Characterization of a plasmid-specified ribosome methylase associated with macrolide resistance

A.G. Shivakumar and D. Dubnau

Department of Microbiology, The Public Health Research Institute of The City of New York, Inc.,
455 First Avenue, New York, NY 10016, USA

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ABSTRACT

The *ermC* gene of plasmid pE194 specifies resistance to the macrolide-lincosamide-streptogramin B antibiotics. This resistance, as well as synthesis of the 29,000 dalton protein product of *ermC*, has been shown to be induced by erythromycin. Weisblum and his colleagues have established that macrolide resistance is associated with a specific dimethylation of adenine in 23 S rRNA. We show that pE194 specifies an RNA methylase that can utilize either 50 S ribosomes or 23 S rRNA as substrates. Synthesis of this methylase is induced by low concentrations of erythromycin, and the enzyme is produced in elevated amounts by strains carrying a high copy number mutant of pE194. The methylase comigrates with the 29K *ermC* product on polyacrylamide gels. The purification and some properties of this methylase are described.

INTRODUCTION

Bacterial resistance to the medically and agriculturally important macrolide-lincosamide-streptogramin B (MLS) group of antibiotics is associated with a specific dimethylation of adenine in 23 S rRNA (1-3). Ribosomes containing such methylated RNA bind MLS antibiotics with reduced affinity, and are hence presumed to be less sensitive to inhibition by these agents *in vivo*. Resistance to the antibiotic thiostrepton is also associated with extra methylation of rRNA (4, 5), whereas kasugamycin resistance is attributed to undermethylation of 16 S rRNA (6, 7). Taken together, these antibiotic resistance and sensitivity mechanisms constitute an interesting set of rRNA modifications, and the enzymes responsible for rRNA methylation might prove to be useful reagents for probing ribosomal structure-function relationships. The kasugamycin related methylase has been isolated and studied (8, 9), but the methylase associated with MLS-resistance has not yet been described.

pE194 is a 3.5 kilobase (kb) multicopy plasmid, derived from *Staphylococcus aureus* and introduced by transformation into *Bacillus subtilis* (10). The resistance conferred by the pE194 MLS resistance determinant, *ermC*, can

be induced by subinhibitory concentrations of erythromycin (Em), a macrolide antibiotic. Also inducible by Em is a 29,000 dalton protein which has been shown to be the product of ermC (11,12). Plasmid mutants (tyc) which confer resistance constitutively express the 29K protein at an elevated level without induction. The ermC gene, together with sequences responsible for inducibility has been localized on the physical map of pE194, the direction of transcription has been determined, and the ermC gene and flanking DNA regions have been sequenced (12,13,14). Recently we have reported that the induction of the 29K ermC product is regulated posttranscriptionally and that interaction of Em with a binding site on a sensitive (undermethylated) ribosome is required for induction (15). Based on these findings, a model for ermC regulation has been proposed (13,14,16). It appeared likely that the 29K protein is an adenine methylase specific for 23 S rRNA. Isolation and characterization of this methylase would clearly be an important step in furthering our understanding both of ermC regulation and the mechanism of MLS-resistance.

In this report we describe the purification of an Em-inducible, pE194 specified methylase which acts on 23 S rRNA as well as on 50 S ribosomes. We present evidence that the reported 29K ermC product is, in fact, the 23 S rRNA methylase.

MATERIALS AND METHODS

[Methyl-³H]AdoMet (70.8 Ci/mMol) was purchased from New England Nuclear, and AdoMet from Sigma. AdoMet was freed of contaminating S-adenosylhomocysteine (AdoHcy) by passage through cellex-P (17). DEAE-Sephadex A-50 and phosphocellulose P-11 were from Pharmacia and Whatman, respectively. Escherichia coli ribosomes, which had been washed with 1 M NH₄Cl, were a gift from Dr. Huey Yang.

Strains, media and growth conditions

The B. subtilis strains used in this study were BD81 (cysA14), BD170 (trpC2 thr-5), BD430 (trpC2 thr-5, pE194), BD431 (trpC2 thr-5, pBD15), and BD488 (trpC2 thr-5, pBD16). pBD15 and pBD16 are derivatives of pE194 carrying the cop-6 and tyc-1 mutations, respectively (10).

B. subtilis strains were grown in VY broth (18) to late logarithmic phase for the isolation of ribosomes, and to mid-log for the induction experiments. For enzyme purification, bacteria were grown in YPT medium (19) containing 2.5% Tryptose (Difco), 2% yeast extract (Difco), 0.3% K₂HPO₄ and 3% glucose, at 34.5°C with vigorous aeration in a Lab Line Hi-density fermentor.

Em (0.05 $\mu\text{g/ml}$) was added when a Klett reading of about 500 was reached, and growth was continued to a Klett reading of 850, at which point the culture was harvested. In YPT medium, under these conditions, stationary phase is reached at a Klett reading of about 1000. The cells were resuspended and washed twice with Buffer A (20 mM Tris-HCl, 1 mM EDTA, 60 mM NH_4Cl , 6 mM β -mercaptoethanol (βME), 10% glycerol, 2 mM p-methyl sulfonyl fluoride (pMSF)) + 1 M KCl, and once with Buffer A + 0.05 M KCl. The pellets were frozen in a dry ice-ethanol mixture and stored at -70°C until needed. The average yield of bacteria under these conditions was 11 grams per liter.

Isolation of ribosomes and rRNA

Salt (1 M NH_4Cl) washed ribosomes were isolated essentially as described by E. Dubnau *et al.* (20). Unmethylated ribosomes were isolated from strains BD81 or BD170. Methylated ribosomes were isolated from strain BD430 after growth in Em (5 $\mu\text{g/ml}$). 50 and 30 S ribosomes were separated in 15-30% sucrose gradients prepared in 0.01 mM MgCl_2 , 10 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl , 6 mM βME . Centrifugation was either at 17,000 rpm for 19 h in an SW27 rotor or at 41,000 rpm for 5 h in an SW41 rotor. In both cases centrifugation was at 5°C . To isolate RNA, pooled 30 and 50 S ribosomes were phenol extracted in the presence of 1% SDS, and the aqueous layers were precipitated with ethanol. The pellets were washed with acetone and the RNA dissolved in 100 mM NaCl, 10 mM NaOAc, pH 5.0.

Assay for methylase activity

Enzyme was assayed by a modification of the procedure described by Poldermans *et al.* (8). The final reaction mixture contained (in a 50 μl volume): 40 mM Tris-HCl (pH 7.5), 4 mM $\text{Mg}(\text{OAc})_2$, 60 mM NH_4Cl , 6 mM βME , 0.02 mM [methyl- ^3H]AdoMet (1.94 Ci/mMol), 15 pMoles of ribosomes, and a suitable amount of enzyme (see below). Crude extracts and high salt washed extracts were assayed in the presence of 6 mM NaCN to inhibit AdoMet decarboxylases (9). After 20 min incubation at 37°C , the reactions were terminated by the addition of an equal volume of assay-buffer-saturated distilled phenol, mixed vigorously and briefly centrifuged to separate the phases. 30 μl was withdrawn from the aqueous phase and mixed with 10 μg bovine serum albumin in 1 ml of water. Two ml of 10%, chilled trichloroacetic acid (TCA) was added and the samples were kept on ice for 10-15 min. They were then filtered through nitrocellulose membrane filters (0.45 μ). The filters were washed with 10 ml of 10% TCA, dried, and [^3H] radioactivity was determined. The counting efficiency was 10.5%. Ribosome-free and enzyme-free blanks were

routinely subtracted from the assay values. The enzyme-free blanks were extremely low (less than 5% of the assay values). The ribosome-free blanks were appreciable (about 20%) only in the assay of crude extracts, probably due to the presence of contaminating ribosomes. The methylation activity was linear in the range from 0.05 to 4.0 units of enzyme per assay. Using 1 unit of enzyme, a linear reaction rate was observed for 40 min. One unit of enzyme activity is defined as the amount of enzyme required for the attachment of 1 pmol of methyl groups/20 min to ribosomes under the conditions of assay described. Protein was determined according to Bradford (21).

Purification of rRNA methylase from BD431 cells

45 grams of *B. subtilis* strain BD431 cells prepared and stored as described above, were resuspended in 90 ml of buffer B (20 mM Tris-HCl, 10 mM Mg(OAc)₂, 6 mM β ME, 10% glycerol, 2 mM PMSF) + 60 mM NH₄Cl. After two passages through a French pressure cell at 20,000 psi, 2 gms of hemoglobin-sepharose (22) and 2 μ g/ml DNase were added, and the preparation was left on ice for 30 min. It was then centrifuged at 30,000 x g for 30 min in a Sorvall RC-2B refrigerated centrifuge. The resulting supernatant, referred to as crude extract, was centrifuged for 4 h at 105,000 x g in a Beckman ultracentrifuge. The ribosomal pellet was resuspended in 80 ml of buffer B + 1 M NH₄Cl, and kept on ice overnight. It was spun at 105,000 x g for 4 h and the supernatant (high salt ribosomal wash) was brought to 60% saturation with (NH₄)₂SO₄ and kept on ice for 2 h, after which it was centrifuged for 20 min at 10,000 rpm. The supernatant from this step was dialyzed overnight against buffer B + 100 mM NH₄Cl, with two changes of buffer. The dialysate was loaded on a phosphocellulose column (1 x 20 cm) that had been equilibrated with buffer B + 100 mM NH₄Cl. After adsorption of the sample, the column was washed with the same buffer until no more A₂₈₀ absorbance was detected in the eluate. A 540 ml linear gradient of NH₄Cl (100-1000 mM NH₄Cl) in buffer B was applied to elute the enzyme. 6 ml fractions were collected and 5 μ l samples were assayed for methylase activity. The enzyme eluted between 530-600 mM NH₄Cl. The active fractions were pooled and dialyzed overnight against buffer B + 50 mM NH₄Cl. The dialysate was loaded onto a DEAE-sephadex A-50 (1 x 8.0 cm) column. The methylase was not adsorbed to the column and appeared in the void volume. Washing with 0.5 M NH₄Cl after this did not elute any more methylase activity. The active fractions were pooled and stored at -70°C with very little loss of activity for up to three months. For electrophoretic analysis of the samples, the

column fractions were concentrated by complete saturation with $(\text{NH}_4)_2\text{SO}_4$, which caused loss of activity.

Electrophoretic procedures

Sample preparations from minicells and polyacrylamide gel electrophoretic procedures have been described (11). Electrophoresis of RNA in 2% acrylamide + 0.5% agarose was essentially by the method of Studier (23).

RESULTS

Methylating activities in crude extracts

Although crude extracts of the plasmid-free MLS-sensitive strain (BD170) contained a low level of methylating activity for BD170 ribosomes, extracts prepared from strains carrying the high copy (cop) mutant and the MLS-constitutive (tyc) derivatives of pE194 exhibited four- to tenfold elevated levels of ribosome methylating activity (Table 1). These activities were measured in extracts of uninduced cultures. Extracts of the strain carrying the cop-6 mutant plasmid (BD431) contained 2.6 times the activity obtained with the tyc mutant plasmid. The low level activity found in extracts of BD170 was the same whether ribosomes from BD170 or from fully induced cultures of BD430 were used as substrate. The elevated methylation levels obtained with the tyc mutant (BD488) extract was only observed when BD170 ribosomes were used as substrate. This has been confirmed using BD430 and BD431 extracts (not shown). We concluded that a pE194-determined activity was present which was specific for unmethylated (MLS-sensitive) ribosomes. In addition, a methylating activity was present which was not plasmid-

TABLE 1. Methylating activity in S30 supernatant fractions.

Enzyme source	Plasmid	Ribosomes used	Enzyme activity (units/mg protein)
BD170	none	BD170	1.2
BD170		BD430	1.2
BD488	pBD16 (<u>tyc-1</u>)	BD170	4.8
BD488		BD430	1.0
BD431	pBD15 (<u>cop-6</u>)	BD170	12.5

Cultures grown in VY to the late log phase without induction were lysed by incubation for 20 min at 37°C with lysozyme (0.5 mg/ml), spun at 30,000 x g for 30 min and the supernatants used for the assays, as described in Materials and Methods.

determined and which acted on ribosomes whether or not they contained the "extra" methyl groups associated with MLS resistance.

Induction of methylating activity by erythromycin

Figure 1 shows that the methylase activities in extracts of BD430 and BD431 were increased by growth in the presence of Em (0.05 $\mu\text{g/ml}$). The activity in the plasmid-free strain (BD170) was not increased. The cop-6 mutant strain (BD431) had higher basal and induced levels than did the strain carrying the wild type plasmid. The strain carrying the tyc-1 mutant plasmid generally exhibited about a twofold higher basal level than the wild type strain and a further slight increase upon induction (not shown). The behavior of the three strains was therefore qualitatively in accord with expectations based on minicell studies of the 29K protein (11). Of particular interest is the approximate congruence of the curves representing the

FIG. 1. Induction of methylating activities by subinhibitory concentrations of erythromycin. Cultures were grown to a Klett reading of 75 in VY. To a portion of each, Em (0.05 $\mu\text{g/ml}$) was added. Time of addition of Em is taken as zero time. Aliquots were withdrawn at the indicated times, chilled, washed, lysed with lysozyme, and the S30 supernatants were tested for enzyme activity as described in Materials and Methods. Solid and dashed lines indicate the presence and absence of Em. (●), BD431; (○), BD430; (Δ), BD170.

uninduced synthesis of enzyme in the cop-6 mutant strain and the induced synthesis in the wild type strain. The extent of this gene dosage effect is thus sufficient to explain the resistance to tylosin conferred by the cop-6 mutant plasmid in the absence of induction (10). Tylosin is a noninducing macrolide.

Sucrose gradient analysis of methylated ribosomes

Ribosomes methylated by these various crude extracts were analyzed by sucrose gradient centrifugation (Fig. 2). The BD170 extracts appeared to methylate the free 30 S ribosomes, whereas the BD488 extract was active on both free 30 S and 50 S ribosomes. When the latter extract was tested on in vivo methylated ribosomes from fully induced BD430 cells, the 50 S ribosome-specific activity was greatly reduced. This experiment served to confirm

FIG. 2. Methylation of ribosomes by crude extracts. Reaction mixtures contained in a 200 μ l volume: 0.13 nMols of ribosomes, 40 mM tris-HCl (pH 7.5), 8 mM $Mg(OAc)_2$, 40 mM NH_4Cl , 6 mM β ME, 0.02 mM $[^3H]$ methyl AdoMet, (70.8 Ci/mMol), and crude enzyme extract (30,000 $\times$ g supernatant) containing 1.3 units of activity. After 20 min incubation at 37°C, the mixtures were chilled and layered on 12 ml 15-30% sucrose gradients (10 mM tris-HCl, pH 7.5, 8 mM $Mg(OAc)_2$, 60 mM NH_4Cl , 6 mM β ME). The gradients were centrifuged for 5 hr at 41,000 rpm in a SW41 rotor at 5°. The fractions were collected from the top, and the A_{260} (●) and the TCA-precipitable radioactivity (○) were determined.

A. BD81 ribosomes methylated with BD488 extract. B. BD81 ribosomes methylated with BD170 extract. C. BD430 ribosomes from a fully induced culture, methylated with BD488 extract.

the presence of two methylating activities and suggested that the pE194-determined activity was specific for 50 S ribosomes. No methylation of 70 S ribosomes was observed.

Purification of the enzyme

The purification of the pE194-specific activity is described in Table 2. Pilot experiments showed that the supernatant fraction following centrifugation at 100,000 x g contained negligible activity, while about half of the activity could be recovered in a 1 M NH_4Cl wash of the ribosome pellet. The supernatant obtained after discarding the material that precipitated at 60% saturation with $(\text{NH}_4)_2\text{SO}_4$, was dialyzed and fractionated on a phosphocellulose column. (Attempts to recover the activity by $(\text{NH}_4)_2\text{SO}_4$ precipitation resulted in major losses of activity.) The elution profile from phosphocellulose is shown in Fig. 3. The methylase activity eluted at 580 mM NH_4Cl . This material was further purified by passage through a DEAE-Sephadex A-50 column. Although the activity did not stick to DEAE-Sephadex A-50, we included this step to get rid of several minor contaminating protein bands detected by electrophoresis. Final purification was about 81-fold with an overall recovery of 8%.

Polyacrylamide gel electrophoresis of the final preparation revealed the presence of a single major band (Fig. 4). Occasionally one or two minor, low molecular weight protein bands have been observed as well (not shown). The purified protein comigrated on SDS-polyacrylamide gels with the 29K protein from a [^{32}S]methionine-labeled minicell extract prepared from a strain carrying pBD15 (Fig. 4).

TABLE 2. Purification of ribosome methylase.

Purification step	Protein (mg.)	Activity (units)	Sp. activity (units/mg.)
S30 supernatant	3818	212960	55.8
High salt ribosomal wash	388.5	117767	303
Ammonium sulfate supernatant (60% saturation)	23.5	82649	669
Phosphocellulose	5.7	23379	4093
DEAE-sephadex A-50	3.8	17143	4511

The assay and the definition of enzyme units are described in Materials and Methods.

FIG. 3. Elution of methylase activity from a phosphocellulose column. Absorbance at 280 nm (●) and methylase activity (○) were determined. Details of column procedure are described in Materials and Methods. NH_4Cl concentrations in the fractions were determined by refractometry.

Characterization of the ribosome methylase

BD170 ribosomes were methylated using the purified enzyme and fractionated on a 15-30% sucrose gradient. Fig. 5 shows that the bulk of the [^3H] methyl radioactivity is associated with the 50 S subunit, demonstrating that the *B. subtilis* 30 S ribosome methylating activity has been removed during purification. When individually pooled 30 S and 50 S ribosomes isolated from 15-30% sucrose gradients containing 10^{-5} M Mg^{++} were used as substrates for the purified methylase, 50 S subunits were found to be the preferred substrates (Table 3). The slight activity obtained with isolated 30 S ribosomes is most likely due to the presence of contaminating 50 S ribosomes. rRNA isolated from 50 S ribosomes was methylated in preference to 16 S RNA. Agarose-acrylamide gel electrophoresis of the rRNA isolated from 50 S ribosomes after methylation *in vitro* showed that the 23 S rRNA was methylated preferentially and that no radioactivity was associated with 5 S rRNA (not shown). Similarly, when rRNA was isolated from unfractionated or from 50 S ribosomes and then methylated with purified enzyme, electrophoretic analysis

FIG. 4. Electrophoretic comparison of *ermC* product and purified ribosome methylase. A [35 S]methionine labeled extract of Em-induced minicells from a strain carrying pBD15 and a sample of purified ribosome methylase were analyzed by electrophoresis on a 15% polyacrylamide gel. (A,a), minicell extract; (B,b), purified ribosome methylase; (C,c), methylase + minicell extract. Lanes A,B, and C show a fluorograph, and a,b, and c the Coomassie blue-stained pattern of the same gel after drying. Two radioactive ink spots used to establish register of the photographs are shown at the top. The photographs were made at the same magnification. The arrow indicates the position of the 29K product of *ermC*.

revealed radioactivity associated only with 23 S rRNA (not shown). Finally, we have observed that *E. coli* ribosomes were a poor substrate (Table 3).

Adenosine (2 mM) inhibited the enzyme activity strongly (Table 4). AMP and ADP had no effect at 2 mM, while ATP was perhaps slightly inhibitory. AdoHcy was found to be a potent inhibitor, the inhibition being complete at 2 mM. Probably, both adenosine and S-AdoHcy interfere with the binding site for AdoMet on the enzyme. Neither Em nor tylosin (Ty) had any effect on the activity. Sulfhydryl reagents (pCMB, iodoacetamide and N-ethyl maleimide) had little or no effect on the activity of the enzyme. The pH optimum for the pE194 ribosome methylase was found to be 7.5. The activities

FIG. 5. Methylation of ribosomes with purified methylase. The reaction mixture was the same as described in the legend to Fig. 2, except that four units of purified methylase were used together with [³H]methyl AdoMet at 1.94 Ci/mMol. The assay mixture was layered on a 38 ml 15-30% sucrose gradient (10 mM tris-HCl, pH 7.5, 8 mM Mg(OAc)₂, 60 mM NH₄Cl, 6 mM βME). Centrifugation was for 19 hr at 17,000 rpm at 5°C in a SW27 rotor. Fraction collection and processing were as described in Fig. 1. (●), A₂₆₀; (○), radioactivity.

were reduced by 85% and 40% at pH 5.6 and 9.5, respectively (not shown). The enzyme was found to be slightly more active in tris-HCl buffer than in phosphate buffers.

DISCUSSION

The ribosome methylase described in this report has been purified essentially to homogeneity (Fig. 4) and has been separated from a *B. subtilis* methylase which acts on 30 S ribosomes (Figs. 1 and 5). This purified methylase appears to be the product of the *ermC* gene of pE194. The evidence leading to this conclusion is as follows. (1) The methylase was detected only in strains carrying pE194 and its mutant derivatives. (2) In a strain carrying

TABLE 3. Substrate specificity of the pE194 ribosome methylase.

rRNA or ribosomes used as substrate	pmol of [^3H -CH $_3$] incorporated/20 min
<u>B. subtilis</u>	
unfractionated ribosomes	1.9
50 S ribosomes	3.5
30 S ribosomes	0.3
Total rRNA	2.0
5 S + 23 S rRNA	9.4
16 S rRNA	0.6
<u>E. coli</u>	
unfractionated ribosomes	0.4

15 pmols of ribosomes or of rRNA were used in each assay together with 2 units of enzyme. Assays were done as described in Materials and Methods.

a high copy number derivative of pE194 (pBD15) an elevated level of methylase was expressed. This behavior is similar to that of the 29K ermC product.

(3) In a strain carrying pBD16, a tyc mutant which expresses MLS-resistance and the 29K protein constitutively, an elevated basal level of methylase was detected. (4) Like MLS-resistance and like the 29K ermC product, increased synthesis of methylase was induced by exposure to a subinhibitory concentration of Em. (5) The methylase and the 29K protein product of ermC comigrated in SDS-polyacrylamide gels. (6) MLS resistance is associated with the addition of methyl groups to 23 S rRNA (1-3). The purified methylase acted on free 50 S ribosomes and 23 S rRNA but not on 30 S ribosomes or 16 S rRNA. (7) Ribosomes methylated in vivo (isolated from strains carrying pE194 following growth in the presence of Em) were not active as substrates for the methylase, indicating that the in vitro and in vivo methylation sites are probably the same.

The substrate specificity of the pE194-methylase is interesting. It acts on free 50 S but not on 70 S ribosomes. Probably access to the methylation site is blocked by association with a 30 S ribosome. Access is not blocked by the presence of Em or Ty even when the Em is present at a concentration about seven-fold higher than that required to bind to 50% of the ribosomes under similar conditions (Table 4) (15). We suggest that the converse is also true: that methylation does not directly block Em binding, but acts by inducing a conformational change in 23 S rRNA. Separation of the

TABLE 4. The effect of substrate analogs and inhibitors on methylation.

Additions	Percent activity
None	100
Adenosine, 0.2 mM	61
2.0 mM	13
5.0 mM	0
AMP, 2.0 mM	100
ADP, 2.0 mM	100
ATP, 2.0 mM	84
Methionine, 2.0 mM	100
AdoHcy, 0.1 mM	50
0.2 mM	20
2.0 mM	0
p-chloromercuribenzoate, 0.5 mM	100
1.0 mM	88
Iodoacetamide, 0.5 mM	94
1.0 mM	94
N-ethyl maleimide, 0.5 mM	92
1.0 mM	76
Erythromycin, 0.7 μ M	100
7.0 μ M	100
Tylosin, 0.48 μ M	100
4.8 μ M	100

Assays were done under standard conditions, as described in Materials and Methods, using 1 unit of enzyme.

methylation and Em-binding site is clearly adaptive, since Em could not act effectively as an inducer of resistance if it prevented the methylase from gaining ready access to the Em-binding site. The substrate specificity of this enzyme (for 23 S rRNA) is observed using free rRNA. Like most enzymes that recognize specific sites on RNA, the methylase probably responds to secondary structure. It follows that the structural features recognized by the ermC methylase in the 50 S ribosome are probably retained by free 23 S rRNA. It has been reported that 23 S rRNA in solution and in the ribosome are folded similarly (24). It will be interesting to understand the basis of the specific interaction of the ermC methylase with its substrate.

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