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Properties and Developmental Roles of the Lysyl- and Tryptophanyl-Transfer Ribonucleic Acid Synthetases of *Bacillus subtilis*: Common Genetic Origin of the Corresponding Spore and Vegetative Enzymes

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The lysyl-transfer ribonucleic acid synthetase (LRS) and tryptophanyl-transfer ribonucleic acid synthetases (TRS) (L-lysine:tRNA ligase [AMP], EC 6.1.1.6; and L-tryptophan:tRNA ligase [AMP], EC 6.1.1.2) have been purified 60- and 100-fold, respectively, from vegetative cells and spores of *Bacillus subtilis*. There are no significant differences between the corresponding spore and vegetative enzymes with respect to their elution characteristics from columns of phosphocellulose or hydroxylapatite, their molecular weight (~130,000 for LRS and ~87,000 for TRS as determined by gel filtration), their kinetic constants for substrates (in the amino acid-dependent adenosine triphosphate-pyrophosphate exchange reaction), and the kinetics of inactivation by heat and by antibody. The Mg^{2+} requirement for optimal enzyme activity of the corresponding spore and vegetative enzyme differ slightly. Mutants having defective (temperature sensitive) vegetative LRS or TRS activities produce spores in which these enzymes are also defective. The mutant spores are more heat sensitive than the parental type, but contain normal levels of dipicolinic acid. They germinate normally at the restrictive temperature (43 C), but are blocked at specific developmental stages in outgrowth. No modification in temperature sensitivity phenotype occurs during outgrowth, nor is there a change in molecular weight of the two enzymes. The implication is that the LRS and TRS activities of the vegetative and spore stages are each coded (at least in part) by the same structural gene. The temperature sensitivity of mutant spores is discussed with respect to those factors which are involved in the formation of the heat-resistant state.

Any attempt to understand the regulatory controls operating in *Bacillus* sporogenesis must account for both the diversity in spore and vegetative cell activities and, at the same time, provide an explanation for why some spore and vegetative proteins carrying out the same functions are related, but apparently not identical. Outside of two notable exceptions (9, 11), it is uncertain whether these proteins are synthesized by the same structural gene, which might then be subject to changes at a secondary level (translation, assembly, modification, etc.), or whether they represent the products of two different and distinct structural genes. Certainly as Kornberg et al. (17) and Sadoff (29) have pointed out, were the spore and vegetative enzymes distinctive in their primary structure (i.e., determined by separate sporogenic and vegetative genes), then a considerable portion of the genetic information borne by the cell would have to be reserved for the formation of spore-specific products with functions identical to their vegetative homologues. Unless selective pressures forced it to do so, a cell would not conserve so much genetic information. Consequently, one is led to suspect that the majority of spore enzymes would be identical or modified versions of the homologous vegetative activities, a suspicion which has been confirmed for several enzymes (17, 29).

Attention in the past has been directed primarily towards enzymes which participate in metabolic activities relating to energy needs and catabolic processes. In only two cases, that of the deoxyribonucleic acid (DNA) polymerase I (6) and the DNA-dependent ribonucleic acid (RNA) polymerase (22), has a comparison been made of spore and vegetative enzymes which

participate directly in macromolecular synthesis. The roles of another group of proteins, the aminoacyl-transfer RNA (tRNA) synthetases, have recently begun to assume new importance in studies of developmental processes (14, 36, 42). These enzymes are indispensable components of a cell's protein-synthesizing machinery (25), they are intimately involved in the repression regulation of amino acid biosynthetic pathways (24), and they may offer a means for exerting translational control in cell development by restricting the use of particular codons (iso-accepting-tRNAs) to specific periods of the life cycle (36). Because of these proven (and hypothetical) functions, this class of proteins might represent an enzyme system with a unique relationship to the biochemical events in sporulation as well as outgrowth. Genetic alterations in the inherent specificity of an aminoacyl-tRNA synthetase, for either its cognate amino acid or tRNA, can produce drastic results with regard to the structure of the proteins synthesized, and also significantly alter the regulatory properties of the cell (24). Printz and Gross (27) have shown that a mutation resulting in a modified leucyl-tRNA synthetase of *Neurospora crassa* leads to "mistranslation" with the result that a large proportion of the organism's proteins are functionally defective. A mutant derepressed for enzymes of the arginine pathway was found by Williams and Williams (45) to be completely defective in the aminoacylation of one of the iso-accepting arginine transfer RNAs.

This report is a comparative study of two aminoacyl-tRNA synthetases isolated from spores and vegetative cells of *Bacillus subtilis*. The study is confined to the lysyl- and tryptophanyl-tRNA synthetases (LRS and TRS) for the following reasons. (i) The synthesis of dipicolinic acid (DPA), a unique spore component, is a product of one branch of the lysine biosynthetic pathway (10). (ii) L-Lysine and its immediate precursor, *meso*-diaminopimelic acid are major constituents of the spore coat and spore cortex (34, 37). (iii) The $tRNA^{lys}$ iso-accepting species undergo both quantitative and qualitative changes at specific times during sporogenesis (18, 43, 44). Although some asporogenic mutants do not show these changes, others are blocked in these events (19, 44). In the case of TRS, (i) there is only one $tRNA^{trp}$ species in vegetative cells, but a second is found in dormant spores (43); (ii) tryptophan deprivation after the completion of vegetative growth completely inhibits sporulation in a tryptophan auxotroph (4) and, therefore, it represents an amino acid which is required in amounts above that which a cell can supply itself through protein turnover.

As a prerequisite for the study of the possible involvement of TRS and LRS in sporogenesis, it was necessary to establish whether the corresponding enzyme found at the two end points of the life cycle (vegetative cell and spore) have a common genetic origin and structure. It was also anticipated that structural gene mutations in these enzymes which produced a temperature-sensitive phenotype in the vegetative state could provide some insight as to how the innate structure of the spore participates in the establishment of heat resistance, a striking characteristic of the dormant state.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All strains are derivatives of *B. subtilis* 168M. The isolation of the temperature-sensitive TRS mutant (*trpS1*; strain GSY1306) has been described previously (35). The temperature-sensitive LRS mutants (*lysS1* and *lysS2*; strains VB151 and VB106, respectively) were isolated from *N*-methyl-*N'*-nitro-*N*-nitrosoquanidine-treated cells by a modification of the method of Kaplan and Anderson (16; Racine and Steinberg, unpublished data). These temperature-sensitive strains grow well at 30 C, but growth is terminated when the temperature is raised to 43 C (the restrictive temperature). They are defective in both the amino acid-dependent adenosine triphosphate-inorganic-pyrophosphate exchange reaction (ATP-PP_i exchange) and the ATP-dependent aminoacylation of tRNA (35; Racine and Steinberg, unpublished data). Strains were maintained in the sporulation phase on AK-2 agar (BBL, Baltimore, Md.) supplemented with 10 μg of thymine per ml.

Media and growth of vegetative cells. Auxotrophic requirements of wild-type and mutant strains were tested on the glucose minimal medium of Spizizen (33). The temperature sensitivity phenotype was checked on NAT, a nutrient agar medium (Difco, Detroit, Mich.) supplemented with 10 mg of thymine and 1 g of glucose per liter.

Vegetative cells of *B. subtilis* strain 168M were grown in 300 liters of PYE medium at 37 C in a stainless-steel fermentor. The PYE medium contained (per liter): K_2HPO_4, 3.68 g; NaCl, 3.5 g;

TABLE 1. *Bacterial strains*

Strain	Genotype	Origin
168M	*trpC2*	C. Anagnostopoulos
GSY266	*metB3*	C. Anagnostopoulos
GSY1306	*trpS1 metB3*	Steinberg and Anagnostopoulos (35)
VB106	*lysS2 metB3*	Racine and Steinberg (unpublished data)
VB151	*lysS1 metB5 leu-8*	Racine and Steinberg (unpublished data)

peptone (Difco), 5 g; yeast extract (Difco), 2 g; and glucose, 4 g. Cells were harvested in the late exponential phase and washed three times by centrifugation in buffer A [10^{-2} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.3) containing 10% glycerol, 10^{-2} M $MgCl_2$, and 5×10^{-3} M 2-mercaptoethanol]. The cell paste was spread on aluminum sheets and frozen at -42 C.

Preparation of spores. Spores of wild-type and mutant strains were obtained after a 36- to 60-h growth period (at 30 C) in the synthetic sporulation medium of Freese and Fortnagel (8). Glutamate (0.18%) and lactate (0.2%) were the main carbon sources. The medium was supplemented with 50 μg each of L-tryptophan, L-methionine, L-lysine, and auxotrophic requirements per ml. The free spores and endospores from 30 liters of medium were collected by centrifugation in a Sharples super centrifuge (Sharples-Stokes, Warminster, Pa.). The cell paste was suspended in 1 liter of warm (37 C) 0.05 M Tris-hydrochloride buffer (pH 8.0) containing 0.15 M NaCl. Lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added at 200 μg/ml, and the suspension was incubated with mixing for 1.5 to 2 h. When distruction of sporangia and vegetative cells was nearly complete, 1 ml of 1 M $MgCl_2$ and 2 μg of pancreatic deoxyribonuclease per ml were added. Incubation was continued for 10 min and then 10 ml of a 10% sodium dodecyl sulfate solution was added. The mixture was centrifuged at 9,000 $\times$ g for 30 min. The pellet was suspended in distilled water, and free spores were separated from the remaining debris by repeated centrifugation in distilled water (8 to 10 times) at 9,000 $\times$ g for 10 min. The dormant spores were lyophilized and stored at -18 C.

Procedure for initiation of germination and outgrowth. Lyophilized spores were suspended in distilled water at 1 mg/ml (if less than 100 mg) or 10 mg/ml (if less than 1.5 g). The suspension (immersed in an ice-water bath) was homogenized by ultrasonic vibration for 3 min at 1-min intervals, at setting 4 of a Branson Sonicator (Heat Systems Ultrasonics, Plainview, N.Y.). After sonic treatment, the spores were warmed to 37 C, heat-activated at 75 C for 30 min, centrifuged at 5,000 $\times$ g for 10 min, and then suspended in sterile, distilled water at 5 mg/ml. Germination was initiated by adding the suspension (final concentration was either 100 μg or 1 mg of spores per ml) to a filter-sterilized germination medium composed of (per liter): antibiotic medium #3 (Difco), 17 g; L-alanine, 1.8 g; adenosine, 0.5 g; and glucose, 2.5 g. Germination was monitored by the decrease in optical density at 525 nm on a Gilford spectrophotometer (Gilford Instruments, Oberlin, Ohio).

Preparation of enzyme extracts from vegetative cells. All operations were carried out at 0 to 4 C. A 50-ml amount of buffer A containing 0.1 M KCl was added to 50 g of frozen cell paste. The suspension was passed twice through a French pressure cell at 20,000 lb/in^2. To reduce the viscosity and increase cell disruption, the extract was subjected to ultrasonic vibration for 5 min at 1-min intervals (setting 8, Branson Sonicator). The extract was kept in an ice-alcohol bath. Treated extract was centrifuged at 12,000 $\times$ g for 20 min, then at 27,000 $\times$ g for 30 min, and finally for 2 h at 105,000 $\times$ g, the supernatant fluid being retained from each step. The final supernatant fluid, or crude extract (52.5 ml, 1,974 mg of protein), was passed through a column of Bio-Gel P-6 (5 by 45 cm) which was equilibrated in buffer A plus 0.1 M KCl. This procedure removed small molecules which interfered with the determination of proteins and the ATP-PP_i exchange assay. An LKB Uvicord II (LKB Instruments, Rockville, Md.) was used to monitor the optical density (OD_{280}) elution profile.

Preparation of extracts from dormant and germinated spores. Spores were disrupted with a Braun MSK mechanical cell homogenizer (Bronwill Scientific Co.). Lyophilized spores (0.5 to 1.5 g) were mixed in a 75-ml MSK sample flask with either 15 g of acid-washed glass beads (470 μm diameter type 070, 3M Co., St. Paul, Minn.) or 15 g of crystallized anhydrous glucose (Difco). This mixture was kept at -18 C overnight and then placed in the MSK mixing chamber which was cooled with CO_2. The homogenizer was operated at 4000 rpm for 5 min with continuous CO_2 purging. Spore disruption, observed with a microscope, was greater than 90%. The disrupted contents were placed into a chilled beaker and mixed with 12 ml of buffer A containing 0.1 M KCl. The slurry was sonically treated and centrifuged as described for the vegetative extract. The ribosome-free supernatant fluid (~10 ml) was applied to a Bio-Gel P-6 column (2.5 by 40 cm) equilibrated in buffer A plus 0.1 M KCl.

Purification of the vegetative TRS and LRS activities. The fractions comprising the void volume of the Bio-Gel P-6 column were pooled (241 ml, 1,879 mg of protein) and applied (50 ml/h) to a phosphocellulose column (2.5 by 38 cm) equilibrated in buffer A plus 0.1 M KCl. The column was washed with 3 column volumes of the equilibrating buffer, and the sample was eluted with buffer A plus 0.6 M KCl. Tubes exhibiting TRS and LRS activity were pooled (200 ml, 148 mg of protein), diluted with buffer A to 0.5 M KCl, and then adjusted to 0.02 M potassium phosphate. This diluted fraction was applied (30 ml/h) to the first hydroxylapatite column (HA-1, 2.2 by 9 cm) equilibrated in buffer A containing 0.5 M KCl and 0.02 M potassium phosphate. The column was washed with 3 column volumes of equilibrating buffer, and the sample was eluted with buffer A supplemented with 0.5 M KCl and 0.12 M potassium phosphate. Tubes having TRS and LRS activity were pooled (140 ml, 34.9 mg of protein) and diluted 2.5-fold with buffer A. This fraction was applied (20 ml/h) to a second hydroxylapatite column (HA-2, 0.9 by 10 cm) which was equilibrated in buffer A containing 0.2 M KCl and 0.046 M potassium phosphate. The column was washed with 2 column volumes of the equilibrating buffer, and LRS and TRS activities were eluted with a linear gradient of potassium phosphate. The mixing chamber contained 150 ml of equilibrating buffer, and the other chamber contained 150 ml of buffer A plus 0.2 M KCl and 0.36 M potassium phosphate. The fractions containing LRS activity (30 ml, 5.7 mg of protein) and TRS activity (57 ml, 8.4

mg of protein) were pooled separately. Each activity was concentrated by ultrafiltration at 50 lb/in^2 (nitrogen) to 5 ml with a PM 30 membrane system (Amicon Corp., Lexington, Mass.). An equal volume of glycerol was added and the samples were stored at −18 C. Under these conditions, there was no loss of TRS or LRS activity for 2 months.

Purification of the spore LRS and TRS activities. Purification of spore LRS and TRS was done by the procedures used for the vegetative enzyme. Since smaller amounts of material (less than 1.5 g dry weight) were involved, the columns of phosphocellulose and hydroxylapatite (HA-1) had bed volumes of 2 ml each (packed in a 5-ml syringe). No attempt was made to fractionate the spore activities on HA-2. The spore enzymes were identical to the vegetative moieties in their elution characteristics from phosphocellulose and hydroxylapatite.

Determination of molecular weights. Molecular weight estimations were made by gel filtration. A 2-ml amount of the phosphocellulose fraction, adjusted to contain 3% sucrose and the molecular weight standards, was applied to a column of Bio-Gel P-200 (1.6 by 90 cm) equilibrated in buffer A plus 0.1 M KCl. Molecular weight standards employed were: whale skeletal muscle myoglobin (17,800), ovalbumin (45,000), horse liver alcohol dehydrogenase (80,000), and yeast alcohol dehydrogenase (151,000). The alcohol dehydrogenases were assayed spectrophotometrically at 340 nm by the method of Vallee and Hoch (40). The myoglobin and ovalbumin were assayed at wave lengths of 410 and 280 nm, respectively. Estimation of molecular weights of the TRS and LRS were made by the procedure of Andrews (2), assuming globular configurations for these enzymes.

Assay of LRS and TRS. TRS and LRS activity was determined by the amino acid-dependent exchange of ^{32}P-labeled sodium pyrophosphate with ATP. The reaction mixture contained (per ml): Tris-hydrochloride, (pH 8.0), 100 μmol; $MgCl_2$ (for TRS and LRS assays, respectively), 5 and 8 μmol; KF, 10 μmol; 2-mercaptoethanol, 10 μmol; disodium ATP, 2 μmol; L-amino acid (for TRS and LRS assays, respectively), 2 and 5 μmol; bovine serum albumin, 200 μg; and [^{32}P]sodium pyrophosphate, 2 μmol ($\sim 10^6$ counts/min). Final volume for each assay was 0.125 ml. Enzyme as specified (0.025 ml) was added to start the reaction, and the mixture was incubated at 25 C for 30 min. The reaction was terminated by the addition of 0.2 ml of cold 5.7% perchloric acid, and the sample was placed on ice. This was followed by the addition of 0.4 ml of sodium acetate buffer (0.05 M, pH 4.5) containing 0.1 M sodium pyrophosphate (PP_i-acetate buffer), and then by 0.1 ml of an aqueous suspension of activated charcoal (Norit, 100 mg/ml) and 2 ml of PP_i-acetate buffer. The suspension was mixed and filtered through a Gelman type E fiber glass filter (25 mm diameter). The filters were washed with distilled water, glued (inverted) to aluminum planchets, and dried at 70 C. A Nuclear-Chicago thin end window, gas-flow counter was used to monitor activity. A blank (no amino acid) was subtracted from each assay. Activity monitored by ATP-PP_i exchange corresponded with that obtained by the ATP-dependent attachment of ^{14}C-labeled L-amino acid to tRNA (35).

One unit of enzyme is defined as that amount incorporating 1 μmol of ^{32}P-PP_i into ATP per min at 25 C. Specific activity is defined as units per milligram of protein.

Immunological techniques. For each antiserum (anti-TRS and anti-LRS), two male New Zealand white rabbits weighing 1.5 to 2.0 kg each were injected intradermally at eight sites on the back and flanks with 1 mg of purified vegetative enzyme (HA-2 fraction) emulsified in Freund complete adjuvant (0.2 ml/site). The adjuvant was prepared by emulsifying the appropriate volume of enzyme with mineral oil—Arlacel A—killed lyophilized *Mycobacterium tuberculosis* strain H37RV (1:1:0.4:2, vol/vol/vol/wt). Sixty days later the animals were restimulated by a course of two intravenous injections divided over 2 days (0.5 mg of enzyme on day 1, and 0.5 mg on day 2). The intravenous injection protocol was repeated 60 days after the last injection. On days 7 and 9 after the first intravenous injection of each series, blood was collected from the marginal ear vein. The blood was allowed to clot at room temperature for 1 h and then stored at 4 C overnight. The clot was removed and the serum was centrifuged at 10,000 × *g* for 15 min. The supernatant fluid was dialyzed for 12 h at 4 C against 50 volumes of PBS buffer (0.15 M KCl in 0.005 M potassium phosphate, pH 6.8) with two buffer changes. Dialyzed serum was stored as 0.5-ml volumes at −42 C. All antisera preparations were found to contain antibodies which inhibited TRS or LRS activity. Control serum taken prior to immunization exhibited none of these activities even when used undiluted.

Tests for the inhibition of enzyme activity by antisera were conducted by the method of Fangman et al. (7). A constant quantity of appropriately diluted enzyme solution (0.05 ml of the HA-1 fraction diluted in PBS buffer) was mixed with twofold serial dilutions of antibody. The mixture (0.1 ml final volume) was incubated for 20 min at 30 C and then cooled on ice. Samples of 0.025 ml were then assayed by the ATP-PP_i exchange assay.

Other procedures. Protein concentration was determined by the method of Lowry et al. (23), with crystalline bovine serum albumin (Calbiochem) as a standard. The ultraviolet absorption (at 280 nm) of column fractions was monitored on a Gilford spectrophotometer. The phosphate gradient in the column fractions was measured by a modification of the method of Fiske and SubbaRow (39), with Elon (*p*-methyl amino phenol sulfate, Eastman Chemicals) as the color developer. The DPA content of dormant spores was assayed as described by Janssen et al. (15). Protease activity was determined by the hydrolysis of azocasein (26).

Materials. Whatman phosphocellulose P-11 (7.4 meq/g) was purchased from Reeve Angel (Clifton, N.J.). It was prepared according to the procedure for carboxymethyl cellulose (Whatman Technical Bulletin IE2). Bio-Gel P-6 (100 to 200 mesh) was a product of Bio-Rad Laboratories (Rockville Center, N.Y.). Hydroxylapatite was prepared by the method of Levin (21). ^{32}P-labeled sodium pyrophosphate in

aqueous solution (carrier free) was obtained from New England Nuclear Corp. The sodium salt of ATP and whale skeletal muscle myoglobin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, and pancreatic deoxyribonuclease I (2400 U/mg) were products of Worthington Biochemicals (Freehold, N.J.). Azocasein and L-amino acids (A grade) were obtained from Calbiochem. Arlacel A (mannide mono-oleate) was a product of Atlas Chemicals (Wilmington, Del.). DPA was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Ovalbumin was obtained from Schwarz-Mann. The sources of other materials are indicated in the text.

RESULTS

Purification of the vegetative TRS and LRS. The purification procedure for LRS and TRS from vegetative cells of *B. subtilis* strain 168M is summarized in Table 2. The methodology permitted simultaneous partial purification of both TRS and LRS, with a 48% yield of TRS and a 19% yield of LRS. The lower yield of LRS presumably is due to its relative instability during purification as compared with TRS. Chromatography of the Bio-Gel fraction on phosphocellulose results in a 10-fold purification for TRS and a 7.5-fold purification for LRS (Fig. 1 and Table 2). On phosphocellulose chromatography, both LRS and TRS elute together as a single component even when a KCl gradient (0.1 to 0.6 M KCl) is employed (Steinberg, unpublished data). If the phosphocellulose fraction is subjected to dialysis or ultrafiltration, approximately 30% of the total LRS activity precipitates; thus, subsequent steps relied upon dilution in buffer A to lower the salt concentration. The LRS and TRS activities elute together in the first hydroxylapatite chromatography step (Fig. 2), whereas in the second step (HA-2) both LRS and TRS elute separately, each as a single zone of activity (Fig. 3). A survey for other aminoacyl-tRNA synthetases which could be detected in the two HA-2 fractions revealed the following. The lysyl-tRNA synthetase fraction contained ATP-PP_i exchange activity (expressed as the percentage of the L-lysine-dependent exchange) for L-tryptophan (4.2%), L-alanine (2.4%), L-histidine (1.6%), and L-leucine (0.7%). The TRS fraction contained ATP-PP_i exchange activity (expressed as the percentage of the L-tryptophan-dependent exchange) for L-histidine (84%), L-leucine (1.5%), and L-alanine (1.3%). No other aminoacyl-tRNA synthetases were present in significant amounts in these two fractions. The

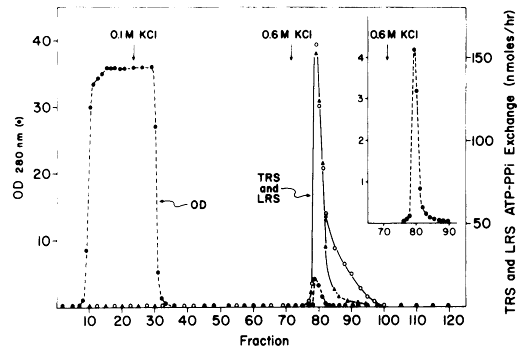

FIG. 1. *Phosphocellulose chromatography of the vegetative extract. Fractions of 10 ml were collected. Symbols:* ●, OD_{280} *of eluate;* △, *LRS activity;* ○, *TRS activity. Enzyme activities of 0.025-ml samples were determined by ATP-PP_i exchange and are expressed as nanomoles of [^{32}P]pyrophosphate into ATP per hour. Fractions 77 through 97 were pooled for the next step. Insert;* OD_{280} *profile of 0.6 M KCl elution step.*

TABLE 2. *Purification of tryptophanyl- and lysyl-tRNA synthetases from vegetative cells*

Fraction	Total protein (mg)	Total activity (U)	Yield of activity (%)	Sp act (U/mg)	Fold purification
A. Tryptophanyl-tRNA synthetase					
Crude extract	1,974	24.43		0.012	
BioGel P-6	1,879	26.37	100	0.014	1.0
Phosphocellulose	148	21.62	82	0.146	10.4
Hydroxylapatite-1	34.9	16.87	64	0.477	34
Hydroxylapatite-2	8.4	12.65	48	1.500	107
B. Lysyl-tRNA synthetase					
Crude extract	1,974	22.85		0.012	
BioGel P-6	1,879	23.01	100	0.012	1.0
Phosphocellulose	148	13.58	59	0.092	7.5
Hydroxylapatite-1	34.9	8.55	37	0.245	20
Hydroxylapatite-2	5.7	4.37	19	0.772	64

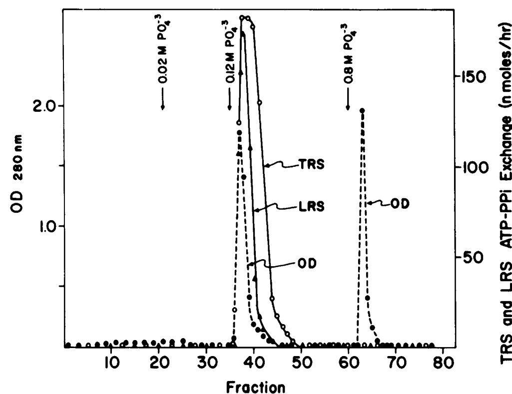

FIG. 2. *Hydroxylapatite chromatography (HA-1) of the phosphocellulose fraction. Fractions of 10 ml were collected. Symbols: ●, OD_{280} of eluate; △, LRS activity; ○, TRS activity. Fractions 36 through 50 were pooled for the next step. Enzymes were assayed as in Fig. 1.*

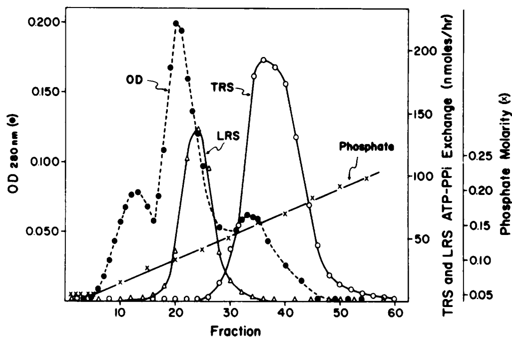

FIG. 3. *Hydroxylapatite chromatography (HA-2) of the HA-1 fraction. Fractions of 3 ml were collected. Symbols: ●, OD_{280} nm of eluate, △, LRS activity; ○ TRS activity; ×, the potassium phosphate molarity of the eluting gradient. Fractions 18 through 38 and 30 through 50 were pooled separately and concentrated by ultrafiltration. Enzymes were assayed as in Fig. 1.*

L-histidine-dependent activity also co-purifies with TRS when the HA-2 fraction is subjected to diethylaminoethyl-cellulose chromatography (Steinberg, unpublished data). The co-purification of these two activities might be of significance in interlocking the regulation of histidine and tryptophan biosynthesis (28), particularly since tryptophanyl-tRNA synthetase participates in the repression control of enzymes of the tryptophan pathway (Steinberg, in press).

The Bio-Gel P-6 fraction obtained from dormant spores failed to produce significant ATP-PP_i exchange activity for either lysine or tryptophan. Activity could only be measured after the first step of purification on phosphocellulose (PC). An inhibiting effect of crude extracts of spores on aminoacyl-tRNA synthetase activity has been observed by Vold (in press).

Heat-inactivation characteristics of vegetative and spore LRS and TRS. A study of the kinetics of heat inactivation of spore and vegetative TRS at 50 C showed that the HA-1 fraction obtained from spores was inactivated at nearly the same rate as its vegetative counterpart (Fig. 4). But, a sixfold difference was noted in the rate of inactivation when the phosphocellulose fractions were employed. The kinetics of heat inactivation of PC fractions of LRS also showed a significant difference between the spore and vegetative fractions (Fig. 4). For the first 3 min the rate of inactivation of spore activity was 4.5-fold greater than that of the vegetative enzyme. Thereafter, the rates of inactivation for these enzymes were the same. Hydroxylapatite fractions (HA-1) of spore and vegetative LRS activity had identical rates of inactivation (Fig. 4).

The PC fraction of the spore enzyme preparation had measurable levels of protease activity (about 1% of that of the crude extract as measured by the hydrolysis of azocasein), whereas the HA-1 fraction was devoid of protease. Thus, it was possible that the inactivation rates observed in PC fractions were, in part, due to the action of proteases. In support of this view was the finding that extract obtained from sporulating cells had the highest protease levels, and LRS activity from sporulating cells had the

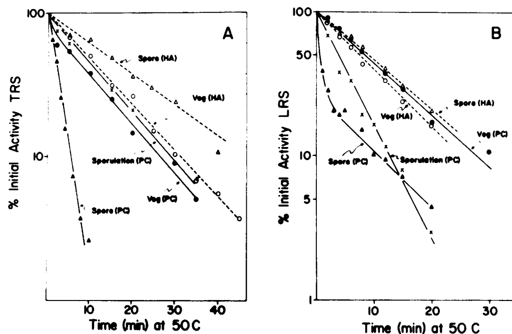

FIG. 4. *Heat-inactivation kinetics of partially purified spore, sporulation, and vegetative LRS and TRS activities. Partially purified enzyme (0.1-ml volumes, 15 µg of protein) either from the phosphocellulose (PC) or hydroxylapatite step (HA-1) was incubated at 50 C. At the times indicated, samples were chilled and assayed in triplicate as in Fig. 1. Sporulation enzyme was purified from a French press extract of a sporulating culture (80 to 90% refractile spores) grown in the medium described by Leighton and Doi (20). (A) Heat inactivation of TRS. (B) Heat inactivation of LRS. Symbols: (▲, △) spore enzyme, PC and HA-1 fractions; (●, ○) vegetative enzyme, PC and HA-1 fractions; (×) sporulation enzyme, PC fraction.*

highest rate of inactivation (Fig. 4). On the other hand, sporulation TRS had a greater heat stability than did the spore enzyme. If inactivation at 50 C was due to the presence of proteases, then incubation of PC fractions at 35 C should permit the protease inactivation of LRS and TRS. There was, however, no significant loss of enzyme activity under these conditions.

The addition of 3 mM $CaCl_2$ had no effect on the kinetics of heat inactivation of either spore or vegetative TRS. Higher concentrations were inhibitory.

Effect of Mg^{2+} concentration on vegetative and spore LRS and TRS activities. A distinct difference was observed between spore and vegetative enzymes with respect to the effect of Mg^{2+} concentration on enzyme activity (Fig. 5). For the vegetative TRS and LRS, the optimal Mg^{2+} concentrations were 5 and 8 μmol of $MgCl_2$ per ml, respectively. At lower concentrations, there was a precipitous drop in activity. For the corresponding spore enzymes (Fig. 5), the LRS activity had a plateau between 2 and 10 μmol, whereas TRS activity decreased significantly at values above 2 μmol of Mg^{2+} per ml.

K_m values of the vegetative and spore LRS and TRS. The kinetic constants for amino acids and ATP of the wild-type synthetase activities purified from spores and vegetative cells are essentially the same (Table 3). These values are also similar to K_m values obtained for other aminoacyl-tRNA synthetases isolated from *Escherichia coli* and *Salmonella typhimurium*.

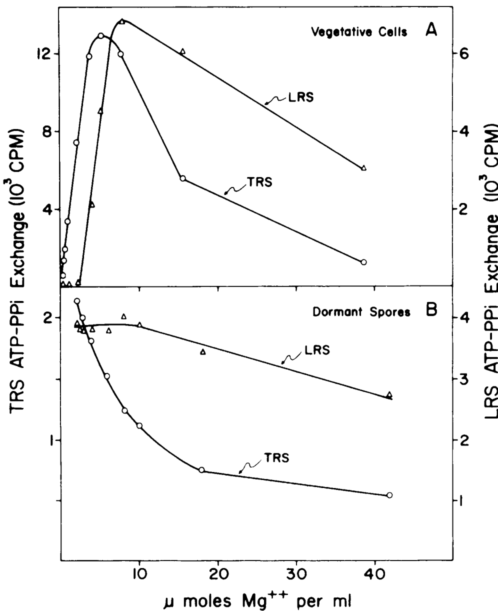

FIG. 5. *Effect of the variation of Mg^{2+} concentration on the LRS and TRS activities isolated from spores and vegetative cells. The assay conditions were the same as in Materials and Methods, except that varying concentrations of $MgCl_2$ were used. (A) Vegetative enzyme, HA-1 fraction; (B) dormant spore enzyme, HA-1 fraction. Symbols: △, LRS activity; O, TRS activity.*

Molecular weight determination. As measured by gel-filtration, the molecular weights (assuming globular configuration) of the vegetative LRS and TRS are ~130,000 and ~87,000, respectively (Fig. 6 and Table 4). The synthetases found in dormant spores, spores germinated under conditions where protein synthesis was inhibited, and cells at the stage of elongation (outgrowing cells harvested just prior to the first cell division) did not deviate significantly from these values. The enzymes purified from spores germinated in the presence of chloramphenicol had slightly lower molecular weights, but these values were well within the reproducibility that is expected by this method (1). It should be noted that, because of variation in molecular configuration among proteins, the gel filtration method has limited accuracy for molecular weight determinations of undenatured proteins.

Immunological relatedness of the spore and vegetative enzymes. Antibody produced against vegetative LRS or TRS (anti-LRS^{veg} or anti-TRS^{veg}) will specifically inactivate the corresponding spore or vegetative enzymes (Fig. 7). Anti-TRS^{veg} was completely inactive against vegetative LRS, but did inactivate TRS from spores or vegetative cells with nearly identical kinetics of inactivation. Similarly, anti-LRS^{veg} did not react with vegetative TRS, but did inhibit LRS activity isolated from spores or vegetative cells (Fig. 7). These results indicate

TABLE 3. *Kinetic constants of the lysyl- and tryptophanyl-tRNA synthetases from dormant spores and vegetative cells*[a]

Enzyme	Source	K_m (M) for:	
		ATP	Amino acid
Lysyl-tRNA synthetase	Spore	0.88×10^{-4}	2.6×10^{-5}
	Vegetative	1.8×10^{-4}	1.6×10^{-5}
Tryptophanyl-tRNA synthetase	Spore	3.5×10^{-4}	1.9×10^{-5}
	Vegetative	4.5×10^{-4}	2.5×10^{-5}

[a] Kinetic constants were determined with the HA-1 fraction. Enzyme preparations were made from strain GSY266. Assay conditions were the same as in Materials and Methods, except that either L-tryptophan, L-lysine, or ATP concentrations were varied. For each substrate concentration, samples were assayed at 10-min intervals over a period of 60 min (25 C). K_m values were calculated by the method described by Hofstee (13).

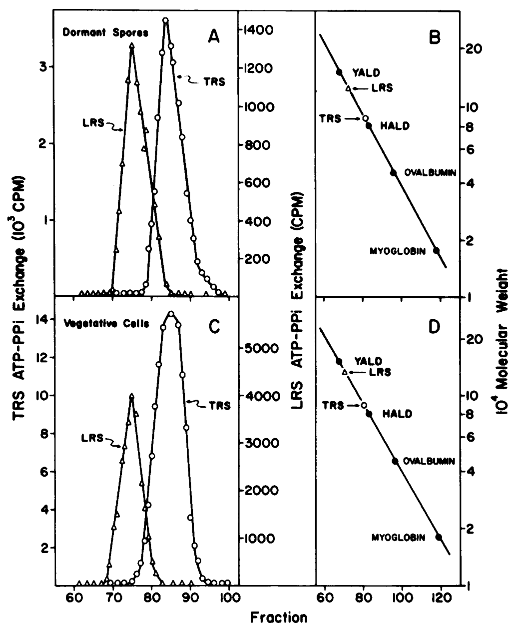

FIG. 6. *Gel filtration of dormant spore and vegetative (exponential phase) LRS and TRS activities. Fractions of 1 ml from a Bio-Gel P-200 column (1.6 by 90 cm) were collected. Each tube was assayed for the internal molecular weight marker and TRS and LRS activities. (A and B) Elution of dormant spore enzymes; (C and D) elution of vegetative enzymes. Symbols: Δ, LRS; O, TRS; YALD, yeast alcohol dehydrogenase; HALD, horse liver alcohol dehydrogenase.*

TABLE 4. *Estimation of the molecular weights of the lysyl- and tryptophanyl-tRNA synthetases*

Developmental stage[a]	Mol wt	
	LRS	TRS
Dormant spore	~130,000	~87,000
Germinated spore + CAM[b]	~125,000	~85,000
Outgrowing cells[c]	~130,000	~85,000
Exponential phase	~130,000	~87,000

[a] Dormant and germinated spores were disrupted with a Braun MSK homogenizer. The French press was used for outgrowth and exponential stages.

[b] The germination medium (1 liter) contained 100 μg of chloramphenicol (CAM) and 1 mg of heat-activated spores per ml. After incubating 60 min at 30 C, the germinated spores were harvested and washed by centrifugation in 0.01 M Tris-hydrochloride (pH 7.3) containing 0.15 M NaCl. They were lyophilized prior to disruption.

[c] The cells from 1 g of heat-activated spores (germinated at 1 mg/ml at 30 C) were harvested by centrifugation just prior to the onset of the first division in the germination medium (210 min). They were washed in buffer A by centrifugation and resuspended in 15 ml of buffer A prior to disruption.

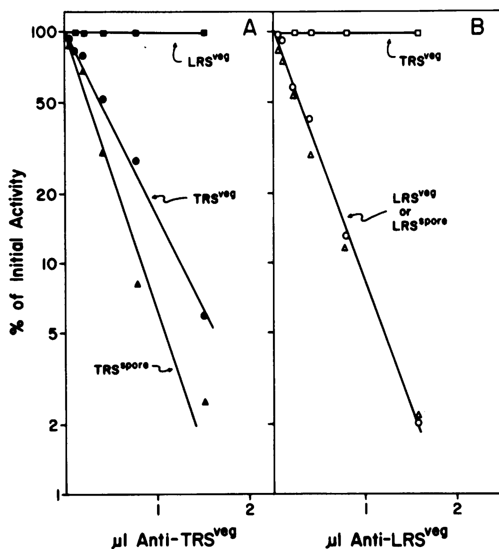

FIG. 7. *Kinetics of antiserum inactivation of the partially purified LRS and TRS activities of spores and vegetative cells. Increasing amounts of antibody prepared against vegetative LRS (anti-LRS^{veg}) and vegetative TRS (anti-TRS^{veg}) were incubated with a constant amount of enzyme (6.6 μg of vegetative enzyme; 8.4 μg of spore enzyme, HA-1 fractions). Residual activity was measured by ATP-PP$_i$ exchange. (A) Inactivation by anti-TRS^{veg}. Symbols: ■, vegetative LRS; O, vegetative TRS; ▲, spore TRS. (B) Inactivation by anti-LRS^{veg}. Symbols: □, vegetative TRS; O, vegetative LRS; Δ, spore LRS.*

that the spore and vegetative enzymes are immunologically related, at least at the site(s) which participates in the ATP-PP$_i$ exchange reaction.

Identical genetic origin of the spore and vegetative enzymes. If an enzyme present in both the spore and vegetative states were coded by the same structural gene, then it would be expected that any enzyme defect present at one stage should also be manifested in the other stage (excluding enzyme modification at a posttranslational level). Spores of mutants having defective vegetative LRS or TRS were therefore examined (Table 5). In every spore enzyme preparation (whether glass beads or crystallized glucose was used as the abrasive), it was found that a mutant with a defective vegetative enzyme produced spores in which that specific enzyme was also defective. Furthermore, the defect carried over into the spore state appeared to be identical to that found in the vegetative cell. First, in the case of VB106 (*lysS2*) a temperature-sensitive vegetative enzyme is produced which has an altered K_m for lysine (Racine and Steinberg, unpublished data). In Table 5 we see that the presence of LRS activity

TABLE 5. *Tryptophan- and lysine-dependent ATP-PP$_i$ exchange activities of dormant spore extracts*[a]

Strain	Relevant genotype	Dry wt of spores	nmol of [^{32}P]PP$_i$ in ATP/mg of protein/h				
			Trp (2)[b]	Trp (5)	Lys (2)	Lys (5)	Lys (40)
GSY266	*trpS*$^+$ *lysS*$^+$	0.5 g[c]	213		558		
		0.5 g[c]	594		3194		
		0.5 g[c]		449			
		1.0 g[c]		1341		965	
		0.5 g[d]	826			1630	
GSY1306	*trpS1 lysS*$^+$	0.5 g[c]		11.2		484	
		0.5 g[d]		4.9		981	
VB151	*trpS*$^+$ *lysS1*	0.5 g[c]	478		4.9		
VB106	*trpS*$^+$ *lysS2*	0.5 g[c]	211		7.8		
		0.5 g[d]	511		4.8		
		1.5 g[e]			5.9		76.4

[a] The phosphocellulose fraction was assayed. Each value represents the average of two or three independent assays on the same preparation. Mutant spores had activities from 1.0 to 1.3 times the background level. All assays were carried out within 6 h after the spores were disrupted.
[b] Amino acid addition (micromoles per milliliter).
[c] The abrasive was glass beads (15 g).
[d] The abrasive was anhydrous cyrstallized glucose (15 g).
[e] The extraction buffer was supplemented with 0.1 M KCl, 2×10^{-3} M L-lysine, and 1.6×10^{-4} M ATP.

in spores of the *lysS2* mutant could only be demonstrated when the ATP-PP$_i$ exchange assay was carried out at high lysine concentrations. Second, if the enzyme present in both the spore and vegetative cell were the same, then we would expect the same phenotypic response (with respect to growth temperature) from spores as has been observed for vegetative cells (35). Spores which are genotypically *lysS1*, *lysS2*, or *trpS1* germinated at 43 C, a temperature which restricts vegetative growth (Fig. 8 and 9). Prolonged incubation at 43 C blocked the temperature-sensitive mutants at specific stages of morphological development. Under these same conditions, wild-type spores completed the outgrowth sequence (germination, swelling, emergence, elongation, and cell division). Spores of the *trpS1* strain resembled class III germination mutants (blocked at the swollen stage), whereas *lysS1* and *lysS2* spores were blocked at stage IV (emergence) (46). Similarly, *lysS1* spores germinated at 30 C and shifted to 43 C during outgrowth were also subject to developmental arrest (Fig. 9). These results indicate that the mutant enzyme present in dormant spores is not modified with respect to the temperature sensitivity phenotype and that no modification of this phenotype takes place during germination or outgrowth.

Heat resistance of trpS and lysS mutant spores. Additional evidence as to the nature of the LRS and TRS proteins present in the spore

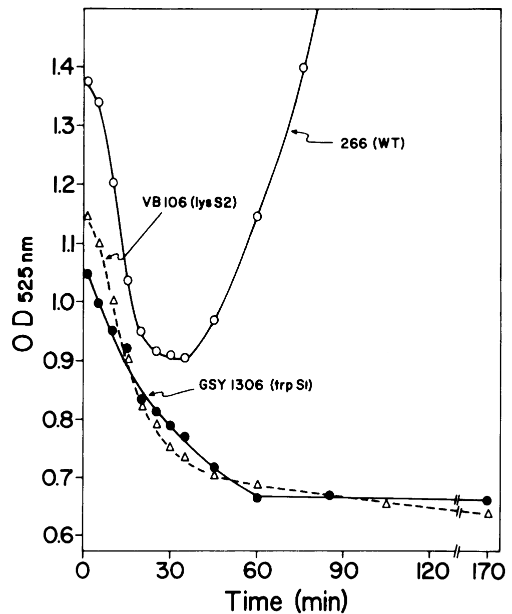

FIG. 8. *Germination and outgrowth of wild-type and mutant spores at the restrictive temperature. Spores of wild-type strain GSY266 (○) and the temperature-sensitive lysS2 (strain VB106, △) and trpS1 (strain GSY1306, ●) mutants were heat-activated and suspended at 100 µg/ml in 100 ml of germination medium (43 C). The optical density (525 nm) of the cultures was recorded at the indicated intervals.*

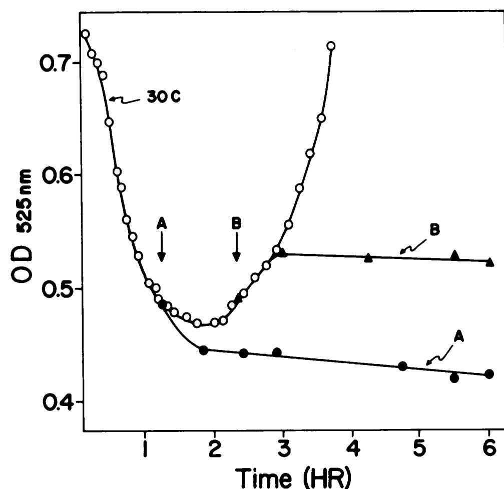

FIG. 9. *Germination and outgrowth of spores of the lysS1 mutant. Spores of strain VB151 (lysS1) were heat-activated and suspended at 100 μg/ml in 100 ml of germination medium (30 C). At the time points indicated by A and B, a portion of the culture (30 ml) was transferred to 43 C. The optical density (525 nm) of the cultures was recorded at the indicated intervals.*

was provided by a study of the kinetics of heat inactivation of dormant spores. In the dormant state, many normal vegetative enzymes are stabilized against the deleterious effects of heat (17, 29). In spite of the extreme lability of the defective vegetative LRS and TRS, both the *lysS* and *trpS* mutants produce spores which can survive a temperature of 80 C for 10 min. This treatment also had no injurious effect on wild-type spores, but it completely destroyed a wild-type vegetative cell population (Fig. 10). On this basis, there appeared to be no essential difference in the heat resistance properties of wild-type and mutant spores. However, prolonged incubation at 80 C revealed a dramatic difference between the two spore types. Within the first 2 h of heat treatment, the survival level of a wild-type spore population decreased to approximately 60% of its initial value (presumably the 40% loss was due to the presence of immature spores). The viability count then remained constant for the next 7 h. In contrast, similar treatment of a suspension of mutant spores yielded a surviving population which represented only 1 to 2% of the initial level. The DPA content of the wild-type and mutant spore populations was found to be the same (Table 6). These results provide additional evidence that the TRS and LRS present in dormant spores are derived from the same structural gene as the corresponding vegetative enzymes. The data also indicate that the packaging of a vegetative protein into the dormant spore structural network does not guarantee that the protein will be stabilized against the effects of heat.

DISCUSSION

Are the spore and vegetative enzymes identical? The finding that mutants having defective vegetative LRS or TRS produce spores in which these enzymes are also defective indicates that the spore and vegetative enzymes are coded (at least in part) by the same structural gene. Additional evidence for structural identity includes similar kinetics of heat and antibody inactivation and similar kinetic constants and molecular weights. No modification in

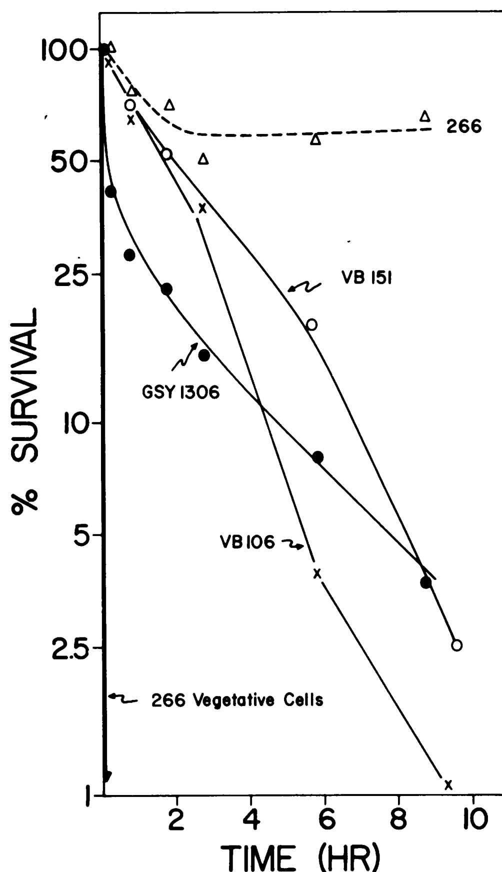

FIG. 10. *Heat inactivation of spores of the wild-type and aminoacyl-tRNA synthetase mutants. Spore suspensions (1 mg/ml in 5 ml of distilled water) or vegetative cells (2 × 10⁸ colony-forming units/ml) were incubated at 80 C. At intervals, samples were removed, diluted in distilled water, and plated in duplicate on NAT medium. The plates were incubated at 30 C for 2 days. Symbols: Δ, wild-type strain GSY266; ○, strain VB151 (lysS1); ●, strain GSY1306 (trpS1); ×, strain VB106 (lysS2).*

TABLE 6. *Dipicolinic acid content of spores of wild type and temperature-sensitive aminoacyl-tRNA synthetase mutants*

Strain	Concn of Ca^{2+} in sporulation medium (M)	μg of DPA per 5 mg dry wt[a]	Avg percent DPA
GSY266	6×10^{-4}	375; 377	7.5
B. cereus T[b]	9×10^{-5}	418; 430	8.5
GSY1306	6×10^{-4}	365; 382	7.5
VB151	3×10^{-4}	367; 380	7.5
VB106	3×10^{-4}	418; 425	8.4

[a] The values represent independent determinations.

[b] Spores of *Bacillus cereus* strain T were produced in G medium and prepared by the method of Vary and Halvorson (41).

mutant phenotype or enzyme molecular weight occurs during spore germination and outgrowth. These results suggest, but do not prove, that the spore and vegetative enzymes are structurally identical. Only the effect of Mg^{2+} concentration on enzyme activity would appear to indicate any difference in the enzymes from the two stages.

The genetic approach taken here should represent one of the definitive methods for proving an identical amino acid sequence of specific spore and vegetative proteins, but there are other explanations for the results. Although the genetic evidence indicates that only one species of TRS and LRS exists in *B. subtilis* spores and vegetative cells (35; Steinberg and Racine, unpublished data), it is conceivable that there could be more than one species of each enzyme. This could arise if we allow for the possibility of enzyme modification at a secondary level which either influences the structure, assembly, or conformation of the translated polypeptide(s). Alternatively, the gene(s) could code for a common indispensable structural component of both the spore and vegetative enzymes.

For many enzymatic activities found in both spores and vegetative cells, it is still unclear whether the homologous activities are represented by the identical or only structurally related proteins (17, 29). There are good reasons to believe that most, if not all, homologous vegetative and spore activities have identical genetic origins. Principal among these is Demain's observation (3) that auxotrophic mutants produce spores which can germinate but cannot undergo further development unless the end product of the defective biosynthetic pathway is supplied exogenously. In only two instances has it been specifically shown that the same cistrons are involved in the synthesis of spore and vegetative cell protein (9, 11). Structural gene mutants of the vegetative purine nucleoside phosphorylase of *B. cereus* (11) and the L-alanine dehydrogenase of *B. subtilis* (9) produce a defective spore enzyme. Although one might expect structural identity in the vegetative and spore forms of purine nucleoside phosphorylase, this is clearly not the case. The two forms are genetically and structurally related, but depending on ionic conditions they exist in different aggregational states (5). Similar physical properties have been found for the glucose dehydrogenase of *B. cereus* (30) and the inorganic pyrophosphatase of *B. megaterium* (38). In these cases, the evidence is consistent with the idea that the spore enzyme is a modified form of the vegetative enzyme. In agreement with this general hypothesis, it has been found that the vegetative fructose 1,6-diphosphate aldolase of *B. cereus* can be converted by a sporulation-associated protease to the form isolated from spores (31, 32).

Nature of the lysS and trpS enzymes and heat resistance in the dormant state. In the dormant spore, many normal vegetative enzymes are stabilized against the injurious effects of heat. Although prime factors in heat stabilization appear to be the unique environmental conditions present in the intact spore and in what form an enzyme is packaged into this structure, the finding that spores of the *lysS* and *trpS* mutants are not as thermoresistant as the wild type suggests that the heat resistance is also a function of the intrinsic structure of the *lysS* and *trpS* proteins. The data presented in Fig. 10 and Table 6 indicate that the packaging of a defective (temperature sensitive) vegetative protein into, what is for all practical purposes, a normal spore milieu does not guarantee that the protein will be stabilized. The defect in the protein may prevent the establishment of the protective state because of a failure in secondary reactions associated with the packaging event (e.g., ion complexing, dehydration, ultrastructural association). Clearly, the heat resistance of wild-type spores is not due to inherently resistant spore proteins since purified vegetative and spore enzymes have identical heat inactivation characteristics (Fig. 4).

It is obvious that heat resistance requires more than just a specific protein structure. There are numerous enzymes (proteins) in the spore (17), and heat inactivation of any one of a number of them could be a lethal event if the end product of their activity was not made available. Indeed, of all the molecules of which

a cell is composed, proteins are among the most heat labile. If a wild-type cell composed of diverse proteins can still produce a heat resistant spore, why weren't the *lysS* and *trpS* mutant proteins stabilized in the spore state? It could be argued that the *lysS* or *trpS* phenotype indirectly affects the production or activity of some other spore component or somehow delays maturation to heat resistance. This seems improbable for the following reasons. First, the mutants have a conditional phenotype; the enzymes can function at the permissive temperature (30 C) at which the spores were produced (35, Steinberg, in press; Racine and Steinberg, unpublished data). Second, to the extent that DPA levels can be used as an indication of spore maturity, the mutant strains had the same DPA levels as the wild type (Table 6). Third, at the time of spore isolation, about 50% of the cell types of each culture were free spores. Finally, it is unlikely that all three mutant strains affect the same spore component. Thus, two remaining explanations for the thermal sensitivity of *trpS* and *lysS* mutant spores are either (i) a modification in these enzymes occurs as part of the stabilization process and this cannot take place with the mutants, or (ii) the structure of the mutant proteins prevents a proper packaging event.

Spores do contain some enzymes which are more heat stable than their vegetative cell counterparts (29). Several, including glucose dehydrogenase (30), fructose 1,6-diphosphate aldolase (32), and purine nucleoside phosphorylase (5), exist as lower-molecular-weight classes in the spore. This structural change, in association with a high ionic environment, provides the enzyme with greater thermal stability. In other enzyme systems examined, the basis of resistance apparently resides, at least in part, in the special protective surroundings furnished by the spore. Enzymes such as catalase and alanine racemase are stabilized by virtue of their being bound in a structural association with the highly organized spore matrix (29). They are not intrinsically heat stable molecules.

The high content of DPA in spores has often been implicated in heat resistance. However, in recent years this role for DPA has become questionable. Several laboratories have isolated mutants which produce spores devoid of DPA (12) but as heat resistant as the wild type. In the present study, spores having similar levels of DPA have been found to differ in their temperature sensitivity as a result of a structural change in a single essential protein. Further study of the nature of these temperature-sensitive enzymes in the dormant state and during sporogenesis could provide some indication of the interaction(s) between the "vegetative" protein and the spore environment which leads to the formation of a heat-resistant entity.

Level of LRS and TRS activity in spores and vegetative cells. The data obtained in this study are in agreement with that of Vold (42) in that the specific activities of the spore enzymes are lower than that found in exponential-phase cells. The results cannot be more directly compared, however, because different methods of purification and enzyme assay were involved.

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