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Isolation of a developmental gene of *Bacillus subtilis* and its expression in *Escherichia coli*

(glucose dehydrogenase/λ Charon phage/cloning)

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ABSTRACT **Glucose dehydrogenase of *Bacillus subtilis* is a developmental enzyme that is not found in growing (vegetative) cells but is synthesized after the differentiation process that leads to the production of endospores has started. We have isolated the gene coding for this enzyme from a λ Charon 4A phage library of *B. subtilis* DNA. It is transcribed and translated in vegetative cells of the nondifferentiating organism *Escherichia coli* into enzymatically active glucose dehydrogenase that has the same physicochemical properties as the enzyme produced in *B. subtilis* during sporulation. Subcloning of the λ DNA insert into pBR322 plasmid derivatives showed that the glucose dehydrogenase gene was transcribed in *E. coli* from a promoter within the *B. subtilis* genome.**

Bacilli respond to adverse nutritional conditions by developing heat-resistant endospores. This differentiation process involves sequential morphological and biochemical changes whose interdependence has been characterized by mutants blocked at different developmental stages (1, 2). The first morphological event following the deprivation of nutrients is the formation of an asymmetric septum instead of the symmetric division septum. The two membranes of the asymmetric septum grow around the small cell compartment until this developing "forespore" has been completely engulfed. Because the two membranes have opposite polarity, preventing normal active transport, the metabolic milieu of the forespore probably differs drastically from that of the mother cell (3–5).

Only when this development has occurred does glucose dehydrogenase (GlcDH; β-D-glucose: NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.47) appear (6): this enzyme is apparently made only in the forespore (7). This raises the question of how the transcription and translation of the *GlcDH* gene is controlled. Attempts to study this control mechanism by isolating mutants that are altered in the structural gene or produce GlcDH constitutively have not succeeded; this failure results in part from the fact that any mutant blocking forespore development also pleiotropically prevents the synthesis of GlcDH. In the work described here, we raised polyclonal antibodies against pure GlcDH (ref. 7; unpublished data) and used them to detect the presence of the *GlcDH* gene in *Escherichia coli* cells infected by λ Charon 4A phages that contained fragments of the *Bacillus subtilis* genome. We report here that the *GlcDH* operon of *B. subtilis*, which does not function in vegetative cells of *B. subtilis*, produces active GlcDH in vegetative cells of *E. coli*.

MATERIALS AND METHODS

Bacteria and Growth Media. The *E. coli* strains used were LE392 and HB101/λ (8). The *B. subtilis* strains are listed in Table 1. Plasmids used were pBR322 (8) and pK06; the latter was derived from pK01 (9) by insertion of an *Eco*RI linker into the *Sma* I site upstream of *galK*. *E. coli* strains were grown in Luria–Bertani (LB) medium (8). *B. subtilis* strains were grown in nutrient sporulation medium (10).

Preparation of Antibody Against GlcDH. Fifty micrograms of GlcDH was dissolved in 0.5 ml of 50 mM imidazole·HCl, pH 6.5/20% glycerol, mixed with 0.5 ml of complete Freund's adjuvant (Difco), and injected subcutaneously (on the dorsal side) into male New Zealand rabbits. Three wk later, and every 8th day thereafter, booster injections of 1 ml of an emulsion of 0.8 ml of Freund's incomplete adjuvant (Difco) and 20 μg of GlcDH in 0.2 ml of buffer were given. One week after each booster injection, blood was obtained from the rabbit's ear and the serum was stored at −70°C. The antibodies (IgG) were partially purified at room temperature by Na_2SO_4 precipitation. Antiserum was diluted three times with phosphate buffer [4.28 mM Na_2HPO_4/1.46 mM KH_2PO_4, pH 7.3/136 mM NaCl/2.6 mM KCl (buffer P)] and dialyzed overnight against 18% (wt/vol) Na_2SO_4. The resulting precipitate was washed with 18% Na_2SO_4, dissolved in buffer P and dialyzed against 15% (wt/vol) Na_2SO_4 overnight. This precipitate was washed with 15% (wt/vol) Na_2SO_4, dissolved in buffer P, and dialyzed against buffer P with one change of buffer P. Antibodies were iodinated by the chloramine-T method (11).

Preparation of *B. subtilis* Extracts. *B. subtilis* cells were grown in nutrient sporulation medium, harvested, and washed as described (10). The cells were suspended in 2 ml of 50 mM imidazole·HCl, pH 6.5/20% (vol/vol) glycerol and lysed by passage through a French pressure cell (Aminco, Silver Spring, MD) at 1,400 kg/cm^2. The lysate was centrifuged at 100,000 × *g* for 60 min, and the supernatant was stored at −20°C. The enzyme remained active and retained its antigenicity for at least 1 year. GlcDH was assayed spectrophotometrically as described (10).

Screening of the λ Charon 4A Library for GlcDH. The library of *B. subtilis* DNA in λ Charon 4A phages was a gift from E. Ferrari, J. D. Henner, and J. A. Hoch (12). *E. coli* LE392 cells (10^9/ml) were grown overnight in LB medium, infected by the phages (10^3/ml) and, after 20 min at 37°C, plated onto tryptone broth (TB) plates (8) to obtain about 100 individual plaques per Petri dish. The lysates of individual plaques were picked up with Pasteur pipets and transferred to "U-shaped" wells in a flexible polyvinyl chloride microtiter plate (Dynatech Laboratories, Alexandria, VA) containing λ dilution buffer (10 mM Tris·HCl, pH 7.5/10 mM $MgSO_4$). After overnight incubation at 4°C, ensuring optimal adsorption of proteins from the

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Abbreviations: GlcDH, glucose dehydrogenase; buffer P, 4.28 mM Na_2HPO_4/1.46 mM KH_2PO_4, pH 7.3/136 mM NaCl/2.6 mM KCl; amp^r, ampicillin resistant; tet^r, tetracycline resistant; kb, kilobase(s).

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cell lysate to the well, the nonadsorbed material, including phages, was transferred to a corresponding well in another microtiter plate (a rigid plastic plate with flat-bottomed wells; Falcon Div., Becton Dickinson) and stored at 4°C as a master plate for subsequent isolation of positive clones. The material that crossreacted with anti-GlcDH antibody in the microtiter plate was detected by radioimmunoassay. For this purpose, the wells were washed once with buffer P, and 3% bovine serum albumin (fraction 5; Armour Pharmaceuticals, Kankakee, IL) was added to saturate all binding sites. After removal of the liquid followed by washing the wells twice with buffer P, 50 μl of iodinated anti-GlcDH antibody [50,000 cpm (1–5 μCi of ^{125}I/μg of IgG; 1 Ci = 3.7 GBq), enough antibody to produce maximal counts with pure GlcDH] was added to each well, and the plate was incubated overnight at room temperature. The wells were washed five times with buffer P, dried, and cut with a razor blade, and radioactivity was measured in a gamma counter (Packard).

To determine whether the purified phages from the positive clones could produce GlcDH activity in *E. coli*, strain LE392 was infected at a multiplicity of 0.01, and 10^8 of these cells were plated on TB plates to produce confluent lysis. After 6 to 7 hr at 37°C, the plates were transferred to 4°C and 2 ml of 50 mM imidazole·HCl, pH 6.5/20% glycerol was added to each one. Four hours later, the buffer and the top agar layer were removed and centrifuged at 5,200 × *g* for 20 min. The supernatant was assayed for GlcDH enzyme activity.

To determine the molecular weight of the GlcDH made in *E. coli*, 1 vol of the lysate was mixed with 9 vol of ice-cold acetone and left at 4°C for 30 min to complete protein precipitation. After centrifugation, the pellet was dissolved in $NaDodSO_4$ sample buffer (62 mM Tris·HCl, pH 6.8/2.3% $NaDodSO_4$/5 mM dithiothreitol/10% glycerol) to a protein concentration of 2 mg/ml, and 20 μl of this preparation was loaded onto a 10% polyacrylamide gel (18 cm × 13 cm × 0.8 mm) that had been prepared by using 0.35 M Tris·HCl, pH 8.8/0.1% $NaDodSO_4$. The proteins were separated at a constant current of 20 mA for 3 hr. The separated proteins were electroblotted to a nitrocellulose sheet (0.45-μm pore size in roll form; Millipore), the sheet was incubated with 3% bovine serum albumin in Tris/saline buffer (10 mM Tris·HCl, pH 7.4/0.9% NaCl), and the paper was incubated with anti-GlcDH antibody in Tris/saline buffer. Then, the paper was washed with Tris/saline buffer and exposed to peroxidase-conjugated goat anti-rabbit IgG, and the peroxidase was visualized by fluorescence staining (13).

Characterization of the Phage and Plasmid DNA. To obtain recombinant phage DNA, *E. coli* LE392 cells were grown in 800 ml of LB broth/10 mM $MgSO_4$ and infected with the various (GlcDH-positive or -negative) clones of the λ Charon 4A library at a multiplicity of 0.01. As soon as the culture had lysed, it was centrifuged at 10,000 × *g* and the phage titer in the supernatant was measured. If the titer was greater than 10^{11}, the phages were precipitated by the addition of polyethyleneglycol 6000 (6% final concentration) and purified on a CsCl step gradient (8). The DNA was extracted by formamide treatment, digested with *Eco*RI, and electrophoresed in 0.7% agarose gel in Tris borate/EDTA buffer (8).

To subclone the *GlcDH* gene, λEF2 DNA was digested with *Eco*RI and ligated with *Eco*RI-digested alkaline phosphatase-treated plasmid pBR322 or pK06; this ligated DNA was used to transform HB101/λ cells (8). For pBR322 plasmids, ampicillin-resistant (amp^r) tetracycline-resistant (tet^r) transformants were isolated; for pK06 plasmids, amp^r transformants were isolated. Drug-resistant transformant colonies were inoculated into 100 μl of LB medium containing 50 μg of ampicillin per ml in wells of rigid plastic microtiter plates. The plates were slowly shaken at 37°C for 4 hr and then centrifuged using a swinging bucket rotor for microtiter plates (Sorvall). The supernatants were removed by aspiration, and the pellets were suspended in 50 μl of 50 mM Tris·HCl buffer, pH 8.0/1 mM EDTA containing 100 μg lysozyme per ml. After 30 min at 37°C, the plates were centrifuged; the supernatants were transferred to wells of a polyvinyl chloride microtiter plate and left overnight at 4°C for complete adsorption. The presence of GlcDH antigenic activity was detected by radioimmunoassay. Plasmid DNA was isolated by cell lysis and alkaline extraction as described by Birnboim and Doly (14). The DNA was digested with *Eco*RI and *Hin*dII and electrophoresed in a 0.7% agarose gel as described above.

RESULTS

The Synthesis of GlcDH Is Regulated at the Genetic Level. Earlier studies in *B. subtilis* correlated the appearance of GlcDH activity with completion of the forespore (stage 3) during spore development (2, 7, 10). Conceivably, the production of an active enzyme could be regulated either by the control of transcription of the *GlcDH* gene or by the controlled conversion of an inactive protein precursor to a functional enzyme. The possibility of stable mRNA has been rendered unlikely by studies indicating that mRNA of *B. subtilis* is as labile during sporulation as during growth (half-life, 3–5 min) (15). To determine whether an inactive GlcDH precursor was produced much earlier than the active enzyme, we examined a standard strain and various sporulation mutants using iodinated anti-GlcDH antibody. In vegetative cells of the standard strain (60015), we detected neither GlcDH crossreactive material nor GlcDH activity (Table 1). The same was true for all sporulation mutants blocked up to stage 2 of development, even when they were cultured into late stationary phase (5 hr after the end of exponential growth), at which time the standard strain produced maximal GlcDH activity. Because mutant cells whose differentiation is blocked at a certain stage produce most or all developmental proteins normally made before this stage, one would expect a GlcDH precursor to be present in at least some of the mutants if it were normally made before stage 3. However, significant amounts of both GlcDH crossreactive material and GlcDH activity were produced only by sporulation mutants blocked at stage 3 or later—i.e., after forespore development (Table 1). Therefore, *B. subtilis* does not seem to produce a precursor for GlcDH during vegetative growth or at sporulation stages preceding the completion of a forespore.

Isolation of the *GlcDH* Gene from a λ Charon 4A Library of *B. subtilis* DNA. DNA of *B. subtilis* had been partially methylated, digested with *Eco*RI, and packaged *in vitro* into λ Charon 4A by Ferrari *et al.* (12). We obtained these phages and screened 2,000 plaques, produced on a lawn of *E. coli* strain LE392, by a radioimmunoassay designed to detect material that crossreacted with anti-GlcDH antibody. The lysates of most plaques produced background counts (less than 700 cpm) whereas those of six plaques produced 10- to 20-fold higher counts (Table 2). From each of the clones with high antigenic activity, phages were isolated, amplified in *E. coli* LE392, and rescreened; the phages of positive clones were called λEF1–λEF6. All six clones produced active GlcDH when *E. coli* LE392 cells were infected by these phages and incubated until confluent lysis occurred. The specific GlcDH activity in these lysates was 1.5- to 15-fold higher than that observed during sporulation of *B. subtilis* (Table 2).

Comparison of the GlcDH Enzymes Obtained from λEF2-Infected *E. coli* and Sporulating *B. subtilis*. To determine whether the GlcDH produced by these λ clones in *E. coli* had the same molecular weight as the *B. subtilis* enzyme, the lysate

Table 1. Strains of *B. subtilis* and their enzymatic and antigenic GlcDH activities

Strain	Genotype	Sporulation block	Cpm per well*	GlcDH activity	Origin of strain
61731	*phe-1 trpC2*	*spo0A12*	343	<0.15	Hoch JH 646
61732	*phe-1 trpC2*	*spo0E11*	278	<0.15	Hoch JH 647
61733	*phe-1 trpC2*	*spo0B136*	155	<0.15	Hoch JH 648
61734	*phe-1 trpC2*	*spo0F221*	255	<0.15	Hoch JH 649
61735	*phe-1 trpC2*	*spo0H81*	282	<0.15	Hoch JH 651
61616	*tre-12*	*spo2*	352	<0.15	Heinze 701
61624		*spo2*†	425	<0.15	Schaeffer 1PF
61294		*spo2A*†	365	<0.15	Schaeffer 108H
61295		*spo3A*†	3,599	59.08	Schaeffer 83U
61625		*spo4*†	4,597	97.24	Schaeffer 83T
61297		*spo5*†	1,542	99.15	Schaeffer 65N
60015	*metC7 trpC2*	*spo*$^+$	1,025	206.4	Nester SB26
		spo$^+$ Veg‡	300	<0.15	

GlcDH activity is reported in (nmol/min)/mg of protein.

* Microtiter wells were cut from the plate, and radioactivity was determined in a gamma counter. Each value is a mean of triplicate samples.

† Statistical data about the developmental stage reached by these mutants have been published (16).

‡ Cells grown in nutrient sporulation medium were harvested 5 hr (t_5) after the end of exponential growth. Veg, vegetative cells harvested during exponential growth at an OD_{600} of 1.0. The harvested cells were suspended in 2 ml of 50 mM imidazole·HCl, pH 6.5/20% glycerol and lysed with a French press at 1,400 kg/cm^2. The extracts were centrifuged for 60 min at 100,000 × *g*; the supernatants were assayed for GlcDH activity and for the presence of GlcDH antigens by radioimmunoassay.

proteins were electrophoresed in a $NaDodSO_4$/polyacrylamide gel, and the location of the GlcDH antigen was determined by reaction with antibodies and peroxidase staining (Fig. 1). In all cases, the observed molecular weight was similar to that of the subunit of *B. subtilis* GlcDH, which is 31,500. This suggests that the *GlcDH* gene was translated as such and had not been fused to the gene of another protein.

The GlcDH of a 1-liter lysate of *E. coli* LE392 cells infected by λEF2 was purified by DEAE-cellulose chromatography followed by chromatofocusing, where it eluted at a pI of 4.7; after further hydrophobic chromatography, the enzyme was electrophoretically pure and had a specific activity of 595 (μmol/min)/mg of protein. Silver staining of the pure protein on a polyacrylamide gel showed the presence of a single band with an apparent subunit molecular weight of 31,500 (not shown). The K_m values of the enzyme were 11.4 mM with 2-deoxy-D-glucose and 12.8 mM with D-glucose. The enzyme could use NAD or NADP as cofactor. All these properties were the same as those of the GlcDH isolated from sporulating *B. subtilis* cells (7).

The *GlcDH* Gene Is Transcribed from a Promoter Within the *B. subtilis* Genome. The size of the *B. subtilis* DNA insert in λEF2 was measured by *Eco*RI digestion of the λEF2 DNA followed by agarose gel electrophoresis (Fig. 2). There were distinct DNA bands of sizes 5.0, 3.5, and 1.8 kilobases (kb) in addition to the bands representing the left and right arms derived from the λ Charon 4A parent phage (lanes 2 and 3). *Eco*RI digestion of the DNAs of the other λEF1–λEF6 phages produced the same five-band pattern (data not shown). To determine which DNA band contained the *GlcDH* gene, the *Eco*RI-digested λEF2 DNA was ligated into *Eco*RI-treated pBR322, which has only one *Eco*RI site and carries the markers amp^r and

Table 2. GlcDH antigenic and enzymatic activities of different clones of *B. subtilis* DNA in λ Charon 4A phages

Phage clone	Cpm per well	GlcDH activity
λ Charon 4A*	586	<0.15
λEF11†	651	<0.15
λEF1	8,637	2,480
λEF2	14,882	5,100
λEF3	6,869	510
λEF4	8,741	960
λEF5	8,674	2,030
λEF6	13,027	2,280

E. coli LE392 cells were infected with the original λ Charon 4A phage or with λ Charon phages containing *B. subtilis* DNA. GlcDH activity is reported in (nmol/min)/mg of protein.

* λ Charon 4A is the original phage.

† λEF11 is one of the many clones containing *B. subtilis* DNA but producing no GlcDH antigenic or enzymatic activity.

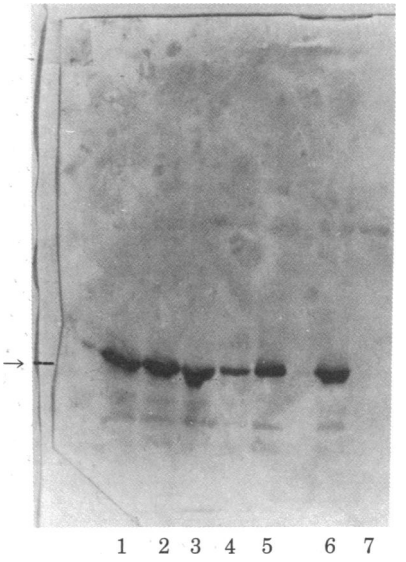

FIG. 1. Identification of the crossreacting protein after electrophoresis in a $NaDodSO_4$-containing gel. Proteins in extracts of *E. coli* LE392 cells, plated together with a phage preparation to produce confluent lysis, were electrophoretically separated in a 0.1% $NaDodSO_4$/10% polyacrylamide gel. Then the proteins were electroblotted onto nitrocellulose paper, the paper was exposed to anti-GlcDH antibody and washed, and the antibody was detected with goat anti-rabbit antibody and peroxidase staining. Lanes: 1–5, soluble proteins from phage clones λEF1–λEF5 containing the *GlcDH* gene; 6, pure GlcDH from *B. subtilis*; 7, soluble proteins from phage clone λEF11, which does not contain the *GlcDH* gene. →, M_r 31,000.

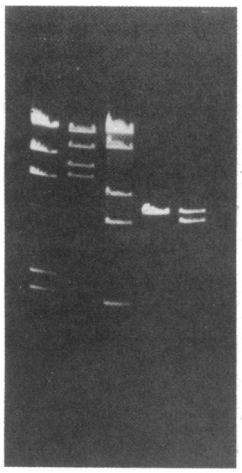

FIG. 2. Electrophoresis of *Eco*RI digests of phage and plasmid DNA. DNA of phage and plasmid clones was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lanes: 1, M_r standards (*Hin*dIII-digested λ DNA); 2, λ Charon 4A; 3, λEF2; 4, pBR322; 5, pEF1.

tet^r (8). Cells of ampicillin-sensitive *E. coli* HB101/λ were transformed with these recombinant plasmids. Two hundred amp^r transformants were cultured, lysed, and screened for GlcDH antigenic activity by radioimmunoassay; 40 had GlcDH activity, and the activity range was 400–689 (nmol/min)/mg of protein. Plasmid pEF1 is one of these transformants and was further studied. *Eco*RI digestion of pEF1 DNA followed by electrophoresis revealed a 3.5-kb fragment. This fragment, which was also present in the original λEF2 phage DNA (lane 5), must therefore contain the *GlcDH* gene.

To determine whether GlcDH was transcribed from a promoter present in the *B. subtilis* chromosome or was a run-off product depending on a promoter of the pBR322 plasmid, we subcloned the 3.5-kb fragment into plasmid pK06, which does not contain a promoter that can transcribe a gene inserted into the *Eco*RI site; i.e., the *Eco*RI site is transcriptionally silent: pK06 contains a *galK* structural gene on one side of the *Eco*RI site and a translational terminator upstream from it on the other side of the *Eco*RI site. The *Eco*RI-digested λEF2 DNA was ligated into the *Eco*RI site of pK06 and used to transform strain HB101/λ. Of 150 amp^r transformants screened for GlcDH antigenic activity by radioimmunoassay, 47 produced GlcDH. Digestion of the 3.5-kb fragment (in pEF1) by *Hin*dII had shown one asymmetric site (not shown). When we digested the DNA of 11 of the 47 GlcDH clones in pK06 with *Hin*dII and electrophoresed the digests, the patterns revealed that five clones (e.g., pEF2 and pEF3) had been inserted in one orientation and six clones (e.g., pEF4 and pEF5) had been inserted in the opposite orientation (Fig. 3). All 11 clones produced GlcDH, and the activity range was 526–726 (nmol/min)/mg of protein.

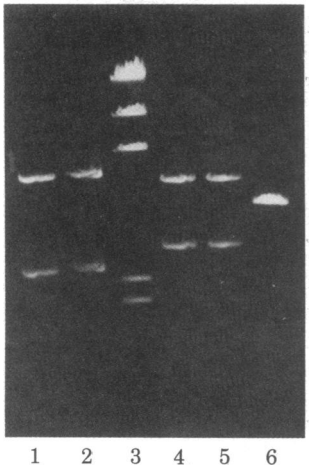

FIG. 3. Electrophoresis of *Hin*dII-digested plasmids in a 0.7% agarose gel. The 3.5-kb DNA of *B. subtilis* that contains the *GlcDH* gene was inserted into the *Eco*RI site of plasmid pK06. Transformants containing *amp*^{r} recombinant plasmids were obtained with the insert in both orientations (compare pEF2 and pEF3 vs. pEF4 and pEF5). Lanes: 1, pEF2; 2, pEF3; 3, M_r markers (*Hin*dIII-digested λ DNA); 4, pEF4; 5, pEF5; 6, plasmid pK06.

DISCUSSION

Several developmental genes of *B. subtilis* that are required for sporulation have previously been cloned and amplified in *E. coli* and *B. subtilis* (17–20). However, for none of these genes can the product be assayed. The cloning of the gene for GlcDH, whose product can be assayed easily, will make it possible to study the mechanisms controlling the expression of a developmental gene at the genetic and functional level.

The *GlcDH* gene is transcribed and translated in *B. subtilis* only after forespore membrane engulfment has been completed, and it seems to be made only inside the forespore (6, 7). Presumably, the synthesis of GlcDH is repressed in vegetative *B. subtilis* and in the mother cell of the sporangium and derepressed or induced in the forespore. Because the GlcDH operon is active in vegetative cells of *E. coli*, for which an excellent cell-free transcription and translation system has been developed, one can now proceed to characterize and purify the repressor of GlcDH that we expect to be present in vegetative *B. subtilis*.

Studies of *B. subtilis* transcription have shown the presence of at least three σ factors associated with RNA polymerase during vegetative growth and the appearance of one or more additional σ factors during sporulation (20, 21). Novel promoter sequences may be used for the transcription of certain sporulation genes (22). It is therefore possible that GlcDH is produced only when a new σ factor appears exclusively in the forespore. The DNA region of the *B. subtilis* chromosome isolated by us contains not only the structural gene for GlcDH but also a promoter that can be used to transcribe the gene, at least in *E. coli*. We cannot yet say whether the same promoter is also used in *B. subtilis*. The specific activity of GlcDH observed in *E. coli* is 2- to 15-fold higher than that in sporulating *B. subtilis*. This probably results from a higher gene copy number in phage-infected or plasmid-transformed *E. coli* rather than from a higher efficiency of the *GlcDH* promoter in *E. coli*. It will be possible to study these control mechanisms *in vitro* when the nucleotide sequence of the *GlcDH* gene together with the associated promoter and other control regions have been determined.

Also, it is now feasible to map the *GlcDH* gene in the *B. subtilis* chromosome and to isolate mutants constitutive in GlcDH activity or deficient in the structural gene. Thereby, the functional role of this enzyme in sporulation or germination can be studied. Although cells may not require a functional *GlcDH* gene during sporulation, the similarity of the carbohydrate specificity of GlcDH and that of compounds able to initiate *B. subtilis* germination (in the presence of asparagine, fructose, and potassium) (23) suggests a possible role of GlcDH in glucose-initiated germination (ref. 24; unpublished data).

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