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Purification and Characterization of a Kanamycin Nucleotidyltransferase from Plasmid pUB110-Carrying Cells of *Bacillus subtilis*

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The nucleotidyltransferase encoded by plasmid pUB110 was purified to greater than 95% purity with a 33% yield. The enzyme is a monomeric protein with a molecular weight of 34,000. The optimum pH for activity is 5, and the optimum $MgCl_2$ concentration for activity is 18 mM. The enzyme, which is synthesized constitutively, is stable for several weeks at 4°C. This enzyme would appear to be a good model gene product for the development of a pUB110 deoxyribonucleic acid-dependent in vitro protein-synthesizing system from *Bacillus subtilis*.

Plasmid pUB110 confers kanamycin resistance on *Bacillus subtilis* (6, 7) and has been the subject of investigation recently as a molecular cloning vehicle (7, 11). The number of copies of pUB110 can be amplified by growth of certain DNA temperature-sensitive mutants of *B. subtilis* containing pUB110 at nonpermissive temperatures (18) or by growth of pUB110-containing strains of *B. subtilis* in the presence of 0.2 M hydroxyurea (17). The culture conditions allow the isolation of large quantities of pUB110 plasmid DNA. This fact suggested to us that pUB110 DNA could be used as a suitable template for our RNA polymerase studies (3, 4) and that it could potentially serve as a model template for a DNA-dependent *B. subtilis* in vitro protein-synthesizing system. The development of such a system would be useful in future studies on the regulation of gene expression from specific *B. subtilis* DNA fragments containing genes for growth or sporulation (3, 4).

As an approach to these studies, we have examined one of the products of the pUB110 genome which is coded by the kanamycin resistance gene. The results show that the pUB110 kanamycin gene codes for a nucleotidyltransferase which transfers a nucleotide residue to the hydroxyl groups of amino glucose moieties of kanamycin (2) and inactivates the antibiotic. The enzyme can be purified by a relatively simple procedure in good yield, is assayed readily, and is quite stable in storage. The enzyme also is a monomeric low-molecular-weight protein and thus has all the properties favorable for its synthesis in vitro.

MATERIALS AND METHODS

Bacterial strain and media. *B. subtilis* NIG1121 *met his* (15) was transformed with pUB110 isolated from OSB529(pUB110) to *B. subtilis* NIG1121 *met his*(pUB110). For the isolation of enzyme, the cells were grown in veal infusion broth (Difco Laboratories) containing 5 μg of kanamycin per ml at 37°C for 18 to 20 h.

Enzyme assay. The method described by Benveniste and Davies (1) was used to assay acetylase activity. The nucleotidyltransferase and phosphotransferase were assayed as described by Ozanne et al. (14). For the nucleotidyltransferase assay during enzyme purification, the following components were present in a final volume of 20 μl: 0.86 mM kanamycin, 0.2 mM and 1 μCi of [^{3}H]ATP, 4 mM $MgCl_2$, 10 mM mercaptoethanol, 62.5 mM Tris-maleate buffer (pH 6.25), and 8 μl of enzyme. The reaction was incubated at 37°C for 10 min. A 5-μl sample of the reaction mixture was placed on phosphocellulose paper (7 mm by 7 mm), washed with 50 ml of distilled water, dried, and counted in a scintillation spectrometer. The reaction was linear for 20 min with suitable enzyme concentrations and was completely dependent on the presence of kanamycin and enzyme.

Polyacrylamide gel electrophoresis. The sodium dodecyl sulfate-polyacrylamide gel method of Laemmli (12) was used for the analysis of nucleotidyltransferase as was the nondenaturing gel method of Hedrick and Smith (10). The molecular weight standards used were bovine serum albumin, *B. subtilis* RNA polymerase δ and σ factors, ovalbumin, aldolase, carboxypeptidase A, DNase I, chymotrypsinogen A, trypsin, RNase A, hemoglobin, and lysozyme.

Protein assay. Protein concentration was determined by the method of Sedmak and Grossberg (16).

Buffers. Buffer A contained 30 mM Tris-hydrochloride (pH 7.5), 1 M KCl, and 2 mM EDTA. Buffer N contained 20 mM Tris-hydrochloride (pH 7.5), 20 mM $MgCl_2$, 10% glycerol, 0.2 mM dithiothreitol, and 50 mM NaCl.

Reagents. [^{3}H]ATP, [^{3}H]GTP, and [^{3}H]UTP were obtained from New England Nuclear Corp.; phenylmethylsulfonyl fluoride and kanamycin sulfate were obtained from Sigma Chemical Co.; diisopropylfluorophosphate was obtained from Aldrich Chemical Co.; and dithiothreitol was obtained from Calbiochem.

RESULTS

Kanamycin-modifying activity in crude extracts. The crude extracts from *B. subtilis* NIG1121 *met his*(pUB110) were tested for three potential kanamycin modification activities, including acetylase, nucleotidyltransferase, and phosphotransferase. The results in Table 1 indicate that only the nucleotidyltransferase activity was increased in extracts of cells carrying pUB110. The enzyme is present in cells grown either in the presence or absence of kanamycin. The enzyme has been found in mid-log, late log, and stationary-phase cells. The presence of pUB110 did not affect the sporulation properties of this strain. Furthermore, when the substrate specificity of the enzyme was examined in the crude extracts, it was shown that the enzyme could use either ATP, GTP, or UTP (Table 2). Other nucleotides were not tested. All subsequent studies were carried out with ATP as the substrate.

Purification of the nucleotidyltransferase. A purification procedure for nucleotidyltransferase was developed to study the properties of the purified enzyme. A typical purification experiment will be described for 10 g (wet weight) of cells. The cells were washed four times with buffer A containing 2 mM phenylmethylsulfonyl fluoride and then three times with buffer N containing 2 mM phenylmethylsulfonyl fluoride by centrifugation. The cells were then suspended in 50 ml of buffer N plus 2 mM phenylmethylsulfonyl fluoride and broken by passage through a French pressure cell three times. To the crude extract was added 25 ml of buffer N plus 2 mM phenylmethylsulfonyl fluoride, and the extract was centrifuged at 13,000 rpm for 30 min (Sorvall SS-34 rotor). To the crude supernatant (69 ml) was added diisopropylfluorophosphate to a final concentration of 5 mM, and the mixture was kept at 0°C for 20 min. This step served to inactivate serine proteases. The crude supernatant was then loaded onto the first DEAE-cellulose column (2.5 by 28.5 cm) and eluted with a 500-ml linear gradient of NaCl from 0 to 0.2 M. The peak activity fractions were pooled and dialyzed against buffer N and then loaded onto a second DEAE-cellulose column (2.5 by 15 cm) and eluted with a 1,000-ml linear NaCl gradient from 0 to 0.3 M. The peak activity fractions from this second DEAE-cellulose column were pooled and dialyzed against buffer N without the glycerol, and then the protein was precipitated with 70% ammonium sulfate.

TABLE 1. *Assays for aminoglycoside-modifying enzymes of pUB110*

Enzyme assayed	Extract −pUB110[a] (cpm)	Extract +pUB110[a] (cpm)	+pUB110/ −pUB110
Acetylase	290	280	0.96
Nucleotidyltransferase	21	201	9.41
Phosphotransferase	34	31	0.90

[a] −pUB110 and +pUB110 indicate the crude extracts prepared from cells without and with pUB110, respectively. Cells from late log phase cultures grown in Penassay broth (Difco) containing 5 μg of kanamycin per ml were collected by centrifugation, suspended in 1:50 volume of 50 mM Tris-hydrochloride buffer, pH 7, and disrupted by sonic oscillation; the supernatant obtained after centrifugation of the crude extract at 8,000 × *g* for 10 min was used for the enzyme assays. [^{14}C]acetyl coenzyme A, [^{14}C]ATP, and [γ-^{32}P]ATP were used for the acetylation, nucleotidylation, and phosphorylation assays as described in the text.

TABLE 2. *Substrate specificity of pUB110 nucleotidyltransferase*

Substrate	Extract, −pUB110[a] (cpm)	Extract, +pUB110[a] (cpm)	+pUB110/ −pUB110
[^{3}H]ATP	108	2,381	22
[^{3}H]GTP	60	1,019	17
[^{3}H]UTP	82	2,554	31

[a] Crude extracts were prepared as described in Table 1, footnote *a*.

The ammonium sulfate precipitate was collected by centrifugation and suspended in 5 ml of buffer N, dialyzed against buffer N, and then loaded onto a Sephadex G-75 column. The enzyme was eluted with buffer N, and the peak activity fractions (Fig. 1, fractions 42 through 54) were pooled and concentrated by a collodion bag apparatus (Schleicher & Schuell Co.).

A summary of the purification results is shown in Table 3. A 75-fold purification was obtained with a yield of 33%. In Fig. 2 is shown sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of the enzyme at various steps of purification. After the Sephadex G-75 column purification step, the enzyme was greater than 95% pure when analyzed by gel electrophoresis (Fig. 2, fractions 44+). The enzyme was quite stable when kept at 4°C with no loss of activity for several weeks, and the enzyme could be kept at −20°C for several months.

Properties of the enzyme and the reaction. The analysis of the purest preparations by nondenaturing gel electrophoresis revealed only one major band (Fig. 3) which had a mobility between that of *B. subtilis* RNA polymerase σ factor (molecular weight, 55,000) and δ factor (molecular weight, 21,000), when examined at various gel concentrations (1). The molecular weight of the enzyme polypeptide, as determined by the sodium dodecyl sulfate-polyacrylamide

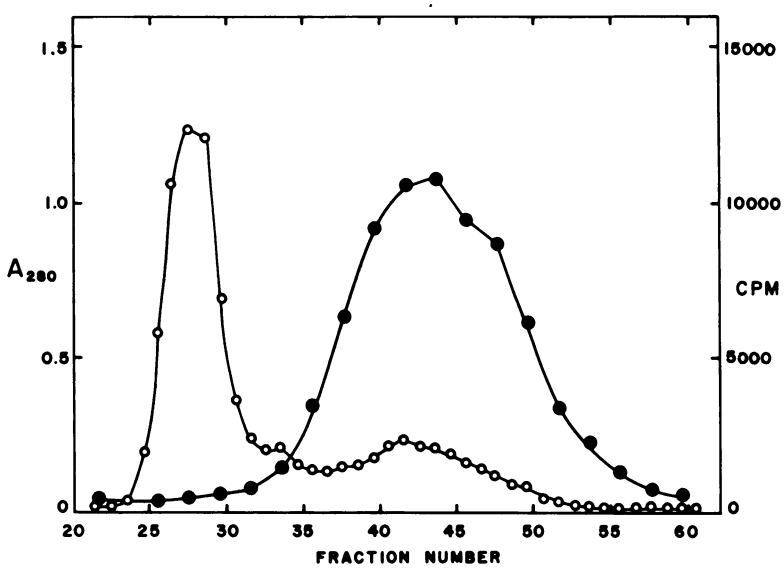

FIG. 1. *Purification of nucleotidyltransferase: Sephadex G-75 column. The peak activity fractions (no. 71 through 96) from the second DEAE-cellulose column were pooled and precipitated with 70% ammonium sulfate, concentrated by centrifugation, suspended in 5 ml of buffer N, dialyzed against buffer N, and loaded onto the Sephadex G-75 column (2.5 by 79 cm). The enzyme was eluted with buffer N and 2.8-ml fractions were collected every 5 min and assayed for A_{280} (○) and activity (●).*

TABLE 3. *Purification procedure for nucleotidyltransferase*

Step	Total protein (mg)	Total activity (units)[a]	Sp act (units/mg)	Purification	Yield (%)
Crude extract	380	2,449	6.4	1.0	100
First DEAE-cellulose column	82	4,530	55.2	8.6	185
Second DEAE-cellulose column	25	2,400	96	15.0	98
Sephadex G-75 column	1.7	816	480	75.0	33

[a] Unit, 1 nmol of AMP-kanamycin formed/10 min.

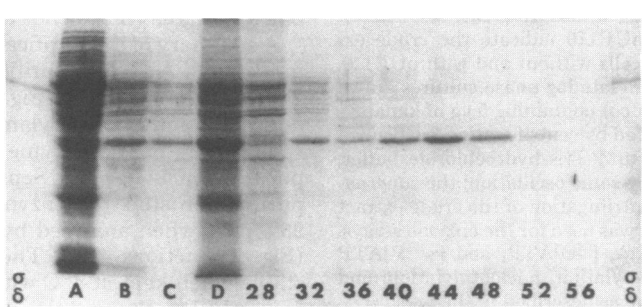

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of nucleotidyltransferase at various steps of purification. The acrylamide concentration was 12%. The sample (25 μl) was combined with 25 μl of sample application buffer, heated, and loaded onto the gel. A, crude extract; B, first DEAE-cellulose column pooled fractions; C, second DEAE-cellulose column pooled fractions; D, 70% ammonium sulfate fraction of the second DEAE-cellulose column pooled fractions; fractions 28 through 56 from the Sephadex G-75 column; and σ (molecular weight, 55,000) and δ (molecular weight, 21,000) RNA polymerase factors from B. subtilis.*

gel electrophoresis method, was 34,000. Therefore, the enzyme appears to be a monomeric protein with a molecular weight of 34,000.

The optimum $MgCl_2$ concentration for the reaction was 18 mM (Fig. 4), and the optimum pH for the reaction was 4.9 (Fig. 5). These two properties of the enzyme should be considered in light of the original purpose for characterizing

this enzyme. If this enzyme is to be synthesized in vitro from a pUB110 DNA-dependent protein-synthesizing system and assayed directly in the reaction mixture, then the $MgCl_2$ concentration and the pH of the in vitro reaction system will have to be changed before assaying for this

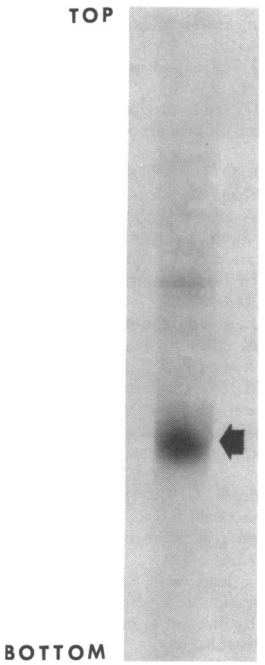

FIG. 3. *Nondenaturing gel electrophoresis of purified nucleotidyltransferase. The acrylamide concentration was 12%. A sample (10.5 μg) of the purified enzyme was loaded on the gel. The proteins moved from top to bottom. The arrow indicates the position of the enzyme.*

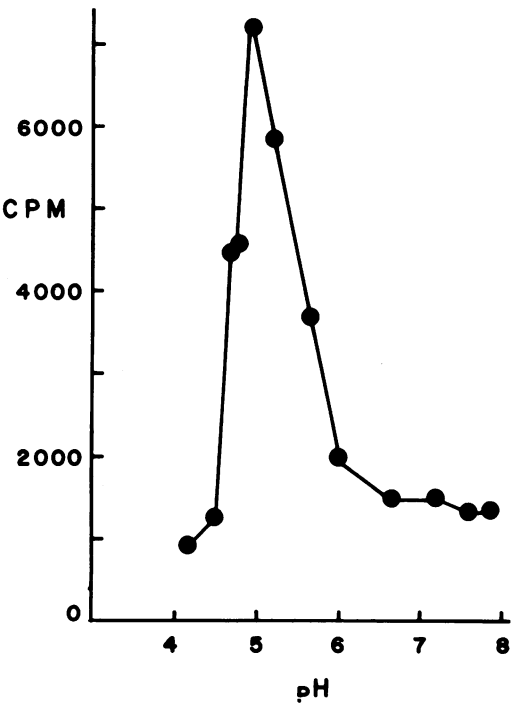

FIG. 5. *The optimum pH for nucleotidyltransferase activity. Trismaleate buffer at various pH's was used for these analyses. The $MgCl_2$ concentration was 18 mM.*

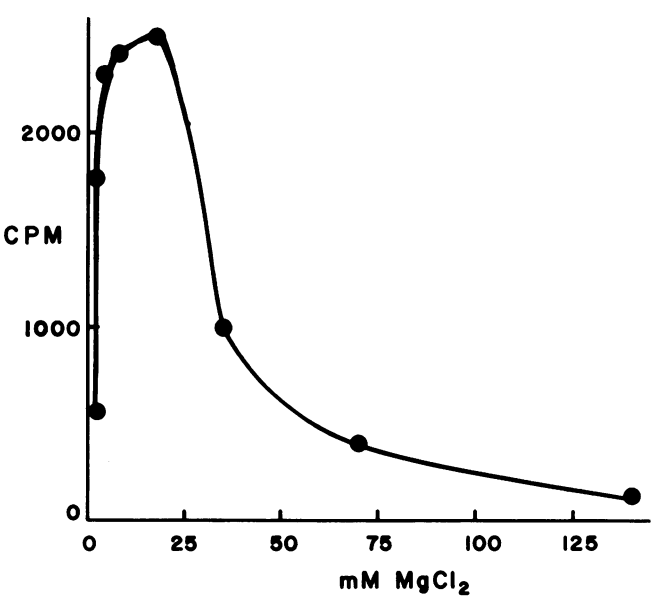

FIG. 4. *The optimum $MgCl_2$ concentration for nucleotidyltransferase activity. The reaction was carried out at pH 5 with various concentrations of $MgCl_2$.*

enzyme, since the $MgCl_2$ concentration and the pH of the in vitro system are usually around 5 mM and 7.6, respectively. The latter conditions are definitely unsuitable for assaying the putative in vitro synthesized nucleotidyltransferase. The effect of salt concentration on enzyme activity was not dramatic, since 200 mM NaCl reduced enzyme activity by only 50%.

DISCUSSION

These studies have shown that the nucleotidyltransferase coded by pUB110 plasmid DNA can be readily purified and characterized. Some of the properties of this enzyme are similar to those reported for a partially purified enzyme from *Staphylococcus aureus* (13), which was the original source of pUB110 (5, 6). Similarities were noted for some of the nucleotide substrates and for optimal pH. However, significant differences were noted during the purification procedure, e.g., we were unable to use a kanamycin-Sepharose 4B affinity column successfully for purification since a large number of contaminating proteins were bound to this column. The knowledge obtained from these studies will be a distinct advantage of future studies, since the enzyme activity has been correlated specifically with plasmid pUB110 and the key parameters of optimum $MgCl_2$ concentration and pH have been determined.

The relative ease of purifying large amounts of this enzyme and several properties of this enzyme make it a suitable candidate for future studies. The low molecular weight, the monomeric composition, the sensitive method for assaying the activity, and the stability of the enzyme indicate that this enzyme could be a useful model gene product for an in vitro DNA-dependent protein-synthesizing system derived from *B. subtilis*. In addition to assaying for enzymatic activity, we should be able to produce antibodies against the enzyme and test for in vitro production of the protein by immunochemical means. The availability of large amounts of pUB110 DNA (17, 18) and of active RNA polymerase from *B. subtilis* (8, 9) should allow such a system to be perfected.

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LITERATURE CITED

1. **Benveniste, R., and J. Davies.** 1971. Enzymatic acetylation of aminoglycoside antibiotics by *Escherichia coli* carrying an R factor. Biochemistry **10**:1787–1796.
2. **Davies, J., and D. I. Smith.** 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32**:469–518.
3. **Doi, R. H.** 1977. Genetic control of sporulation. Annu. Rev. Genet. **11**:29–48.
4. **Doi, R. H.** 1977. Role of ribonucleic acid polymerase in gene selection in procaryotes. Bacteriol. Rev. **41**:568–594.
5. **Ehrlich, S. D.** 1977. Replication and expression of plasmids from *Staphylococcus aureus* in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **74**:1680–1682.
6. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
7. **Gryczan, T. J., and D. Dubnau.** 1978. Construction and properties of chimeric plasmids in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **75**:1428–1432.
8. **Halling, S. M., K. C. Burtis, and R. H. Doi.** 1977. Reconstitution studies show that rifampicin resistance is determined by the largest polypeptide of *Bacillus subtilis* RNA polymerase. J. Biol. Chem. **252**:9024–9031.
9. **Halling, S. M., K. C. Burtis, and R. H. Doi.** 1978. β' subunit of bacterial RNA polymerase is responsible for streptolydigin resistance in *Bacillus subtilis*. Nature (London) **272**:837–839.
10. **Hedrick, J. L., and A. J. Smith.** 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126**:155–164.
11. **Keggins, K. M., P. S. Lovett, and E. J. Duvall.** 1978. Molecular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of vector plasmid pUB110. Proc. Natl. Acad. Sci. U.S.A. **75**:1423–1427.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **LeGoffic, F., A. Martel, M. L. Capmau, B. Baca, P. Goebel, H. Chardon, C. J. Soussy, J. Duval, and D. H. Bouanchaud.** 1976. New plasmid-mediated nucleotidylation of aminoglycoside antibiotics in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **10**:258–264.
14. **Ozanne, B., R. Benveniste, D. Tipper, and J. Davies.** 1969. Aminoglycoside antibiotics: inactivation by phosphorylation in *Escherichia coli* carrying R factors. J. Bacteriol. **100**:1144–1146.
15. **Sadaie, Y., and K. Narui.** 1976. Repair deficiency, mutator activity, and thermal prophage inducibility in *dna-8132* strains of *Bacillus subtilis*. J. Bacteriol. **126**:1037–1041.
16. **Sedmak, J. J., and S. E. Grossberg.** 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. **78**:544–552.
17. **Shivakumar, A. G., and D. Dubnau.** 1978. Differential effect of hydroxyurea on the replication of plasmid and chromosomal DNA in *Bacillus subtilis*. J. Bacteriol. **136**:1205–1207.
18. **Shivakumar, A. G., and D. Dubnau.** 1978. Plasmid replication in DNA Ts mutants of *Bacillus subtilis*. Plasmid **1**:405–416.