Aminoglycoside Resistance Genes *aph(2″)-Ib* and *aac(6′)-Im* Detected Together in Strains of both *Escherichia coli* and *Enterococcus faecium*

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Escherichia coli SCH92111602 expresses an aminoglycoside resistance profile similar to that conferred by the *aac(6′)-Ie-aph(2″)-Ia* gene found in gram-positive cocci and was found to contain the aminoglycoside resistance genes *aph(2″)-Ib* and *aac(6′)-Im* (only 44 nucleotides apart). *aph(2″)-Ib* had been reported previously in *Enterococcus faecium* SF11770. *aac(6′)-Im* had not been detected previously in enterococci and was found to be present also 44 nucleotides downstream from *aph(2″)-Ib* in *E. faecium* SF11770. *aph(2″)-Ib* and *aac(6′)-Im* are separate open reading frames, each with its own putative ribosome binding site, whereas *aac(6′)-Ie-aph(2″)-Ia* appears to be a fusion of two genes with just one start and one stop codon. The deduced AAC(6′)-Im protein exhibits 56% identity and 80% similarity to the AAC(6′)-Ie domain of the bifunctional enzyme AAC(6′)-APH(2″). Our results document the existence of a member of the *aph(2″)* family of genes in gram-negative bacteria and provide evidence suggesting the horizontal transfer of *aph(2″)-Ib* and *aac(6′)-Im* as a unit between gram-positive and gram-negative bacteria.

The aminoglycoside resistance gene, *aac(6′)-Ie-aph(2″)-Ia*, encodes a bifunctional enzyme, AAC(6′)-APH(2″), that confers resistance to a broad spectrum of aminoglycosides and has to date been detected only in gram-positive bacteria, including *Enterococcus* spp., *Staphylococcus aureus*, *Streptococcus agalactiae* (group B), *Streptococcus mitis*, and group G *Streptococcus* (3, 6, 8, 13, 19). Several amikacin-resistant gram-negative bacterial clinical isolates from Slovakia and Germany express an aminoglycoside resistance profile similar to that conferred by *aac(6′)-Ie-aph(2″)-Ia* (11). In order to determine whether an aminoglycoside resistance gene similar to *aac(6′)-Ie-aph(2″)-Ia* was responsible for the resistance profile in these gram-negative bacteria, we chose one of these resistant isolates, *Escherichia coli* SCH92111602, for further study. We describe here the characterization of the *aac(6′)-Im* and *aph(2″)-Ib* genes from this *E. coli* isolate, and the detection of this pair of genes also in clinical isolates of *Enterococcus faecium*.

MATERIALS AND METHODS

E. coli SCH92111602 is a clinical isolate from University Hospital, Bratislava, Slovakia. Aminoglycoside MICs were determined by a standard broth microdilution method (16). Gentamicin was obtained from Fluka (Buchs, Switzerland). Netilmicin, dibekacin, and arbekacin were donated by Meiji Seika Kaisha (Tokyo, Japan). All other antibiotics were purchased from Sigma Chemical Company (St. Louis, Mo.). Plasmid DNA from clinical strains was isolated using a

Qiagen (Chatsworth, Calif.) plasmid column or a modified alkaline lysis method (26). Plasmid DNA from transformants was prepared with either a Wizard miniprep (Promega, Madison, Wis.) or a Qiagen column. DNA was digested with restriction enzymes and ligated with T4 ligase from New England Biolabs (Beverly, Mass.) or Life Technologies (Rockville, Md.) according to the manufacturers' recommendations, and products were analyzed in an agarose gel system of Tris-borate-EDTA. Cloned *Pfu* DNA polymerase enzyme (Stratagene) or recombinant *Taq* DNA polymerase (Life Technologies) was used for PCRs. Oligonucleotide primers were synthesized by Research Genetics (Huntsville, Ala.) or by Life Technologies. The vectors pBluescript II KS(+) (Stratagene Cloning Systems, La Jolla, Calif.) and pACYC184 (New England Biolabs) were used in standard cloning experiments (2). Competent *E. coli* DH5α cells were used as the recipients in transformation experiments. The phosphocellulose paper-binding assay was performed as previously described (18). Nucleotide sequencing was performed by Lark Technologies, Inc. (Houston, Tex.), and by the DNA Sequencing Core, University of Michigan. Computer analysis was performed with Mac Vector software, version 6.0, and OMIGA software, version 2.0 (Genetics Computer Group, Madison, Wis.). The GenBank database was searched with the BLAST program from the National Center for Biotechnology Information (1). Amino acid sequences were compared by using the Gap Analysis Program from the University of Wisconsin Genetics Computer Group (5).

Conditions for dot blot hybridizations were performed at 42°C and have been described previously (21). DNA hybridization was performed with a collection of 4,625 aminoglycoside-resistant bacterial clinical isolates acquired by one of the coauthors (G. H. Miller) within the past decade. Some of these isolates had been included in previous studies to detect the presence of other aminoglycoside resistance genes (15). These 4,625 isolates were from Belgium ($n = 455$), the Czech Republic ($n = 49$), France ($n = 907$), Guatemala ($n = 123$), Mexico ($n = 516$), the Philippines ($n = 41$), Portugal ($n = 378$), Slovakia ($n = 71$), South Africa ($n = 868$), Thailand ($n = 255$), Turkey ($n = 725$), the United States ($n = 81$), and Venezuela ($n = 156$). A 303-bp internal fragment (nucleotides 1282 to 1584) of the *aac(6′)-Im* gene was used as the probe for DNA hybridizations. This fragment was generated by PCR amplification using primers 5′-GGCTGACAGATGACCGTGTTCTTG-3′ and 5′-GTAGATATTGGCATACTACTCTGC-3′. PCR conditions were as follows: DNA was initially denatured for 4 min at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C. The amplified DNA fragment was removed from 1% agarose gels by repeated electroelution in dialysis tubing and purified by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipi-

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TABLE 1. Susceptibility profiles conferred by *aph(2″)-Ib* and/or *aac(6′)-Im* cloned in the vector pBluescript in *E. coli* DH5α

Aminoglycoside	MIC (µg/ml)				
	aph(2″)-Ib + aac(6′)-Im	*aph(2″)-Ib*	*aac(6′)-Im*	DH5α(pBluescript)	*E. coli* SCH92111602
Gentamicin	64	64	0.25	0.125	128
Tobramycin	512	32	8.0	0.5	256
Amikacin	32	2.0	8.0	0.25	32
Neomycin	2.0	0.5	1.0	0.5	1.0
Dibekacin	512	32	16	0.5	≥512
Netilmicin	128	64	4.0	0.125	64
Kanamycin A	≥2,048	512	64	0.5	≥512
Arbekacin	4.0	4.0	0.25	0.125	4.0

tation. The purified DNA fragment was labeled with [α-^{32}P]ATP using an oligonucleotide labeling kit from Amersham Pharmacia Biotech, Inc. (Piscataway, N.J.). Unincorporated nucleotides were separated from labeled product by column chromatography on Sephadex G-50 columns (5 Prime-3 Prime, Inc., Boulder, Colo.). The PCR primers used for detecting the *aac(6′)-Im* gene in enterococcal isolates were 5′-GCGAGTTTCCTTTCGCCC-3′ and 5′-CACCGCATC GGCATCC-3′. PCR conditions were as follows: an initial 5-min denaturation at 94°C, followed by 30 cycles of 30 s of denaturation at 94°C, 20 s of annealing at 58°C, and 1 min of extension at 72°C.

Nucleotide sequence accession number. The nucleotide sequence for *aac(6′)-Im* has been deposited in GenBank under accession number AF337947.

RESULTS AND DISCUSSION

SCH92111602 is an *E. coli* clinical isolate resistant to a number of aminoglycoside antibiotics, including gentamicin, tobramycin, and amikacin (Table 1), and contains an approximately 50-kb plasmid. Plasmid DNA isolated from this strain was introduced into *E. coli* DH5α by transformation, and colonies were selected on Luria-Bertani agar plates containing 10 µg of tobramycin (Eli Lilly & Co., Indianapolis, Ind.) per ml. Analysis of restriction digests on agarose gels of DNA from a tobramycin-resistant transformant confirmed the presence of the same 50-kb plasmid that was isolated from *E. coli* SCH92111602. The 50-kb plasmid was digested with *Xmn*I, and the fragments were ligated into the *Eco*RV site of the vector pACYC184. After electroporation, selection for tobramycin-resistant transformants yielded an *E. coli* DH5α derivative that contained a 3.7-kb cloned fragment. Further subcloning experiments using the vector pBluescript II KS(+) yielded a tobramycin-resistant *E. coli* DH5α transformant that contained a 2.5-kb *Ava*I fragment ligated to pBluescript (designated pSCH075). Nucleotide sequencing revealed the presence of an 897-bp open reading frame (ORF), whose predicted amino acid sequence was identical to that of the APH(2″)-Ib aminoglycoside-modifying enzyme reported in *E. faecium*, except for three amino acid changes that resulted from four nucleotide differences (12). A putative promoter sequence composed of TTGAAA and TATAAT (−35 and −10) was noted 36 bases upstream of the ATG start codon, which was identical to that noted in *E. faecium*. APH(2″)-Ib from *E. faecium* has 33% identity and 51% similarity with the deduced APH(2″)-Ia domain of the bifunctional enzyme AAC(6′)-APH(2″) (12).

Beginning 44 nucleotides downstream from the *aph(2″)-Ib* gene in the DNA cloned from the *E. coli* plasmid, a second ORF of 534 nucleotides was detected (G+C content of 40%), which exhibited 65% nucleotide identity with the *aac(6′)-Ie* portion of *aac(6′)-Ie-aph(2″)-Ia*. A putative ribosome binding site (GAGG) was located 7 nucleotides upstream from the start codon of this ORF, but no nucleotide sequences consistent with a −35 and −10 promoter sequence were detected. The predicted 178-amino acid sequence of this ORF showed similarity to sequences of aminoglycoside acetyltransferases in the GenBank database. The highest homology was seen with the deduced protein of the *aac(6′)-Ie-aph(2″)-Ia* gene from *Enterococcus* [56% identity and 80% similarity to the AAC(6′)-Ie domain] (6). Other acetyltransferases with lower degrees of homology include AAC(6′)-IIa from *Pseudomonas aeruginosa* (20) and AAC(6′)-Ib, initially detected in *Serratia marcescens* (24) and subsequently in *Klebsiella pneumoniae, Salmonella enterica* Serovar Typhimurium, *P. aeruginosa*, and *Pseudomonas fluorescens* (7, 9, 14, 17). Our newly observed ORF has been designated *aac(6′)-Im* (23). The *aac(6′)-Im* designation has been used by others to describe another aminoglycoside acetyltransferase, which was initially named *aac(6′)-Il* (10), then had its name changed to *aac(6′)-Im* (25), and subsequently was renamed *aac(6′)-Ip* (4).

Since the *aac(6′)-Im* gene was found in such close proximity downstream from the *aph(2″)-Ib* gene in *E. coli* SCH92111602, we attempted to determine if *aac(6′)-Im* was also present in the *E. faecium* SF11770 isolate in which we had initially detected *aph(2″)-Ib* (12). A 3.3-kb *Hin*dIII fragment from *E. faecium* SF11770 cloned in the vector pWM119 in previous experiments was known to contain the *aph(2″)-Ib* gene (S. J. Kao, I. You, and J. W. Chow, unpublished data). Nucleotide sequencing of this fragment downstream from *aph(2″)-Ib* determined that the *aac(6′)-Im* gene was also present, as in *E. coli*, 44 bases from *aph(2″)-Ib*. The nucleotide sequence of *aac(6′)-Im* from *E. faecium* was identical to that from *E. coli* except for two nucleotides, whose presence did not alter the predicted amino acid sequence. The sequence of the 200 nucleotides upstream of *aph(2″)-Ib* and that of the 44 nucleotides between the *aph(2″)-Ib* and *aac(6′)-Im* ORFs were identical in the *E. faecium* and *E. coli* strains. The 200 nucleotides downstream from *aac(6′)-Im* were identical in the two strains except for one base. PCR results showed that all of the nine other *E. faecium* clinical isolates in our collection known to possess the *aph(2″)-Ib* gene were also positive for the *aac(6′)-Im* gene.

The *aac(6′)-Im* gene was subcloned by digesting with *Hin*cII the 3.3-kb *Hin*dIII fragment [derived from *E. faecium* SF11770 and containing both *aph(2″)-Ib* and *aac(6′)-Im*]. A 1.5-kb *Hin*cII fragment [which contained *aac(6′)-Im* but not *aph(2″)-Ib*] was ligated to pBluescript digested with *Hin*cII, and the ligation products were transformed into *E. coli* DH5α. The

aminoglycoside MICs for the *E. coli* DH5α transformant containing *aac(6′)-Im* are listed in Table 1. As seen in Table 1, the presence of both the *aph(2″)-Ib* and *aac(6′)-Im* genes in *E. coli* confers a higher level of resistance to some aminoglycosides than the presence of either gene alone. The aminoglycoside acetyltransferase activity, designated AAC(6′)-Im, was confirmed by the phosphocellulose paper-binding assay (data not shown). Crude extracts from *E. coli* DH5α containing only *aac(6′)-Im* exhibited acetyltransferase activity with tobramycin, amikacin, dibekacin, netilmicin, and kanamycin A, but not with gentamicin, neomycin, or arbekacin. The aminoglycoside phosphotransferase activity of APH(2″)-Ib from *E. faecium* has been reported previously (12).

A 303-bp internal fragment of the *aac(6′)-Im* gene from *E. coli* SCH92111602 was amplified by PCR and used as a probe. DNA hybridization results showed that 46 of 4,625 (1%) bacterial isolates hybridized to the *aac(6′)-Im* probe. The isolates that hybridized with this probe were from Slovakia (*n* = 38), the Czech Republic (*n* = 6), and Belgium (*n* = 2). Several genera were represented among the isolates that hybridized to the *aac(6′)-Im* probe, including *Aeromonas* (1 of 6), *Citrobacter* (1 of 132), *Enterobacter* (9 of 616), *Escherichia* (5 of 590), *Klebsiella* (16 of 1,117), *Pseudomonas* (4 of 765), and *Serratia* (10 of 267).

Although the *aph(2″)-Ib* and *aac(6′)-Im* genes show a high degree of homology with the *aac(6′)-Ie-aph(2″)-Ia* gene from *Enterococcus*, there are significant differences. *aac(6′)-Ie-aph(2″)-Ia* appears to be a fusion of two genes and has one start (ATG) and one stop (TAA) codon, whereas *aph(2″)-Ib* and *aac(6′)-Im* are separate ORFs, each with its own putative ribosome binding site, although we have some preliminary (unpublished) data suggesting that transcription of *aph(2″)-Ib* and *aac(6′)-Im* may come from a single promoter. In addition, the order of the AAC and the APH domains of *aac(6′)-Ie-aph(2″)-Ia* is reversed compared to the set of *aph(2″)-Ib* and *aac(6′)-Im* genes. Furthermore, the AAC(6′)-Ie domain of *aac(6′)-Ie-aph(2″)-Ia* confers resistance to fortimicin (22), whereas AAC(6′)-Im does not (J. Petrin, Kuvelkar, M. Kettner, R. S. Hare, G. H. Miller, and K. J. Shaw, Abstr. Cold Spring Harbor Bacteria Phage Meet., abstr. 197, 1995). Finally, whereas *aac(6′)-Ie-aph(2″)-Ia* has been detected only in gram-positive cocci, *aph(2″)-Ib* and *aac(6′)-Im* have now been detected in both enterococci and gram-negative bacilli. Our results show that a member of the *aph(2″)* family of genes is present in gram-negative bacteria and provide evidence for the horizontal transfer of *aph(2″)-Ib* and *aac(6′)-Im* as a unit between gram-positive and gram-negative bacteria. The G+C content of the *aph(2″)-Ib* (32%) and *aac(6′)-Im* (40%) genes suggests that they may have originated in enterococci (G+C content, approximately 35%) or another bacterial genus and were subsequently transferred to *E. coli* (G+C content, approximately 50%). The presence of these two linked aminoglycoside resistance genes that appear to have been transferred together between such diverse bacterial species is another example of how bacteria evolve to become more resistant to a broader spectrum of antimicrobial agents.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1995. Current protocols in molecular biology, vol 1. John Wiley & Sons, Inc., New York, n.y.
3. **Buu-Hoï, A., C. Le Bouguenec, and T. Horaud.** 1990. High-level chromosomal gentamicin resistance in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. **34:**985–988.
4. **Centrón, D., and P. H. Roy.** 1998. Characterization of the 6′-N-aminoglycoside acetyltransferase gene *aac(6′)-Iq* from the integron of a natural multiresistance plasmid. Antimicrob. Agents Chemother. **42:**1506–1508.
5. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **Ferretti, J. J., K. S. Gilmore, and P. Courvalin.** 1986. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J. Bacteriol. **167:**631–638.
7. **Galimand, M., T. Lambert, G. Gerbaud, and P. Courvalin.** 1993. Characterization of the *aac(6′)-Ib* gene encoding an aminoglycoside 6′-N-acetyltransferase in *Pseudomonas aeruginosa* BM2656. Antimicrob. Agents Chemother. **37:**1456–1462.
8. **Galimand, M., T. Lambert, G. Gerbaud, and P. Courvalin.** 1999. High-level aminoglycoside resistance in the beta-hemolytic group G *Streptococcus* isolate BM2721. Antimicrob. Agents Chemother. **43:**3008–3010.
9. **Hall, R. M., D. E. Brookes, and H. W. Stokes.** 1991. Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point. Mol. Microbiol. **5:**1941–1959.
10. **Hannecart-Pokorni, E., F. Depuydt, L. De Wit, E. Van Bossuyt, J. Content, and R. Vanhoof.** 1997. Characterization of the 6′-N-aminoglycoside acetyltransferase gene *aac(6′)-Ib* associated with a *sulI*-type integron. Antimicrob. Agents Chemother. **41:**314–318.
11. **Kallova, J., T. Macickova, A. Majtanova, A. Aghova, D. Adam, and M. Kettner.** 1995. Transferable amikacin resistance in gram-negative bacterial isolates. Chemotherapy **41:**187–192.
12. **Kao, S. J., I. You, D. B. Clewell, S. M. Donabedian, M. J. Zervos, J. Petrin, K. J. Shaw, and J. W. Chow.** 2000. Detection of the high-level aminoglycoside resistance gene *aph(2″)-Ib* in *Enterococcus faecium*. Antimicrob. Agents. Chemother. **44:**2876–2879.
13. **Kaufhold, A., and E. Potgieter.** 1993. Chromosomally mediated high-level gentamicin resistance in *Streptococcus mitis*. Antimicrob. Agents Chemother. **37:**2740–2742.
14. **Lambert, T., M.-C. Ploy, and P. Courvalin.** 1994. A spontaneous point mutation in the *aac(6′)-Ib* gene results in altered substrate specificity of aminoglycoside 6′-N-acetyltransferase of a *Pseudomonas fluorescens* strain. FEMS Microbiol. Lett. **115:**297–304.
15. **Miller, G. H., F. J. Sabatelli, R. S. Hare, Y. Glupczynski, P. Mackey, D. Shlaes, K. Shimizu, K. J. Shaw, and the Aminoglycoside Resistance Study Groups.** 1997. The most frequent aminoglycoside resistance mechanisms—changes with time and geographic area: a reflection of aminoglycoside usage patterns? Clin. Infect. Dis. **24:**(Suppl. 1):S46–S62.
16. **National Committee for Clinical Laboratory Standards.** 1999. Approved standard M7–A5: methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. NCCLS, Wayne, Pa.
17. **Nobuta, K., M. E. Tolmasky, L. M. Crosa, and J. H. Crosa.** 1988. Sequencing and expression of the 6′-N-acetyltransferase gene of transposon Tn*1331* from *Klebsiella pneumoniae*. J. Bacteriol. **170:**3769–3773.
18. **Perlin, M. H., and S. A. Lerner.** 1979. Amikacin resistance associated with a plasmid-borne aminoglycoside phosphotransferase in *Escherichia coli*. Antimicrob. Agents Chemother. **16:**598–604.
19. **Rouch, D. A., M. E. Byrne, Y. C. Kong, and R. A. Skurray.** 1987. The *aacA-aphD* gentamicin and kanamycin resistance determinant of Tn*4001* from *Staphylococcus aureus*: expression and sequence analysis. J. Gen. Microbiol. **133:**3039–3052.
20. **Shaw, K. J., C. A. Cramer, M. Rizzo, R. Mierzwa, K. Gewain, G. H. Miller, and R. S. Hare.** 1989. Isolation, characterization, and DNA sequence analysis of an AAC(6′)-II gene from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33:**2052–2062.
21. **Shaw, K. J., R. S. Hare, F. J. Sabatelli, M. Rizzo, C. A. Cramer, L. Naples, S. Kocsi, H. Munayyer, P. Mann, G. H. Miller, L. Verbist, H. Van Landuyt, Y. Glupczynski, M. Catalano, and M. Woloj.** 1991. Correlation between aminoglycoside resistance profiles and DNA hybridization of clinical isolates. Antimicrob. Agents Chemother. **35:**2253–2261.
22. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.
23. **Shaw, K. J., F. J. Sabatelli, L. Naples, P. Mann, R. S. Hare, and G. H. Miller.** 1998. The application of molecular techniques for the study of aminoglyco-

side resistance, p. 555–577. *In* N. Woodford and A. P. Johnson (ed.), Methods in molecular medicine, vol. 15. Molecular bacteriology: protocols and clinical applications. Humana Press Inc., Totowa, N.J.

24. **Tran Van Nhieu, G., and E. Collatz.** 1987. Primary structure of an aminoglycoside 6′-*N*-acetyltransferase, AAC(6′)-4, fused in vivo with the signal peptide of the Tn*3*-encoded β-lactamase. J. Bacteriol. **169:**5708–5714.

25. **Vanhoof, R., E. Hannecart-Pokorni, and J. Content.** 1998. Nomenclature of genes encoding aminoglycoside-modifying enzymes. Antimicrob Agents Chemother. **42:**483.

26. **Wirth, R., F. Y. An, and D. B. Clewell.** 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165:**831–836.