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Coordinate Synthesis of the Enzymes of Pyrimidine Biosynthesis in *Bacillus subtilis*

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Strains of *Bacillus subtilis* that were resistant to repression of pyrimidine nucleotide biosynthetic enzymes were selected by isolating spontaneous uracil-tolerant derivatives of a uracil-sensitive strain, which lacks arginine-repressible carbamyl phosphate synthetase. The relative content of all six enzymes of uridylic acid biosynthesis de novo in these strains was in a constant ratio over a 10-fold range of derepression, which indicates that synthesis of these enzymes is coordinately regulated.

The six enzymes of de novo pyrimidine biosynthesis are repressible by pyrimidines and coded for by adjacent genes in *Bacillus subtilis* (8), but it is not clear that synthesis of these enzymes is coordinately regulated. In the course of studies on the carbamyl phosphate synthetases of *B. subtilis* (6, 7), we found a simple means of isolating strains that are genetically derepressed for the enzymes of pyrimidine biosynthesis. These strains have been used to demonstrate coordinate synthesis of all six enzymes. We have developed optimal assay conditions for four enzymes of pyrimidine biosynthesis, dihydroorotase (EC 3.5.2.3), dihydroorotate dehydrogenase (EC 1.3.3.1), orotate phosphoribosyltransferase (EC 2.4.2.10), and OMP decarboxylase (EC 4.1.1.23), in crude *B. subtilis* extracts, and we report them here.

B. subtilis JH861 (*trpC2 urs-1*) fails to grow in the presence of uracil because it lacks the arginine-repressible carbamyl phosphate synthetase (6). As a result, growth in the presence of uracil represses the remaining carbamyl phosphate synthetase and creates a condition of starvation for precursors to arginine synthesis. As expected, strain JH861 grows well in the presence of both uracil and arginine. Spontaneous revertants of strain JH861 to uracil tolerance were picked at a frequency of about 1 in 10^7 cells from minimal medium (1) agar plates supplemented with 0.1% glucose and 50 μg each of uracil and tryptophan per ml. Seventeen such colonies were picked and characterized. All grew at normal rates in the presence of 50 μg of uracil per ml, but nine were still sensitive to uridine. All of the revertants grew normally on a medium containing both uridine and arginine. Each revertant was grown to the midexponential phase in the presence of 50 μg of uracil per ml, and the cells were assayed for aspartate transcarbamylase. All 17 isolates produced elevated levels of the enzyme, although they varied in the extent of derepression over a 10-fold range. Thus, it appears that none of the revertants were true revertants. That is, none had become uracil tolerant by regaining the arginine-repressible carbamyl phosphate synthetase; if any had, the pyrimidine biosynthetic enzymes would be fully repressible by uracil. This conclusion was confirmed by direct assay for the arginine-repressible carbamyl phosphate synthetase in the seven isolates used in all subsequent studies; none had regained this activity.

The strains apparently had become uracil tolerant by secondary mutations that prevented repression by uracil. For many of the revertants these mutations probably rendered the cells unable to take up uracil or to convert uracil to the true repressing metabolite, which is likely to be UTP or CTP or both (10). This appeared to be the case for those revertants that are uracil tolerant but uridine sensitive. The revertants sensitive to both uracil and uridine may result from mutations in regulatory genes, but they may also result from defects in the conversion of UMP to UTP or CTP. Such mutants in *pyrH* (UMP kinase) of *Salmonella typhimurium* synthesize pyrimidine biosynthetic enzymes constitutively (3, 4). Further characterization will be required to establish whether any of the strains described here result from true regulatory mutations, but the approach described here should be a good one for searching for such mutants, which have proven difficult to isolate in enteric bacteria (4).

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The availability of the strains that produced pyrimidine enzymes constitutively enabled us to examine whether such synthesis was coordinate in cells that were grown under identical conditions in the presence of a constant excess of exogenous uracil and harvested during exponential growth. This was important because interference by inactivation of pyrimidine enzymes in the stationary phase or during pyrimidine starvation (7, 11) could be avoided. Simultaneous synthesis and degradation of aspartate transcarbamylase during uracil starvation of *B. subtilis* cells has been demonstrated (5), and each of the other enzymes is inactivated in stationary cells (7; unpublished data). Seven revertants (R-1, R-2, R-9, R-10, R-11, R-12, and R-13) and the isogenic sibling of strain JH861, strain JH862, which has normal carbamyl phosphate synthetase activities (6), were grown to the midexponential phase in the presence of 50 μg of uracil per ml. The cells were harvested and assayed for all six enzymes of pyrimidine biosynthesis. The specific activities were plotted as coordinate plots (Fig. 1). It is evident that synthesis of all six enzymes was coordinately controlled over the greater-than-10-fold range of derepression in these strains. Together with the observation that the genes for these enzymes map in a cluster (8), this result suggests that the *pyr* genes in *Bacillus* may constitute an operon. Proof of this suggestion will require the demonstration that derepression can result from a true regulatory mutation.

The studies reported here required preliminary characterization of several of the enzymes for optimal stability and activity during assays in crude extracts. This resulted in assay procedures and a number of kinetic properties that are substantially different from those previously employed in studies with *B. subtilis* extracts (8). A major difference was that, with the exception of pyrimidine-repressible carbamyl phosphate synthetase, none of the enzymes were inhibited to a significant degree by nucleotides. Important results of the characterization studies are reported along with the assay procedures.

The procedures used for washing cells to remove extracellular protease and for disrupting cells by sonic disruption in stabilizing buffer have been described previously (7). The cells were suspended in a volume of buffer equal to 0.1 to 0.033 of the culture volume from which they were harvested, except for assays of orotate phosphoribosyltransferase and OMP decarboxylase, when the volume of extract was 0.02 to 0.01 of the culture volume. Extracts to be assayed for pyrimidine-repressible carbamyl phosphate synthetase were dialyzed as previously described (7): When dihydroorotase, dihyroorotate dehydrogenase, orotate phosphoribosyltransferase, and OMP decarboxylase were to be assayed, the cell washing procedure differed by the use of 50 mM Tris buffer (pH 8.1) containing 1 M KCl and 1 mM phenylmethylsulfonyl fluoride. Furthermore, cells to be assayed for the latter two enzymes were sonically disrupted in 50 mM Tris Buffer (pH 8.1) containing 10 mg of bovine serum albumin per ml, 5 mM $MgCl_2$, and 20% glycerol. Crude extracts to be assayed for orotate phosphoribosyltransferase were supplemented with 3 mg of 5'-phosphoribosyl-1-PP (PRPP) per ml to stabilize that enzyme. Extracts to be used for assay of OMP decarboxylase were diluted into an equal volume of 25 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES), pH 7.7, containing 10 mg of bovine serum albumin per ml, 20% glycerol, and sufficient OMP to give a final concentration of 0.1 mM. Extracts were centrifuged as described previously, and most were assayed without storage, although carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities were stable to storage at −20°C for several days (7).

Methods for the assay of pyrimidine-repressible and arginine-repressible carbamyl phosphate synthetases (6) and aspartate transcarbamylase (11) have been described elsewhere.

Reaction mixtures for assay of dihydroorotase contained 0.28 M K^+-glycine (pH 10), 10 mM dihydroorotate, 5 mM $ZnCl_2$, and 100 μl of extract in a final volume of 1.0 ml. Assays were incubated at 37°C for 30 min and terminated by the addition of 5% $HClO_4$. The blank had dihydroorotate added after $HClO_4$. The formation of carbamyl aspartate was then determined by the procedure of Prescott and Jones (9). *B. subtilis* dihydroorotase had a pH optimum of 9.8 and a K_m for dihydroorotate of 1.1 mM. Co^{2+} and Zn^{2+} at optimal concentrations stimulated activity 2.3- and 1.7-fold, respectively. No inhibition by 5 mM UMP, UTP, ATP, AMP, CTP, OMP, GMP, or GTP was observed under our assay conditions.

Dihydroorotate dehydrogenase was assayed by following the decrease in absorbance at 420 nm in a reaction mixture containing 0.3 M Tris-hydrochloride (pH 8.1), 1 mM $K_3Fe(CN)_6$, 5 mM dihydroorotate, and 100 to 200 μl of extract in a final volume of 1.0 ml. A decrease in absorbance of 1.076 corresponded to 0.5 μmol of orotate formed per ml. Reactions were incubated at 37°C and initiated by the addition of enzyme. The blank was a sample lacking dihydroorotate. The assay was linear with time for only 5 to 10 min. The activity of dihydroorotate dehydrogenase in extracts was routinely stabilized by inclusion of 10 mg of bovine serum albumin per ml of the extracts. The activity and stability of the enzyme were not altered by the

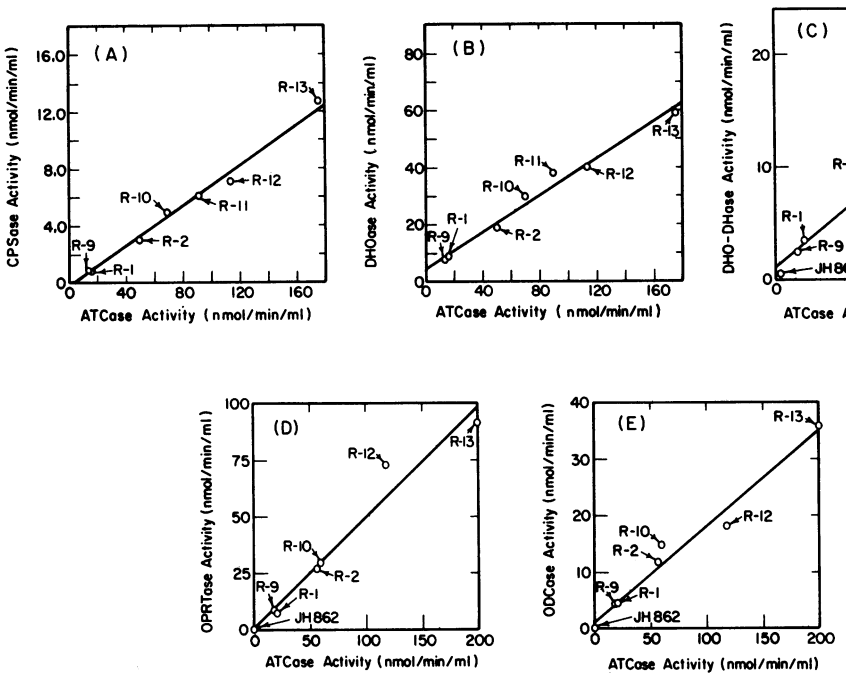

FIG. 1. Coordinate plots for the activities of pyrimidine biosynthetic enzymes in a series of mutant strains that were resistant to repression by uracil. (A) and (B), (C), and (D) and (E) show the results of separate experiments in which several of the strains described in the text were grown at 37°C with vigorous aeration on the minimal salts described by Cox and Hanson (1) supplemented with 0.1% glucose, 50 μg of uracil per ml, and a mixture of 50 μg of each of 19 amino acids (excluding arginine) per ml. The cells were harvested during exponential growth when they had reached a cell density of 80 to 100 U as determined with a Klett-Summerson colorimeter with a no. 66 filter. Activities are presented as nanomoles per minute per milliliter of culture, where 1 ml of culture corresponds to about 1.8×10^8 cells. The aspartate transcarbamylase activity of the parent strain (JH861) grown in the absence of uracil was about 16 nmol/min per ml under these conditions. Strains R-1, R-2, and R-9 are resistant to both uracil and uridine; strains R-10, R-11, R-12, and R-13 are uracil resistant but sensitive to uridine. the abscissa shows the activity of aspartate transcarbamylase (ATCase) versus (A) pyrimidine-repressible carbamyl phosphate synthetase (CPSase), (B) dihydroorotase (DHOase), (C) dihydroorotate dehydrogenase (DHO-DHase), (D) orotate phosphoribosyltransferase (OPRTase), and (E) orotidine-5'-monophosphate decarboxylase (ODCase). The lines represent least-squares fits to the datum points.

inclusion of 0.1% Triton X-100 in the buffers. The enzyme displayed a pH optimum of 7.8 and a K_m for dihydroorotate of 0.2 mM. The enzyme was 30% inhibited by 1 mM orotate under our assay conditions, but 1 mM UMP, UTP, GMP, AMP, ATP, and IMP were not inhibitory.

OMP decarboxylase was assayed by a determination of the rate of release of $^{14}CO_2$ from [*carboxy*-^{14}C]OMP. The reaction vessel, sealed with a serum cap, and the CO_2 trapping procedure of Fox (2) were used. The reaction mixture contained 0.5 mM [^{14}C]OMP (37,500 cpm), 25 mM K^+-HEPES (pH 7.7), 10 mg of bovine serum albumin, and 20% (vol/vol) glycerol in a final volume of 0.5 ml. Reactions were initiated by the addition of 25 to 100 μl of extract and incubated at 30°C. The assays were terminated by injecting 0.5 ml of 1N HCl and incubated for 1 h at 45°C to trap $^{14}CO_2$. The trapped CO_2 was counted in 15 ml of scintillation fluid (xylene–Triton X-114–water [12:4:1 vol/vol] containing 0.4% 2,5-diphenyloxazole) after standing overnight to minimize chemiluminescence. The activity of the decarboxylase could be stabilized by extracting cells and assaying in buffers containing 10 mg of bovine serum albumin per ml, 20% glycerol, and 0.1 mM OMP. Under these conditions the enzyme lost activity at 5% per h at 0°C. The activity of the enzyme was nearly constant from pH 6.7 to 9.5. The K_m for OMP was 1.9×10^{-6} M. The enzyme was not affected by 4 mM AMP, ATP, GMP, GTP, CMP, CTP, UMP, UDP, or UTP, PP_i, carbamyl phosphate, carbamyl aspartate, or 6-azauracil under standard assay conditions. Interestingly, the enzyme was completely inhibited by 1 mM phenylmethylsulfonyl fluoride.

Orotate phosphoribosyltransferase was as-

sayed by a radioisotopic procedure similar to that used for OMP decarboxylase, except that [*carboxy*-^{14}C]orotate and PRPP were used as substrates and an excess of partially purified orotate decarboxylase [protein precipitating between 40 and 65% of saturation with $(NH_4)_2SO_4$] from *S. typhimurium* S-88, which lacks orotate phosphoribosyltransferase, was added. The reaction vessels were exactly the same as for the decarboxylase assay, except that the reaction mixture contained 0.5 mM [^{14}C]orotate (175,000 cpm), 4 mM PRPP, sufficient decarboxylase from *S. typhimurium* S-88 to catalyze decarboxylation of 50 to 75 nmol of OMP per min, extract to be assayed (10 to 50 μl), and buffer (50 mM Tris-hydrochloride [pH 8.1] containing 10 mg of bovine serum albumin per ml, 20% glycerol, and 5 mM $MgCl_2$) to bring the final volume to 0.5 ml. Both extracts, 2 mM PRPP, and buffer were incubated together for a few mintues at 30°C. Then an additional 2 mM PRPP and the [^{14}C] orotate were added to initiate the reaction. This protocol was necessary to obtain assays that were linear with time. It was necessary to stabilize the phosphoribosyltransferase activity in the extracts by adding 10 mg of bovine serum albumin per ml, 20% glycerol, and 3 mg of PRPP per ml to buffers. Under these conditions the enzyme lost activity at a rate of 15% per h at 0°C; it was stable under assay conditions at 30°C for 30 min, however. The pH optimum for the phosphoribosyltransferase was between 8 and 9.5; pH 8.1 was used for assays to minimize PRPP breakdown. The enzyme required Mg^{2+} for activity and was inhibited by Ca^{2+} or EDTA. Under standard assay conditions the enzyme was weakly inhibited by 10 mM ATP, UTP, CTP, and GTP and almost totally inhibited by 10 mM dihydroorotate.

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