ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1987, p. 667–670
0066-4804/87/040667-04$02.00/0

Vol. 31, No. 4

More than One DNA Sequence Encodes the 2″-*O*-Adenylyltransferase Phenotype

STEPHEN C. LEE,[1]†* P. P. CLEARY,[1] AND D. N. GERDING[2,3,4]

Departments of Microbiology,[1] Medicine,[2] and Laboratory Medicine,[3] University of Minnesota, and Minneapolis Veterans Administration Medical Center,[4] Minneapolis, Minnesota 55417

Received 28 July 1986/Accepted 30 December 1986

Biochemical and phenotypic assays indicate that three enterobacterial R plasmids isolated in a single hospital encode 2″-*O*-adenylyltransferase [ANT(2″)], and an ANT(2″)-specific probe was developed from one plasmid. Southern hybridization revealed the three plasmids to be unrelated and, further, showed their ANT(2″)-encoding genes to be different.

The aminoglycoside-modifying activity 2″-*O*-adenylyltransferase [ANT(2″)] was first described in 1971 (2). ANT(2″) has since been shown to be widely distributed in the United States and abroad (15, 16, 20). ANT(2″) confers resistance to the clinically important antibiotics gentamicin and tobramycin and is frequently encoded by plasmids from members of the family *Enterobacteriaceae* (7). Although ANT(2″) is among the most common plasmid-encoded gentamicin resistances of enteric bacteria (15, 20), little is known about heterogeneity among ANT(2″) genes. An ANT(2″) protein with novel properties has been identified (5), but since no characterization was made of the gene encoding it, it remains possible that its gene is homologous to the more widely distributed ANT(2″) gene. The question of DNA sequence heterogeneity among ANT(2″) genes is important if DNA hybridization methods are to be used for typing of aminoglycoside resistance genes in the clinical laboratory.

pSCL14, pSCL29, and pSCL35 are gentamicin R plasmids that were involved in nosocomially acquired infections at the Minneapolis Veterans Administration Medical Center (11). pSCL14 is identical to an epidemic R plasmid that was introduced to the Minneapolis Veterans Administration Medical Center in 1975 and was responsible for an outbreak of gentamicin resistance that persists at the hospital to date (8, 10, 11, 19). On a molecular level, pSCL14 differs noticably from pSCL29 and pSCL35. As determined by summing the sizes of their restriction fragments, the sizes of pSCL14, pSCL35, and pSCL29 are approximately 85, 123, and 48 kilobases, respectively (S. C. Lee, unpublished data). Additionally, the pSCL29 and pSCL35 restriction patterns differ greatly from one another and from that of pSCL14 (11). In this paper, we investigate the relatedness of the three plasmids and the relatedness of their gentamicin resistance genes by biochemical and molecular means.

We cloned the pSCL14 gentamicin resistance gene on a 3.3-kilobase *Hin*dIII fragment. Transformed *Escherichia coli* MC1000 (4) containing the resultant chimeric plasmid pSCL36 were selected directly on L agar containing 5 μg of gentamicin per ml. Figure 1 shows the restriction map of the pSCL36 insert. By a series of subcloning experiments, a 0.1-kilobase fragment, cloned in pSCL53, was identified that is internal to the pSCL14 gentamicin resistance gene (Fig. 1). The cloning vector pBR322 (3) was used in the construction of all chimeric plasmids except pSCL53. The vector used for pSCL53 was pUC19 (14).

Both of the commonly used methods for identifying aminoglycoside-modifying enzymes, the aminoglycoside resistance pattern method (AGRP) (6, 15, 20) and the aminoglycoside substrate specificity method (also known as the phosphocellulose-binding assay [6, 9]), were used to identify the mechanism of gentamicin resistance of nosocomial and chimeric gentamicin resistance plasmids. AGRP data (Table 1) indicated the possession of ANT(2″) by *E. coli* 36X3 (pSCL14), 80X4(pSCL29), 178X14(pSCL35), GRC-1(pSCL36), GRC-2(pSCL48), and GRC-3(pSCL51). 178X14 apparently also possesses 6′-*N*-acetyltransferase activity. Substrate specificity data (Table 2) confirmed the presence of ANT(2″) adenylating activity in extracts of 36X3, 80X4, 178X14 and GRC-1. In general, the AGRP and substrate

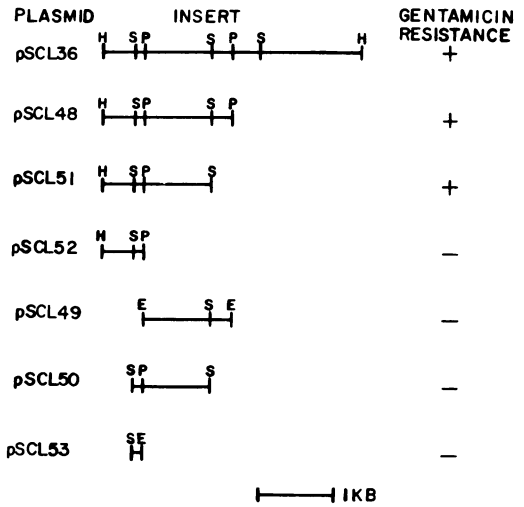

FIG. 1. Restriction maps of overlapping inserts of chimeric plasmids containing portions of the gentamicin resistance gene of pSCL14. The ability of each plasmid to confer gentamicin resistance is indicated at the right. pBR322 (3) was the cloning vector used for all chimeric plasmids except pSCL53. The pSCL53 vector was pUC19 (14). Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II, S, *Sph*I. The *Eco*RI sites of pSCL49 and pSCL53 result from the addition of *Eco*RI linkers to *Pvu*II sites (12).

* Corresponding author.
† Present address: Department of Genetics, Stanford University Medical Center, Stanford, CA 94305.

TABLE 1. Enzymatic modes of aminoglycoside resistance encoded by gentamicin resistance plasmids as determined by aminoglycoside resistance patterns of *E. coli* plasmid-bearing and host bacterial strains

Strain (plasmid)	MIC (μg/ml)[a]										Aminoglycoside-modifying activity
	GM	TOB	SISO	AMK	DKB	NET	2′-NET	6′-NET	Sch 21420	Sch 22591	
JC411[b]	0.50	0.50	0.25	0.50	1.0	0.125	0.50	0.25	1.0	0.50	None
36X3(pSCL14)	16	8	8	0.25	64	0.25	0.50	0.25	0.50	0.25	ANT(2″)
80X4(pSCL29)	32	256	8	0.25	128	0.125	1.0	0.50	1.0	2	ANT(2″)
178X14(pSCL35)	16	32	16	16	32	64	256	1.0	4	32	ANT(2″) + AAC(6′)[c]
MC1000[d]	0.50	0.50	0.25	0.50	1.0	0.25	0.25	1.0	1.0	0.50	None
GRC-1(pSCL36)	32	32	16	0.50	64	0.50	1.0	1.0	0.50	1.0	ANT(2″)
GRC-2(pSCL48)	64	64	16	2	256	1.0	1.0	1.0	0.50	1.0	ANT(2″)
GRC-3(pSCL51)	64	32	8	2	128	0.50	0.50	1.0	1.0	2	ANT(2″)

[a] Abbreviations: GM, gentamicin; TOB, tobramycin; SISO, sisomicin; AMK, amikacin; DKB, dibekacin; NET, netilmicin; 2′-NET, 2′-*N*-ethyl-netilmicin; 6′-NET, 6′-*N*-ethyl-netilmicin; Sch 21420, HAPA-gentamicin B; Sch 22591, 5-episisomicin.
[b] JC411 is the *E. coli* host strain (1) used for the nosocomial plasmids in this study.
[c] AAC(6′), 6′-*N*-acetyltransferase.
[d] MC1000 is the *E. coli* cloning vector host strain (4) used for chimeric plasmids in this study.

specificity data agreed with results typical for ANT(2″)-possessing strains (6, 15, 20). However, 80X4 showed unusually high levels of tobramycin resistance relative to its gentamicin resistance (Table 1). 80X4 extracts also showed higher levels of tobramycin than gentamicin-adenylating activity (Table 2); this may be a novel property of the pSCL29-encoded ANT(2″) protein. All resistant strains exhibit some amikacin-adenylating activity (Table 2). Amikacin-modifying ANT(2″) enzymes have been described (5), but since extracts of all resistant strains adenylated gentamicin C1a (Table 2) and the novel ANT(2″) described by Coombe and George does not (5), the plasmids tested here probably do not encode that particular novel enzyme. The gentamicin resistance gene cloned in pSCL36 is an ANT(2″) gene (Table 1). The low-level butirosin and paromomycin-adenylating activities it confers (Table 2) may have been made apparent because of the increased copy number of the cloned ANT(2″) gene. Additionally, pSCL14, pSCL48, pSCL51, and pSCL53 hybridize to the previously described (22) pFCT3103 ANT(2″) probe (J. J. Plorde, personal communication). pFCT3103 is derived from pLST1000 (22), an intercontinentally distributed R plasmid (16). Thus, the ANT(2″) gene cloned from pSCL14 is related to a widely disseminated ANT(2″) sequence.

We examined the relatedness of pSCL14, pSCL29, and pSCL35 by DNA hybridization. *Eco*RI digests of the three plasmids were electrophoresed through 0.7% agarose gels, transferred to nitrocellulose by the method of Southern (21), and hybridized to radiolabeled (i.e., nick-translated [18]) pSCL14 or pSCL29. Hybridizations and washes were carried out at 68°C in 2× SSPE (1× SSPE is 1 mM disodium EDTA, 5.5 mM NaOH, 10 mM NaH_2PO_4, and 180 mM NaCl [pH 7.0]). When the digests were hybridized to labeled pSCL14, only one homologous pSCL35 *Eco*RI fragment was detected (Fig. 2, lane A). Three pSCL29 fragments hybridized faintly to the pSCL14 probe (lanes C and D). Only one pSCL14 fragment hybridized to labeled pSCL29 (lane F). pSCL35 did not hybridize to the pSCL29 probe (lane E). These results indicate that the three nosocomial plasmids are not closely related and suggest that the three plasmids were introduced to the Minneapolis Veterans Administration Medical Center independently.

We then investigated the homology of the pSCL29 and pSCL35 ANT(2″) genes to that of pSCL14. Southern transfers of *Eco*RI digests of pSCL14, pSCL29, and pSCL35 were prepared and hybridized to the radiolabeled pSCL53 insert. Hybridization and washes were performed at low stringency (15× SSPE, 68°C), which would give hybridization at T_m − 60°C (13) with *E. coli* DNA (51% G+C [17]). Neither pSCL35 nor pSCL29 hybridized to the pSCL53 probe (Fig. 3, lanes A and C). Therefore, the pSCL35 and pSCL29 ANT(2″) genes differ in DNA sequence from the pSCL14 gene. Considering the very low stringency, it is likely that the amino acid sequences of the pSCL29 and pSCL35 ANT-2″ proteins differ from that of the pSCL14 protein as well. Furthermore, the pSCL29 gene differs from the

TABLE 2. Aminoglycoside adenylyltransferase activities in osmotic-shock extracts of *E. coli* host and plasmid-bearing strains to a battery of aminoglycosides

Strain (plasmid)	Phosphocellulose-bound cpm of ^{14}C-labeled ATP (% relative activity)[a] for:							Aminoglycoside-adenylating enzyme
	Control assay with no aminoglycoside	Gentamicin C1a	Kanamycin A	Tobramycin	Amikacin	Butirosin	Paromomycin	
JC411[b]	265 (ND[c])	548 (ND)	336 (ND)	524 (ND)	412 (ND)	364 (ND)	206 (ND)	None
36X3(pSCL14)	314 (22)	1,452 (100)	739 (51)	1,739 (120)	941 (65)	339 (23)	320 (22)	ANT(2″)
80X4(pSCL29)	127 (5)	2,707 (100)	6,069 (224)	5,955 (220)	1,517 (56)	698 (26)	257 (9)	ANT(2″)
178X14(pSCL35)	123 (7)	1,652 (100)	3,445 (209)	3,399 (206)	1,013 (61)	325 (20)	257 (16)	ANT(2″)
MC1000(pBR322)[d]	209 (ND)	426 (100)	337 (ND)	285 (ND)	244 (ND)	358 (ND)	238 (ND)	None
GRC-1(pSCL36)	306 (1)	31,862 (100)	14,248 (45)	22,429 (70)	3,331 (10)	1,225 (4)	763 (2)	ANT(2″)

[a] Aminoglycoside adenylyltransferase activities are expressed as a percentage of the activity of the same strains to gentamicin C1a.
[b] JC411 is the *E. coli* host strain (1) used for the nosocomial plasmids in this study.
[c] ND, Not determined.
[d] MC1000 is the *E. coli* cloning vector host strain (4) used for chimeric plasmids in this study.

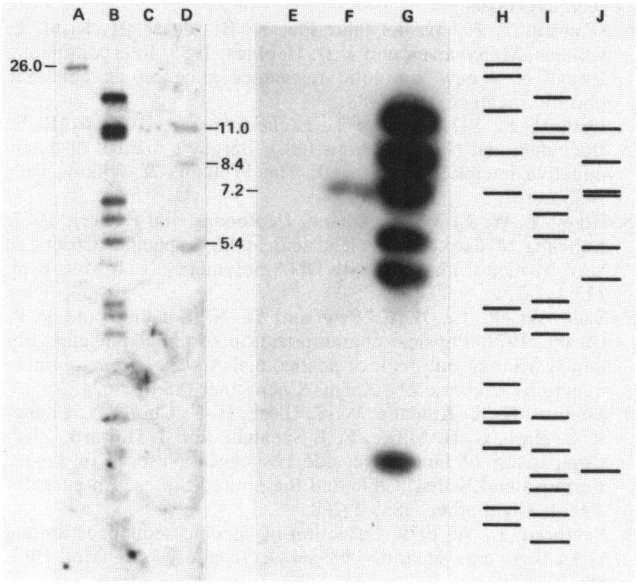

FIG. 2. Southern transfers of *Eco*RI-digested pSCL14, pSCL29, and pSCL35. Lanes A to D were hybridized to intact pSCL14 and lanes E to G were hybridized to intact pSCL29 in an aqueous solution containing 2× SSPE at 68°C. Lanes H to J are schematic drawings of *Eco*RI cleavage patterns (11). All lanes contain equimolar amounts of plasmid except lane D, which contains a 10-fold molar excess of pSCL29. Fragment sizes are indicated in kilobases. Lanes: A, pSCL35; B, pSCL14; C, pSCL29; D, pSCL29; E, pSCL35; F, pSCL14; G, pSCL29; H, pSCL35; I, pSCL14; J, pSCL29.

pSCL14 gene along much of its length. pSCL29 did not hybridize to the insert of pSCL48 (data not shown), although that insert contains the intact pSCL14 ANT(2″) gene (Fig. 1). Both pSCL29 and pSCL35 failed to hybridize to the pFCT3103 ANT(2″) probe (22; Plorde, personal communication).

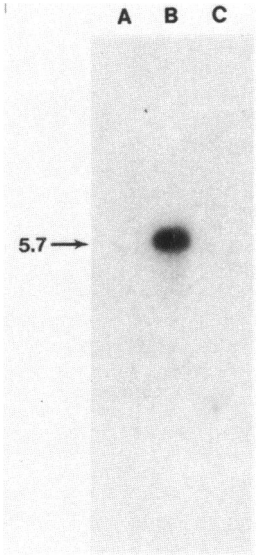

FIG. 3. Southern transfers of *Eco*RI-digested pSCL14, pSCL29, and pSCL35 hybridized to the 0.1-kilobase insert of pSCL53 in aqueous solution containing 15× SSPE at 50°C. All lanes contain equimolar amounts of plasmid. Fragment sizes are indicated in kilobases. Lanes: A, pSCL35; B, pSCL14; C, pSCL29.

In summary, pSCL14, pSCL29, and pSCL35 are very dissimilar R plasmids, which were probably introduced to the Minneapolis Veterans Administration Medical Center independently of one another. Both AGRP and aminoglycoside substrate specificity data indicate that all three encode gentamicin resistance by ANT(2″). However, only the ANT(2″) gene of pSCL14 is highly homologous to the widely disseminated ANT(2″) gene. The ANT(2″) genes of pSCL29 and pSCL35 differ significantly in sequence from the pSCL14 gene. The observation that pSCL35 does not hybridize to radiolabeled pSCL29 (Fig. 2, lane E) suggests that the ANT-2″ genes of pSCL29 and pSCL35 may also differ in sequence from one another.

This is the first clear demonstration of DNA sequence heterogeneity among ANT(2″) genes. The pSCL14 ANT(2″) gene is probably related by descent to the widely distributed ANT(2″) gene (16, 22). If the pSCL29 or pSCL35 genes are likewise related to the widely distributed gene, the relationships are distant. Perhaps not all ANT(2″) genes have a common ancestor. In addition to those described here, still other ANT(2″) genes might exist. For instance, the relationship between the ANT(2″) genes discussed here and the gene encoding the novel ANT(2″) enzyme described by Coombe and George (5) has not yet been tested.

The frequency at which novel ANT(2″) genes like those of pSCL29 and pSCL35 occur is uncertain. Since typing of aminoglycoside-modifying genes by AGRP and DNA hybridization methods are reported to agree well (20), it is likely that novel ANT(2″) genes are rare. However, this situation could change over time. Therefore, methods for identifying aminoglycoside-modifying genes of clinical isolates by DNA hybridization will have to take into account our observation of DNA sequence heterogeneity among ANT(2″) genes.

This work was supported by Public Health Service training grant 1T32HL107114 from The National Institute of Heart and Lung Disease, by Public Health Service grant AI6722 from The National Institutes of Health, and by the Veterans Administration.

We thank S. N. Cohen, R. Hare, K. J. Kendall, B. S. M. Lee, C. A. Miller, and C. A. Omer for helpful discussions. We are grateful to Roberta Hare of Schering Corp. for performing AGRP experiments and to David Bobey and Vi Rossomano of Bristol-Myers for performing the aminoglycoside substrate specificity assay. We thank Donna Geryk for manuscript preparation. We also thank S. N. Cohen for kindly allowing his laboratory facilities to be used in the completion of this work.

LITERATURE CITED

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
2. **Benveniste, R., and J. Davies.** 1971. R-factor mediated gentamicin resistance: a new enzyme which modifies aminoglycoside and antibiotics. FEBS Lett. **14**:293–296.
3. **Bolivar, F., R. L. Rodrigues, P. J. Greene, M. C. Betlatch, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
5. **Coombe, R. G., and A. M. George.** 1981. New plasmid-mediated aminoglycoside adenylyltransferase of broad substrate range that adenylylates amikacin. Antimicrob. Agents. Chemother. **20**:75–80.
6. **Davies, J.** 1980. Aminoglycoside-aminocyclitol antibiotics and

their modifying enzymes, p. 474–489. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.

7. **Davies, J., and D. I. Smith.** 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32**:469–506.
8. **Gerding, D. N., A. E. Buxton, R. A. Hughes, P. P. Cleary, J. Arbaczawski, and W. E. Stamm.** 1979. Nosocomial multiply resistant *Klebsiella pneumoniae*: epidemiology of an outbreak of apparent index case origin. Antimicrob. Agents Chemother. **15**:608–615.
9. **Hass, M. J., and J. E. Dowding.** 1975. Aminoglycoside modifying enzymes. Methods Enzymol. **43**:611–628.
10. **Lee, S. C., D. N. Gerding, and P. P. Cleary.** 1984. Plasmid macroevolution in a nosocomial environment: demonstration of a persistent molecular polymorphism and construction of a cladistic phylogeny on the basis of restriction data. Mol. Gen. Genet. **194**:173–178.
11. **Lee, S. C., D. N. Gerding, and P. P. Cleary.** 1986. Hospital distribution, persistence, and reintroduction of related gentamicin R plasmids. Antimicrob. Agents Chemother. **29**:654–659.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
14. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–321.
15. **Miller, G. H., E. J. Sabatelli, R. S. Hare, and J. A. Waitz.** 1980. Survey of aminoglycoside resistance patterns. Dev. Ind. Microbiol. **21**:91–104.
16. **O'Brien, T. F., M. del Pilar Pla, K. H. Meyer, H. Kishi, E. Gilleece, M. Syvanen, and J. D. Hopkins.** 1985. Intercontinental spread of a new antibiotic resistance gene on an epidemic plasmid. Science **230**:87–88.
17. **Ørskov, F.** 1974. Genus I. *Escherichia*, p. 100. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
18. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling of deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
19. **Sadowski, P. L., B. C. Peterson, D. N. Gerding, and P. P. Cleary.** 1979. Physical characterization of ten R plasmids obtained from an outbreak of nosocomial *Klebsiella pneumoniae* infections. Antimicrob. Agents Chemother. **15**:616–624.
20. **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.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
22. **Tenover, F. C., T. D. Gootz, K. P. Gordon, L. S. Tompkins, S. A. Young, and J. J. Plorde.** 1984. Development of a DNA probe for the structural gene of the 2″-*O*-adenyltransferase aminoglycoside modifying enzyme. J. Infect. Dis. **150**:678–687.