gel, plasmid-encoded proteins labeled with ^{35}S-methionine were visualized by exposure of an X-ray film to the dried gel. Proteins in the lanes were from a Pharmacia calibration kit for molecular weight determination of low-molecular-weight proteins (lanes 1 and 5), CSR603 containing pUC19 (Ap^r) (lane 2), CSR603 containing pDL269 (Sp^r Ap^r) (lane 3), CSR603 containing pDL270 (Sp^r Ap^s) (lane 4), and CSR603 (lane 6). Numbers on the left designate the locations of molecular weight markers in thousands.

(lane 4) that migrated in the same region of the gel as the single band in lane 2 and the double band in lane 3. The size of the protein represented by this band was calculated to be approximately 29 kDa, close to the size predicted for AAD(9) from the ORF. The plasmid-free parent strain CSR603 produced no detectable radiolabeled protein (lane 6). The faster-migrating bands seen in lanes 3 and 4 probably represented breakdown products of the AAD(9) protein, since they were produced only by the two transformants encoding AAD(9) activity.

Comparison of Sp^r and Sp^r Sm^r determinants. The deduced amino acid sequence of the streptococcal AAD(9) protein was compared with that of AAD(9) encoded by Tn*554* (21) and with that of the AAD protein encoded by Tn*7* of *E. coli* (9). Optimal alignments were obtained by the introduction of gaps in all three amino acid sequences (Fig. 4). The streptococcal and staphylococcal proteins shared approximately 39% amino acid identity, which was expanded to 53% when conservative amino acid changes were included. The staphylococcal and *E. coli* proteins were related similarly, with 36% identity overall, which was extended to 54% when conservative replacements were considered. When the same approach was used to compare the streptococcal and *E. coli* proteins, the degrees of identity were 27 and 47%, on the basis of actual amino acids and conservative replacements, respectively.

As noted in previous comparisons of the staphylococcal and *E. coli* amino acid sequences (21) and extended here to include the protein encoded by the streptococcal Sp^r gene, four regions were fairly well conserved among the three enzymes (designated a, b, c, and e in Fig. 4). When conservative amino acid replacements were included, analyses of these regions revealed that the staphylococcal and *E. coli* sequences were more related to each other (71 to 86% homology) than to the streptococcal sequence, with the exception of region b. In the latter region, the streptococcal and staphylococcal sequences were 88% homologous. Amino acids in region d were conserved in the streptococcal and staphylococcal sequences (80% homology, with conservative replacements) but not in the protein encoded by *aadA*.

DISCUSSION

E. faecalis LDR55 was able to transfer all three of its antibiotic resistance traits, i.e., Sp^r, Em^r, and Tc^r, to *E. faecalis* OG1-RF. However, only the Tc^r trait was transferred alone. The transfer of Sp^r and/or Em^r was always accompanied by the transfer of Tc^r. Among the transconjugants exhibiting different resistance phenotypes, plasmid DNA was detected only in those expressing Tc^r and Sp^r. A plasmid of the same size as that detected in one of the Tc^r Sp^r transconjugants, pDL55 (26 kb) of strain DL186, was also the only plasmid ever detected in parent strain LDR55. Curiously, none of the resistant transconjugants, including that containing pDL55, were able to serve as resistance

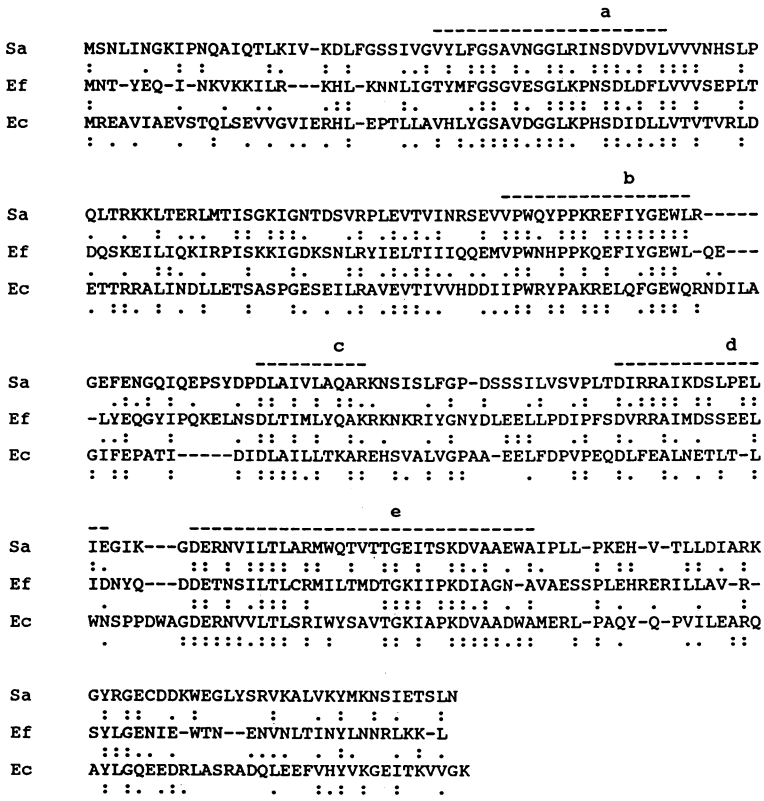

FIG. 4. Comparison of deduced AAD(9) proteins produced by *E. faecalis* LDR55 and *S. aureus* containing Tn*554* and the AAD(3")(9) protein produced by *E. coli* containing Tn*7*. a, b, c, d, and e indicate highly conserved regions (delineated by lines above the single-letter amino acid designations) of all three proteins. Colons represent identical amino acids, and periods represent conservative replacements. Sa, *S. aureus*; Ef, *E. faecalis*; Ec, *E. coli*. Symbols under the deduced *S. aureus* protein designate a comparison between *S. aureus* and *E. faecalis*, those under *E. faecalis* designate a comparison between *E. faecalis* and *E. coli*, and those under *E. coli* designate a comparison between *E. coli* and *S. aureus*.

donors in subsequent filter mating experiments. It is possible that the resistance traits of strain LDR55 were mobilized by a chromosomal conjugative element present in the donor, by an undetectable conjugative plasmid, or even by transduction due to an undetected phage.

The MIC of spectinomycin for transconjugant strain DL186 of *E. faecalis* OG1-RF was 10-fold higher than was that for the original Sp^r donor strain, LDR55, and the former strain expressed a 3-fold-higher level of spectinomycin-specific adenylating activity than did the latter strain (Table 1). The reasons for these differences are not known. They may simply reflect differences in the expression of the same gene, *spc*, by the two strains of *E. faecalis*, OG1-RF and LDR55.

Although the MICs for each set of strains, *S. sanguis* Challis and *E. coli* TB1, carrying recombinant plasmids containing the *spc* gene were equivalent, plasmids pDL265 and pDL269 effected the production of approximately threefold and sevenfold more spectinomycin-specific adenylating activities, respectively, than did the other *spc*-containing recombinant plasmids. There is no ready explanation for the increased activity observed with pDL269, since it was derived by deletion of sequences distal to the *spc* gene (Fig. 1). In the case of pDL265, however, the possibility exists that it contains a strong promoter sequence that is located upstream of the *spc* gene and that is missing in the other streptococcal recombinant plasmids.

The ORFs corresponding to the *spc* structural genes from *E. faecalis* LDR55 and *S. aureus* transposon Tn*554* exhibited approximately 40% nucleotide base identity, distributed throughout the respective sequences. Unlike other cloned determinants encoding resistance to aminoglycoside antibiotics in *E. faecalis* strains (15), the *spc* gene from strain LDR55 did not hybridize to the corresponding gene from Tn*554*. At the amino acid levels of the two deduced AAD(9) proteins, there was 36% identity overall, which was extended to 54% when conservative replacements were considered. Clearly, if the two genes compared here share a common evolutionary origin, it is not due to a recent genetic transfer event.

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