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Identification of *tms-26* as an Allele of the *gcaD* Gene, Which Encodes *N*-Acetylglucosamine 1-Phosphate Uridyltransferase in *Bacillus subtilis*

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The temperature-sensitive *Bacillus subtilis tms-26* mutant strain was characterized biochemically and shown to be defective in *N*-acetylglucosamine 1-phosphate uridyltransferase activity. At the permissive temperature (34°C), the mutant strain contained about 15% of the wild-type activity of this enzyme, whereas at the nonpermissive temperature (48°C), the mutant enzyme was barely detectable. Furthermore, the *N*-acetylglucosamine 1-phosphate uridyltransferase activity of the *tms-26* mutant strain was much more heat labile in vitro than that of the wild-type strain. The level of *N*-acetylglucosamine 1-phosphate, the substrate of the uridyltransferase activity, was elevated more than 40-fold in the mutant strain at the permissive temperature compared with the level in the wild-type strain. During a temperature shift, the level of UDP-*N*-acetylglucosamine, the product of the uridyltransferase activity, decreased much more in the mutant strain than in the wild-type strain. An *Escherichia coli* strain harboring the wild-type version of the *tms-26* allele on a plasmid contained increased *N*-acetylglucosamine 1-phosphate uridyltransferase activity compared with that in the haploid strain. It is suggested that the gene for *N*-acetylglucosamine 1-phosphate uridyltransferase in *B. subtilis* be designated *gcaD*.

The *Bacillus subtilis tms-26* lesion was originally isolated during a search for temperature-sensitive mutants (4). The molecular defect was not discovered, nor was a physiological function assigned to the gene, although it appeared from the original studies that the mutant was altered in cell morphology. Later, the wild-type allele was cloned, and the promoter driving the expression of the gene was studied in appreciable detail. The promoter appears to be recognized by RNA polymerase holoenzyme containing σ^A (5, 6, 9, 10, 17, 18, 29). Furthermore, the nucleotide sequence of the gene has been reported, and the gene has been shown to be located immediately upstream of and possibly expressed as an operon with the *prs* gene, encoding phosphoribosylpyrophosphate synthetase (11, 19). A search of data bases for sequences homologous to the wild-type version of the *tms-26* mutant allele revealed an open reading frame in *Escherichia coli* designated ECO*urf-1* (19). The gene encoding this open reading frame in *E. coli* is located between the *atp* operon and the *glmS* gene, encoding glucosamine 6-phosphate synthase, at 84 min on the linkage map (28). To gain further insight into the function of the gene product of the *B. subtilis tms-26* mutant allele as well as that of the wild-type allele, I initiated a detailed physiological study of the mutant.

The data presented in this article demonstrate that *B. subtilis* strains harboring the *tms-26* mutant allele are defective in the enzyme *N*-acetylglucosamine 1-phosphate uridyltransferase (EC 2.7.7.23). I suggest the designation *gcaD* for the gene for this enzyme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used were ED334 (*metB5 leuA8*) and ED336 (*metB5 leuA8 gcaD26* [*tms-26*]). They were derived from BC50 (*metB5 leuA8 nic-38 purA16*) (4), which was transformed to Nic$^+$ and Pur$^+$, and BC102 (*metB5 leuA8 purA16 gcaD26* [*tms-26*]) (4), which was transformed to Pur$^+$, respectively, with DNA isolated from strain 168. BC50 and BC102 were obtained from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus. As the host strain for plasmids, *E. coli* HO340 (18) was used. *B. subtilis* was grown in Spizizen minimal medium (26) or in L broth (16). Supplements to the minimal medium were glucose (0.5%), Casamino Acids (0.2%), and leucine and methionine (40 mg/liter each). *E. coli* was grown in L broth. Cell growth was monitored as the A_{436} in an Eppendorf PCP6121 photometer. An A_{436} of 1 (1-cm light path) corresponds to approximately 0.2 mg (dry weight) per ml.

Preparation of cell extracts. Cells were grown in L broth. At an A_{436} of 0.8, 40 ml of the culture was harvested by centrifugation, washed in 0.9% saline, and centrifuged. Pellets were usually frozen before analysis. For preparation of extracts, pellets were resuspended in 0.5 ml of buffer, sonicated in an MSE ultrasonic disintegrator four times for 15 s each time, and centrifuged in a Sorvall SS-34 rotor at 5,000 rpm for 20 min.

Enzyme assays. *N*-Acetylglucosamine 1-phosphate uridyltransferase activity was assayed as follows. Ten microliters of extract (in 50 mM Tris-HCl [pH 7.5]) containing uridyltransferase activity of 0.5 to 5 nmol/min, prewarmed to 37°C, was added to 40 μl of a reaction cocktail, prewarmed to 37°C, to yield the following final concentrations: 4 mM *N*-acetylglucosamine 1-phosphate, 3 mM [α-^{32}P]UTP (10 TBq/mol), 8 mM $MgCl_2$, 20 mM NaF, and 50 mM Tris-HCl (pH 8.0). At 1, 3, and 6 min, 10-μl samples were removed and mixed on a piece of Parafilm with 5 μl of 0.33 M HCOOH containing 5 mM UDP-*N*-acetylglucosamine. This 15 μl was applied to a polyethyleneimine-cellulose thin-layer chromatographic plate. The plate was dried and, to separate UTP and UDP-*N*-acetylglucosamine, developed in system 1, which consisted of methanol (2 cm), 1 M CH_3COOH (2 cm), and 0.9 M CH_3COOH–0.3 M LiCl (15 cm) (22). Occasion-

ally, the chromatograms were developed in another solvent, system 2, consisting of 6 g of $Na_2B_4O_7 \cdot 10H_2O$, 3 g of H_3BO_3, and 25 ml of ethylene glycol in 70 ml of water (22). These chromatograms were developed for 18 cm. After development, the chromatograms were dried and submitted to autoradiography for approximately 15 h or visualized under a UV mineral lamp (254 nm) to locate the UTP and the UDP-*N*-acetylglucosamine spots. The spots were cut out, and the radioactivity was determined in a Packard Tri-Carb 2000 liquid scintillation analyzer. For determination of the *N*-acetylglucosamine 1-phosphate-dependent formation of UDP-*N*-acetylglucosamine, incubation mixtures that lacked *N*-acetylglucosamine 1-phosphate were run in parallel. Sampling in HCOOH served to stop the reaction, and the UDP-*N*-acetylglucosamine present in HCOOH served as a marker. The thin-layer plates used were prepared as described before (23); commercially available polyethyleneimine-cellulose thin-layer plates from Baker-flex were also applicable.

Phosphoribosylpyrophosphate synthetase activity was assayed by a modification of the procedure described by Arnvig et al. (1). Ten microliters of extract (in 50 mM potassium phosphate buffer [pH 7.5]) was added to 90 μl of a reaction mixture (both prewarmed to 37°C) to yield the following final concentrations: 50 mM potassium phosphate–50 mM triethanolamine buffer (pH 8.0), 5 mM ribose 5-phosphate, 3 mM [γ-^{32}P]ATP (1 TBq/mol; prepared as described previously [13]), 5 mM $MgCl_2$, 20 mM NaF, 17 mM phosphoenolpyruvate, and 0.2 μg of pyruvate kinase (Boehringer). Sampling and chromatographic separation of [^{32}P]ATP and [^{32}P]phosphoribosylpyrophosphate were performed as previously described (13).

Glucose 6-phosphate dehydrogenase activity was determined as described by Kornberg and Horecker (14). Protein content was determined by the bicinchoninic acid procedure with chemicals provided by Pierce (25). Bovine serum albumin was used as the standard.

^{32}P labelling of sugar phosphates, sugar nucleotides, and ribonucleotides. Cells were grown in 3-(*N*-morpholino)propanesulfonic acid-buffered medium (7) supplemented with glucose, methionine, leucine, Casamino Acids, and 2 mM phosphate. After several generations of exponential growth, carrier-free $^{32}P_i$ was added to a specific radioactivity of 0.56 TBq/mol. After at least two doubling times, cells were harvested by rapid filtration on Sartorius SM30 filters, and the sugar nucleotides, sugar phosphates, and ribonucleoside triphosphates were extracted by treatment of the cells with ice-cold 0.33 M HCOOH. A sample to be chromatographed was mixed with unlabelled *N*-acetylglucosamine 1-phosphate and *N*-acetylglucosamine 6-phosphate (50 nmol each) and with UDP-*N*-acetylglucosamine and UMP (5 nmol each), and separation was performed by two-dimensional thin-layer chromatography on polyethyleneimine-cellulose plates by the system described by Randerath and Randerath (22). Chromatograms were submitted to autoradiography. The positions of uracil-containing compounds were visualized under a UV mineral lamp, while the positions of *N*-acetylglucosamine 1-phosphate and *N*-acetylglucosamine 6-phosphate were located by dipping the chromatograms in a solution of $FeCl_3$ and 5-sulfosalicylic acid in ethanol (27). The radioactive spots were cut out, and the radioactivity was quantitated by liquid scintillation counting. Ribonucleoside triphosphates were determined as described by Jensen et al. (13).

[^{14}C]uracil labelling of nucleotides. Cells were grown exponentially in Spizizen minimal medium supplemented with glucose, methionine, leucine, Casamino Acids, and [2-^{14}C] uracil (88 μM; 1.42 TBq/mol). After at least two doubling times, cells were harvested by filtration, and labelled compounds were extracted with ice-cold 0.33 M HCOOH. Extracts were mixed with unlabelled UDP-*N*-acetylglucosamine and UDP-glucose before chromatography. Following two-dimensional thin-layer chromatography and autoradiography as described above, the spots of these compounds were cut out, and the radioactivity was determined by liquid scintillation counting. In addition, the UTP content was determined by one-dimensional thin-layer chromatography in 0.85 M KH_2PO_4 (pH 3.4).

Genetic procedures and DNA manipulation. Transformation of *B. subtilis* was performed essentially as described by Boylan et al. (3). Transformation of *E. coli* has also been described (15). *E. coli* transformants were selected, and plasmids were maintained by the presence of ampicillin (50 mg/liter). The DNA species used were pDA2 (18) and pHO143, which was constructed by digestion of pDA2 DNA with restriction endonuclease *Eco*RI and ligation to *Eco*RI-treated pBR322 DNA (2). Plasmid pHO143 contains the *gcaD* gene in a 1,664-nucleotide DNA fragment (nucleotides 1 to 1664 of the sequence published by Nilsson et al. [19]). Restriction endonuclease *Eco*RI and T4 DNA ligase were obtained from Boehringer.

Materials. *N*-Acetylglucosamine 1-phosphate and UDP-*N*-acetylglucosamine were obtained from Sigma Chemical Co. Other nucleotides and phosphoenolpyruvate were obtained from Boehringer. [α-^{32}P]UTP and [2-^{14}C]uracil were purchased from New England Nuclear Corp., and carrier-free $^{32}P_i$ was purchased from Amersham.

RESULTS

Growth of the *B. subtilis gcaD26* strain. It was reported originally that the mass doubling time was slightly increased (growth was decreased) for the *gcaD26* strain grown at 48°C in enriched salts medium and also that the mutant strain entered the stationary growth phase at a lower cell density than did the parent strain (4). In my experiments, however, the *gcaD26* strain (ED336) grew almost like the *gca*$^+$ (wild-type) strain (ED334) at the permissive temperature and adopted a lower mass doubling time (growth was increased) following a temperature shift to 48°C. Cultures of the *gcaD26* strain became stationary about 1.5 h after the temperature shift and, moreover, lysed at this time, causing a loss in absorbance and cell viability (data not shown). A loss of cell viability was also observed on solid media. Thus, there was no survival of cells seeded on L broth agar plates after incubation at 34°C, following an overnight incubation at 48°C. Incubation of the *gcaD26* strain at 48°C apparently is lethal. In the experiments described below, care was taken to sample cultures of the *gcaD26* strain at the restrictive temperature before cell lysis occurred.

Sugar nucleotide and sugar phosphate pools. Given the altered cell shape of the *gcaD26* strain following a temperature shift to 48°C (4), the fact that *gcaD26* cells lyse after the temperature shift, and the close proximity and eventual operon structure of the *E. coli gcaD* and *glmS* (glucosamine 6-phosphate synthase) genes, I inferred that the mutant strain might be defective in cell wall synthesis and that a likely candidate for a defective enzyme was one catalyzing a step in the pathway leading from glucosamine 6-phosphate to UDP-*N*-acetylglucosamine. To study this pathway, I determined the pool sizes of the metabolites *N*-acetylglucosamine 6-phosphate, *N*-acetylglucosamine 1-phosphate, and UDP-

TABLE 1. Sugar nucleotide and sugar phosphate pool sizes in *gcaD26* and *gca*$^+$ strains at permissive and nonpermissive temperatures[a]

Strain	Growth temp (°C)	Pool size (μmol/g [dry weight]) for:			
		UDPGlcNAc[b]	GlcNAc1P[c]	UDPGlc[d]	UMP
ED336 (*gcaD26*)	34	1.33	2.22	1.45	0.57
	48	0.14	1.43	1.31	0.14
ED334 (*gca*$^+$)	34	2.83	<0.05	2.99	0.94
	48	2.02	<0.05	2.18	0.48

[a] Cells growing exponentially in the presence of $^{32}P_i$ at 34°C were diluted 10-fold at an A_{436} of 0.8 into identical media prewarmed at 48°C, and growth was continued at 48°C. Samples were removed at an A_{436} of 0.8. Extraction and chromatography were performed as described in Materials and Methods. Duplicate experiments showed less than 10% variation.
[b] UDPGlcNAc, UDP-*N*-acetylglucosamine.
[c] GlcNAc1P, *N*-acetylglucosamine 1-phosphate.
[d] UDPGlc, UDP-glucose.

N-acetylglucosamine by ^{32}P labelling of the metabolites of mutant and wild-type cells growing at 34 and 48°C. *N*-Acetylglucosamine 6-phosphate was very difficult to quantitate by the chromatographic system used because of cochromatography with other compounds, but from the autoradiograms there appeared to be very little *N*-acetylglucosamine 6-phosphate present, and there was no major alteration in the amount of this compound (data not shown). The pool sizes of the other two compounds are shown in Table 1, together with determinations of UDP-glucose and UMP. It is evident from these data that the level of *N*-acetylglucosamine 1-phosphate was elevated more than 40-fold in the mutant strain grown at the permissive temperature compared with the level in the wild-type strain. Furthermore, the pool of UDP-*N*-acetylglucosamine in the mutant strain dropped to a very low level following the shift to the nonpermissive temperature, much more so than in the wild-type strain. In general, all the levels were reduced after the temperature shift. The ribonucleoside triphosphate pools were almost identical in the two strains, both before and after the temperature shift (data not shown). The data presented in Table 1 indicate that the utilization of *N*-acetylglucosamine 1-phosphate is reduced in the mutant strain at both temperatures and that the formation of UDP-*N*-acetylglucosamine is greatly reduced at the nonpermissive temperature, and therefore the activity of *N*-acetylglucosamine 1-phosphate uridyltransferase is impaired.

Kinetics of pool changes during the temperature shift. For analysis of the pool changes in more detail during the temperature shift, the cells were labelled with [2-^{14}C]uracil, and nucleotides were extracted and quantitated (Fig. 1). The mutant strain showed a decrease in the pool size of UDP-*N*-acetylglucosamine immediately after the temperature shift, in contrast to the immediate increase in the pool size in the wild-type strain. In addition, the relative changes in the pool sizes of UDP-glucose and UTP were almost identical in the two strains. These results also indicate an impairment of UDP-*N*-acetylglucosamine uridyltransferase in the mutant strain.

Activity of *N*-acetylglucosamine 1-phosphate uridyltransferase. For assaying the activity of *N*-acetylglucosamine 1-phosphate uridyltransferase, a new assay procedure was developed. With this procedure, the *N*-acetylglucosamine 1-phosphate-dependent formation of [^{32}P]UDP-*N*-acetylglucosamine from [^{32}P]UTP is determined by chromatographic

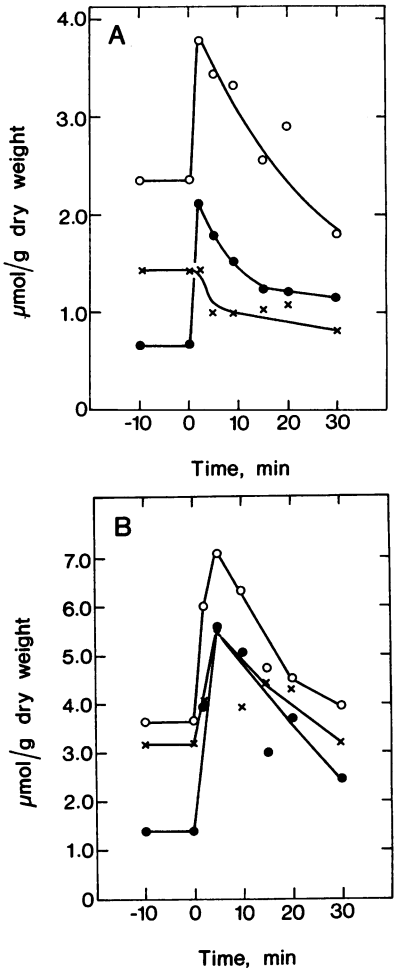

FIG. 1. Kinetics of pool changes for UDP-*N*-acetylglucosamine (×), UDP-glucose (●), and UTP (○) in *gcaD26* (A; ED336) and *gca*$^+$ (B; ED334) strains during a temperature shift. Cells were grown in the presence of [2-^{14}C]uracil at 34°C for at least two doublings as described in Materials and Methods. At an A_{436} of 0.3 (zero time), the cultures were rapidly heated to 48°C. Samples were removed at intervals, and nucleotides were extracted and quantitated as described in Materials and Methods. Note the difference in the units on the ordinates. The data are the results of a typical experiment. Results of duplicate experiments were virtually identical.

separation of the two labelled compounds. An example of the determination of the activity of this uridyltransferase is shown in Fig. 2. The results of uridyltransferase assays of the *gcaD26* and *gca*$^+$ strains are given in Table 2. In addition to uridyltransferase activity, the activities of other selected enzymes are given. It appears from these data that the activity of uridyltransferase in the mutant strain was reduced to approximately 15% of that in the wild-type strain at the permissive temperature. At the nonpermissive temperature, uridyltransferase activity was barely detectable in the mutant strain. The activities of phosphoribosylpyrophosphate synthetase and glucose 6-phosphate dehydrogenase were almost identical in the two strains.

Heat inactivation was performed by heating extracts of the two strains grown at 34°C to 56°C as shown in Fig. 3. The results show that the *gcaD26* strain contains an *N*-acetylglu-

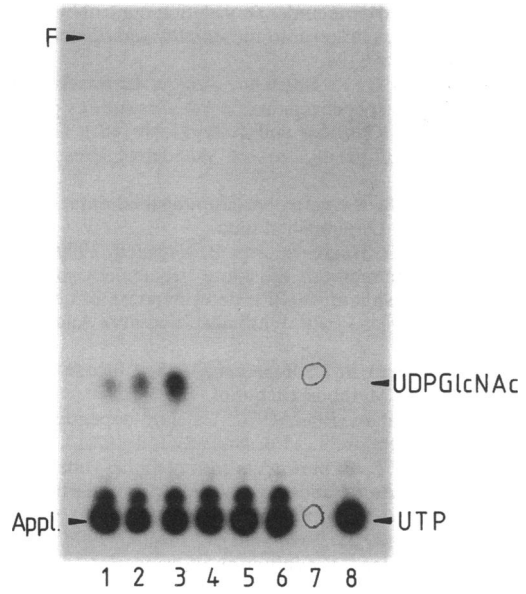

FIG. 2. Assay of *N*-acetylglucosamine 1-phosphate uridyltransferase activity. An autoradiogram of a chromatogram used to separate UTP and UDP-*N*-acetylglucosamine (UDPGlcNAc) is shown. Incubation, sampling, and chromatography (system 1) were as described in Materials and Methods. Lanes: 1 to 3, samples taken at 1, 3, and 6 min from the standard assay; 4 to 6, samples taken at 1, 3, and 6 min from an assay that lacked *N*-acetylglucosamine 1-phosphate; 7, circles indicate the positions of unlabelled markers; 8, sample of an assay mixture to which no extract was added. Appl., application line; F, solvent front.

cosamine 1-phosphate uridyltransferase that is much more heat labile than the enzyme of the *gca*$^+$ strain.

Finally, an *E. coli* strain was transformed with the plasmid that contained the wild-type *gcaD* allele (pHO143). The construction and structure of this plasmid are described in Materials and Methods. The uridyltransferase activity determined in extracts of strain HO340/pHO143 was 179 nmol/min/mg of protein; that in the haploid host strain, HO340, was 41 nmol/min/mg of protein. Thus, the activity in the former was four times that in the latter.

TABLE 2. Enzyme activities in *gcaD26* and *gca*$^+$ strains at permissive and nonpermissive temperatures[a]

Strain	Growth temp (°C)	Activity (nmol/min/mg of protein) of:		
		GlcNAc1P uridyltransferase[b]	PRibPP synthetase[c]	Glucose 6-phosphate dehydrogenase
ED336 (*gcaD26*)	34	29	123	161
	48	7	83	151
ED334 (*gca*$^+$)	34	184	101	163
	48	132	99	144

[a] Cells growing exponentially in L broth at 34°C were diluted 10-fold at an A_{436} of 0.8 into identical media prewarmed at 48°C, and growth was continued at 48°C. Cell extracts were prepared and enzyme activities were determined as described in Materials and Methods. The data are results of typical experiments. Results of duplicate experiments were virtually identical.

[b] GlcNAc1P, *N*-acetylglucosamine 1-phosphate.

[c] PRibPP, phosphoribosylpyrophosphate.

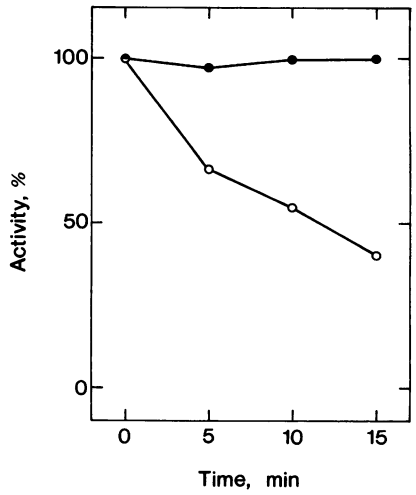

FIG. 3. Heat inactivation of the *N*-acetylglucosamine 1-phosphate uridyltransferase activities in *gcaD26* (○; ED336) and *gca*$^+$ (●; ED334) strains. Cultures were grown in L broth at 34°C. Cell extracts were prepared and assays were performed as described in Materials and Methods. At zero time, extracts were heated to 56°C. Samples were removed at intervals, cooled in ice, and centrifuged to remove denatured protein, and the residual enzyme activities were assayed. Heat inactivation was done at a protein concentration of 5 mg/ml with 50 mM Tris-HCl (pH 7.5). The enzyme activities corresponding to 100% were those given in Table 2.

DISCUSSION

In bacteria, UDP-*N*-acetylglucosamine is the precursor for the peptidoglycan components *N*-acetylglucosamine and *N*-acetylmuramic acid (20), as well as for other polysaccharides (24). In gram-negative bacteria, UDP-*N*-acetylglucosamine also is the precursor for the di-*N*-acetylglucosamine moiety of lipid A (21). Thus, UDP-*N*-acetylglucosamine is an important donor of acetylglucosamine residues in di- and polysaccharide synthesis. The synthesis of UDP-*N*-acetylglucosamine, however, has been only poorly characterized. UDP-*N*-acetylglucosamine is believed to be synthesized from fructose 6-phosphate by four enzyme-catalyzed reactions. The *B. subtilis* gene (*gcaA*) encoding the enzyme for the first of these reactions, glucosamine 6-phosphate synthase, has been identified, and mutants defective in *gcaA* have been characterized (8). The enzymes for the next two reactions, glucosamine 6-phosphate acetyltransferase and phospho-*N*-acetylglucosamine mutase, as well as their genes, remain to be characterized. I have shown here that the *gcaD26* (*tms-26*) mutant strain of *B. subtilis* is in fact defective in *N*-acetylglucosamine 1-phosphate uridyltransferase, the enzyme catalyzing the fourth step of the pathway, the synthesis of UDP-*N*-acetylglucosamine. The salient characteristics of the mutant strain include (i) a heat-labile uridyltransferase, (ii) reduced uridyltransferase activity at the permissive temperature, (iii) lack of uridyltransferase activity at the nonpermissive temperature, (iv) increased levels of *N*-acetylglucosamine 1-phosphate, the substrate of the uridyltransferase, and (v) decreased levels of UDP-*N*-acetylglucosamine, the product of the uridyltransferase, at the nonpermissive temperature. In addition, an *E. coli* strain harboring the *B. subtilis gcaD* gene on a plasmid showed increased uridyltransferase specific activity.

One of the prominent properties of the *gcaD26* strain is the lysis of cells following a shift to the nonpermissive temper-

ature. This property is in contrast to the properties originally described by Copeland and Marmur (4), who found only a 50% reduction in viability and apparently no loss in cell density following a temperature upshift. The reason for this discrepancy is not clear at present but may involve growth medium differences. I have found that the temperature sensitivity of the *gcaD26* strain can be restored to temperature resistance by transformation with plasmid pHO143, which harbors a 1,664-nucleotide DNA fragment carrying the wild-type *gcaD* allele (12). Furthermore, congression of a variety of *B. subtilis* strains with DNA isolated from BC102 (the original *gcaD26* strain) resulted in the isolation of a number of temperature-sensitive transformants, all of which could be complemented by pHO143 (12). Thus, the temperature sensitivity of the *gcaD26* strain appears to be caused by a single mutation.

E. coli host cells harboring a plasmid-borne *gcaD* gene show a modest increase in uridyltransferase activity only, approximately a one-unit contribution per replicon. The reason for this low gene dosage effect is not understood, but this effect may be caused by autoregulation of *gcaD* gene expression. Attempts to overproduce uridyltransferase activity in *B. subtilis* have so far been unsuccessful (11). Extracts of *E. coli* cells harboring the *E. coli gcaD* gene also show a low gene dosage effect, approximately fourfold (12).

A question that needs to be addressed is the apparent operon organization of the *gcaD* and *prs* (phosphoribosylpyrophosphate synthetase) genes, which both encode essential "housekeeping" enzymes that may be regulated by a common phenomenon, e.g., the growth rate.

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