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Detection of the aminoglycoside-streptothricin resistance gene cluster *ant(6)-sat4-aph(3´)-III* in commensal viridans group streptococci

Paula Cerdá,[1] Pilar Goñi,[1] Leticia Millán,[1] Carmen Rubio,[1,2] Rafael Gómez-Lus[1]*

[1]Department of Microbiology, Preventive Medicine and Public Health, Zaragoza University School of Medicine, Spain. [2]Microbiology Service, Lozano Blesa University Clinic Hospital, Zaragoza, Spain

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Summary. High-level aminoglycoside resistance was assessed in 190 commensal erythromycin-resistant α-hemolytic streptococcal strains. Of these, seven were also aminoglycoside-resistant: one *Streptococcus mitis* strain was resistant to high levels of kanamycin and carried the *aph(3´)-III* gene, four *S. mitis* strains were resistant to high levels of streptomycin and lacked aminoglycoside-modifying enzymes, and two *S. oralis* strains that were resistant to high levels of kanamycin and streptomycin harbored both the *aph(3´)-III* and the *ant(6)* genes. The two *S. oralis* strains also carried the *ant(6)-sat4-aph(3´´)-III* aminoglycoside-streptothricin resistance gene cluster, but it was not contained in a Tn*5405*-like structure. The presence of this resistance gene cluster in commensal streptococci suggests an exchange of resistance genes between these bacteria and enterococci or staphylococci. [**Int Microbiol** 2007; 10(1):57-60]

Key words: viridans group streptococci · aminoglycosides · streptothricin · resistance genes

Introduction

Viridans group streptococci (VGS) are important pathogens in endocarditis, which most often arises from hematogenous seeding from the oral cavity. Aminoglycosides are essential chemotherapeutic agents. Although the resistance of VGS to high levels of aminoglycosides is not common, resistance has been reported in some clinical strains [7]. The molecular basis of this resistance has been widely studied in gram-positive bacteria, but there is little information about the resistance of VGS and especially of commensal isolates, which are thought to act as reservoirs of resistance genes [2]. Aminoglycoside resistance in gram-positive cocci is generally due to the synthesis of aminoglycoside-modifying enzymes (AMEs). Three AMEs are of particular significance among enterococci and staphylococci, since they modify and thus inactivate traditional aminoglycosides of therapeutic importance: (i) AAC(6´)/APH(2´´), encoded by *aac(6´)/aph(2´´)*, which inactivates gentamicin, kanamycin, tobramycin, neomycin, and amikacin; (ii) ANT(4´)-I, encoded by *ant(4´)-Ia*, which inactivates kanamycin, neomycin, tobramycin, and amikacin; and (iii) APH(3´)-III, encoded by *aph(3´)-IIIa*, which inactivates kanamycin and neomycin. These genes may be located on a plasmid or in the chromosome, and are often carried on transposable elements [5,12,14,15]. Streptomycin resistance by enzymatic modification is associated with the *ant(6)-Ia* gene, which encodes an ANT(6)-I enzyme [15]; with the *aph(3´´)* gene, which encodes an

***Corresponding author:** R. Gómez-Lus
Department of Microbiology, Zaragoza University School of Medicine
Domingo Miral, s/n
50009 Zaragoza, Spain
Tel. +34-976761692. Fax +34-976761693
E-mail: gomezlus@unizar.es

APH(3´´) enzyme; and with the *ant(3´´) (9)* gene, which confers streptomycin and spectinomycin resistance by encoding an ANT(3´´) (9) enzyme [6]. Ribosomal mutations have also been found for streptomycin resistance [14]. Furthermore, bacteria carrying these resistance genes may also have an *ant(6)-sat4-aph(3´)-III* gene cluster, conferring resistance to aminoglycosides and streptothricin. In some strains, this cluster is linked to the *erm*(B) gene [9,18]. In addition, it is part of a transposon structure, Tn*5405*, found on the chromosome of staphylococci [18].

The aim of the present work was to assess the frequency and molecular basis of high-level aminoglycoside resistance in α-hemolytic streptococci from normal microbiota with different macrolide resistance phenotypes, thereby contributing to our knowledge of the potential role of these bacteria as reservoirs of resistance genes.

Materials and methods

Bacterial strains. The sample consisted of 190 erythromycin-resistant α-hemolytic streptococci (189 VGS and 1 *Streptococcus acidominimus*) that were previously studied for macrolide resistance phenotypes and genes. The bacteria were mainly isolated from nasopharyngeal samples in the Microbiology Service of the Lozano Blesa University Clinic Hospital, (Zaragoza, Spain) [4]. Streptococci carrying either constitutive or inducible resistance to macrolides, lincosamides, and streptogramin B ($cMLS_B$, $iMLS_B$) phenotypes had the *erm*(B) gene, either alone or in combination with the *mef*(A/E) gene (52.6%). Strains were identified on the basis of colony morphology, α-hemolysis, optochin, susceptibility and Gram stain. Species were identified using the API20 Strep System (API System, bioMerieux, Marcy-l'Etoile, France). The isolates were assigned to a group/species according to the criteria of Facklam [10].

Antimicrobial susceptibility testing. Antimicrobial susceptibility was tested using a standard agar diffusion test with commercially available disks (Bio-Rad, La Coquette, France). Minimum inhibitory concentrations (MICs) were determined by a standard agar dilution method according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [8]. *Steptococcus pneumoniae* ATCC 49619 was used as a control strain. The antibiotics tested included the aminoglycosides kanamycin (Amersham Life Science, China), gentamicin, streptomycin, and spectinomycin (Sigma, USA), and streptothricin (obtained from Dr. Jens Jacob, Robert Koch Institut, Berlin, Germany). The disks contained 500 μg of antibiotic, except for kanamycin (1000 μg). In the evaluation of MICs, resistance to streptothricin was set at ≥1024 μg/ml, according to the method of Werner et al. [18]. The resistance ranges for the other antibiotics were those recommended by the CLSI [8].

Genetic analysis of resistance genes. Total DNA was isolated as described by Ausubel et al. [1]. Plasmid DNA was prepared using a modification of the alkaline lysis procedure [13]. Resistance genotypes were determined by PCR amplification of total DNA obtained from the high-level aminoglycoside-resistant isolates. Oligonucleotide primers specific to the genes *aph(3´)-III*, *ant(6)*, *ant(4´)*, *ant(3´´)(9)*, *aph(3´´)*, and *sat4* were used [3,6,7,14,15,17]. Strains harboring each of the antibiotic resistance genes were included as positive controls and were obtained from our collection. The presence of the *ant(6)-sat4-aph(3´)-III* gene cluster was investigated with three additional PCRs using the following primer pairs: agc1 (5´-TATATCCGAGGATTTGTG-3´, *ant(6)* gene)/agc2 (5´-CTTTTCAGGGCTTTGTTCAT-3´, *aph(3´)-III* gene); Ps1/*ant(6)*R [16,18]; and *ant(6)*F/*aph(3´)-III*R [16,17]. The PCR conditions were 35 cycles at 95ºC (1 min), 58ºC (Ps1/*ant(6)*R and *ant(6)*F/*aph(3´)-III*R) or 51ºC (agc1/agc2), and 72ºC (2 min). All reactions were done in 2.5 mM $MgCl_2$, with 0.25 mM deoxynucleoside triphosphates, 0.5 μM of each primer, and 2.5 U *Taq* DNA polymerase.

The presence of the transposon Tn*5405* was studied by PCR as described by Derbise et al. [9]. A direct linkage between the *erm*(B) and *ant*(6) genes was screened according to the procedure of Werner et al. [18]. Amplifications were done in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The PCR products were resolved by electrophoresis on 1.5% agarose gels. *Taq* DNA polymerase (Promega, Madison, WI, USA) was used to amplify fragments <2 kb, and *LA Taq* polymerase (TaKaRa, Shiga, Japan) to amplify fragments >2 kb. The PCR products were purified using GFX PCR DNA and the Gel Band Purification Kit and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (Amersham Biosciences, Freiburg, Germany).The Basic Local Alignment Search Tool (BLAST) software was used to conduct identity searches of the GenBank database, available at the website of the National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov].

Results and Discussion

Aminoglycoside antibiotics are essential for the treatment of infective endocarditis. Bacterial resistance to this group of antibiotics appears to be associated with resistance to other antibiotics, such as macrolide antibiotics. Genes encoding AMEs, which confer aminoglycoside resistance, provide excellent markers to detect the transfer of resistance genes and to identify bacteria serving as resistance reservoirs.

Of the 190 strains of commensal erythromycin-resistant α-hemolytic streptococci tested for high-level aminoglycoside resistance, seven (3.68%) were aminoglycoside-resistant. Three strains (1.6%) were resistant to high levels of kanamycin and five strains (3.2%) were resistant to high levels of streptomycin (Table 1). One strain of *S. mitis* showed high-level resistance to kanamycin and carried the *aph(3´)-III* gene. Four (2.1%) *S. mitis* strains showed high-level resistance to streptomycin but no AMEs were detected. Two *S. oralis* strains (57 and 58b) showed high-level resistance to kanamycin and streptomycin, and harbored both the *aph(3´)-III* and the *ant(6)* genes. The *ant(4´)* gene was not found in kanamycin-resistant isolates. Resistance to gentamicin or spectinomycin was not exhibited by any of the strains, consistent with failure to detect the *ant(3´´)(9)* gene in the sample. Streptomycin resistance in the four strains lacking AMEs might have been due to ribosomal mutations, usually associated with MICs >32,000 μg/ml or to other, as-yet-unidentified resistance mechanisms [16].

Table 1 shows the association between aminoglycoside, streptothricin and macrolide resistance patterns in VGS and lists the corresponding genes. Other authors have also reported

Table 1. Aminoglycoside, streptothricin, and macrolide resistance patterns and genes in commensal viridans group streptococci.

	Aminoglycoside resistance			Macrolide resistance	
Strain	Resistance pattern	MIC (μg/ml)	Genotype	Phenotype	Genotype
Streptococcus oralis 57	Sm Km St	Sm: 4096	*ant(6)*	$cMLS_B$	*erm*(B), *mef*(E)
		Km: 8192	*aph(3´)-III*		
		St : >2048	*sat4*		
S. oralis 58b	Sm Km St	Sm: 4096	*ant(6)*	$cMLS_B$	*erm*(B), *mef*(E)
		Km: 8192	*aph(3´)-III*		
		St: >2048	*sat4*		
S. mitis 128	Sm	>32,000	–	$iMLS_B$	*erm*(B)
S. mitis 138	Sm	16,000	–	$iMLS_B$	*erm*(B), *mef*(E)
S. mitis 191	Sm	>32,000	–	$cMLS_B$	*erm*(B)
S. mitis 223	Km	>8192	*aph(3´)-III*	$iMLS_B$	*erm*(B), *mef*(E)
S. mitis 257	Sm	32,000	–	$cMLS_B$	*erm*(B)

Km, kanamycin; Sm, streptomycin; St, streptothricin.

the presence of high-level aminoglycoside resistance in this group of bacteria as well as an association with macrolides, lincosamides, and streptogramin B (MLS) resistance markers [7]. The two *S. oralis* isolates, 57 and 58b, were tested for resistance to streptothricin (Table 1) and the presence of the *sat4* gene was demonstrated.

Three additional PCRs were carried out to investigate the presence of the *ant(6)-sat4-aph(3´)-III* gene cluster in these two strains. The nucleotide sequences of the gene clusters of *S. oralis* strains 57 and 58b were identical and showed 100% identity with the sequences of ORF44 (*ant(6)*), ORF45 (*sat4*), and ORF46 (*aph(3´)-III*) of *Enterococcus faecalis* plasmid pRE25 DNA (GenBank accession no. X92945), and with the same cluster from different canine *S. intermedius* isolates (GenBank accession no. AF299292), and 99% identity with the aminoglycoside-streptothricin resistance gene cluster described in two strains of *E. faecium* (GenBank accession no. AF330699). The *S. oralis* sequence showed a point mutation at nucleotide 2136, which results in replacement of the amino acid Ile by Val at position 225 in the putative *E. faecium* APH(3´)-III protein; however, this substitution does not seem to influence the kanamycin resistance level. As far as we know, this is the first description of the *ant(6)-sat4-aph(3´)-III* gene cluster in VGS.

In *Staphylococcus* sp., the *ant(6)-sat4-aph(3´)-III* gene cluster has been described as a part of a Tn*5405*-like structure [9]. To determine whether the *S. oralis* cluster was also part of a Tn*5405*-like structure in the two *S. oralis* strains, PCR assays were done. PCR amplifications of the left end of the element and from the *aph(3´)-III* gene to the ORFz were negative; however, a 6-kb fragment was obtained for the right end of the transposon. Seven hundred bp of the 5´ end of the amplicon and 900 bp of the 3´ end were sequenced. The former showed 62% amino-acid identity to the corresponding fragment of lambda S2 prophage site-specific recombinase (GenBank accession no. NP_688849.1), and the latter displayed 51% amino-acid identity to the corresponding fragment of the portal protein of bIL285 bacteriophage (GenBank accession no. NP_4719081). These results suggested that the *ant(6)-sat4-aph(3´)-III* gene cluster in the *S. oralis* strains was inserted in a prophage. This possibility is currently being further investigated in our laboratory. Although the aminoglycoside-streptothricin resistance gene cluster is generally part of the transposon Tn*5405*, a high level of heterogeneity of Tn*5405*-related elements, with different arrangements including deletions and insertions of ORFs, has been described in enterococci and staphylococci [9,18]. Nonetheless, the *ant(6)-sat4-aph(3´)-III* gene cluster is a conserved region, as was the case in the *S. oralis* strains.

Linkage between *erm*(B) and this gene cluster was recently described in *E. faecium* and *Staphylococcus intermedius* [18]. The *S. oralis* isolates used in the present study also had the *erm*(B) gene, but PCR analysis revealed no association between this gene and the aminoglycoside-streptothricin resistance gene cluster. The alkaline lysis technique did not provide evidence for the presence of plasmids in our isolates, which suggests either that high-level resistance to kanamycin, streptomycin, and streptothricin is chromosomally mediated, as reported by other authors for VGS, or that the cluster is located on a large plasmid [7]. High-level resist-

ance to aminoglycosides in VGS is of concern. In fact, this group of bacteria might act as a reservoir of other antibiotic resistances, perhaps including aminoglycosides [2].

Many studies have been conducted to demonstrate the potential role of commensal flora as reservoirs of resistance genes [4], and the exchange of these genes between strains of human and animal origin [11]. For these purposes, the study of aminoglycoside-resistance genes has been especially useful. Our results demonstrated the presence in commensal *S. oralis* of an aminoglycoside-streptothricin resistance gene cluster found in staphylococci and enterococci, which suggests an exchange of resistance genes between these bacteria. This type of exchange might also occur with other pathogenic bacteria sharing a habitat with *S. pneumoniae*. The presence in commensal *S. oralis* of the *sat4* gene, which confers resistance to streptothricin, an antibiotic never used in humans or for ergotropic purposes in Spain, leads us to think that there is probably a co-selection of this gene via the antibiotic resistances encoded by the *aph(3´)-III* and/or the *ant(6)* genes.

The results confirm the need to continue surveillance of the resistance genes harbored by commensal bacteria. The DNA sequence of the *ant(6)-sat4-aph(3´)-III* gene cluster of *S. oralis* 58b has been assigned GenBank accession no. AY712687.

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