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Heterogeneity of 6′-*N*-Acetyltransferases of Type 4 Conferring Resistance to Amikacin and Related Aminoglycosides in Members of the Family *Enterobacteriaceae*

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DNA-DNA hybridization and immunoblotting were used to assess the relatedness between the 6′-*N*-acetyltransferases of type 4 encoded by plasmid pAZ007 from a clinical isolate of *Serratia marcescens* and those encoded by NR79 and R5. The absence of detectable DNA-DNA homology and of immunological cross-reactivity suggests the existence of at least two distinct 6′-*N*-acetyltransferase type 4 genes that mediate amikacin resistance in gram-negative bacilli.

Resistance to amikacin in gram-negative bacilli is usually mediated by aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] (17, 21). These enzymes, which have the broadest substrate range within the AAC(6′) group and which typically modify amikacin, were assigned to the AAC(6′)s of type 4 (14), which was subsequently also termed type I (13). Ideally, for a precise epidemiological survey of aminoglycoside resistance, a given type should include only structurally closely related enzymes.

In contrast with the incidence of resistance to other aminoglycosides, a continuing low incidence of resistance to amikacin has been observed in large areas in various places in the world, although not worldwide (21). Furthermore, high-level use of amikacin does not seem to increase the rates of amikacin resistance in several individual institutions (2, 3, 9, 15). Links between the use of amikacin and the occurrence of AAC(6′)-producing resistant strains may, however, exist (10; D. M. Shlaes, R. S. Hare, G. H. Miller, A. Yessayan, F. J. Sabatelli, and W. J. Weiss, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 521, 1986), and some localized outbreaks of amikacin resistance in gram-negative bacilli have been reported (7, 27; A. Buré, C. Carlier, L. Slim, B. Pangon, E. Rouveix, and P. Courvalin, Program Abstr. 14th Int. Congr. Chemother., Kyoto, Japan, abstr. no. P-42-20, 1985). Several AAC(6′) genes found in various bacterial genera have been mapped on plasmids, have been cloned, or both (5, 12, 24), but their possible structural relationships are not known. It was the purpose of this study to examine whether such a relationship exists between an AAC(6′) type 4 [AAC(6′)-4] gene involved in a recent outbreak of amikacin resistance (27) and known AAC(6′)-4 genes (5, 12).

The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pMH1 (18.5 megadaltons), which is part of the R factor R5, and plasmid NR79 (63 megadaltons) were found to be related and to carry similar, if not identical, AAC(6′) genes whose expression may, however, be under the control of different regulatory sequences (5). The pMH1-encoded enzyme was the first enzyme described to confer resistance to aminoglycosides, including amikacin, via acetylation (16), and its biochemical and enzymatic properties have been studied in detail (1, 18, 19). The AAC(6′) gene of NR79 was later shown to be part of the multiple resistance transposon Tn*2424* (12).

The multiple resistance plasmid pAZ007, which encodes an AAC(6′) which fits type 4 of the nomenclature of Mitsuhashi and Kawabe (14), was isolated from a clinical *Serratia marcescens* strain during a localized outbreak in one hospital in which closely related plasmids were found in five enterobacterial genera (27). The AAC(6′)-4 gene of pAZ007 was cloned into pBR322 to form plasmid pAZ505, and its sequence was determined (26). The corresponding acetyltransferase was purified to homogeneity and was used to raise antibodies in rabbits (G. Tran Van Nhieu and E. Collatz, Program Abstr. 27th ICAAC, abstr. no. 392, 1987).

We prepared a ca. 0.5-kilobase (kb) *Dde*I-*Hae*III fragment, which covered most of the AAC(6′) gene of pAZ505 (26), from a low-melting-point agarose gel (11) and radiolabeled it with deoxyadenosine 5′-α-[^{35}S]thiotriphosphate (>14.8 TBq/mmol) by the standard multiprime DNA labeling protocol provided by Amersham Corp. (Arlington Heights, Ill.). This fragment was used to probe the DNA of NR79, pUB307::Tn*2424*, pMH1, and pAZ505. Plasmid DNA was digested with *Eco*RI, separated by electrophoresis in a 1% agarose gel, and transferred to a nitrocellulose filter as described previously (22).

Prehybridization and hybridization were carried out for 6 and 16 h, respectively, at 68°C in sixfold-concentrated SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), fivefold-concentrated Denhardt solution, and 0.5% sodium dodecyl sulfate or at a lower stringency at 30°C in the presence of 20% formamide. Washes were performed as described previously (11) at 68°C or at a lower stringency at 42°C. Hybridization with the DNA of plasmids pMH1, pUB307::Tn*2424*, or NR79 was not observed at a high stringency (Fig. 1, lanes a′, b′, and d′) or at a lower stringency (data not shown). In a reverse experiment, the ca. 4-kb *Eco*RI-*Pst*I fragment of pMH1 (5), which conferred resistance to amikacin after it was cloned into pHSS6 (20), hybridized with ca. 4.6-kb *Eco*RI fragments of pMH1, pUB307::Tn*2424*, and NR79, but not with DNA from phage M13mp8 containing the ca. 0.5-kb *Dde*I-*Hae*III fragment of pAZ505 (data not shown).

We obtained qualitatively similar results when we probed the acetyltransferases by immunoblotting with the antibodies raised against the pAZ505-encoded AAC(6′)-4. Cells containing the plasmids shown in Fig. 1 from 200 μl of

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TABLE 1. Bacterial strains and plasmids used in this study[a]

E. coli strain (relevant marker)	Plasmid (relevant marker)	MIC (μg/ml)				Reference or source[b]
		Ak	Tm	Gm	Nt	
C600	pMH1 (Ak Cm Sm Su Tra$^+$)	32	32	1	16	5
W677	NR79 (Ak Cm Sm Su Tc Tra$^+$)	32	64	2	32	5
W3110 (Nal)	pUB307::Tn*2424* (Ak Cm Sm Su Tc Tra$^+$)	256	128	2	128	12
BM694 (Nal)	pAZ007[c] (Ak Ap Sm Su Tp Tra$^+$)	32	32	1	32	27
	pAZ505 (Ak Ap)	128	128	2	128	pBR322 containing ca. 1.5-kb *Hpa*II fragment from pAZ007 (26)

[a] Abbreviations: Ak, amikacin; Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Nal, nalidixic acid; Nt, netilmicin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim.

[b] *E. coli* C600(pMH1) (5) was kindly provided by P. Courvalin, and W677(NR79) and W3110(pUB307::Tn*2424*) were provided by B. Wiedemann (pUB307 is Pm^r and Tc^r).

[c] Plasmids were not numbered in reference 27; plasmid pAZ007 had the endonuclease digestion profile of the type IA plasmids described previously (27).

exponentially growing cultures (A_{650}, ca. 0.4) were suspended in sample buffer (8) and heated at 100°C for 10 min. The proteins were separated by polyacrylamide gel electrophoresis (8) and transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) by electroblotting (25). The filters were incubated with anti-AAC(6′)-4 and peroxidase-labeled anti-rabbit immunoglobulin G (Diagnostics Pasteur) antisera, both of which were diluted 500-fold, under the conditions described previously (6). No cross-reaction was observed with the heterologous acetyltransferases (Fig. 2, lanes A′, B′, and D′).

These results suggest the existence of at least two nonhomologous AAC(6′)-4 genes that mediate resistance to amikacin in gram-negative bacilli by synthesis of immunologically distinct AAC(6′)-4. In addition, while the apparent molecular weight and that calculated from the length of the structural gene of the pAZ505-encoded acetyltransferase were 22,400 and 24,500, respectively (26), the pMH1-encoded AAC(6′)-4 was thought to be a tetrameric enzyme (molecular weight, 60,000 to 70,000) with an apparent subunit molecular weight of 14,500 (19). Substrate profiles which might reflect the structural differences between the AAC(6′)s encoded by plasmids NR79 and pMH1 and derivatives (4, 18) and pAZ007 (27) have not been performed in parallel.

Since the submission of this report, the nucleotide sequence of a second AAC(6′) gene that mediates resistance to amikacin has been established, and the gene was found, under stringent conditions, to be homologous to that carried by R5 (23). Comparison of the nucleotide sequences of the R5-related AAC(6′) gene (23) and that of pAZ007 (26) did not reveal a structural relationship.

We have recently screened, by immunoblotting, approximately 70 amikacin-resistant, gram-negative bacteria from various geographical areas, with resistance profiles indicative of AAC(6′) synthesis (21). We found ca. 40% of them to contain cross-reacting proteins. The apparent molecular weights showed slight variations in the vicinity of 24,000 (unpublished data). We must await the use of additional probes, at least of that specific for the R5-type AAC(6′) (23), to evaluate the extent of heterogeneity which exists among this type of aminoglycoside acetyltransferase. The availability of comparative structural data should also allow the establishment of a more specific nomenclature for the molecular epidemiological study of this clinically important mechanism of resistance.

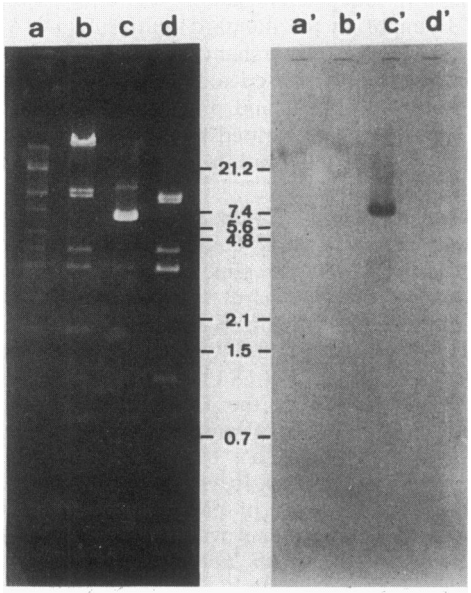

FIG. 1. *Eco*RI digestion of plasmids NR79 (lane a), pUB307::Tn*2424* (lane b), pAZ505 (lane c), and pMH1 (lane d). The autoradiogram of the corresponding nitrocellulose filter obtained after Southern blotting, which was probed with the ^{35}S-labeled *Dde*I-*Hae*III fragment of the *aacA* gene of pAZ505, is shown in lanes a′ through d′. The positions of the molecular weight markers (in thousands) are indicated between the two sets of lanes.

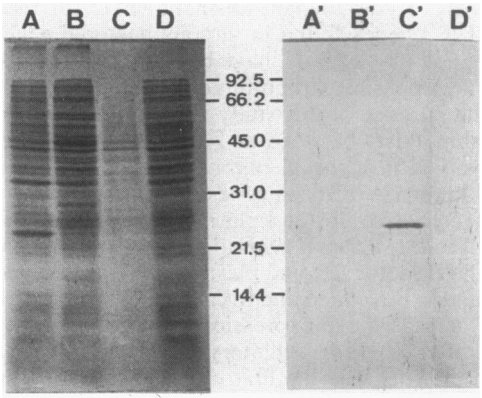

FIG. 2. Electrophoresis in a 12% polyacrylamide gel of proteins from crude extracts of strains harboring NR79 (lane A), pUB307::Tn*2424* (lane B), pAZ505 (lane C), and pMH1 (lane D). The corresponding immunoblot obtained with antibodies raised against the pAZ505-encoded AAC(6′)-4 is shown in lanes A′ through D′. The positions of the molecular weight markers (in thousands) are indicated between the two sets of lanes.

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LITERATURE CITED

1. **Benveniste, R., and J. Davies.** 1971. Enzymatic acetylations of aminoglycoside antibiotics by *Escherichia coli* carrying an R factor. Biochemistry **10**:1781–1796.
2. **Betts, R. F., W. M. Valenti, S. W. Chapman, T. Chonmaitree, G. Mowrer, P. Pincus, M. Messner, and R. Robertson.** 1984. Five-year surveillance of aminoglycoside usage in a university hospital. Ann. Intern. Med. **100**:219–222.
3. **Gerding, D. N., and T. A. Larson.** 1986. Resistance surveillance programs and the incidence of gram-negative bacillary resistance to amikacin from 1967 to 1985. Am J. Med. **80**(Suppl. 6B): 22–28.
4. **Haas, M., S. Biddlecome, J. Davies, C. E. Luce, and P. J. L. Darniels.** 1976. Enzymatic modification of aminoglycoside antibiotics: a new 6′-*N*-acetylating enzyme from a *Pseudomonas aeruginosa* isolate. Antimicrob. Agents Chemother. **9**:945–950.
5. **Haas, M. J., and J. Davies.** 1980. Characterization of the plasmids comprising the R factor R5 and their relationships to other R plasmids. Plasmid **3**:260–277.
6. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
7. **Krcméry, V., and J. Havlik.** 1986. Emergence of transferable amikacin resistance in Enterobacteriaceae in a Czechoslovakian clinic. J. Antimicrob. Chemother. **18**:429–433.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
9. **Larson, T. A., C. R. Garrett, and D. N. Gerding.** 1986. Frequency of aminoglycoside 6′-*N*-acetyltransferases among *Serratia* species during increased use of amikacin in the hospital. Antimicrob. Agents Chemother. **30**:176–178.
10. **Levine, J. F., M. J. Maslow, R. E. Leibowitz, A. A. Pollock, B. A. Hanna, S. Schaefler, M. S. Simberkoff, and J. Rahal, Jr.** 1985. Amikacin-resistant gram-negative bacilli: correlation of occurrence with amikacin use. J. Infect. Dis. **151**:295–300.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Meyer, J. F., B. A. Nies, and B. Wiedemann.** 1983. Amikacin resistance mediated by multiresistance transposon Tn*2424*. J. Bacteriol. **155**:755–760.
13. **Meyer, J. F., and B. Wiedemann.** 1985. Characterization of aminoglycoside 6′-N-acetyltransferases (AAC(6′)) from gram-negative bacteria and *Streptomyces kanamyceticus*. J. Antimicrob. Chemother. **15**:271–282.
14. **Mitsuhashi, S., and H. Kawabe.** 1982. Aminoglycoside antibiotic resistance in bacteria, p. 97–122. *In* A. Whelton and H. C. Neu (ed.), The aminoglycosides. Marcel Dekker, Inc., New York.
15. **Moody, M., C. A. de Jongh, S. C. Schimpff, and G. L. Tillman.** 1982. Long-term amikacin use: effects on aminoglycoside susceptibility patterns of gram-negative bacilli. J. Am. Med. Assoc. **248**:1199–1202.
16. **Okamoto, S., and Y. Suzuki.** 1965. Chloramphenicol, dihydrostreptomycin, and kanamycin-inactivating enzymes from multiple drug resistant *Escherichia coli* carrying episome "R." Nature (London) **208**:1301–1303.
17. **Price, K. E., P. A. Kresel, L. A. Farchione, S. B. Siskin, and S. A. Karpow.** 1981. Epidemiological studies of aminoglycoside resistance in the USA. J. Antimicrob. Chemother. **8**(Suppl. A): 89–105.
18. **Radika, K., and D. B. Northrop.** 1984. Substrate specificities and structure-activity relationships for acylation of antibiotics catalyzed by kanamycin acetyltransferase. Biochemistry **23**: 5118–5122.
19. **Radika, K., and D. B. Northrop.** 1984. Purification of two forms of kanamycin acetyltransferase from E. coli. Arch. Biochem. Biophys. **233**:272–285.
20. **Seifert, S. H., M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of introducing transposons into transformable organisms, p. 123–134. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering, principles and methods, vol. 8. Plenum Publishing Corp., New York.
21. **Shimizu, K., T. Kumada, W.-C. Hsieh, H.-Y. Chung, Y. Chong, R. S. Hare, G. H. Miller, F. J. Sabatelli, and J. Howard.** 1985. Comparison of aminoglycoside resistance patterns in Japan, Formosa, and Korea, Chile, and the United States. Antimicrob. Agents Chemother. **28**:282–288.
22. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–517.
23. **Tenover, F. C., D. Filpula, K. L. Phillips, and J. J. Plorde.** 1988. Cloning and sequencing of a gene encoding an aminoglycoside 6′-*N*-acetyltransferase from an R factor of *Citrobacter diversus*. J. Bacteriol. **170**:471–473.
24. **Tolmasky, M. E., M. Roberts, M. Woloj, and J. H. Croza.** 1986. Molecular cloning of amikacin resistance determinants from a *Klebsiella pneumoniae* plasmid. Antimicrob. Agents Chemother. **30**:315–320.
25. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
26. **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.
27. **Tran Van Nhieu, G., F. W. Goldstein, M. E. Pinto, J. F. Acar, and E. Collatz.** 1986. Transfer of amikacin resistance by closely related plasmids in members of the family *Enterobacteriaceae* isolated in Chile. Antimicrob. Agents Chemother. **29**:833–837.