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A *Bacillus subtilis* Malate Dehydrogenase Gene

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A *Bacillus subtilis* gene for malate dehydrogenase (*citH*) was found downstream of genes for citrate synthase and isocitrate dehydrogenase. Disruption of *citH* caused partial auxotrophy for aspartate and a requirement for aspartate during sporulation. In the absence of aspartate, *citH* mutant cells were blocked at a late stage of spore formation.

Enzymes of the Krebs citric acid cycle are known to play critical roles in the growth and differentiation of *Bacillus subtilis* (4, 8, 10, 15, 16, 25). Mutations in genes for Krebs cycle enzymes typically cause auxotrophy for amino acids and vitamins derived from Krebs cycle intermediates and a failure to metabolize substrates that cannot pass through the glycolytic pathway. Moreover, in most cases studied, mutations in Krebs cycle genes have been shown to cause a significant defect in spore formation even when cells are supplied with nutrients derived from the cycle (8, 10, 25).

With the exception of the genes for succinate thiokinase and malate dehydrogenase (MDH), the isolation of all *B. subtilis* Krebs cycle genes has been reported (12, 16), and the phenotypes of mutants defective in the various gene products have been analyzed (12, 16). In the course of studying citrate synthase genes, we found that the gene for the major citrate synthase (*citZ*) is located upstream of and is cotranscribed with the gene for isocitrate dehydrogenase (*citC*) (16, 17). Just downstream of *citC* we found a DNA sequence that could encode part of an MDH-like enzyme. Since the physical location of this cluster was consistent with prior mapping studies of the MDH gene (*citH*) and since Hulett and colleagues had found that an insertion mutation in this region (14) causes partial aspartate auxotrophy (13), we undertook completion of the sequencing and determination of whether the cloned DNA truly encodes MDH.

The 1.85-kb insert of *B. subtilis* DNA in plasmid pCS54 (Fig. 1) contains the 3′ end of the *citC* gene and the 5′ end of the *phoP* gene (19), surrounding a region of about 1 kb that was not previously reported. The sequence of this region was determined on both strands by using Sequenase version 2.0 and Taquence enzymes (U.S. Biochemicals) with double- or single-stranded DNAs of pCS54 and pMJB4 (Fig. 1) as templates. Analysis of the sequence (Fig. 2), using the University of Wisconsin Genetics Computer Group programs (7), revealed that an open reading frame (ORF) of 312 codons, preceded by an apparent ribosome binding site, lies downstream of *citC*. By comparison with sequences in the EMBL-GenBank collection, we found that the putative product of this ORF is similar to proteins described as lactate dehydrogenases (LDHs) and MDHs. The greatest similarity (35 to 38% identity) was found to LDHs from *Bacillus* and *Lactococcus* spp. The only MDH in the same range of similarity (37% identity) was from *Haloarcula marismortui* (a halophilic archaebacterium) (5).

LDH and MDH catalyze analogous chemical reactions (2). While the product of the *B. subtilis* ORF appears to be more similar to LDHs than to MDHs, it seemed likely that a cluster of Krebs cycle genes would include a gene for MDH rather than one for LDH. In fact, a conserved residue (Arg-87 in the *B. subtilis* ORF) involved in substrate specificity for both types of enzymes (5, 11, 24) is always arginine in MDH enzymes but is glutamine in LDHs (5) (Fig. 3). Moreover, two residues in the same region of the protein, Met-91 and Asp-94 in the *B. subtilis* ORF, are often Met and Asp in MDHs but are invariably Glu and Leu in LDHs (Fig. 3). On the other hand, a region between residues 127 and 158 in the *B. subtilis* ORF is much more similar to the corresponding region in LDHs than in MDHs (except for MDH of *H. marismortui*) (Fig. 3). Cendrin et al. (5) have noted that the sequence of *H. marismortui* MDH is sufficiently different from those of most eubacterial MDHs and LDHs to cause it to be placed on a distinct branch of the evolutionary tree; the product of the *B. subtilis* ORF may belong on the same branch.

To establish the function of the *B. subtilis* ORF unambiguously, we cloned a spectinomycin resistance cassette (18) between the *Hin*cII and *Sty*I sites within *citH* in pCS60, creating pCS77 (Fig. 1), and integrated this insertion-deletion mutation into the *B. subtilis* chromosome by double-crossover recombination. Southern blot hybridization of restriction enzyme-digested chromosomal DNA of the resultant strain, SJB238, verified that the expected recombination event had occurred. Strain SJB238 and its isogenic *citH*$^+$ parent, JH642 (22), were grown in nutrient broth sporulation (DS) medium (9) until early stationary phase (Krebs cycle enzymes are usually at their peak specific activity at this time). Extracts of cells were tested for MDH and LDH activities. As shown in Table 1, wild-type cells had both MDH and LDH activities; strain SJB238 had nearly normal LDH activity but a very low level of MDH activity. We conclude that the *B. subtilis* ORF encodes MDH. Since, in prior work, a mutation causing a deficiency in MDH activity was mapped to the *phoP* region of the chromosome and called *citH* (1), we assume that the same genetic locus is affected in strain SJB238. Repeated attempts to acquire and culture the original *citH* mutant strain have been unsuccessful.

The *citH*::*spc* mutation had only a slight effect on cell growth in minimal medium (and no effect in DS medium). In minimal-glucose-salts medium, the mutant had a doubling time of about 116 min, while the wild-type strain doubled every 94 min.

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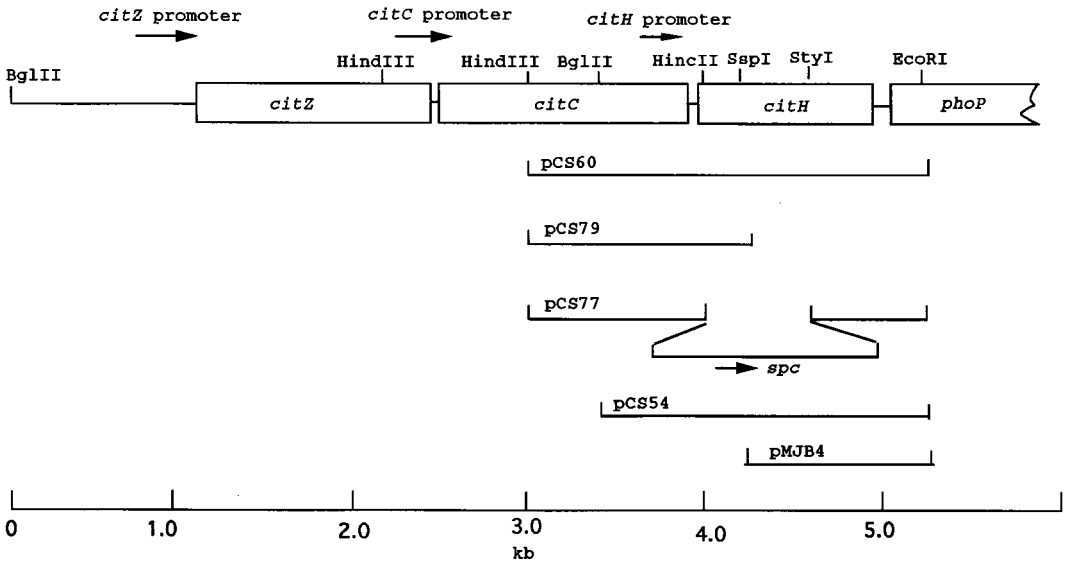

FIG. 1. Physical map of the *citZCH* operon. The lines below the map show the *B. subtilis* DNA contents of various plasmids. The arrows show the approximate locations of promoter sites. Vectors were as follows: pCS54, pBluescript SK− (Stratagene); pMJB4, pBluescript SK+ (Stratagene); pCS60, pJPM1 (21); pCS79, pCS53 (pBluescript SK− plus *neo* cassette); pCS77, pBluescript SK−.

Addition of aspartate (0.2%) to the medium decreased the doubling times of both the wild-type and mutant strains to 72 min. If strain SJB238 is totally devoid of MDH activity, there must be a second source of oxaloacetate that allows synthesis of aspartate, albeit at a reduced rate, in the mutant. Pyruvate carboxylase activity is the most likely source.

The sporulation efficiency of strain SJB238 in DS medium was reduced 1,000-fold compared with that of the parental strain (Table 2). This defect was overcome, in part, by addition of aspartate to the medium, suggesting that higher levels of aspartate are required for sporulation than for growth. It is uncertain whether the aspartate is needed as such, as a precursor of other metabolites (e.g., isoleucine, threonine, methionine, lysine, diaminopimelic acid, dipicolinic acid, or pyrimidines), as a source of oxaloacetate for gluconeogenesis, or as a source of oxaloacetate to drive the Krebs cycle.

To assess the stage at which blockage of sporulation occurs in strain SJB238, we tested the activities of various sporulation gene promoters fused to *lacZ*. The promoters were chosen to reflect the activities of sporulation-specific forms of RNA polymerase containing σ^E, σ^G, and σ^K. Expression of *spoIID*, *sspB*, and *cotA* fusions in wild-type and *citH* mutant cells grown in DS medium without added aspartate occurred at the same time and at the same level as in parental cells (Fig. 4). Thus, if the *citH* mutation causes any defect in transcription, it is a very late block.

The location of the *citH* gene suggests that it might be cotranscribed with *citZ* and *citC*. If so, polar mutations in the

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citC--> I  K  N  M  D  *                 citH --->        rbs        M  G  N  T  R  K  K  V  S  V  I  G  A
2901  TGATCAAAAACATGGACTAAGCAAGGAAAAAGCCTAAAACTAGCCATAAAGGAGAAGAGACATGGGAAATACTCGTAAAAAAGTTTCTGTTATCGGAG
        G  F  T  G  A  T  T  A  F  L  I  A  Q  K  E  L  A  D  V  V  L  V  D  I  P  Q  L  E  N  P  T  K  G
3001  CAGGTTTTACCGGAGCTACAACTGCATTTTTAATCGCTCAAAAAGAGCTGGCAGACGTTGTTCTTGTTGACATTCCGCAATTGGAGAACCCGACAAAGGG
        K  A  L  D  M  L  E  A  S  P  V  Q  G  F  D  A  K  I  T  G  T  S  N  Y  E  D  T  A  G  S  D  I  V
3101  AAAAGCGCTTGATATGCTTGAAGCAAGCCCGGTTCAAGGCTTTGACGCAAAAATTACGGGAACATCCAATTACGAGGATACAGCCGGCTCTGACATTGTT
        V  I  T  A  G  I  A  R  K  P  G  M  S  R  D  D  L  V  S  T  N  E  K  I  M  R  S  V  T  Q  E  I  V  K
3201  GTCATTACAGCCGGTATCGCAAGAAAACCTGGTATGAGCAGAGATGATCTGGTCTCTACAAACGAAAAGATTATGAGAAGCGTTACGCAGGAAATCGTGA
        Y  S  P  D  S  I  I  V  V  L  T  N  P  V  D  A  M  T  Y  A  V  Y  K  E  S  G  F  P  K  E  R  V  I
3301  AATATTCTCCTGACTCTATTATTGTGGTGCTGACAAATCCTGTTGATGCAATGACATACGCGGTGTACAAAGAATCAGGCTTCCCTAAAGAGCGTGTAAT
        G  Q  S  G  V  L  D  T  A  R  F  R  T  F  V  A  E  E  L  N  L  S  V  K  D  V  T  G  F  V  L  G  G
3401  CGGCCAGTCAGGTGTGCTTGATACGGCAAGATTCAGAACATTTGTGGCAGAGGAATTAAACCTGTCAGTGAAAGATGTGACTGGTTTCGTACTCGGCGGA
        H  G  D  D  M  V  P  L  V  R  Y  S  Y  A  G  G  I  P  L  E  T  L  I  P  K  E  R  I  D  A  I  V  E  R
3501  CACGGTGACGATATGGTTCCGCTTGTGCGTTATTCTTATGCTGGCGGTATCCCGCTTGAAACTCTTATTCCGAAAGAACGGATTGACGCAATTGTGGAGC
          T  R  K  G  G  G  E  I  V  N  L  L  G  N  G  S  A  Y  Y  A  P  A  A  S  L  T  E  M  V  E  A  I  L
3601  GCACTAGAAAAGGCGGAGGCGAAATCGTGAATCTTCTTGGAAACGGAAGCGCGTATTATGCGCCTGCGGCTTCTCTGACAGAAATGGTCGAAGCGATCTT
        K  D  Q  R  R  V  L  P  T  I  A  Y  L  E  G  E  Y  G  Y  E  G  I  Y  L  G  V  P  T  I  V  G  G  N
3701  GAAAGATCAGCGCCGCGTCCTTCCTACAATTGCTTATCTTGAAGGGGAATACGGCTATGAAGGCATCTACCTTGGTGTTCCTACAATTGTAGGCGGCAAC
        G  L  E  Q  I  I  E  L  E  L  T  D  Y  E  R  A  Q  L  N  K  S  V  E  S  V  K  N  V  M  K  V  L  S  *
3801  GGTCTTGAGCAAATCATTGAACTTGAACTGACAGACTATGAAAGAGCGCAGCTGAATAAATCAGTTGAATCTGTCAAAAATGTCATGAAAGTATTATCCT
          *  >>> >>>>>>>>>>>          <<<<<<<<<<< <<<
3901  AATAAAAAGAGAGAAAGGCTTGCTTAATACAGCCTTTCTCTTTTTACTATAAATGAAAGCGCTATCATAAACGTCTTTATTTCTTTTAAAAATGATGTAA

4001  AAGGCGAATTGTCGGAATGTCCTGCTTTCGCTAAAATAAAATCATGAAACATGTTAAGATGACATAAAATAGAGAAATAGGATTGCGGGGAATTATAATA
        phoP  --->  M  N  K  K  I  L
4101  CTGGAGGCACAGCATGAACAAGAAAATTTTA
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FIG. 2. Sequence of the nontemplate strand of the *citH* gene and its putative product. The putative ribosome binding site (rbs) for *citH* translation is underlined, and a potential factor-independent transcription termination site between *citH* and *phoP* is indicated by arrowheads. The asterisks denote stop codons. The active-site arginine residue conserved in MDHs is in boldface. The numbering of the DNA residues follows that of the upstream *citZ* and *citC* genes (16).

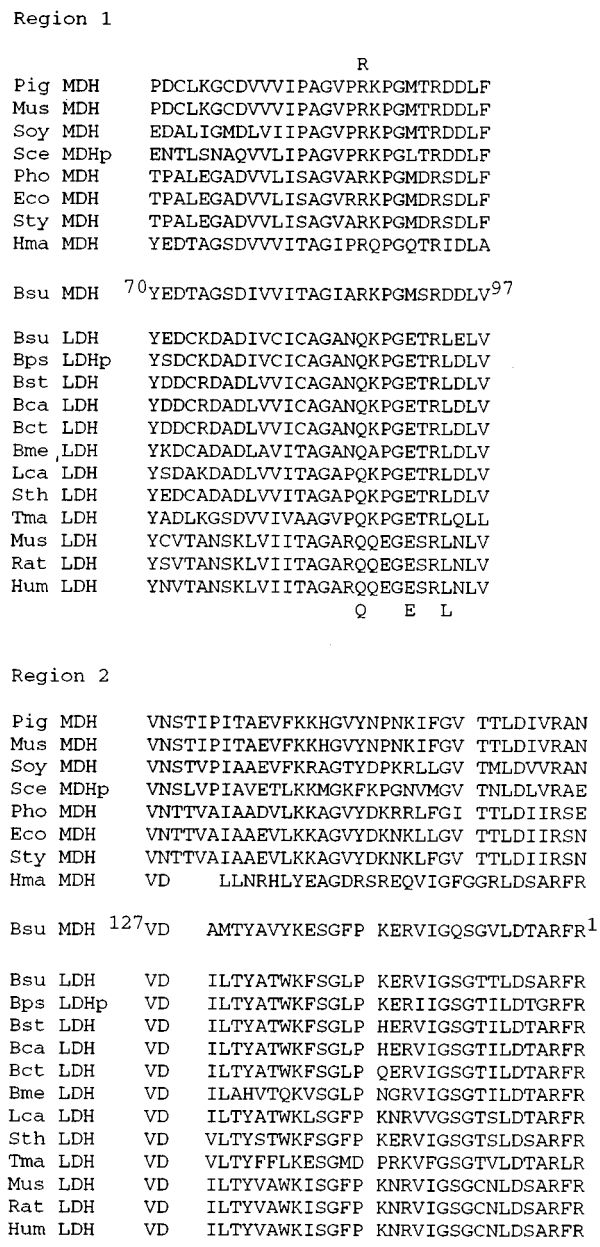

FIG. 3. Alignments of protein sequences for MDH and LDH from various organisms. Two conserved regions are compared, and selected highly conserved residues in region 1 are indicated above and below the sequence. The accession numbers for the sequences cited are as follows: pig mitochondrial MDH, P00346; mouse mitochondrial (Mus) MDH, M16229; soybean glyoxysomal (Soy) MDH, L01628; *Saccharomyces cerevisiae* (Sce) MDHp, the peroxisomal enzyme, P32419; *Photobacterium* sp. (Pho) MDH, L13319; *Escherichia coli* (Eco) MDH, P06994; *Salmonella typhimurium* (Sty) MDH, P25077; *H. marismortui* (Hma) MDH, M97218; *Bacillus subtilis* (Bsu) MDH, U05257; *B. subtilis* (Bsu) LDH, P13714; *Bacillus psychrosaccharolyticus* (Bps) LDHp, one of two LDH isozymes in this organism, P14561; *Bacillus stearothermophilus* (Bst) LDH, A91726; *Bacillus caldolyticus* (Bca) LDH, B29704; *Bacillus caldotenax* (Bct) LDH, S00019; *Bacillus megaterium* (Bme) LDH, P00345; *Lactobacillus casei* (Lca) LDH, A43944; *Streptococcus thermophilus* (Sth) LDH, D13405; *Thermotoga maritima* (Tma) LDH, S36863; mouse (Mus) LDH M chain, S06290; rat LDH M chain, P04642; and human (Hum) LDH M chain, A00347.

upstream genes should decrease *citH* expression. In fact, *spc* insertions in *citZ* or *citC* reduced MDH activity by about 70% (Table 1). Since the *spc* cassette is expected to block transcriptional read-through, such a result is consistent with partial cotranscription. To test whether *citH* has its own promoter, we integrated into the *B. subtilis* chromosome a plasmid, pCS79, carrying the 3′ end of *citC* and the 5′ end of *citH* (Fig. 1). (This construction does not disrupt either the *citC* gene or the *citH* gene per se but separates the *citH* coding sequence from any promoter that lies upstream of the *Hin*dIII site within *citC*.) The resultant strain, SJB258, had no defect in cell growth (data not shown) and sporulated more efficiently than did the *citH*::*spc* strain (Table 2), suggesting that a promoter for *citH* lies within the cloned segment. (The vector does not supply a promoter for read-through transcription in the orientation used.) Strain SJB258 still sporulated less efficiently than did the wild-type strain, however, unless the medium was supplemented with aspartate. Taken together, these results suggest that *citH* has its own promoter but that this promoter is not able to achieve a rate of MDH accumulation adequate for sporulation. Read-through transcription from the *citZ* or *citC* promoter may be needed to provide the level of MDH expression needed for efficient sporulation.

The less-than-total blockage of sporulation caused by a *citH* null mutation might be explained by the presence of a second MDH-encoding gene in *B. subtilis*. E. M. Bryan and C. P. Moran, Jr. (3), have isolated a σ^E-dependent gene whose putative product is similar to a minority class of MDHs and LDHs (distinct from those listed in Fig. 3).

Nucleotide sequence accession number. The nucleotide sequence reported here has been given GenBank accession number U05257.

TABLE 1. L-MDH and L-LDH activities[a]

Strain	Relevant genotype	Sp act[b]	
		MDH	LDH
JH642	*cit*$^+$	1.00	1.00
SJB194	Δ*citZ*::*spc*	0.28	ND
SJB219	Δ*citC*::*spc*	0.35	0.72
SJB238	Δ*citH*::*spc*	0.02	0.86

[a] Cell cultures were grown in DS medium to approximately 1.5 h after exponential phase. Crude extracts were prepared as described previously (16) in a buffer containing 0.1 M Tris (pH 7.5), 2 mM EDTA, 0.2 M dithiothreitol, and 10% (vol/vol) glycerol.

[b] Enzyme-specific activities of the wild-type strain were normalized to 1.00, and the ratios of enzyme activities in mutant strains to those in the wild-type strain are reported. Actual specific activities in the wild-type strain were as follows: MDH, 63.1 U/mg; LDH, 61.2 U/mg. One unit of enzyme activity produced 1 μmol of NADH per min at 25°C. Enzyme assay solution contained 50 mM Tris (pH 7.5 [for MDH] or pH 8.8 [for LDH]), 2 mM NAD^+, and 50 mM L-malate or 100 mM L-lactate. ND, not determined.

TABLE 2. Sporulation frequencies of *B. subtilis* strains with or without 0.2% aspartate[a]

Strain	Relevant genotype	Addition to medium	Heat-resistant CFU at 17.5 h	Relative sporulation frequency
JH642	*cit*$^+$		2.6×10^8	1.00
SJB258	*'citC citH'*::*neo*		3.7×10^7	0.14
SJB238	Δ*citH*::*spc*		3.0×10^5	1.15×10^{-3}
JH642	*cit*$^+$	Aspartate	4.6×10^8	1.00
SJB258	*'citC citH'*::*neo*	Aspartate	3.4×10^8	0.74
SJB238	Δ*citH*::*spc*	Aspartate	5.0×10^7	0.11

[a] Cell cultures were grown in DS medium (9) with or without the addition of 0.2% aspartate. All cultures grew to approximately the same cell density (4×10^8 to 5×10^8 cells per ml). Spore formation was assayed by heating a sample of each culture to 80°C for 10 min and then plating each on DS medium. Samples were taken 17.5 h after the end of the exponential growth phase.

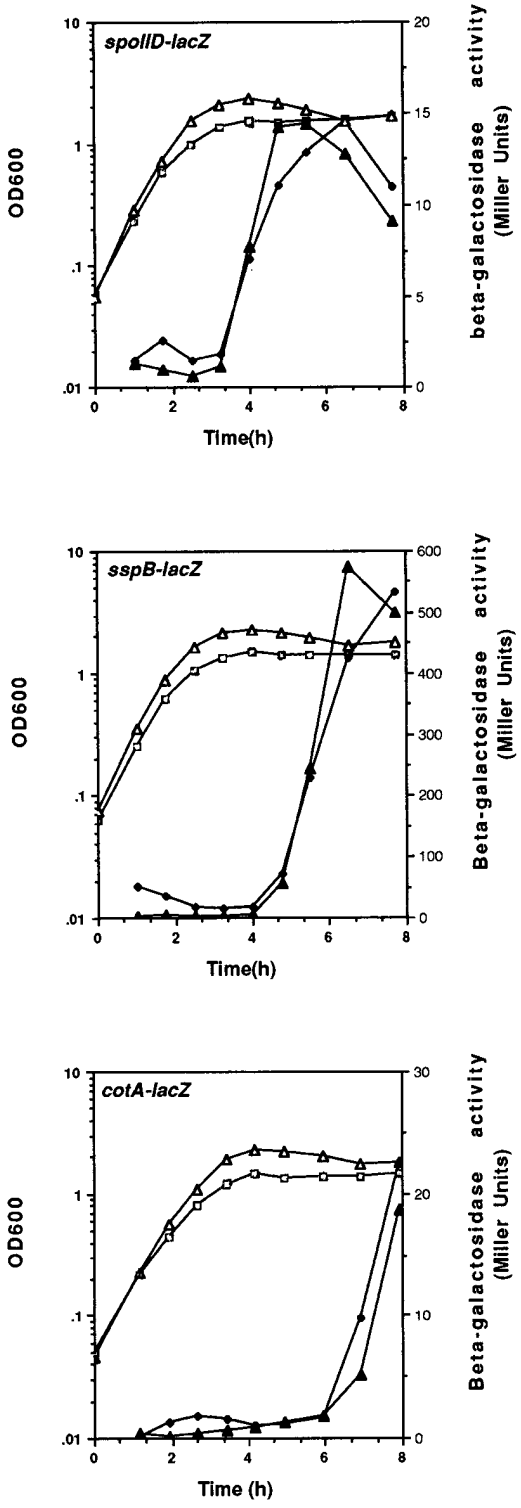

FIG. 4. β-Galactosidase activity in *B. subtilis* strains carrying fusions of sporulation gene promoter sites to *lacZ*. In each case, a *citH*$^+$ and an isogenic *citH*::*spc* mutant strain were compared. The isogenic strains were constructed by transformation with DNA isolated from strain SR10 (23) for the *spoIID-lacZ* fusion, plasmid pJF751 (20) for the *sspB-lacZ* fusion, and DNA from strain SC432 (6) for the *cotA-lacZ* fusion. Cells were harvested, made permeable by exposure to toluene, and assayed for β-galactosidase activity as described previously (17). Open symbols indicate culture turbidity (optical density at 600 nm [OD600]); closed symbols indicate β-galactosidase activity. The triangles reflect data obtained with the *citH*$^+$ strain; the squares and diamonds indicate data for the Δ*citH* strain.

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