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A macrolide–lincosamide–streptogramin B resistance determinant from *Bacillus anthracis* 590: cloning and expression of *ermJ*

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The inducible macrolide–lincosamide–streptogramin B resistance determinant, *ermJ*, from *Bacillus anthracis* 590 was cloned in *Escherichia coli* CSH26. The DNA sequence of *ermJ* was similar to that of *ermK* or *ermD* from *B. licheniformis*, suggesting that *ermK*-like genes have been distributed in *Bacillus* strains by transposition. Expression of *ermJ* was achieved in a *B. subtilis* minicell system, and the rRNA methyltransferase product of *ermJ* was purified. The molecular mass of the enzyme was 58 kDa, and it was concluded to be a homodimer. Its biochemical characteristics were different from those of *ermC* methyltransferase.

Introduction

MLS antibiotics (macrolides, lincosamides and substances related to streptogramin B) inhibit protein biosynthesis, their site of action being the 50S ribosomal subunit. Resistance to MLS antibiotics can be brought about by two mechanisms (reviewed by Dubnau 1984): one is through modification of ribosomal proteins L4 or L22 in *Escherichia coli* or L17 in *B. subtilis*; the other is through alteration of the secondary structure of 23S rRNA causing a loss of affinity for MLS antibiotics. The latter involves base substitution by mutation, and results in N^6-mono- or N^6,N^6-dimethylation at a specific adenine residue. If the resistance is caused by alteration of the target site of erythromycin (Em) on 23S rRNA, cross-resistance to MLS antibiotics should be obtained. Resistance through inactivating MLS antibiotics or impermeability to cell membranes has been shown to occur in *E. coli*, *Staphylococcus aureus*, *Staph. epidermidis* and *Streptomyces* spp.; here resistance was limited to structurally related antibiotics (reviewed by Leclercq & Courvalin, 1991).

Mechanisms for regulating expression of MLS resistance genes are classified as translational attenuation (Weisblum, 1985), translational feedback inhibition (Shivakumar *et al.*, 1980) or autoregulation (Breidt & Dubnau, 1990). Translational attenuation is the most important mechanism and has been studied intensively. In the case of *ermC* (Weisblum, 1985) induction occurs, and the process entails activating mRNA encoding a 23S rRNA methyltransferase. Activation involves altering the secondary structure of mRNA, and is brought about by stalling an Em–ribosome complex on a leader region encoding a short polypeptide. The existence of translational attenuation has also been suggested in *ermA* (Murphy, 1985), *ermD* (Gryczan *et al.*, 1984), *ermG* (Monod *et al.*, 1987), *ermSF* (Kamimiya & Weisblum, 1988) and *ermAM* (Horinouchi *et al.*, 1983). In contrast to the translational attenuation associated with most MLS resistance genes, transcriptional regulation has been found in *ermK*, the MLS-resistant element isolated from *B. licheniformis*. Kwak *et al.* (1991) suggested that synthesis of the *ermK* message is initiated constitutively upstream of the proposed ρ factor-independent terminator, but is completed inducibly downstream of this site.

We have isolated an MLS-resistant strain identified as *B. anthracis* from a soil sample in Korea (Choi *et al.*, 1989). By colony hybridization, the MLS-resistant element, designated *ermJ*, has now been cloned from the chromosomal DNA of this strain, and its nucleotide sequence determined. Its leader sequence is compared with those of other *erm* genes and a possible control mechanism for *ermJ* expression is proposed. Expression of *ermJ* was obtained in a *B. subtilis* minicell system. The *ermJ* gene product (rRNA methyltransferase) was

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Abbreviations: Em, erythromycin; MLS, macrolide–lincosamide–streptogramin B; SAM, *S*-adenosylmethionine.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number L08389.

isolated, and its molecular mass and biochemical characteristics were determined.

Methods

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Cloning of ermJ. Total chromosomal DNA isolated from *B. anthracis* 590 was digested with various restriction enzymes and hybridized with a *MspI–MspI* fragment (660 bp) of *ermK* containing the upstream region of the structural genes (Southern, 1975; Kwak *et al.*, 1991). In a *BclI* digest, fragments about 3·0 kb long hybridized with the probe. These fragments were electroeluted and ligated with pBS42 linearized with *Bam*HI. *E. coli* CSH26 was transformed with the recombinant plasmids and the transformants were screened by colony hybridization (Davis *et al.*, 1986) with the same probe used in the Southern procedure. Plasmid DNA isolated from four colonies showing positive signals was used to transform *B. subtilis* UOTO277, and the resulting inducible resistance patterns were compared with those of the original *B. anthracis* 590. The cloned plasmid was named pBA423. For subcloning, *Hin*cII–*Hin*cII (1·4 kb), *Taq*I–*Taq*I (2·0 kb) and *Bam*HI–*Eco*RV (1·8 kb) fragments were isolated by electroelution, ligated with appropriately digested pBS42, and used to transform *B. subtilis* UOTO277 (Gryczan *et al.*, 1978). Transformant colonies were selected after 2 days incubation on LB agar containing Em (10 μg ml^{-1}) and chloramphenicol (10 μg ml^{-1}).

DNA sequencing of ermJ. The nucleotide sequence of the 1·4 kb *Hin*cII–*Hin*cII fragment containing *ermJ* was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). A sequencing system with the pGEM-3Zf phagemid vector (Mead & Kemper, 1988) and the Klenow fragment was used.

Minicell purification. Minicells were purified by the method of Shivakumar *et al.* (1979). *B. subtilis* CU403 transformed with pBA423 was grown at 37 °C in medium GM-1 (Shivakumar *et al.*, 1979) to late exponential phase; then penicillin G (25 U ml^{-1}) was added and incubation continued for 1 h more. The cultures were quickly chilled and subsequent steps were performed at 0 °C. The cells were harvested by centrifugation at 12000 *g* and resuspended with a few ml of GM-1 medium lacking methionine and glucose (wash medium); the resulting suspension was layered on a 5–30% sucrose gradient and centrifuged (Beckman SW28 rotor at 3500 *g*) for 20 min. The cloudy minicell layer was removed with a serum separator and centrifuged for 10 min at 20000 *g*. The pellet was resuspended in 1–2 ml wash medium, layered on another 5–30% (w/v) sucrose gradient and centrifuged as before. The sharper minicell layer was removed, pelleted and resuspended in 2 ml of wash medium; its absorbance at 660 nm was measured. The minicells were repelleted and resuspended in preservation medium (GM-1 containing 10%, v/v, glycerol) at an A_{660} of 2·0. These stock suspensions were quick-frozen and stored at −70 °C.

Incorporation of [^{35}S]methionine into B. subtilis minicells. The frozen minicells (100 μl) were thawed, resuspended in 1 ml GM-1 medium lacking methionine (−Met), and centrifuged at 10000 *g* for 5 min. The minicell pellets were resuspended in 100 μl GM-1 (−Met) medium to which the inducer, Em (0·1 μg ml^{-1}), was added and were incubated at 37 °C for 15 min. Next, 1 μl [^{35}S]methionine [1150 μCi $mmol^{-1}$ (1 Ci = 37 GBq)] was added, and the mixture was incubated for a further 30 min. The reaction was stopped by adding trichloroacetic acid (final concentration 10%, w/v), then 20–30 μl sample buffer (20 mM-Tris/HCl, 8 mM-NaH_2PO_4, pH 7·8, 1%, w/v, SDS, 1%, w/v, β-mercaptoethanol) was added to the reaction mixture. The sample was electrophoresed in a 12% (w/v) SDS-polyacrylamide gel and the dried gel was exposed to X-ray film.

Isolation of 50S ribosomal subunits from B. subtilis. 50S ribosomal subunits were prepared from *B. subtilis* UOTO277 by modifying the methods of Fahnestock *et al.* (1974). 50S and 30S ribosomal subunits were separated from salt-washed 70S ribosomes in 10–30% sucrose gradients prepared in a buffer containing 10 mM-Tris/HCl (pH 7·4), 30 mM-NH_4Cl, 0·3 mM-$MgCl_2$ and 6 mM-β-mercaptoethanol. Centrifugation was carried out at 50000 *g* for 15 h.

Purification of rRNA methyltransferase from B. subtilis transformed with pBA423. The enzyme was purified by the method of Shivakumar & Dubnau (1981). *B. subtilis* UOTO277 transformed with pBA423 was grown in 10 l LB broth to mid-exponential phase and then Em (0·1 μg ml^{-1}) was added. After a further 3 h incubation, cells were harvested and washed twice with buffer A (20 mM-Tris/HCl, pH 7·4, 1 mM-EDTA, 60 mM-NH_4Cl, 6 mM-β-mercaptoethanol, 10% glycerol, 2 mM-PMSF) containing 1 M-KCl, then once with buffer A containing 0·05 M-KCl. The cells (20–30 g) were ground in a mortar with twice their weight of sea sand and resuspended in 80 ml buffer B (20 mM-Tris/HCl, pH 7·4, 10 mM-magnesium acetate, 6 mM-β-mercaptoethanol, 10% glycerol, 2 mM-PMSF) containing 60 mM-NH_4Cl. DNAase (2 μg ml^{-1}) was added to the suspension and the mixture was left on ice for 30 min. It was then centrifuged at 30000 *g* for 30 min. The resulting supernatant (S30 crude extract) was centrifuged for 4 h at 105000 *g*. The ribosomal pellet was resuspended in 50 ml buffer B containing 1 M-NH_4Cl and kept on ice overnight. It

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Description
Bacterial strains	
B. anthracis 590	Source of *ermJ* (this study)
B. subtilis UOTO277	Transformable host for studying expression of plasmid constructs (Band & Henner, 1984)
B. subtilis CU403	Transformable host for minicell study (Reeve *et al.*, 1973)
E. coli CSH26	Transformable host for preparation of *B. anthracis* DNA library for colony hybridization (Miller, 1972)
Plasmids	
pBS42	Plasmid vector for cloning *ermJ* from *B. anthracis* DNA (Band & Henner, 1984)
pBA423	Derived from pBS42: contains 2·9 kb *BclI* fragment of *B. anthracis* DNA with *ermJ* insert (this study)

was centrifuged again (105000 *g* for 4 h) and the supernatant (high-salt ribosomal wash) was brought to 40% saturation with $(NH_4)_2SO_4$. After 2 h at 0 °C, it was centrifuged for 20 min at 12000 *g*. The supernatant from this step was brought to 80% saturation with $(NH_4)_2SO_4$, kept on ice overnight, and centrifuged. The pellet obtained was dissolved in buffer B containing 100 mM-NH_4Cl. The solution was dialysed against buffer B containing 100 mM-NH_4Cl for 48 h at 4 °C. The dialysate was loaded on a phosphocellulose column (1·5 × 24 cm) that had been equilibrated with buffer B containing 100 mM-NH_4Cl. A linear gradient of 200 ml NH_4Cl (0·1–1·0 M) in buffer B was applied to elute the enzyme. Fractions (2 ml) were collected, and 10 µl samples from each were assayed for methyltransferase activity. The active fractions were pooled, concentrated in an Amicon ultra-filtration cell and stored at −70 °C.

Assay for rRNA methyltransferase activity. Enzyme activity was assayed by the method of Shivakumar & Dubnau (1981). The final reaction mixture, containing 40 mM-Tris/HCl (pH 7·5), 4 mM-magnesium acetate, 60 mM-NH_4Cl, 6 mM-β-mercaptoethanol, 0·02 mM-*S*-adenosyl[methyl-^{3}H]methionine (SAM) (10 Ci mmol^{-1}), 15 pmol of 50S ribosomes, and enzyme solution to give a total volume of 50 µl, was incubated at 37 °C for 20 min. One unit of enzyme activity was defined as the amount of enzyme required to introduce 1 pmol of methyl groups per 20 min to 15 pmol of 50S ribosomes under the assay conditions described.

Results and Discussion

Cloning and DNA sequencing of ermJ

Competent cells of *E. coli* were transformed with a ligated mixture of linearized pBS42 plasmids and sized *Bcl*I-digested *B. anthracis* DNA containing a fragment of approximately 3·0 kb that hybridized with *ermK*. By colony hybridization using *ermK* as the probe, four transformants showing positive signals were chosen from the 1000 colonies examined. The recombinant plasmid isolated from cultures of these transformants was used to transform *B. subtilis* UOTO277. *B. subtilis* transformants

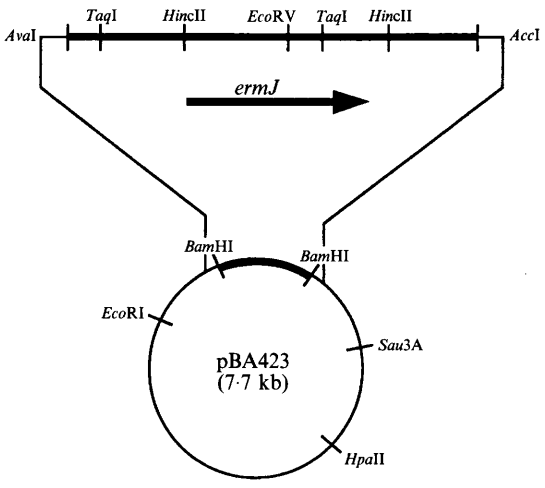

Fig. 1. Restriction map of plasmid pBA423. The thick line indicates cloned *Bcl*I fragments from chromosomal DNA of *B. anthracis* 590. The arrow indicates the direction of transcription of *ermJ*, the MLS resistance determinant.

resistant to both Em and chloramphenicol were chosen. The inducible resistance patterns of these transformants, determined by the agar disk method, were identical to that of the original *B. anthracis* 590. With an Em disk at the centre of each plate D-shaped inhibition zones appeared around disks containing kitasamycin or tylosin. The recombinant plasmid was designated pBA423.

Using information from the restriction endonuclease map of pBA423 (Fig. 1), the various fragments were electroeluted and inserted into the vector pBS42. By transforming *B. subtilis* UOTO277 with the recombinant plasmids, and testing the transformants for resistance to Em and other antibiotics, an MLS resistance gene was located on the *Hinc*II–*Hinc*II (1·4 kb) fragment.

The nucleotide sequence of the *Hinc*II–*Hinc*II fragment was determined (Fig. 2). The 1324 bp DNA fragment contained a small open reading frame (ORF) from nucleotide 70 to 110 encoding a leader peptide of 14 amino acids. A Shine–Dalgarno region (SD-1) was located upstream of the ORF. A large ORF from nucleotide 391 to 1252 was considered to be the *ermJ* structural gene encoding a methyltransferase with a deduced molecular mass of 33 kDa. Another Shine–Dalgarno region (SD-2) was located upstream of this large ORF. When the nucleotide sequence of *ermJ* was compared with the sequences of other *erm* genes, it showed similarity to those of *ermK* and *ermD*, the MLS resistance elements cloned from *B. licheniformis* (Gryczan *et al.*, 1984; Kwak *et al.*, 1991). There are 28 nucleotide and 9 amino acid differences between *ermJ* and *ermK* in a comparison of 1324 nucleotides and 287 amino acids (Table 2). Therefore, the MLS resistance genes are likely to be phylogenetically related. Because the leader sequence of *ermJ* was similar to that of *ermK*, expression of *ermJ* is also likely to be controlled by the transcriptional attenuation mechanism demonstrated for *ermK* (Kwak *et al.*, 1991).

Expression of ermJ in B. subtilis minicells

The *B. subtilis* minicell system was used to identify the polypeptides expressed from *ermJ*. PAGE showed a faint band of 33 kDa in the absence of Em. The intensity of the band increased when the minicells were treated with 0·1 µg Em ml^{-1} (Fig. 3). Since the size of the induced protein corresponded to that deduced from the DNA sequence of *ermJ*, the protein was thought to be the rRNA methyltransferase gene product. Protein bands of 50 and 26 kDa showed the same intensity in the presence and absence of Em. These proteins were presumed to be gene products of pBS42, the vector component of pBA423. The 33 kDa protein product of *ermJ* is evidently produced at a basal level without Em. The presence of low concentrations of Em cause a conformational change

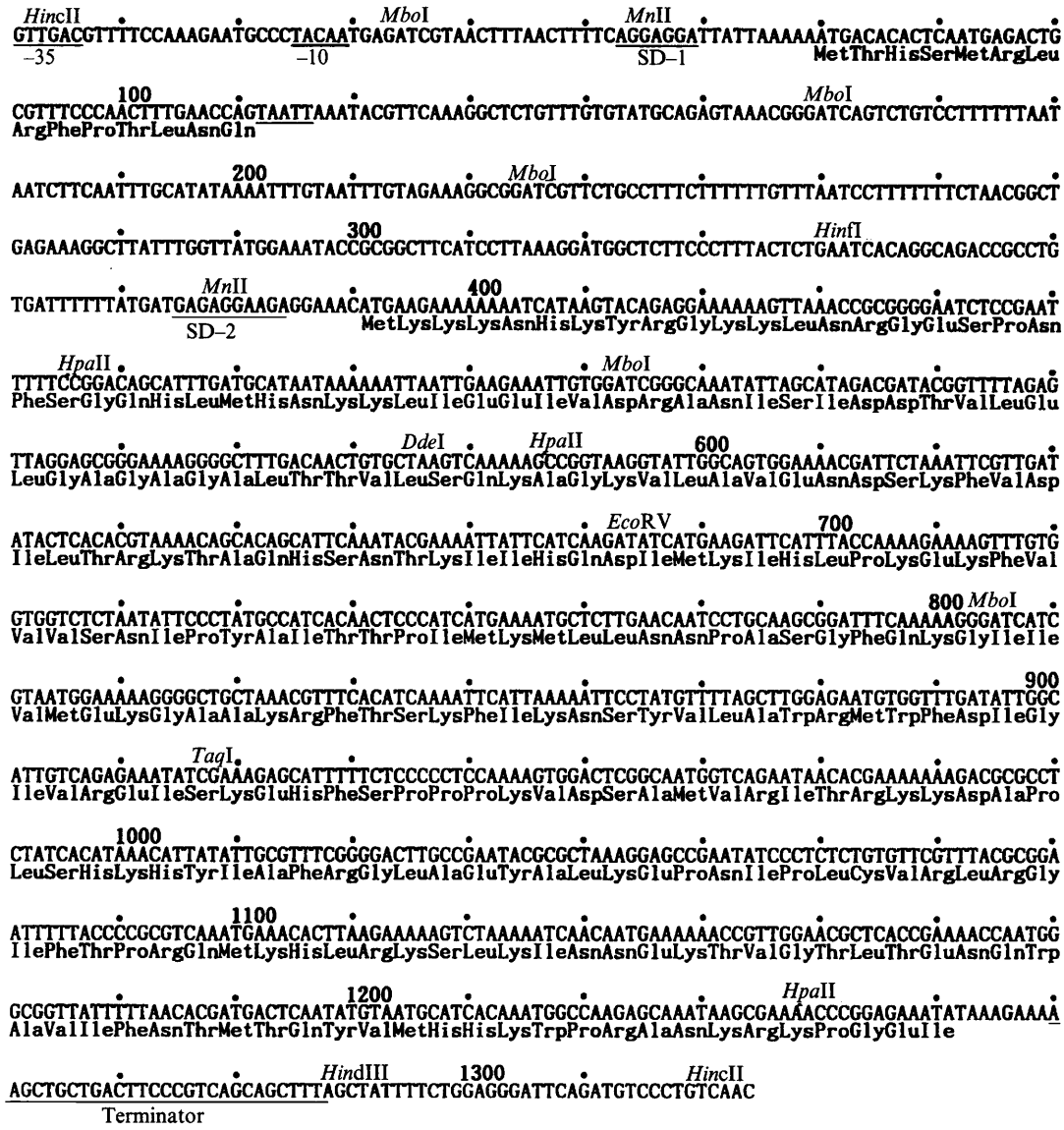

Fig. 2. Nucleotide sequence of *ermJ*. The positions of the *ermJ* promotor (−35 and −10), the probable transcriptional terminator, relevant restriction endonuclease recognition sites, Shine–Dalgarno sequences SD-1 and SD-2 and their associated initiation codons, and the termination codons of the leader peptide and methyltransferase are all underlined.

in the leader mRNA; ribosomes bind to the SD-2 region and increase expression of the rRNA methyltransferase gene.

Purification of rRNA methyltransferase

To further understand the characteristics of the gene product of *ermJ*, rRNA methyltransferase was purified. The amount and activity of protein for each purification step is shown in Table 3. The methyltransferase assay showed negligible activity in the supernatant obtained by centrifugation at 105000 ***g***. Therefore, as reported by Shivakumar & Dubnau (1981), rRNA methyltransferase binds tightly to 70S ribosomes. The enzyme could be separated from the ribosomes by washing with high salt buffer containing 1 M-NH_4Cl. The recovery of methyltransferase activity in the high-salt wash was 50% of that in the S30 extract. The methyltransferase activity in the 40–80% $(NH_4)_2SO_4$ precipitate was only 60%, and the amount of protein was only 5·6% of that in the high-salt wash. The elution profile (Fig. 4) from a phosphocellulose column showed that the methyltransferase was eluted at 0·4–0·6 M-NH_4Cl. The active fractions were collected and concentrated to one tenth volume by ultrafiltration and examined by SDS-PAGE. A single protein band of 33 kDa was identified on the gel (Fig. 5);

Table 2. *Differences in nucleotide and deduced amino acid sequences between ermJ and ermK*

Position		Base (aa) substitution*	
ermJ	*ermK*	*ermJ*	*ermK*
90	471	T (Leu)	G (Leu)
210	591	T	C
305	686	C	A
443	824	C (Ser)	A (Tyr)
558	939	G (Gly)	T (Gly)
571	952	G (Val)	A (Met)
629	1010	A (Asp)	C (Ala)
658	1039	T (Ser)	C (Pro)
1008	1389	T (Tyr)	C (Tyr)
1019–20	1400–1	G, G (Arg)	T, T (Leu)
1023	1404	A (Gly)	G (Gly)
1035	1416	C (Tyr)	T (Tyr)
1051–3	1432–4	A, T (Asn)	C, A (Gln)
1054–5	1435–6	A, T (Ile)	G, C (Ala)
1060	1441	C (Leu)	T (Phe)
1069–70	1450–1	C, G (Arg)	G, C (Ala)
1089	1470	C (Thr)	T (Thr)
1128	1509	C (Ile)	T (Ile)
1203	1584	A (Val)	G (Val)
1209	1590	T (His)	C (His)
1271	1652	T	G
1274	1655	C	T
1287	1671	T	A
1292	1675	A	T

* aa, Amino acid.

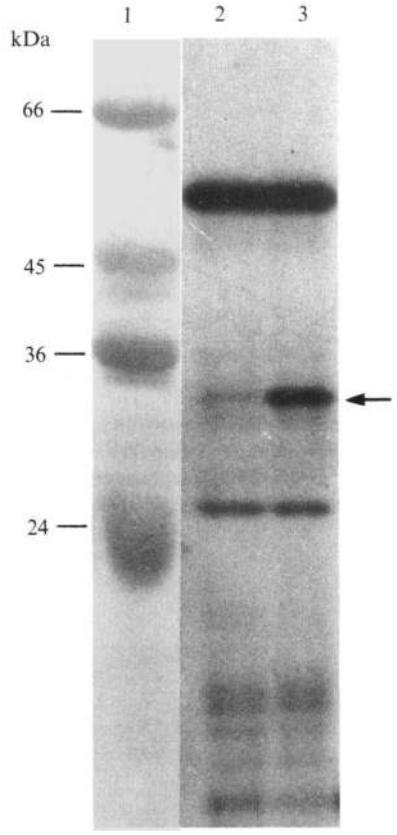

Fig. 3. Autoradiograph of a 12% SDS-polyacrylamide gel of [^{35}S]methionine-labelled minicell products. Lanes: 1, molecular mass markers; 2, products without induction; 3, products after induction with 0·1 μg Em ml^{-1}. The arrow indicates the 33 kDa product of *ermJ*.

this was of the same size as the protein expressed by induction in minicells. Compared with the $(NH_4)_2SO_4$ precipitate, the enzyme in the final eluate showed about 55-fold increase in specific activity and an overall recovery of 6·1%.

Characterization of rRNA methyltransferase

To determine the optimal concentration of Em for induction of *ermJ*, *B. subtilis* UOTO277 transformed with pBA423 was grown to mid-exponential phase, 0·01–10 μg Em ml^{-1} were added to the culture broth, and the cultures were incubated for 3 h more. The activity reached its maximum with 0·01 μg Em ml^{-1} and decreased above 0·1 μg Em ml^{-1}. Therefore, rRNA methyltransferase synthesis is probably induced to a high level at a low concentration of Em, but synthesis of the enzyme itself is inhibited by high concentrations of Em. To identify the optimal culture time after Em induction, 0·01 μg Em ml^{-1} was added to the culture at mid-exponential phase. Assays for methylase activity in the culture at 1 h intervals showed a rapid increase in the first hour, and subsequently a slower increase until the third

Table 3. *Purification of rRNA methyltransferase expressed by ermJ*

Step	Total protein (mg)	Total activity (U)*	Specific activity [U (mg protein)$^{-1}$]*
S30 extract	514	1398	1·2
High-salt wash	104	749	7·2
40–80% $(NH_4)_2SO_4$ precipitate	28·8	432	14·9
Phosphocellulose column eluate	1·3	85·3	65·6

* One unit of enzyme activity is defined as the amount of enzyme required for the attachment of 1 pmol of methyl groups per 20 min to 50S ribosomal subunits under the conditions of the assay.

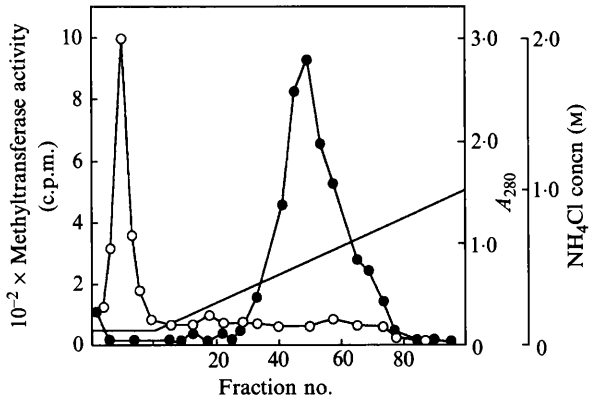

Fig. 4. Elution profile of rRNA methyltransferase during phosphocellulose column chromatography. Material was eluted with a 0·1–1·0 M-NH_4Cl gradient (—) and 2 ml fractions were collected. For each fraction, A_{280} (○) and rRNA methyltransferase activity (●) were determined.

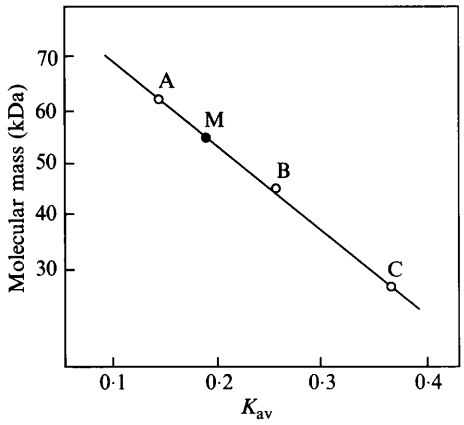

Fig. 6. Molecular mass estimation of rRNA methyltransferase by Sephadex G-100 gel permeation chromatography. Molecular mass markers (○): A, bovine serum albumin (66 kDa); B, egg albumin (45 kDa); C, carbonic anhydrase (29 kDa). M (●), rRNA methyltransferase (58 kDa).

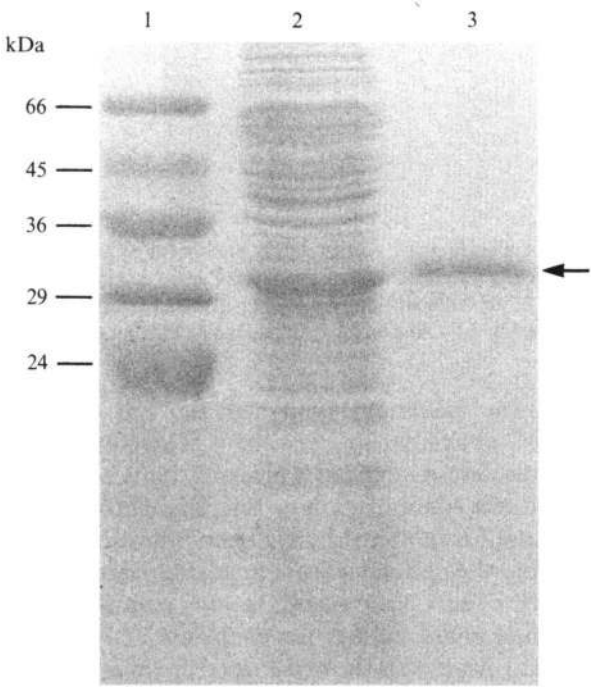

Fig. 5. SDS-PAGE (12%) of proteins from a culture of *B. subtilis* UOTO277 transformed with pBA423. The gel was stained with Coomassie blue. Lanes: 1, molecular mass markers; 2, 40–80% $(NH_4)_2SO_4$ precipitate; 3, rRNA methyltransferase purified by phosphocellulose column chromatography. The arrow indicates the 33 kDa product of *ermJ*.

hour, when it remained constant. Others have invoked feedback inhibition or an autoregulation mechanism to account for the constant level of *ermC* product in bacterial cells (Shivakumar *et al.*, 1980; Gryczan *et al.*, 1978). The optimal culture time for methylase activity, following Em induction of *ermJ* was concluded to be between 1 and 3 h.

The molecular mass of the purified rRNA methyltransferase was determined by gel permeation chromatography using Sephadex G-100. Trypsinogen (24 kDa), egg albumin (45 kDa) and bovine serum albumin (66 kDa) were included as standards. The molecular mass estimated from a log molecular mass versus K_{av} plot was 58 kDa (Fig. 6). Therefore, the enzyme is probably a homodimer of approximately 33 kDa subunits.

The molecular mass discrepancy may be explained by a conformational change during dimer formation. The optimal temperature for the enzyme reaction was 40 °C. Activity was reduced by 90 and 30% at 45 and 60 °C, respectively. Therefore, this rRNA methyltransferase has some thermal stability. The pH optimum for methyltransferase activity was 7·0, and for enzyme storage at 4 °C, pH 5 or 6 was optimum. The enzyme showed little activity loss after 3 months at −70 °C. The above characteristics are somewhat different from those of *ermC* methyltransferase as indicated in Table 4 (Shivakumar & Dubnau, 1981; Denoya & Dubnau, 1987).

To understand the substrate specificity of *ermJ* methyltransferase, the activities of the enzyme on 50S, 30S and 70S ribosomes from *B. subtilis*, and on 50S ribosomes from *B. subtilis* transformed with pBA423 and induced with Em were compared. Three picomoles of C^3H_3 were incorporated into 15 pmol of 50S ribosomes, but only 0·3, 0·6 and 0·4 pmol of C^3H_3 were incorporated into 30S, 70S and methylated 50S ribosomes, respectively. It was concluded that the enzyme methylated 50S ribosomes but not 30S or 70S ribosomes, or methylated 50S ribosomes. Presumably it acted on free 50S ribosomes but not on 70S ribosomes because the methylation site was blocked by 30S ribosomes, as reported for the

Table 4. *Comparison of rRNA methyltransferases expressed from ermJ and ermC*

	ermJ	*ermC*
Molecular mass*	33 kDa	29 kDa
Molecular mass†	58 kDa	NK
Composition	Homodimer	NK
Optimal pH	7·0	7·5
Optimal temperature	40 °C	35–37 °C
K_m for 50S ribosome	210 nM	375 nM
K_m for SAM	3·18 μM	12 μM
Substrate specificity	50S Ribosome	50S Ribosome

NK, Not known.
* Molecular mass of protein deduced from nucleotide sequence.
† Molecular mass of natural form of protein.

ermC enzyme (Shivakumar & Dubnau, 1981). The absence of activity with *in vivo*-methylated 50S ribosomes indicated that *in vivo* and *in vitro* methylation sites are the same. Moreover, since 50S ribosomes prepared from cells expressing *ermJ* were not further methylated *in vitro* by the *ermC* product, the two proteins methylate the same site in 23S rRNA. An enzyme stoichiometry study was performed, and K_m and V_{max} values for each substrate were calculated from a Lineweaver–Burk plot (Segel, 1975). The K_m values for 50S ribosomes and SAM were 210 nM and 3·18 μM, respectively. The *ermC* enzyme has not yet been structurally analysed, and it would be desirable to characterize the *erm* methylases of phylogenetically-related organisms with a view to protein engineering. The interactions of the enzymes with their substrates, 50S ribosomes or 23S rRNAs, would also be of considerable interest.

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