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

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Enterococcus faecalis LDR55, a human clinical isolate, is resistant to tetracycline (Tc'), erythromycin (Em'), and high levels (>2,000 µg/ml) of spectinomycin (Sp') but not streptomycin. Filter matings between strain LDR55 and E. faecalis OG1-RF produced transconjugants with the following resistance phenotypes: Tc' Em' Sp', Tc' Em' Spr', and Tc' only but never Em' or Sp' only. The genetic determinant encoding resistance to spectinomycin was cloned in Streptococcus sanguis Challis from pDL55, a 26-kb plasmid harbored by a Tc' Sp' transconjugant. Subcloning experiments yielded a 1.1-kb ClaI-NdeI fragment that encoded very high-level Sp' in S. sanguis (10 µg/ml) and Escherichia coli (50 µg/ml). Cell extracts of cultures obtained from Sp' strains expressed adenylylating activity for spectinomycin but not for streptomycin, indicating that Sp' was due to an AAD(9) activity. The nucleotide base sequence of the 1.1-kb ClaI-NdeI 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 Tn554. 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' when transferred to S. sanguis or group D streptococci, were presented.

Resistance to high levels (>2,000 µg/ml) of aminoglycoside antibiotics, such as neomycin, kanamycin (Km'), and streptomycin (Sm'), is common among clinical isolates of group D streptococci (16). Resistance to gentamicin (Gm') 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' and Sm' on pH1, 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' in enterococci (6), are also common in Staphylococcus aureus (16, 25). Thus, it seems clear that the staphylococcal and streptococcal share a common pool of genetic determinants encoding antibiotic resistance traits.

Bacterial spectinomycin resistance (Sp'), although much less common than resistance to other aminoglycoside-aminocyclitol antibiotics, is usually due to an adenylylating enzyme, AAD(3')(9), which modifies spectinomycin at the 9-OH position of the spectinomycin actinamidine ring and streptomycin at the 3''-OH position of the streptomycin glucosamine ring (29). Consequently, any bacterial strain exhibiting high-level Sp' also will exhibit Sm'. To date, the AAD(9) activity encoded by the S. aureus transposon Tn554 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' animal isolates were Sm', suggesting that Sp' 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' 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 deoxyribonuclease and dideoxyribonuclease triphosphates were obtained from Bethesda Research Laboratories. [α-35S]ATP (specific activity, >400 Ci/mmole) and [U-14C] ATP (specific activity, 400 to 600 mCi/mmole) were obtained from Amershams 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* Challas 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. *E. coli* transformants were selected in the presence of 50 μg each of spectinomycin and ampicillin per ml.

Aminoglycoside-aminocyclitol adenylase assays. A detailed protocol for the aminoglycoside-aminocyclitol adenyla-

tion assay (1) was provided by B. Meders-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 NH₄Cl, 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 H₂O, and 10 μl of cell extract or TMN buffer. The ATP mixture contained 0.1 μCi of [14C]-ATP and 0.25 mM ATP in 0.5X buffer containing 67 mM Tris-HCl (pH 7.4), 42 mM MgCl₂, 0.4 M NH₄Cl, 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 H₂O for 3 min and washed three times for 2 min each time in distilled H₂O. The papers were dried and counted in a 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 cpn/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' 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-35S]ATP 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' and Ap' [ampicillin resistance]), and pDL270 (expressing Sp' Ap' [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 pyrA98 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 10⁸ 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. 35S-methionine (≥1,050 Ci/mmole; 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 adenylylating activity

<table>
<thead>
<tr>
<th>Strain tested</th>
<th>MIC (mg/ml) of spectinomycina</th>
<th>Spectinomycin-specific adenylylating activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG1-RF</td>
<td>0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>LDR55</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>DL186</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td><em>S. sanguis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL1</td>
<td>0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>DL1(pDL265)</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>DL1(pDL266)</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>DL1(pDL267)</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>DL1(pDL273)</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB1</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>TB1(pDL268)</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>TB1(pDL269)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

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, melibiose, and glycerol (anaerobically); and growth but no acid production in the presence of L-arabinose, sorbose, and glycerol (anaerobically). LDR55 was resistant to erythromycin (25 μg/ml), tetracycline (10 μg/ml) (Tc'), 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' and Sp'. Among transconjugants selected for resistance to tetracycline, 54% were Tc' only, 23% were Tc' Em', 21% were Tc' Em' Sp', and 2% were Tc' Sp'. All transconjugants selected in the presence of 1,000 μg of spectinomycin per ml were also Tc', and 20% of these were also Em'. No transconjugants that were Em' or Sp' only were obtained. Transconjugants which specified Sp' in addition to other markers did not serve as resistance donors in subsequent mating experiments.

One transconjugant exhibiting an Sp' Tc' 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 adenylylating activity was detected in cell extracts of strain LDR55 and was 1,500 times greater in DL186 than in OG1-RF (Table 1). No adenylylating activity was detected in extracts of the transconjugant when spectinomycin (400 μg/ml) was used as the substrate. Spectinomycin-specific adenylylating 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' gene from pDL55. A series of cloning and subcloning experiments, involving both streptococcal and *E. coli* host-vector systems, located the *E. faecalis* Sp' 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' determinant from pDL55. pDL55 and Km' vector pDL413 (14) were digested with *Eco*RI, mixed, and 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' Km' transformant contained pDL413 and a 6.4-kb *Eco*RI fragment from pDL55. A similar experiment involving *ClaI*-digested pDL55 and Sm' plasmid pDL412 (14) provided an Sp' Sm' transformant containing recombinant plasmid pDL266 composed of vector pDL412 and a 4.2-kb *ClaI* fragment from pDL55. Restriction endonuclease maps of recombinant plasmids pDL265 and pDL266 revealed the presence of a common 2.5-kb *ClaI*-*Eco*RI fragment. This fragment was subcloned into *ClaI*- and *Eco*RI-digested pVA380-1 by selection for an Sp' *S. sanguis* transformant which contained pDL267. The expression of the *E. faecalis* Sp' gene in *E. coli* was established when the 2.5-kb *ClaI*-*Eco*RI fragment from pDL267 and *AccI*- and *Eco*RI-digested pUC19 DNA were mixed, treated with DNA ligase, and used to transform *E. coli* TB1, with selection for Sp' transformants. Recombinant plasmid pDL268 was isolated from one of the *E. coli* transformants, digested with *NdeI*, religated, and used to transform strain TB1, again with selection for Sp' 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 *NdeI*, religated, and used to produce Sp' transformants of *S. sanguis* Challis. The recombinant plasmid obtained from this experiment, pDL273, also contained only the 1.1-kb *ClaI*-*NdeI* 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 spectinomycin-specific adenylylating activity. Extracts of the *E. coli* clone containing pDL269, as expected, did not have any detectable streptomycin-specific adenylylating activity.

Sequencing of the 1.1-kb DNA fragment encoding Sp'. A partial restriction endonuclease map of the 1.1-kb *ClaI*-*NdeI* fragment was constructed from single and double digests of the 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' came from a mutation at the *KpnI* 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' 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
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′ gene.**

The size of the protein encoded by the Sp′ 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′ derivative of pDL269 was constructed by digestion of plasmid DNA with *Ava*II, religation, and transformation of *E. coli*, with selection for Sp′. Colonies resistant to spectinomycin were tested for susceptibility to ampicillin. An Ap′ transformant contained a plasmid, pDL270, which was identical to pDL269, with the exception of a 222-bp deletion in the Ap′ 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
FIG. 2. Nucleotide base sequence of 1.1-kb ClaI-NdeI fragment from pDL55 encoding Sp'. 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.

(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' and Sp' Sm' determinants. The deduced amino acid sequence of the streptococcal AAD(9) protein was compared with that of AAD(9) encoded by Tn554 (21) and with that of the AAD protein encoded by Tn7 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 expanded 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.

FIG. 3. Autoradiogram identifying the protein responsible for Sp'. 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 35S-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') (lane 2), CSR603 containing pDL269 (Sp' Ap') (lane 3), CSR603 containing pDL270 (Sp' Ap') (lane 4), and CSR603 (lane 6). Numbers on the left designate the locations of molecular weight markers in thousands.

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' 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', Em', and Tc', to E. faecalis OG1-RF. However, only the Tc' trait was transferred alone. The transfer of Sp' and/or Em' was always accompanied by the transfer of Tc'. Among the transconjugants exhibiting different resistance phenotypes, plasmid DNA was detected only in those expressing Tc' and Sp'. A plasmid of the same size as that detected in one of the Tc' Sp' 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
donors in subsequent filter mating experiments. It is possible that the resistance traits of strain LDR55 were mobilized by a chromosomal conjugal 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+ 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 Tn544 exhibited approximately 40% nucleotide base identity, distributed throughout the respective ORFs. 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 Tn544. 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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