JOURNAL OF BACTERIOLOGY, Nov. 1991, p. 7213–7218
0021-9193/91/227213-06$02.00/0

Vol. 173, No. 22

Site-Specific Methylation of 16S rRNA Caused by *pct*, a Pactamycin Resistance Determinant from the Producing Organism, *Streptomyces pactum*

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Received 13 June 1991/Accepted 6 September 1991

Ribosomal resistance to pactamycin in clones of *Streptomyces lividans* containing DNA (*pct*) from *Streptomyces pactum*, the pactamycin producer, involves methylation of 16S RNA. The modified residue A-941 in *S. lividans* 16S rRNA (A-964 in the homologous *Escherichia coli* sequence) is converted to 1-methyladenosine, and the ribosomal ability to bind pactamycin is reduced or abolished.

Pactamycin is an antibiotic that inhibits protein synthesis by binding directly to the ribosomes of prokaryotes, eukaryotes, or archaebacteria, in each case at a single site associated with the smaller (30S or 40S) ribonucleoprotein subunit (for a review, see reference 13). The pactamycin binding site has evidently been retained throughout ribosomal evolution, doubtless for important functional reasons, so that characterization of that site would be likely to contribute positively to an eventual understanding of ribosomal function. It is also evident that in producing such a toxic compound, *Streptomyces pactum* faces an interesting challenge to its own well-being.

In an initial survey (7), ribosomes from *S. pactum* were shown to be insensitive to pactamycin in vitro, and, when DNA from that organism was subsequently cloned in *Streptomyces lividans*, strains were obtained which, likewise, possessed resistant ribosomes. The designation *pct* was proposed for the gene responsible for such ribosomal resistance. In both the producing organism and the *pct* clones, the 30S ribosomal subunit appeared to be the source of pactamycin resistance, which was eventually localized to some property of the 16S rRNA, as a result of in vitro reconstruction experiments involving rRNA and proteins derived from sensitive and resistant strains (8). At that time, further attempts to analyze the *pct* resistance mechanism proved fruitless, although there were strong precedents to suggest that specific methylation of rRNA might have been involved. For example, resistance to various aminoglycosides in *Micromonospora purpurea*, *Streptomyces tenjimariensis*, and *Streptomyces tenebrarius*, and in resistant clones containing DNA fragments therefrom, involves methylation of specific single nucleotides within 16S rRNA, each characteristic of a given resistance phenotype. Similarly, for drugs such as thiostrepton, macrolides, and lincosamides that normally attack the larger ribosomal subunit, the producing organisms defend themselves via site-specific methylation of 23S rRNA (for a review, see reference 10).

In the present work, we have characterized in detail the mechanism of resistance to pactamycin in clones of *S. lividans* containing *pct* DNA from *S. pactum*.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Prior to the present work, two *Escherichia coli-Streptomyces* shuttle plasmids (vectors pLST 520 and pLST 52-666), both containing *pct* DNA from *S. pactum*, were constructed (Table 1) and introduced into *S. lividans* TK21 (obtained from D. A. Hopwood, John Innes Institute, Norwich, United Kingdom). These plasmids were constructed to facilitate the manipulation of *pct* DNA which, when originally cloned as a 4.9-kb *Kpn*I fragment inserted into pIJ 702, proved to be unstable in *S. lividans* (8). Although pactamycin resistance was not expressed in *E. coli*, the *pct* DNA was stable in that host. Strain TB520 (*S. lividans* TK21 containing pLST 520) gave rise to a mixed population of mycelial units of which about 70% contained pactamycin-resistant ribosomes, indicating residual instability of pLST 520 (for details, see reference 8). However, ribosomes prepared from strain TB666 (*S. lividans* TK21 containing pLST 52-666) were totally resistant to pactamycin (6).

Strains of *S. lividans* were maintained at 30°C on NE agar plates (20) containing thiostrepton (final concentration, 10 μg/ml). For liquid cultures, also grown at 30°C, YEME medium (14) supplemented with 5 mM $MgCl_2$ and 0.5% (wt/vol) polyethylene glycol 6000 was used.

Antibiotics. Pactamycin was obtained from The Upjohn Company, and thiostrepton was from E. R. Squibb and Sons.

Coupled transcription-translation in vitro. Salt-washed ribosomes and their subunits were prepared as described elsewhere (20) and were used together with crude initiation factors and postribosomal supernatant (S100) for coupled transcription-translation directed by pUC 18 DNA (7).

Preparation of crude pactamycin resistance methylase activity. Crude pactamycin resistance methylase activity was obtained as a ribosomal wash fraction, by centrifuging crude ribosomes through high-salt buffer containing 20% (wt/vol) sucrose as described elsewhere (29).

Methylation of 30S ribosomal subunits. Reaction mixtures (100 μl) contained 20 pmol of 30S ribosomal subunits or 70S ribosomes from *S. lividans* TSK1 (control strain), 20 to 30 μl of crude pactamycin resistance methylase (i.e., ribosomal wash fraction) from *S. lividans* 520 or 666, and 2.5 μCi of [*methyl*-^{3}H]*S*-adenosylmethionine ([*methyl*-^{3}H]SAM; 500 mCi/mmol; 18.5 GBq/mmol). Incubation conditions were as

TABLE 1. Strains and plasmids used

Plasmid	Description	Reference or source	*S. lividans* strain[a]
pIJ 702	*Streptomyces* cloning vector; broad host range; multicopy	15	TSK1
pOJ 160	*E. coli-Streptomyces* shuttle vector formed from pUC 18 and SCP 2	B. Schoner, Eli Lilly and Co.	
pLST 520	pOJ 160 with 4.9 kb of *S. pactum pct* DNA	8	TB520
pLST 52-666	*E. coli-Streptomyces* shuttle vector formed from pUC 18 and pIJ 702 with *pct* DNA	6	TB666

[a] Plasmid-containing derivatives of strain TK21.

previously described (20). These conditions were used to establish that the *pct* strains of *S. lividans* possessed clone-specific rRNA methylase activity and to estimate the stoichiometry with which ribosomal particles were modified. For identification of the methylated residue generated within 16S rRNA, the methyl-donor cofactor [*methyl*-^{3}H]SAM was used at higher specific radioactivity (i.e., 15 μCi at 15 Ci/mmol; 555 GBq/mmol) in a reaction mixture scaled up by a factor of 3.

To examine a possible correlation between rRNA methylation and resistance, 30S ribosomal subunits from *S. lividans* TSK1 were incubated for 30 min at 30°C as described above but in the presence (or absence) of unlabelled SAM (final concentration, 0.5 mM). The particles were then examined directly for their response to pactamycin in (i) cell-free coupled transcription-translation systems (7) and (ii) drug-binding assays (see below).

Specific cleavage of 16S rRNA with RNase H. Approximately 1.5 μg of phenol-extracted RNA from ^{3}H-methylated 30S ribosomal subunits was incubated for 5 min at 70°C with 10 to 20 pmol of 17- to 23-mer deoxyoligonucleotide in 10 μl of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–KOH (pH 7.0) plus 50 mM KCl and then cooled slowly to about 37°C. Various deoxyoligonucleotides were used, which were complementary in reverse to defined sequences within 16S rRNA. The assay volume was then made up to 48 μl and the buffer was modified to give 10 mM HEPES-KOH (pH 7.0)–50 mM KCl–9 mM $MgCl_2$–0.2 mM dithiothreitol before the addition of 1 μl of RNase H (2 U; Bethesda Research Laboratories) plus 1 μl of human placental RNase inhibitor (10 U; Bethesda Research Laboratories), and incubation was continued at 37°C for 30 min. After extraction with phenol, RNA fragments were precipitated from the aqueous phase with ethanol, dissolved in 4 μl of H_2O, and separated by electrophoresis in 1% agarose gels. These were then treated with a fluorographic reagent (Amplify; Amersham International), dried, and exposed for fluorography.

Treatment of rRNA with RNase T1 and P1. About 15 μg of ^{3}H-methylated rRNA, in 30 μl of 70 mM sodium acetate (pH 5.3), was incubated at 37°C for 3 h with 1,380 U of RNase T1 and then with 90 U of nuclease P1 (both from Bethesda Research Laboratories) at the same temperature overnight. The digests were then applied directly to the middle of a 50-cm strip of Whatman 3 MM paper and subjected to electrophoresis at 1,200 V for 2.5 h in 70 mM sodium phosphate buffer (pH 6.8). Alternatively, RNA samples were treated with RNase P1 alone, without prior exposure to RNase T1.

Primer extension by reverse transcriptase. Approximately 3 μg of 16S rRNA from pactamycin-sensitive *S. lividans* (strain TSK1) and the resistant strain (TB666) was used as a template for 1 U of avian myeloblastosis virus reverse transcriptase (Anglian Biotechnology Ltd.), primed by about 2 pmol of a 17-mer deoxyoligonucleotide complementary in reverse to residues 1013 to 1029 of the 16S RNA. The conditions were as described elsewhere (2).

Iodination of pactamycin. Pactamycin (30 nmol) in 50 mM sodium phosphate (pH 7.5) plus 50% ethanol was iodinated with 0.5 mCi of $Na^{125}I$ by a modification of the chloramine T method (to avoid direct contact of the drug with oxidant) as previously described (26) and then purified by thin-layer chromatography (24). Nonradioactive iodopactamycin was prepared similarly and added to the radiolabelled drug to give the desired specific radioactivity.

Binding of radiolabelled pactamycin to ribosomes. Ribosomes (25 pmol) were incubated with ^{125}I-labelled pactamycin (100 pmol; 300 cpm/pmol) at 37°C for 30 min in buffer containing 10 mM HEPES–KOH (pH 7.6), 10 mM $MgCl_2$, 60 mM NH_4Cl, and 5 mM 2-mercaptoethanol. Ethanol (1.5 volumes) was then added, and the samples were kept on ice for 15 min. The precipitated ribosomes were collected on Whatman GFC glass fiber filters, which were washed twice with buffer containing 50% ethanol, and their radioactivity was estimated in a gamma counter.

RESULTS

Methylation of rRNA and pactamycin resistance. The two pactamycin-resistant strains of *S. lividans* (TB520 and TB666) contain clone-specific rRNA methylase activity that is responsible for their resistance character. This became apparent as a result of two sets of experiments. In the first, with 30S ribosomal subunits as the substrate, ribosomal wash fractions ("crude methylase") from either of the two clones catalyzed the transfer of radiolabelled methyl groups from [*methyl*-^{3}H]SAM into 16S rRNA from the control strain, *S. lividans* TSK1 (data not shown). Subsequently, intact 70S ribosomes were also shown to serve as substrates for the methylase activity, which routinely occurred with a stoichiometry of 0.4 to 0.5 pmol of methyl groups transferred per pmol of 30S or 70S particles. In other experiments, in which nonradioactive SAM was used, a causal connection was established between the clone-specific methylase activity and ribosomal resistance to pactamycin, thereby also accounting for resistance to the drug in the producing organism, *S. pactum*. Thus, when incubated with crude methylase plus SAM (but to a much lesser extent when the cofactor was omitted), ribosomes became significantly resistant to pactamycin as assayed in a coupled transcription-translation system (data not shown). This evidently resulted from reduced binding of the drug to the methylated ribosomes (Table 2).

Characterization of a methylated base. When radiolabelled methyl groups were incorporated into *S. lividans* 16S rRNA in the presence of crude pactamycin resistance methylase, and the RNA was then subjected to acid hydrolysis, the released radioactivity was found in association with a purine base and comigrated with 1-methyladenine (m^1A) during descending paper chromatography in two different solvent systems (data not shown; for details of methods, see reference 2). Subsequently, the identity of the methylated base

TABLE 2. Binding of radiolabelled pactamycin to ribosomes from *S. lividans* TSK1[a]

Incubation mixture	Radiolabelled pactamycin bound to ribosomes, cpm[b]	Pactamycin bound, % of control
Ribosomes alone (25 pmol)	4,330 (14.4)	100
Ribosomes plus crude methylase	3,729 (12.4)	86
Ribosomes plus crude methylase plus cofactor	1,848 (4.6)	42

[a] Ribosomes (25 pmol) from the control strain, *S. lividans* TSK1, were incubated for 30 min at 30°C either alone or with crude methylase from the *pct* strain, TB666, both in the presence and in the absence of methyl donor cofactor SAM (0.5 mM). ^{125}I-labelled pactamycin (100 pmol; approximately 300 cpm/pmol) was then added, and incubation at 30°C was continued for a further 30 min, prior to the addition of 2 volumes of ice-cold ethanol and retention at 0°C for 15 min. Ribosomes were then collected by filtration on Whatman GF/C glass fiber discs which were subjected to gamma counting to estimate bound radioactivity.

[b] Values in parentheses are picomoles.

was confirmed (Fig. 1) when it was shown to undergo rearrangement to N^6-methyladenine (m^6A) under alkaline conditions (5). This was done with 16S RNA methylated by enzyme derived from either of the two pactamycin-resistant *S. lividans* clones. However, since these strains evidently contained similar methylase activities, the remainder of the

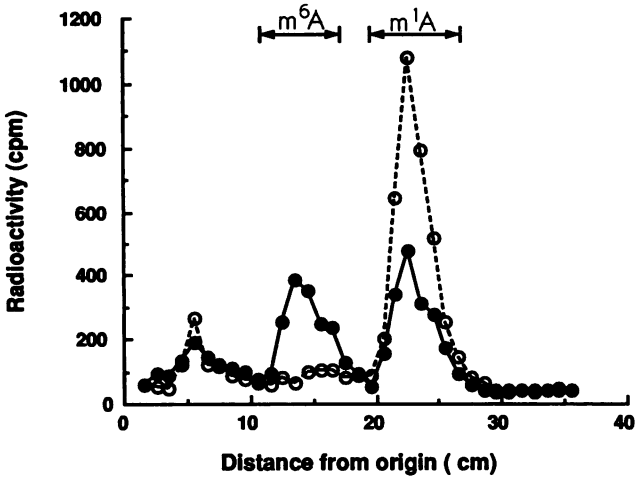

FIG. 1. Identification of the methylated base produced within 16S rRNA by the pactamycin resistance methylase. Ribosomal 30S subunits (60 pmol) from *S. lividans* TSK1 were modified in vitro by using [*methyl*-^{3}H]SAM (1 nmol; 15Ci/mmol) as the cofactor plus the ribosomal wash fraction from *S. lividans* TB666 as the crude methylase. Radiomethylated 16S RNA was then extracted from the particles by using phenol. The resultant RNA preparation was hydrolyzed by using 1 M HCl at 100°C for 60 min, and a portion of the product was subjected directly to high-voltage paper electrophoresis. The remainder of the HCl hydrolysate was dried under vacuum, taken up in 0.5 M NH_4OH, and incubated at 100°C for 3 h. The resultant hydrolysate was fractionated by high-voltage paper electrophoresis at 1,400 V for 105 min on Whatman 3MM paper, using 50 mM ammonium acetate buffer (pH 4.1). Tracks along the paper were then cut into 1 cm wide strips for the estimation of radioactivity by liquid scintillation counting. The positions of the m^6A and m^1A markers are indicated. Symbols: ○, acid hydrolysate; ●, acid hydrolysate treated with NH_4OH.

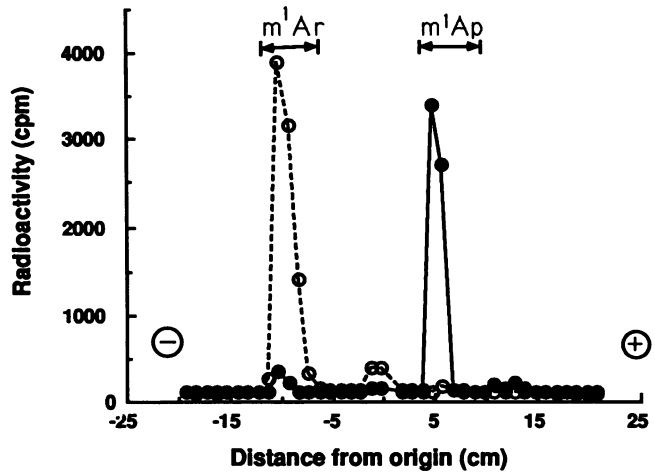

FIG. 2. Identification of the nucleotide preceding the residue methylated by the *pct* product. Radiomethylated 16S RNA (15 μg) was extracted from *S. lividans* 30S ribosomal subunits, previously modified in vitro by using crude pactamycin resistance methylase and [*methyl*-^{3}H]SAM (see legend to Fig. 1), and divided into two portions. Some of the RNA (●) was digested to completion with nuclease P1, while the remainder (○) was digested to completion with RNase T_1 and then subsequently with nuclease P1. Both sets of digestion products were subjected to high-voltage paper electrophoresis for 150 min at 1,200 V on Whatman 3MM paper in 75 mM sodium phosphate buffer (pH 6.8). Radioactivity on the paper was estimated as described in the legend to Fig. 1. Authentic 1-methyladenosine was run alongside the radiolabelled material as a marker. m^1Ar and m^1Ap indicate the positions of 1-methyladenosine and 1-methyl adenosine-5′-monophosphate, respectively.

work described here was carried out by using crude methylase from strain TB666 only.

Sequence at the site of methylation. In a preliminary experiment that turned out to be crucial (see below), the methylated nucleoside (m^1Ar) associated with pactamycin resistance was shown to be preceded by a G residue in 16S rRNA from *S. lividans*. This was established when radiomethylated RNA was digested with nuclease P1 and RNase T1. Nuclease P1 releases nucleoside 5′-monophosphates from RNA and, when used alone in the present studies, released radiolabelled 1-methyladenosine-5′-monophosphate (Fig. 2). However, the radiolabelled material was released as a nucleoside (and not as a nucleotide) when nuclease P1 acted upon 16S RNA predigested with RNase T1. Since RNase T1 cleaves RNA after G residues, giving fragments with 5′-hydroxyl and 3′-phosphate termini, the m^1Ar generated within 16S RNA by the *pct* product must have constituted the 5′-terminal residue of one such RNA fragment.

Site of methylation within 16S rRNA. The site of methylation due to the *pct* product was eventually located by a combination of methods involving the generation of specific fragments of 16S rRNA and the use of reverse transcriptase to scan nucleotide sequences for the presence of modified residues (such as m^1A) at which the enzyme stops or pauses during cDNA synthesis. Thus, by using deoxyoligonucleotides complementary to specific and widely spaced sequences within *S. lividans* 16S rRNA (22), together with RNase H which acts exclusively within RNA-DNA hybrids, the site of methylation associated with pactamycin resistance was shown to lie between residues 909 and 1013. This was established by observing the relative migration in agar-

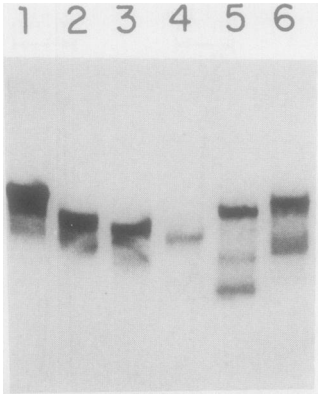

FIG. 3. Approximate location of the site of action of the PCT methylase. Total rRNA from *S. lividans* TSK1 was radiomethylated by using a ribosomal wash fraction from *S. lividans* TB666 (the *pct* strain) and annealed separately to four different deoxyoligonucleotides. After digestion with RNase H, the products were separated by agarose gel electrophoresis and examined by autoradiography. Lanes: 1, intact rRNA; 2 to 6, RNase H digestion products after hybridization of rRNA with deoxyoligonucleotides complementary in reverse to 16S RNA residues 490 to 511 (lane 2), 689 to 711 (lane 3), 890 to 909 (lane 4), 1013 to 1029 (lane 5), and 1306 to 1325 (lane 6). The reversed shift of the major radiolabelled RNA fragment that occurs between lanes 4 and 5 suggests that the methylated adenosine giving pactamycin resistance is located between residues 909 and 1029 of *S. lividans* 16S rRNA.

ose gels of radiolabelled digestion products derived from radiomethylated RNA (Fig. 3). Next, a deoxyoligonucleotide complementary in reverse to residues 1013 to 1029 was used as a primer for reverse transcriptase with nonradioactive 16S rRNA as the template, the latter being derived from the *pct* clone TB666 and, in controls, from strain TSK1 (Fig. 4, lanes 2 and 1, respectively). Reverse transcriptase was also used in the sequencing mode together with dideoxynucleotides to confirm that priming had occurred at the intended site. The absence of any obvious pause site specific to a template derived from strain TB666 indicated that m^1A residues in RNA, known to block reverse transcriptase efficiently (2), were not present between residues G-1010 and G-947 of the 16S RNA sequence. This narrowed down the site of action of the PCT methylase to residues 909 to 946. Unfortunately, reverse transcriptase was unable to proceed along the template (even that derived from the control strain) throughout the whole of the region of interest because of some feature of *Streptomyces* 16S rRNA that terminated cDNA synthesis soon after G-947. Phylogenetic comparison reveals this to be a highly conserved portion of 16S-like RNA within which a tandem pair of nucleotides are typically methylated, e.g., residues 966 and 967 within *E. coli* 16S RNA corresponding to residues 943 and 944 in the *Streptomyces* sequence (19). Quite apart from the pactamycin resistance mechanism, we suspect that *S. lividans* 16S rRNA is also modified within this conserved sequence, most probably at G-943 and C-944.

In a final, high-resolution analysis with RNase H, by using deoxyoligonucleotides more closely spaced along the *S. lividans* 16S rRNA sequence, the site of action of the PCT methylase was shown to lie between residues 940 and 947 (GACGCAAC; Fig. 5). This sequence contains three A residues, only one of which is preceded by G (Fig. 6). We therefore concluded that the pactamycin resistance enzyme acts on residue A-941 within *S. lividans* 16S rRNA, the

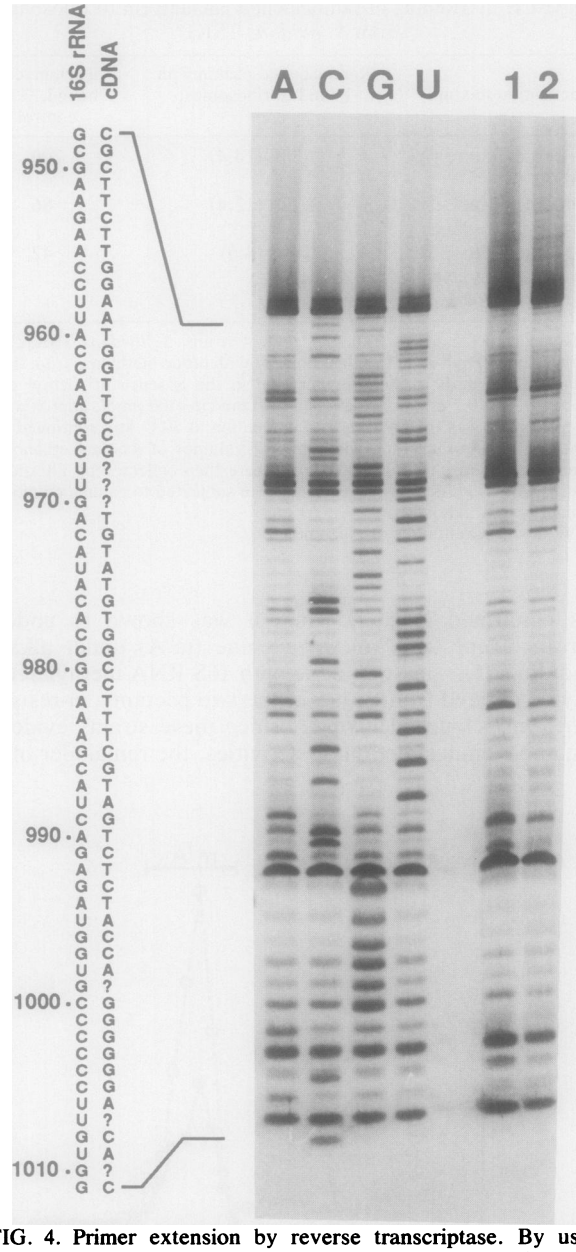

FIG. 4. Primer extension by reverse transcriptase. By using intact rRNA from *S. lividans* as the template, a 17-base deoxyoligonucleotide complementary to residues 1013 to 1029 in 16S rRNA was used to prime reverse transcriptase, and the resultant cDNA transcripts were separated on 10% polyacrylamide sequencing gels. Templates were rRNAs from *S. lividans* TSK1 (lane 1) and *S. lividans* TB666 (lane 2). Lanes A, C, G, and U contained the complementary dideoxynucleoside triphosphates for sequence generation from control rRNA. The failure of sequencing ladder formation beyond residue 948 is attributable to modification of C-944 and G-943 that precede (in the antisense direction) the residue methylated by the pactamycin resistance enzyme (namely, A-941; see text). The sequence read from the gel is indicated together with the complementary sequence in *S. lividans* 16S rRNA.

equivalent of A-964 within the homologous *E. coli* sequence. Again, in this experiment, there was evidence to support the suspicion that *S. lividans* 16S RNA is additionally modified adjacent to this site. Thus, RNase H failed to generate defined cleavage products in the presence of the deoxyoli-

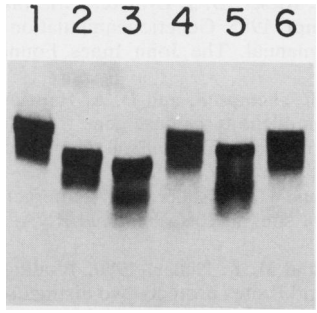

FIG. 5. Precise location of the site of action of the PCT methylase. Radiomethylated 16S rRNA from *S. lividans* TSK1 was hybridized to deoxyoligonucleotides and digested with RNase H as described in the legend to Fig. 3. Lanes: 1 and 6, intact rRNA controls; 2 to 5, RNase H digestion products after hybridization with deoxyoligonucleotides complementary to 16S RNA residues 689 to 711 (lane 2), 924 to 940 (lane 3), 930 to 950 (lane 4), and 947 to 967 (lane 5). The reversed shift in migration of the major radiolabelled RNA fragment that occurs between lanes 3 and 4 suggests that the adenosine modified by the pactamycin resistance enzyme lies between residues 940 and 950 of *S. lividans* 16S rRNA.

gonucleotide complementary to residues 930 to 950 (Fig. 5, lane 4), presumably because of failure of the latter to hybridize efficiently with the template.

DISCUSSION

These findings should be viewed against a background of mounting evidence that the ribosome is fundamentally an RNA enzyme (18) and that antibiotics make important, even primary, contacts with rRNA when inhibiting protein synthesis (for a review, see reference 11). From the present data

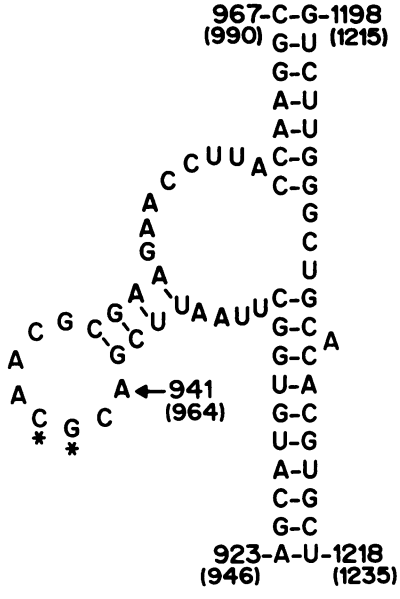

FIG. 6. Proposed secondary structure of *S. lividans* 16S rRNA around the site of action of the PCT methylase. The sequence of *S. lividans* TK21 16S rRNA (22) has been superimposed on the ubiquitous secondary structure model (19). Numbered residues in the *Streptomyces* 16S rRNA sequence are followed by the corresponding *E. coli* sequence positions in parentheses. Asterisks indicate phylogenetically conserved methylated residues.

alone, we cannot conclude that pactamycin recognizes 16S RNA exclusively when binding to the 30S ribosomal subunit (although that may well be so) or that methylation of A-964 (*E. coli* numbering system) totally abolishes such binding. The filter binding assay used in the experiments summarized in Table 1 would have failed to detect weak residual drug binding, and pactamycin has been shown to photoincorporate into various proteins after binding to *E. coli* 30S ribosomal subunits (23–25). Nevertheless, ribosomes methylated on residue A-964 of 16S RNA are insensitive to pactamycin during in vitro protein synthesis (8), and that is the most sensitive assay available to detect meaningful drug binding. We therefore conclude that, in all probability, pactamycin affords one more example of the ability of rRNA to recognize (or be recognized by) small drug molecules of diverse chemical types.

The plausible suggestion that pactamycin interacts with 16S rRNA at or near residue A-964 carries with it the implication that this portion of the RNA might play an active role in ribosomal function. Consistent with this speculation, the nucleotide sequence surrounding A-964 (and its equivalent in 16S-like rRNA from all three phylogenetic kingdoms) has been highly conserved throughout evolution and pactamycin has been shown to inhibit ribosomes from all three kingdoms. The presence of modified nucleosides immediately adjacent to A-964 is also highly suggestive. Others have speculated that such methylated residues may be landmarks for functionally important sites, and, in particular, the conserved sequence surrounding the modified nucleosides 966 and 967 in *E. coli* 16S rRNA has been described as "one of the more interesting regions in the 16S rRNA" (27). The present data contribute to the suspicion that this portion of rRNA is part of an active site within the ribosomal enzyme.

When bound to *E. coli* 30S ribosomal subunits, pactamycin protected residues G-693 and C-795 of 16S RNA from chemical attack (12, 28). These nucleosides are immediately adjacent to residues that have been cross-linked in situ within the 30S particle (1) and, ideally, ought also to be close to the methylation site (A-964) within the tertiary folded molecule. Two principal models for the latter have been proposed (4, 21), and, in both, residues 693 and 795 are positioned in the platform of the 30S particle, adjacent to the cleft. Moreover, at least one of the models (21) places residue 964 fairly close by on the opposite side of the cleft (i.e., on the proximal side of the head). These data may well imply that pactamycin binds into the cleft of the 30S particle and are consistent with the results of photoaffinity labelling studies (24) in which protein S21 was a major target for the drug, in addition to 16S rRNA. Protein S21 sits at the base of the cleft (9) and interacts with 16S RNA close to at least one of the sites that pactamycin protects (3). Other data suggest that such binding may interfere with the attachment of tRNA to the particle, apparently to the P site. Thus, the chemical footprint given by pactamycin partially overlaps that of edeine, a peptide antibiotic that also protects A-794 and G-926 in addition to G-693 and C-795 (16). All four of these sites have been positioned around the cleft of the 30S particle (3), and all have been independently classified as P site related from the manner in which they were protected by specifically bound tRNA (17).

ACKNOWLEDGMENTS

This work was supported by grant PB0450 from Direccion General de Politica Cientifica y Tecnica (to J.P.G.B.) and by a grant from The Wellcome Trust (to E.C.). We thank the British Council

and the Spanish Ministerio de Educacion y Ciencia for travel funds awarded under the Acciones Integradas programme.

We thank M. Iglesias for the radiolabelling of pactamycin.

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