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Restriction and Modification in *Bacillus subtilis*: Two DNA Methyltransferases with *Bsu*RI Specificity

I. PURIFICATION AND PHYSICAL PROPERTIES*

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Two *S*-adenosyl-L-methionine:DNA(cytosine 5)-methyltransferases, termed M.*Bsu*RIa and M.*Bsu*RIb, were purified 3,000- and 4,000-fold, respectively, from *Bacillus subtilis* strain OG3R (r^+m^+) by successive column chromatography. The molecular weights determined by gel filtration were 37,000 for M.*Bsu*RIa and 40,000 for M.*Bsu*RIb. The sedimentation coefficients $s_{20,w}$ were 3.55 for both enzymes as determined by glycerol gradient centrifugation, corresponding to molecular weights of 43,000. Analysis of the two methyltransferases by agarose gel electrophoresis under native conditions, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, showed correspondence of the M.*Bsu*RIa activity with one protein band at a molecular weight of 41,000, whereas M.*Bsu*RIb activity was associated with two protein bands with molecular weights of 42,000 and 39,000, respectively.

Bacillus subtilis r^+m^+[1] strains R and OG3R express a restriction/modification system whose biological parameters have been described previously (Trautner *et al.*, 1974). The restriction and the corresponding modification enzyme recognize the same symmetrical tetranucleotide sequence 5′GG↓CC in double-stranded DNA (Bron and Murray, 1975). The restriction endonuclease cleaves in the middle of the recognition sequence of unmodified DNA (Bron and Murray, 1975), indicated by the arrow; and the modification methyltransferase methylates the internal cytosine of the sequence to produce 5-methylcytosine (Günthert *et al.*, 1978). The R.*Bsu*RI enzyme has been purified to homogeneity (Bron and Hörz, 1980).

The methyltransferase acts on unmodified or semimodified DNA using *S*-adenosyl-L-methionine as methyl group donor (Günthert *et al.*, 1977), while the restriction endonuclease requires only Mg^{2+} to produce double strand scissions within the recognition sequence of unmodified DNA. Thus, the restriction and modification enzymes of *B. subtilis* strains R and OG3R are defined as class II enzymes.

This paper describes the purification and molecular parameters of two host methyltransferases with the same sequence specificity, and the accompanying paper (Günthert *et al.*, 1981) refers to the physicochemical parameters of these enzymes and their mode of action on the DNA substrate.

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[1] The abbreviations used are: r^+m^+, *Bsu*RI restricting and modifying bacterial genotype; M.*Bsu*RI, bacterial DNA modification enzyme of *B. subtilis* r^+m^+ strains; R.*Bsu*RI, bacterial DNA restriction enzyme of *B. subtilis* r^+m^+ strains; SDS, sodium dodecyl sulfate.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains—*B. subtilis* strains used are listed in Table I.

DNA—Bacterial DNA was isolated as described by Bron *et al.*, 1975. The usual DNA methyltransferase assay was performed with calf thymus DNA (Sigma). To test the methylation specificity of the methyltransferases with respect to the 5′GGCC sequence, modified and unmodified chromosomal DNA (*B. subtilis* SB1207, r^-m^-, and *B. subtilis* TB804, r^+m^+) were used as substrates.

Source of Materials—*S*-adenosyl-L-[methyl-^{3}H]methionine (15 Ci/mmol) was purchased from Amersham. Molecular weight markers for gel filtration and for glycerol gradient centrifugation containing ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and ferritin were obtained from Pharmacia. Low and high molecular weight marker kits for SDS-gel electrophoresis were purchased from Pharmacia and Boehringer. Spectral grade glycerol for glycerol gradient centrifugation was from Merck; polyethylene glycol 20,000 for enzyme concentration and Brij 58 were from Serva; Aquasol scintillation liquid was a product of New England Nuclear. The following materials were used for column chromatography: DEAE-cellulose DE52 (Whatman), phosphocellulose P11 (Whatman), Ultrogel AcA54 gel filtration (LKB), phenyl Sepharose CL-4B (Pharmacia), BioRex 70 (BioRad), and hydroxyapatite Biogel HT (BioRad). Most of the chromatographic runs were monitored with a two-channel UV spectraphotometer from Pharmacia. The chemicals for gel electrophoresis were purchased from BioRad; phenylmethylsulfonyl fluoride was obtained from Boehringer; all other chemicals were analytical grade from Merck.

Methods

DNA Methyltransferase Assay—The standard methyltransferase assay measures the incorporation of [^{3}H]-methyl groups from *S*-adenosyl-L-[*methyl*-^{3}H]methionine into appropriate DNA. The reaction mixture contained for M.*Bsu*RIa, in a total volume of 50 µl: 100 mM Tris-HCl, pH 8.4, 5 mM EDTA, 1 mM dithioerythritol, 0.005% Brij 58, 20% glycerol, 5 µg of DNA, 3.3 µM *S*-adenosyl-L-[*methyl*-^{3}H] methionine, and 5 µl of enzyme in appropriate dilutions. For M.*Bsu*RIb, Tris-HCl was at 440 mM, while for testing both enzymes in the same assay, Tris-HCl was at 270 mM, always at pH 8.4. The purification of the DNA from nonbound radioactivity was performed as described previously (Trautner *et al.*, 1980). One unit of DNA methyltransferase activity is defined as that amount of enzyme which incorporates 1 pmol of methyl groups into DNA within 1 h at 37 °C. The efficiency of counting on GF/C filters in Aquasol was 38% in a Nuclear Chicago Isocap 300.

SDS-Polyacrylamide Gel Electrophoresis—Protein samples (0.5 to 10 µg) in volumes of up to 50 µl were adjusted to 2% SDS, 0.02% bromophenol blue, 1% 2-mercaptoethanol, and heated for 2 min at 100 °C (Lugtenberg *et al.*, 1975). Larger volumes were initially precipitated with 10% trichloroacetic acid and washed with ethanol/ether (1:1). Reference proteins were treated in the same way. Electrophoresis was performed on 1.5-mm thick slab gels for 3 h at 200 V. The gels were prepared as described by Laemmli (1970) with the following modifications: the resolving gel contained 15% (w/v) acrylamide,

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TABLE I
List of B. subtilis strains

Strains	Chromosomal markers and lysogeny	Comments and origin
SB1207	r^-m^- su^{3+} leu^-metB5^- thr^-, SPβ^-	Zahler *et al.*, 1977
TB101	r^-m^- su^{3+} leu^-metB5^- thr^-, SPβ^+	Trautner *et al.*, 1980
R	r^+m^+, SPβ^+	Trautner *et al.*, 1974
OG3R	r^+m^+, SPβ^+	Obtained by transformation of a *B. subtilis* 168 derivative with *B. subtilis* R DNA (Bron *et al.*, 1975)
TB804	r^+m^+ su^{3+} leu^-, SPβ^-	Trautner *et al.*, 1980

TABLE II
Purification of M.BsuRIa and M.BsuRIb from B. subtilis OG3R

Fraction	Step	Protein	Specific activity	Total activity	Recovery
		mg	*10[3] units/ mg protein*	*10[3] units*	%
I	Cell lysate	7350	0.28	2060	65
II	High speed supernatant	5700	0.55	3130	97
III	Ammonium sulfate precipitate	5500	0.58	3200	100
IV	DEAE-cellulose	4200	0.73	3070	96
Va	Phosphocellulose	12	24	290	81[a]
Vb		35	66	2300	
VIa	DNA affinity	1.0	170	170	60[a]
VIb		3.1	560	1730	
VIIa	Gel filtration	0.5	232	116	31[a]
VIIb		1.6	540	865	
VIIIa	Phenyl Sepharose	0.07	1000	70	26[a]
VIIIb		0.45	1670	750	
IXa	BioRex 70	0.04	1200	48	22[a]
IXb		0.41	1600	665	
Xa	Hydroxyapatite	0.02	2100	42	19[a]
Xb		0.18	3300	595	
Xa_{conc}	Polyethylene glycol concentration	0.02	1880	38	16[a]
Xb_{conc}		0.18	2570	460	

[a] Percentage of recovery was calculated for the sum of both enzymes.

0.087% (w/v) *N,N'*-methylenebisacrylamide, and the stacking gel was of 5% (w/v) acrylamide and 0.087% (w/v) *N,N'*-methylenebisacrylamide.

Proteins were stained with 50% methanol, 9% acetic acid, 0.25% Coomassie brilliant blue R-250 (Serva) for 1 h at 37 °C, and destained in 20% methanol, 7% acetic acid, with several changes of destaining solution.

Preparation and Use of DNA Polyacrylamide Agarose—200 mg of calf thymus DNA were suspended in 50 mM Tris-HCl, pH 7.6, and mixed with polyacrylamide and agarose (BioRad) as described by Cavalieri and Carroll (1970) with the modifications of Bron and Hörz (1980). About 250 ml of column material was thus obtained which could be reused three to four times for the purification of about 10 mg of DNA-binding proteins. After each chromatographic run, the column was stripped of any adhering proteins by washing with 2 M KCl, 0.4% sodium azide, 0.1 mM EDTA, 20% glycerol, and kept in the cold. Before the next run, the DNA gel was intensively washed with DNA washing buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM dithioerythritol, 0.1 mM EDTA, 20% glycerol) until no optical density was detectable in the effluent. Maximal flow rates of the column were maintained at less than 13 cm/h.

Glycerol Gradient Centrifugation—200-µl samples each of the concentrated enzyme preparations VIa (27 µg) and VIb (diluted 1:10, 12 µg) were dialyzed against 270 mM Tris-HCl, pH 8.4, 1 mM dithioerythritol, 10% glycerol, and layered on top of 12.5-ml 10–30% (w/v) glycerol gradients (optical grade glycerol in the same buffer). 0.5 mg each of marker proteins aldolase (M_r = 158,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), and chymotrypsinogen A (M_r = 25,000) were layered on two separate gradient tubes. Centrifugation was for 39 h at 40,000 rpm in a Beckman SW41 rotor at 4 °C. Fractions were collected by continuously pumping from the bottom of the tubes and monitoring the marker proteins at 280 nm with the Pharmacia UV monitor. 5 µl of each 0.3-ml fraction from the methyltransferase-containing tubes were assayed for methylating activity.

Agarose Gel Electrophoresis—The electrophoretic analysis of the methyltransferases under native and denaturing conditions was performed as described by Lanka *et al.* (1979). After the first electrophoretic step performed in an agarose tube gel under native conditions, the gel was cut into 38–39 2.6-mm thick slices. The slices were incubated for 16 h at 4 °C in 50 µl of methyltransferase assay buffer for elution of the proteins. Aliquots of 5 µl were assayed for methyltransferase activity with a recovery of activity between 10 and 20%. For detection of protein, a parallel gel was stained with Coomassie blue. Agarose slices were heated in 2% SDS, 1% 2-mercaptoethanol, and 0.03% bromophenol blue for 5 min at 100 °C, loaded onto an SDS-polyacrylamide slab gel, and run as described above.

Determinations of Protein Concentration—Protein determinations were carried out according to McKnight (1977) with bovine serum albumin as standard. Protein concentrations down to 1–2 µg/ml could be easily detected without influence of Tris or thiol-reducing agents.

RESULTS

The Purification Procedure

Strain OG3R, a restricting/modifying derivative of strain 168, lysogenic for SPβ, was the source of methyltransferases whose purification is described in this paper (Table II). For comparative purposes, we have also purified methyltransferases from strain R and TB804. Strain R is the *B. subtilis* strain in which the *Bsu*RI restriction/modification system was originally discovered. Strain TB804 is an SPβ nonlysogenic 168 derivative, which had been transformed by R DNA to an r^+m^+ genotype (see Table I).

We have used calf thymus DNA in all assays of methyltransferase activity. The 5'C*G[2] methylation of this DNA (Doskočil and Šorm, 1962) did not interfere with *Bsu*RI methylation. The specificity of *Bsu*RI methylation was routinely verified in assays by the inability of *Bsu*RI-modified DNA (from strain TB804) to serve as substrate for the methylation reaction. For purification steps 1 to 3, the methyltransferase assay was performed with 270 mM Tris-HCl, pH 8.4, while after separation of the two enzyme activities, they were always tested under their optimal ionic strength conditions. Protease inhibitors were not added to cells, since phenylmethylsulfonyl fluoride neither affected the quantity nor the quality of purified material.

Step 1: Cell Growth and Preparation of Cell-Free Extracts—*B. subtilis* OG3R was grown in 100 liters of TY medium (Rottländer and Trautner, 1970) at 37 °C until the end of the logarithmic phase of growth, yielding 430 g (wet weight) of cells. The cells were concentrated by centrifugation, washed twice in DEK buffer (20 mM Tris-HCl, pH 8.4, 500 mM KCl, 1 mM dithioerythritol, 0.1 mM EDTA, 20% glycerol), and suspended in the same buffer to give a thick cell paste. This and all following steps were carried out at 4 °C. The cells were disrupted by passing them twice through a French pressure cell (Amicon). The cell lysate was diluted with an equal volume of DEK buffer and ultrasonicated to reduce viscosity. Cell debris was removed by centrifugation at 48,000 × *g* for 1 h in a Sorvall SS34 rotor (Fraction I, 650 ml). Ribosomes and membranes of the supernatant were removed by centrifugation for 3 h at 160,000 × *g* in a Beckman 60 Ti rotor. The high speed supernatant was diluted with an equal volume of DE buffer (like DEK buffer, but 20 mM KCl) (fraction II, 1,300 ml), and proteins were precipitated with ammonium sulfate (75% saturation) for 2 h with gentle stirring. The precipitate was collected by centrifugation at 28,000

[2] Asterisk denotes methyl group.

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× *g* for 1 h in a Sorvall GSA rotor and resuspended in DE buffer. This material was dialyzed against three changes of 15 liters of DE buffer (fraction III, 1640 ml), and half of this fraction was used for the further purification. The other half was stored in liquid nitrogen. The data for step I to III in Table II have been corrected accordingly.

Strains R and TB804 were grown in 10 liters each of TY medium yielding 30 g of cells. Preparations of methyltransferase from these cells followed the procedure outlined in Table II, except for a proportional scale-down.

Step 2: DEAE-cellulose Ion Exchange Chromatography—Fraction III (820 ml) was applied to a DEAE-cellulose column (8 × 30 cm). The column was washed with 2 liters of DE buffer and then eluted with a 5-liter linear gradient of KCl (0.02 to 0.5 M) in DE buffer. Methyltransferase activity eluted between 0.15 and 0.3 M KCl in a broad peak. Fractions of this peak were pooled (fraction IV, 2,350 ml) and dialyzed against two changes of 15 liters of PC buffer (16 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4, 1 mM dithioerythritol, 0.1 mM EDTA, 20% glycerol). The DEAE-chromatography was intended to separate the methylating activity from the restriction endonuclease (eluting between 0.3 and 0.4 M KCl, Heininger *et al.*, 1977) and the nucleic acids, which remained bound to the column material under these conditions. The A_{260}/A_{280} ratio did not exceed 1.0 within the methyltransferase peak.

Step 3: Phosphocellulose Ion Exchange Chromatography—Fraction IV (2350 ml) was applied to a phosphocellulose column (6 × 30 cm). After washing the column with 1 liter of PC buffer, a linear 3-liter gradient of KCl (0 to 0.7 M) was applied. Two methyltransferase peaks eluted at 0.21 M KCl (M.*Bsu*RIa) and at 0.32 M KCl (M.*Bsu*RIb) using strain OG3R (Fig. 1*A*). Fractions from the two peaks were pooled and dialyzed against two 10-liter changes of DNA buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM dithioerythritol, 0.1 mM EDTA, 20% glycerol). Fraction Va had 193 ml; fraction Vb had 435 ml. The elution profile of the corresponding material from strain R was very similar (Fig. 1*B*), whereas fractionation of methyltransferase derived from strain TB804 showed only activity at the position of M.*Bsu*RIb (Fig. 1*C*).

Methyltransferases M.*Bsu*RIa and M.*Bsu*RIb were stable in their chromatographic behavior: rechromatography of each of the two enzyme fractions on phosphocellulose columns resulted in homogeneous peaks eluting at their characteristic gradient positions. Following this separation, enzymes characterized as M.*Bsu*RIa and M.*Bsu*RIb behaved identically, irrespective of the strain from which they originated.

Step 4: DNA Affinity Chromatography—Fractions Va and Vb were each applied in parallel to columns for DNA affinity chromatography (4 × 13 cm for Va and 4 × 22 cm for Vb), previously washed with DNA buffer. After washing with 150 or 300 ml of DNA buffer, respectively, the methylating activity was subsequently eluted with a linear gradient of 0.1 to 1.0 M KCl (400 or 800 ml, respectively). Both methyltransferases eluted at 0.24 M KCl. The active fractions were pooled (fraction VIa, 125 ml; fraction VIb, 150 ml). Fractions VIa and VIb were concentrated 20- to 30-fold by dialysis against 30% (w/v) polyethylene glycol 20,000 (in 100 mM Tris-HCl, pH 8.4, 1 mM dithioerythritol, 10% glycerol). The concentrated preparations were dialyzed against two 5-liter changes of UG buffer (50 mM Tris-HCl, pH 8.4, 500 mM KCl, 1 mM dithioerythritol, 20% glycerol). The recovery of methyltransferase activity after concentration was without significant loss of activity.

Step 5: Gel Filtration—The concentrated fractions from the DNA affinity chromatography (VIa_{conc}, 7.3 ml; VIb_{conc}, 5.2 ml) were applied to columns of Ultrogel AcA 54 with a fractionation range of M_r between 5,000 and 70,000. The columns (2 × 75 cm) were then eluted with UG buffer at a flow rate of 15 ml/h. The pooled active fractions (fraction VIIa, 20 ml; fraction VIIb, 20 ml) were dialyzed against two changes of 5 liters of PS buffer (100 mM Tris-HCl, pH 8.4, 1 M KCl, 1 mM dithioerythritol).

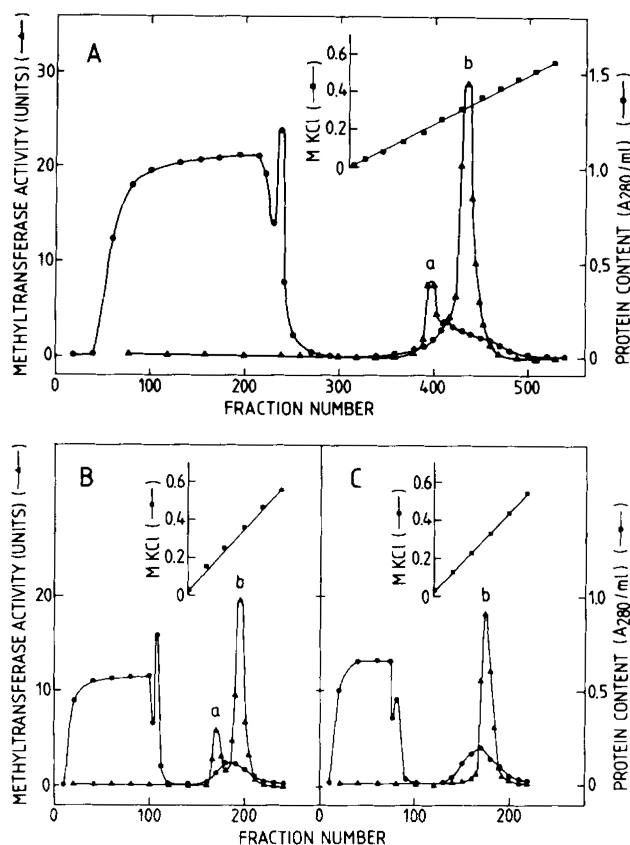

FIG. 1. **Phosphocellulose chromatography of M.*Bsu*RI methyltransferases.** Proteins from purification step IV of Table II were applied to the columns. 13-ml fractions were collected and 5 µl aliquots were tested for methylating activity. *A*, 4200 mg from strain OG3R; *B*, 1100 mg from strain R; *C*, 950 mg from strain TB804 were chromatographed. The column sizes for *B* and *C* were 4 × 24 cm; elution of these columns was with a 1-liter linear gradient from 0.0 to 0.6 M KCl. The positions of M.*Bsu*RIa activity are indicated by *a*, those of M.*Bsu*RIb activity by *b*.

Step 6: Hydrophobic Interaction Chromatography—Fractions VIIa and VIIb were applied to columns of phenyl Sepharose CL-4B. The columns (2 × 3 cm for M.*Bsu*RIa, and 2 × 10 cm for M.*Bsu*RIb) were washed with 20 or 50 ml of PS buffer, respectively, then eluted with a linear decreasing salt gradient of 1 to 0 M KCl, and combined with an increasing linear glycerol gradient from 0 to 50% (30 ml for M.*Bsu*RIa, and 100 ml for M.*Bsu*RIb). M.*Bsu*RIa eluted at 0.42 M KCl; M.*Bsu*RIb eluted at 0.34 M KCl. The active fractions were pooled (fraction VIIIa, 37 ml; fraction VIIIb, 75 ml) and dialyzed against two changes of 2.5 liters of BioRex buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1 mM dithioerythritol, 0.005% Brij 58, 20% glycerol).

Step 7: BioRex Ion Exchange Chromatography—Fractions VIIIa and VIIIb were applied to columns of BioRex 70. The columns (1.6 × 3 cm for M.*Bsu*RIa, and 1.6 × 7 cm for M.*Bsu*RIb) were washed with 15 to 20 ml of BioRex buffer and then eluted with a linear gradient of 0.05 to 0.7 M KCl in BioRex buffer (30 ml for M.*Bsu*RIa and 60 ml for M.*Bsu*RIb). M.*Bsu*RIa eluted at 0.23 M KCl; M.*Bsu*RIb eluted at 0.28 M KCl. The active fractions were pooled (fraction IXa, 11 ml; fraction IXb, 27 ml) and dialyzed against two 2.5-liter changes of HA buffer (16 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4, 100

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mM KCl, 1 mM dithioerythritol, 0.005% Brij 58, 20% glycerol).

Step 8: Hydroxyapatite Chromatography—Fractions IXa and IXb were applied to columns of hydroxyapatite. The columns (1 × 1.3 cm for M.*Bsu*RIa and 1 × 2.5 cm for M.*Bsu*RIb) were washed with 2 to 5 ml of HA buffer and then eluted with a linear salt gradient of 0.04 to 0.4 M KH_2PO_4 in HA buffer (10 ml for M.*Bsu*RIa, and 30 ml for M.*Bsu*RIb). Both methyltransferases eluted at 0.13 M KH_2PO_4. The active fractions were pooled (fraction Xa, 5 ml; fraction Xb, 15 ml) and subsequently concentrated by dialysis against 30% (w/v) polyethylene glycol 20,000 (in 100 mM Tris-HCl, pH 8.4, 1 mM dithioerythritol, 0.005% Brij 58, 10% glycerol). After concentration, the enzyme fractions were dialyzed against the storage buffer (100 mM Tris-HCl, pH 8.4, 1 mM dithioerythritol, 0.005% Brij 58, 50% glycerol) for 3 days (three 5-liter changes). The concentrated enzyme fractions (fraction Xa_{conc}, 0.5 ml; fraction Xb_{conc}, 2.2 ml) were stored at −20 °C. M.*Bsu*RIa lost about 10% activity and M.*Bsu*RIb lost about 30% activity over a period of 3 months.

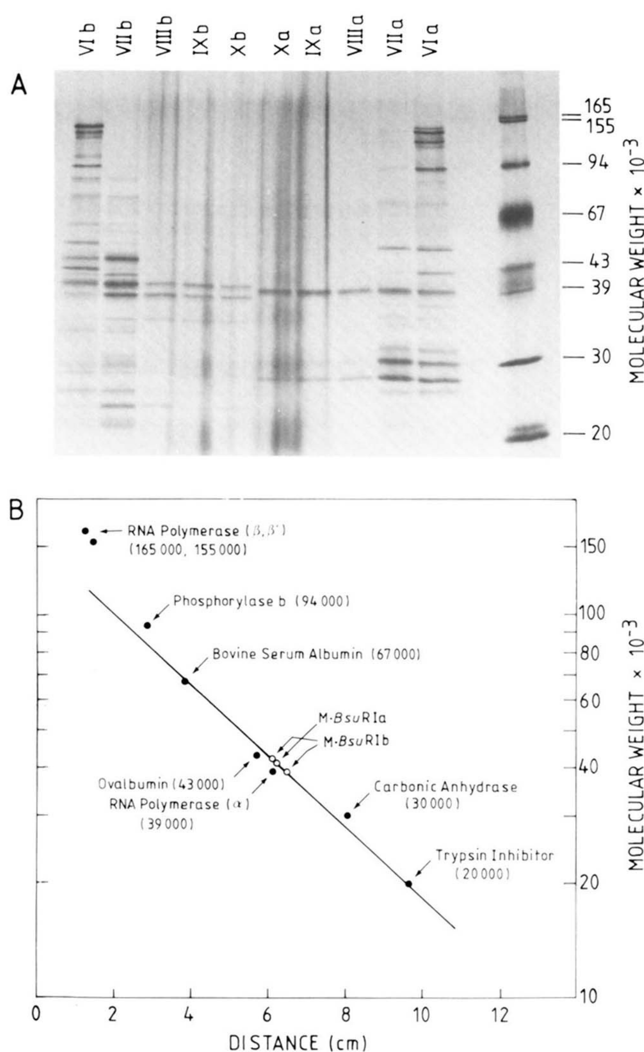

FIG. 2. **Determination of molecular weight of M.*Bsu*RI methyltransferases under denaturing conditions.** *A*, SDS-polyacrylamide (15%) slab gel electrophoresis of different enzyme purification steps from strain OG3R. Each sample, containing approximately 1000 units of methylating activity, was concentrated with trichloroacetic acid and proteins denatured with SDS. *B*, determination of molecular weights of the denatured M.*Bsu*RI methyltransferases, with respect to the indicated molecular weight standards.

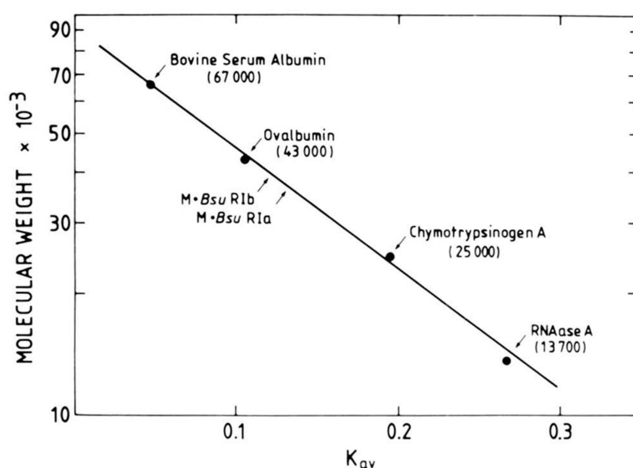

FIG. 3. **Determination of molecular weight of M.*Bsu*RI methyltransferases by gel filtration.** The concentrated enzyme fractions VIa and VIb were applied to columns of Ultrogel AcA54. The columns were calibrated with the indicated standard proteins in varying combinations. Standard proteins were measured by absorbance at 280 nm, and the methyltransferases were tested for activity in 5-μl aliquots of the 4-ml fractions. The partition coefficient (K_{av}) was determined from elution volume.

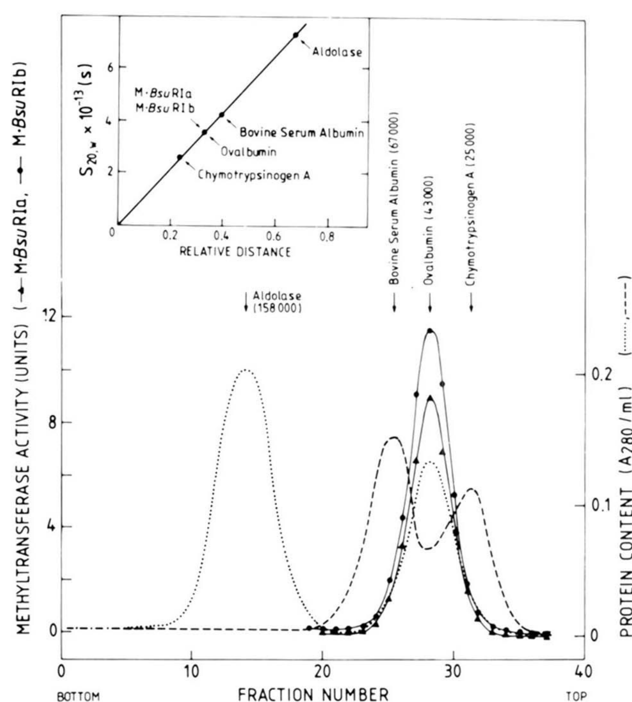

FIG. 4. **Determination of molecular weight of M.*Bsu*RI methyltransferases by glycerol gradient centrifugation.** Aliquots of the concentrated enzyme fractions VIa (27 μg) and VIb (12 μg) were loaded onto 10–30% (w/v) glycerol gradients. Marker proteins were run in parallel tubes (aldolase and ovalbumin, as well as bovine serum albumin and chymotrypsinogen A) and measured by absorbance at 280 nm. 5-μl aliquots of the fractions (0.3 ml) from the enzyme-containing tubes were tested for methylating activity. *Inset*, the sedimentation coefficients $s_{20,w}$ of the marker proteins were plotted against their relative distance in the tube.

Molecular Weight Determinations

SDS-Gel Electrophoresis—To monitor the purification procedure and to determine the molecular weight of the enzymes under denaturing conditions, 1,000 units of methylating activity of fractions VI to X from M.*Bsu*RIa and M.*Bsu*RIb of *B.*

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subtilis OG3R were analyzed by SDS-gel electrophoresis (Fig. 2). Fractions VIIa and VIIb were essentially free of proteins larger than M_r = 70,000 after gel filtration. With phenyl Sepharose chromatography (fractions VIIIa and VIIIb) most of the smaller proteins had been removed. Although about 65% of the total protein was removed during the last two steps (BioRex 70 and hydroxyapatite), there were still minor impurities visible on the gel at M_r = 35,000 in the M.*Bsu*RIb preparation, and at M_r = 25,000 in the *M.Bsu*RIa preparation. With enzyme M.*Bsu*RIa, we observed one major protein band at M_r = 41,000. However, two bands (M_r = 42,000 and 39,000) were found in preparations of M.*Bsu*RIb. The material in both these bands did not decrease during the last five purification steps, showing correlation between methylating activity and amount of stainable material. Besides, the two bands of the M.*Bsu*RIb preparations appeared in equal amounts in the last purification steps.

Gel Filtration—For the determination of native molecular weight and Stokes radius, analytical gel filtration was carried out with the concentrated enzyme fractions VIa (1.0 mg, 7.3 ml) and VIb (3.1 mg, 5.2 ml), respectively. The void volume of the column was established with ferritin (M_r = 450,000), and the column was calibrated with protein markers of known molecular weight. The distribution coefficient k_{av}, as described by Laurent and Killander (1964) was calculated for each protein standard and used to give a standard curve. The apparent molecular weights of the methyltransferases analyzed under these conditions were 37,000 (±3,000) for M.*Bsu*RIa and 40,000 (±3,000) for M.*Bsu*RIb (Fig. 3).

The molecular Stokes radii were also determined graphically by the correlation between partition coefficients k_{av} and the Stokes radii of the marker proteins, as described by Siegel and Monty (1966). The Stokes radius for M.*Bsu*RIa is 2.6 nm; for M.*Bsu*RIb, 2.8 nm. The frictional coefficients f/f_0 were determined from the Stokes radii and the molecular weights obtained by SDS-gel electrophoresis and thus calculated to be 1.1 for M.*Bsu*RIa and 1.2 for M.*Bsu*RIb (Siegel and Monty, 1966). Therefore the shape of the two proteins is almost globular.

Glycerol Gradient Centrifugation—Concentrated enzyme fractions VIa and VIb were subjected to centrifugation in glycerol gradients to determine the sedimentation coefficients of M.*Bsu*RIa and M.*Bsu*RIb (Fig. 4). Both methyltransferases co-sediment with ovalbumin, indicating molecular weights of 43,000 and sedimentation coefficients of 3.55 *S*. Recovery of methylating activity was about 40% in various experiments.

Electrophoretic Analysis under Native and Denaturing Conditions—M.*Bsu*RIa and M.*Bsu*RIb revealed different net surface charges in agarose tube gel electrophoresis. The relative running distance with respect to bromophenol blue of M.*Bsu*RIa was 0.22 for the described conditions, in comparison to 0.17 for M.*Bsu*RIb. Analysis of the protein-containing slices on SDS-polyacrylamide slab gels showed coincidence of the M.*Bsu*RIa activity with the main protein band at M_r = 41,000, while M.*Bsu*RIb activity coincided with the two major protein bands at M_r = 42,000 and 39,000 (*slice 7* in Fig. 5). The proportions of the two bands changed slightly from slice 6 to slice 8 in the agarose gel. Because slice 6 (enriched for the M_r = 42,000 band) was 55% as active as slice 7, while slice 8 (enriched for the M_r = 39,000 band) was only 20% as active as slice 7, the M.*Bsu*RIb enzyme might be represented only by the upper protein band at M_r = 42,000.

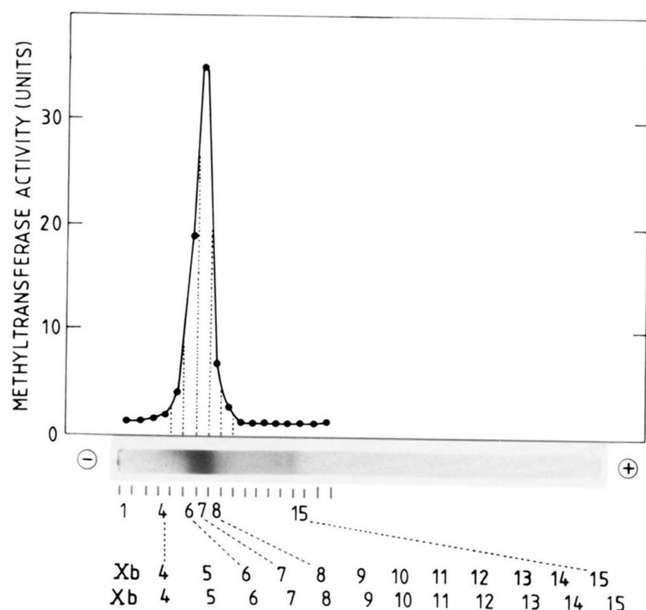

FIG. 5. **Gel electrophoretic analysis of M.*Bsu*RIb under native and denaturing conditions.** 4 μg of the concentrated enzyme fraction Xb were subjected to agarose electrophoresis in a cylindrical gel (0.5 × 10 cm) and cut into 2.6-mm slices. After elution of the slices, 5-μl aliquots were tested for methylating activity, as shown in the *top panel*. A parallel gel was stained with Coomassie blue (*middle panel*). Slices 4 to 15 and 2.5 μg of fraction Xb were electrophoresed on a SDS-polyacrylamide (15%) slab gel (*bottom panel*).

DISCUSSION

The results reported here document the purification of cytosine methyltransferases from *B. subtilis* r^+m^+ strains with high specific activity and high recovery rates. Whereas analysis of restriction revealed a straightforward situation in which the potential of a cell to restrict could be attributed to one enzyme whose presence was correlated with the r^+ genotype of *B. subtilis* (Bron *et al.*, 1975), the expression of modification is more complicated.

Obviously, more than two genes can define the modification potential of a *B. subtilis* cell: chromosomal genes, and as we discovered while this work was in progress, genes specifying *Bsu*RI modification located in prophages. Methyltransferase genes were, *e.g.* identified in phage SPβ (Trautner *et al.*, 1980), which prevails in virtually all *B. subtilis* 168 strains, and also in phage ϕ3T (Noyer-Weidner *et al.*, 1981).

In line with this, a variety of distinct methyltransferases has been discovered, the correlation of which to genetic determinants remains to be analyzed. From the data of Fig. 1, which demonstrate that M.*Bsu*RIb is present in the SPβ nonlysogenic strain TB804, we conclude that this enzyme must be chromosomally determined and not be encoded by SPβ. This also appears to be true of M.*Bsu*RIa. Neither this enzyme nor M.*Bsu*RIb could be detected in the purification of methyltransferases from strain TB101 (r^-m^-, SPβ lysogenic). In-

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stead, such cells produce a methyltransferase after the induction of SPβ with 5′GGCC specificity which is readily distinguishable in its physical and catalytic properties from the enzymes described here.[3] Purification of such SPβ-coded methyltransferase(s) is presently in progress in our laboratory. The absence of such SPβ-coded enzyme(s) in the preparations analyzed here indicates that no induction leading to expression of the SPβ message has occurred during the growth of OG3R and R cells. Neither of the enzymes discussed could be isolated from the r^-m^- SPβ nonlysogenic strain SB1207.

We do not know yet whether processing of one and the same protein is involved in the generation of M.*Bsu*RIa and M.*Bsu*RIb, or whether they are encoded by two separate genes. The latter situation would imply that strain TB804 is distinct in its modifying genotype from strains R and OG3R. The purification of two DNA methyltransferases from *Haemophilus parainfluenzae* with the same specificity (M.*Hpa*II) was recently reported by Yoo and Agarwal (1980). They discussed the possibility that one of the enzymes is the precursor form of the other. This question, related to M.*Bsu*RIa and M.*Bsu*RIb, is further investigated in the subsequent paper in which we compare various reaction parameters of the two purified enzymes (Günthert *et al.*, 1981). An answer concerning the identity of the two enzymes is important also to explain the observation that the proportion of M.*Bsu*RIa and M.*Bsu*RIb varied with the growth phase of cells. In early exponentially growing cells, <5% of the total methylating activity was in the form of M.*Bsu*RIa, with this proportion increasing to 20–30% in stationary phase cells.[4]

We are also concerned with the fact that the purification process of M.*Bsu*RIb leads to the enrichment of two protein bands as analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Because of the close correspondence of molecular weights of the active M.*Bsu*RIb enzymes in gel filtration and sedimentation analysis (Figs. 3 and 4), and the two protein bands observed under denaturating conditions, these cannot represent subunits of a dimeric enzyme. Therefore, there are two alternative ways to interpret our observations. 1) The two bands represent unrelated proteins which fortuitously cochromatograph. Of these, only one band represents the modification enzyme. 2) The two proteins are related. Both may be enzymatically active. The slight difference in molecular weight arises as a consequence of intracellular processing.

Further work is in progress to decide between these alternatives and the question raised before of whether M.*Bsu*RIa and M.*Bsu*RIb are structurally related. Both peptide mapping and immunological tests will be applied.

[3] U. Günthert and M. Freund, unpublished experiments.

[4] U. Günthert, S. Jeep, and M. Freund, unpublished experiments.

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