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Transcriptional and Posttranscriptional Control of the *Bacillus subtilis* Succinate Dehydrogenase Operon

LARS MELIN,[1]* LARS RUTBERG,[2] AND ALEXANDER VON GABAIN[1]

Department of Bacteriology, Karolinska Institutet, Box 60400, S-10401 Stockholm,[1] and Department of Microbiology, University of Lund, Sölvegatan 21, S-223 62 Lund,[2] Sweden

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The amount of succinate dehydrogenase (SDH) in *Bacillus subtilis* varies with growth conditions. In this work we studied the steady-state level and the rate of decay of *B. subtilis sdh* mRNA under different growth conditions. In exponentially growing cells, the steady-state level of *sdh* mRNA was severalfold lower when glucose was present compared with growth without glucose, whereas the rate of decay of *sdh* mRNA was the same with and without glucose. Thus, glucose repression seems to act by decreasing *sdh* mRNA synthesis. When the bacteria entered the stationary phase, the steady-state level of *sdh* mRNA dropped about sixfold. At the same time, *sdh* mRNA half-life decreased from 2.6 to 0.4 min. This result indicates that transcription of the *sdh* operon is initiated at the same rate in exponentially growing and in stationary-phase cells. The start point of the *sdh* transcripts, as measured by primer extension, was the same under all conditions studied, suggesting that the *sdh* operon is solely controlled by the previously identified σ^{43}-like promoter. The increase of SDH activity in stationary phase may be explained by reduced dilution of the SDH proteins as a result of the retarded growth rate. We suggest that enhanced degradation of the *sdh* transcript is a means by which the bacteria adjust expression to the demands of stationary phase.

Succinate dehydrogenase [SDH; EC 1.3.99.1, succinate: (acceptor) oxidoreductase] is present in all aerobic cells. It is a membrane-bound enzyme which is part of both the Krebs cycle and the respiratory chain. SDH is composed of two subunits of different size, a flavoprotein (Fp) and an iron-sulfur protein (Ip). SDH is anchored to the inner mitochondrial membrane in eucaryotic cells and to the inner side of the cytoplasmic membrane in bacteria via a *b*-type cytochrome (11, 12). In *Bacillus subtilis*, the structural genes for the three subunits of the membrane-bound SDH complex are organized in an operon which is transcribed in the order *sdhC* (cytochrome b_{558}), *sdhA* (Fp), and *sdhB* (Ip). During exponential growth, the *sdh* operon is transcribed from a σ^{43}-like promoter initiating transcription at a guanosine residue 90 base pairs upstream from the start codon for *sdhC* (19).

The amount of SDH activity (and SDH protein) found in *B. subtilis* varies with growth conditions and growth stage. Expression of the *sdh* operon is sensitive to glucose repression. When a *B. subtilis* culture growing in the absence of glucose approaches stationary phase (defined as time zero [T_0]), the SDH activity continues to increase. No increase in SDH activity is seen during stationary phase when glucose is present in the medium. Neither the mechanism of glucose repression nor that of the increase in SDH activity at the end of growth is known.

When *B. subtilis* passes through T_0 into stationary phase, a number of new promoters which are transcriptionally silent during growth become active. These promoters may control new gene activities or provide a means of ensuring continued transcription of housekeeping genes at a required rate during stationary phase and sporulation (14, 33). Control of Krebs cycle genes may involve the use of such alternative promoters, as has recently been found for *citG* (coding for fumarase) (7).

Other Krebs cycle enzymes, such as aconitase and 2-oxoglutarate dehydrogenase (ODH), are also glucose repressed and continue to accumulate after T_0 (5, 23). For *citB* (coding for aconitase), it has recently been shown that the steady-state level of *citB* mRNA is higher in cells grown without glucose than in glucose-grown cells (27). This finding was interpreted to mean that glucose repression affects the rate of transcription from the *citB* promoter. An alternative interpretation is that glucose affects the rate of decay of *citB* mRNA. The transcriptional organization of the recently isolated *citK* and *citM* genes, which code for the E_1 and E_2 subunits of the ODH complex, respectively (3), is not known.

In the present experiments, we have examined the steady-state levels and the rate of decay of *sdh* mRNA in *B. subtilis* under different growth conditions. The results show conclusively that in growing cells, glucose repression acts solely by affecting the rate of transcription and does not influence the rate of decay of *sdh* mRNA. Furthermore, we have found that expression of *sdh* does not involve recruitment of any new transcriptional start site other than that identified previously at any growth rate tested. Our results give no indication that the rate of transcription of the *sdh* operon alters as the cells enter stationary phase. Instead, we have found that the steady-state level of *sdh* mRNA decreases as the bacteria enter T_0 and at least up to T_4. During this time, there is a corresponding decrease in the stability of *sdh* mRNA. The experiments indicate that expression of the *Bacillus subtilis sdh* operon under different conditions is regulated at both the transcriptional and posttranscriptional levels.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial strains used were *B. subtilis* 3G18 (*ade met trpC2*) and *B. subtilis* 3G18::*sdp8* (19). pSDP4 is a derivative of plasmid pPL603 in which a *Sau*3AI-*Pst*I DNA fragment containing the *sdh* promoter region has been inserted in front of the promoterless *cat-86* gene (19).

Media and growth of bacteria. The bacteria were kept on

* Corresponding author.

tryptic blood agar base (Difco); when required, chloramphenicol and kanamycin were added at 5 μg/ml each. Liquid cultures were grown in nutrient sporulation medium (NSMP [9]). Competent cells were prepared as described by Arwert and Venema (1).

DNA and RNA techniques. Plasmid DNA was prepared by standard methods (17). pSDP4 DNA was used to create a 96-base-pair deletion upstream of the −35 region of the *sdh* σ^{43}-like promoter (see Results). The deletion was introduced into the *B. subtilis* chromosome by recently described techniques based on homologous recombination (19). RNA was extracted by the hot phenol extraction method described before (32). Primer extension was done with avian myeloblastosis virus reverse transcriptase (New England Biolabs) as described before (18). The primer corresponded to nucleotides 301 to 324 in the published sequence of the *sdh* region (16), which corresponds to the 5′ end of the *sdhC* region (see Fig. 2). In each primer extension experiment, the amount of primer was doubled and no increase of the amount of extended primer was noticed. Thus, the input amount of primer was saturating the complementary mRNA species.

Transfer of nucleic acid fragments to nitrocellulose filters after gel electrophoresis and hybridizations was done according to standard methods (17, 28). Radioactive labeling of DNA was done with a random priming kit obtained from Boehringer.

Restriction enzymes, DNA polymerase I (Klenow fragment), T4 polynucleotide kinase, and T4 ligase were purchased from New England Biolabs, Boehringer, or Pharmacia.

Other methods. SDH enzyme activity was determined as described before (11).

RESULTS

Effect of glucose on steady-state *sdh* mRNA levels and rate of decay in exponentially growing cells. Rifamycin blocks initiation of bacterial transcription but not RNA chain growth. To examine the steady-state level of *sdh* mRNA and its rate of decay, strain 3G18 was grown in NSMP with and without glucose. Rifamycin was added to exponentially growing cultures, and total RNA was extracted at different times after addition of the drug. *sdh* mRNA was then probed by the primer extension technique. The reverse transcripts were analyzed by polyacrylamide gel electrophoresis followed by autoradiography of the gels. The length of the major reverse transcript was 155 to 160 nucleotides. Its size assigns the transcriptional start at or close to the same G residue that has been identified previously as the transcriptional start by nuclease S1 protection experiments (19).

The autoradiographs were evaluated by densitometry to measure the relative amounts of *sdh* mRNA and total RNA at the different time points. As shown in Fig. 1A, the *sdh* transcripts started at the same 5′ end and thus probably initiated at the same promoter with or without glucose. The steady-state level of *sdh* mRNA was about fourfold lower in cells grown with glucose than in cells grown without glucose. There was also a corresponding fourfold decrease in SDH enzyme activity in glucose-grown cells. The *sdh* mRNA level and SDH specific activity were 0.7 versus 3.1 (arbitrary units) and 0.1 versus 0.4 mmol of substrate converted per mg of protein per min for cells grown with versus without glucose, respectively. The ratio of *sdh* mRNA to specific activity was 7 and 8 in the presence and absence of glucose. The half-life of *sdh* mRNA was about 2.5 min irrespective of the presence of glucose (Fig. 1B). From these results, we conclude that glucose repression can be completely attributed to an effect on the rate of transcription of the *sdh* operon.

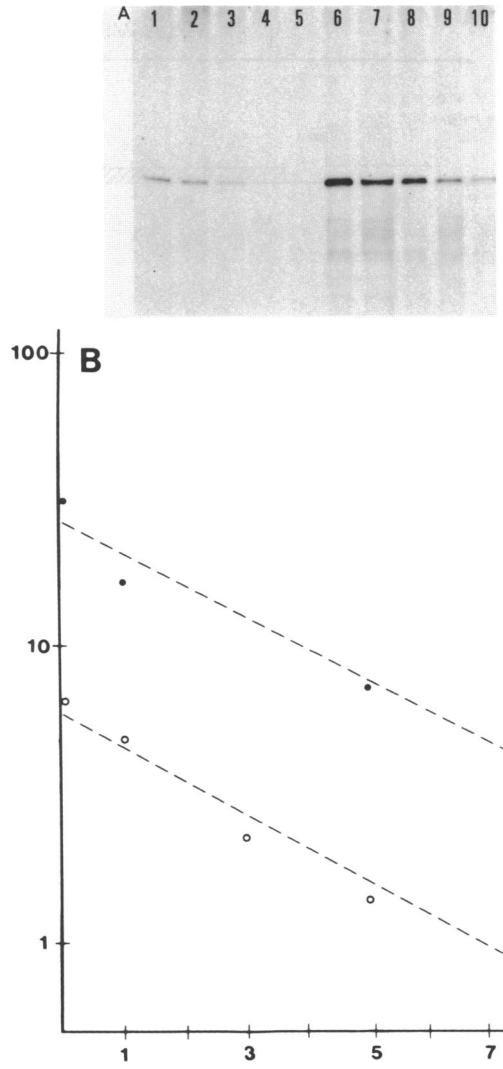
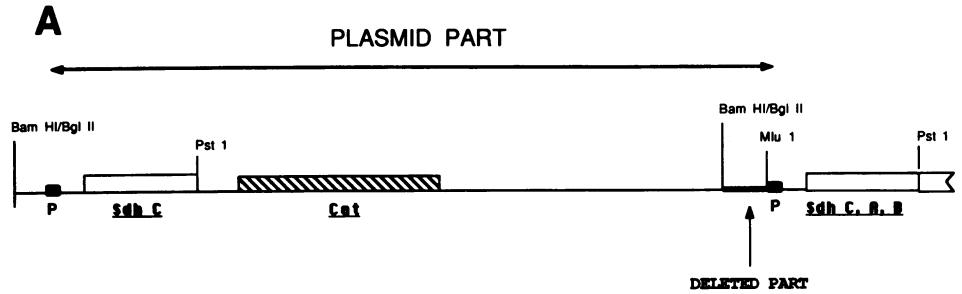


FIG. 1. (A) Strain 3G18 was grown in NSMP with or without 1% glucose at 37°C with shaking (200 rpm) to an A_{600} of 0.4 to 0.6, which is within the exponential growth phase for both growth conditions. Rifamycin was then added at 100 μg/ml, and samples were taken for RNA extraction at different times after addition of the drug. *sdh* mRNA was analyzed by primer extension with a primer corresponding to nucleotides 301 to 324 of the coding region of *sdhC* (16) (Fig. 2B). For each reaction, 5 μmol of primer was added to 10 μg of total RNA, which represents an excess of primer. The reverse transcripts obtained were analyzed by gel electrophoresis in a 6% polyacrylamide–urea gel, followed by autoradiography of the gels. Lanes 1–5, RNA from cells growing in presence of glucose: lanes 6–10, RNA from cells growing without glucose. Lanes correspond to samples taken at different times after addition of rifamycin: 0 min (lanes 1 and 6), 1 min (lanes 2 and 7), 3 min (lanes 3 and 8), 5 min (lanes 4 and 9), and 8 min (lanes 5 and 10). (B) Relative amounts of *sdh* reverse transcript in the samples of panel A were estimated by densitometric analysis of the autoradiogram. The values obtained were plotted in arbitrary units versus time of sampling. Solid circles correspond to samples from cells grown without glucose; open circles correspond to samples from cells grown with glucose. The half-life of the *sdh* mRNA was 2.4 min with glucose and 2.6 min without glucose.

The mechanism of glucose repression is not known for *B.*



B

SAU 3AI
TATAGCTCAAACAGGGGGGAGGATTACAGAATGATCCTGT 40

AATTCTTATGAAAAATTAAGCAAGAAATATATATTGATAA 80

AATAAAATTTTTCAATCAACTAATCAATTCGGAAAATTAT 120

MLU I
AATTTATGTACGCGTTTTCTTGACGCCCTTTTGAGGGAGG 160
-35

AGTAAAATGAAATTGTCAATAAATCTTAATAAAGTGCTTA 200
-10 +1

CAATTGAAAGAAGTGGGGGAAGAGATTTAGCACATTTCGC 240

Sdh C
ACTTATCAAACAGGGGGTAAAGTA ATG TCT GGG AAC 278

AGA GAG TTT TAT TTT CGA AGA TTG CAT TCG 308

TTG CTT GGC GTC ATA CCG TCG GCA TCT TTC 338

subtilis. "Classical" transcriptional repressors are present in limiting amounts and can often be titrated by an excess of operator present on a multicopy plasmid. The presence of the *sdh* promoter region (nucleotides −139 to +385 [16, 19]) on a derivative of pUB110 (copy number, 30 to 40) did not relieve glucose repression of the chromosomal *sdh* operon (data not shown).

Deletion of 96 base pairs upstream of the *sdh* promoter does not relieve glucose repression. Chambliss and co-workers (21) have shown that a mutation in the 5′ noncoding region of *B. subtilis amvE* mRNA relieves glucose repression. To test whether sequences upstream of the *sdh* "core" promoter are important for glucose repression, a *Sau*3A-*Mlu*I fragment covering nucleotides −139 to −43 (Fig. 2) was deleted. The deletion was introduced into the chromosomally located *sdh* operon, and the resulting construction was verified by Southern blotting analysis. In bacteria carrying this deletion, the *sdh* operon was still glucose repressed (Fig. 3). The deletion decreased the signal strength of the *sdh* promoter at least eightfold, as estimated from steady-state levels of mRNA (Fig. 4). Deletion of an upstream A+T-rich region is known to decrease the strength of other *B. subtilis* promoters (2, 20). Accumulation of SDH as growth ceases was affected by the above deletion; however, this may simply reflect the decreased signal strength of the *sdh* promoter.

Same *sdh* transcriptional start used in growing and stationary-phase cells. At least six sigma factors are known which can provide different specificities to the *B. subtilis* RNA polymerase core enzyme (6). The finding that deletion of about 100 nucleotides upstream the *sdh* σ^{43}-like promoter −35 region affects the accumulation of SDH may indicate that alternative promoters located in the deleted region are involved in this regulation. Sequences resembling the con-

FIG. 2. (A) Map of plasmid pSDP4 inserted in front of the *sdh* operon in the chromosome with the deletion upstream of the *sdh* promoter region. The deletion was created by a *Bam*HI-*Mlu*I digest of plasmid pSDP4 (see Materials and Methods), and the large fragment was isolated and recovered from a 1% agarose gel. The ends created were made blunt by standard fill-in reactions with Klenow enzyme and ligated, and the plasmid was transformed into *B. subtilis* 3G18. This plasmid was deleted of its origin of replication, located in a *Bam*HI-*Bgl*II restriction fragment (19). After ligation it was transformed into *B. subtilis* 3G18, and transformants were selected on TBAB with chloramphenicol (CAP; 5 μg/ml). The only way the cells can be transformed to CAP resistance is to integrate the plasmid into the chromosome due to its inability to replicate autonomously. A Campbell-like integration of this plasmid over the homologous region interchanges the plasmid-derived deleted *sdh* promoter region with the nondeleted chromosomally located *sdh* promoter region. This was confirmed by Southern blot analysis experiments, and the strain was designated 3G18 Δ*sdhp*. Indicated are the upstream deletion in front of the *sdh* operon, the *cat* gene, and relevant restriction enzyme sites. P, *sdh* promoter. (B) Nucleotide sequence of the promoter region of the *sdh* operon and the first part of the *sdhC* gene (16). The *Sau*3AI and *Mlu*I restriction sites used to delete the promoter upstream region, the −35 and −10 regions of the *sdh* σ^{43}-like promoter, and the transcriptional start of the *sdh* operon are indicated, as is the translational start of *sdhC*. The nucleotides which are complementary to the primer used in the primer extension experiments are underlined.

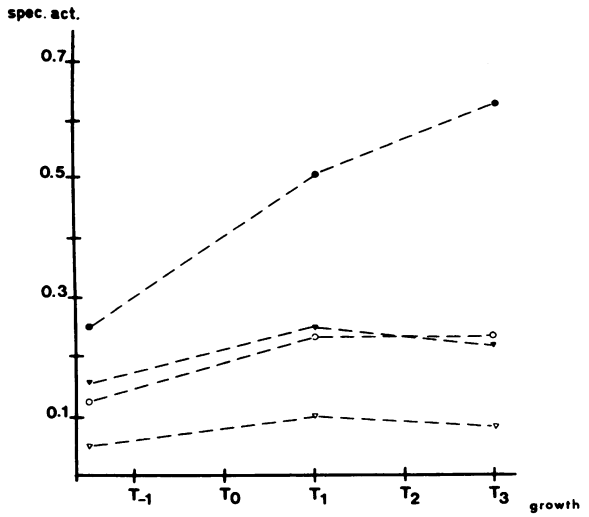


FIG. 3. SDH enzyme activity in *B. subtilis* strains 3G18::*sdp8* (19) and 3G18 Δ*sdhp* (see Fig. 2A). The cultures were grown with shaking at 37°C in NSMP in the presence and absence of 1% glucose. Samples were taken at different time points, and SDH enzyme activity was measured. T_{-1} represents 1 h before the culture entered the stationary phase, which is defined as T_0. T_1, T_2, T_3, and T_4 represent 1, 2, 3, and 4 h, respectively, after T_0. Samples were taken in mid-log phase, T_1, and T_3. Symbols: ●, 3G18::*sdp8* (19) without glucose; ○, 3G18::*sdp8* (19) with glucose; ▲, 3G18 Δ*sdhp* without glucose; △, 3G18 Δ*sdhp* with glucose. SDH activity is expressed as micromoles of substrate converted per milligram of protein per minute.

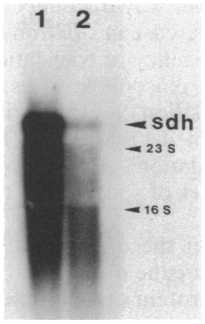


FIG. 4. Strains 3G18::*sdp8* (19) and 3G18 Δ*sdhp* were grown with shaking at 37°C in NSMP without glucose to an A_{600} of 0.6, when total RNA was extracted. RNA (10 μg) was electrophoresed in a 1.2% agarose gel under denaturing conditions and then transferred to a nitrocellulose filter. The filter was probed with a 1.6-kilobase *Pst*I-*Eco*RI fragment, covering the terminal part of *sdhC* and most of *sdhA* (16, 24), radioactively labeled by random priming. Lane 1, RNA from 3G18::*sdp8* (19); lane 2, RNA from 3G18 Δ*sdhp*. The probe hybridized with the ca. 3,400-nucleotide *sdh* transcript (19) and to some extent also to 23S and 16S rRNA.

sensus sequences of a σ^{32} promoter are located within the region (16). To analyze the possible use of such alternative promoters, the 5′ ends of the *sdh* transcripts from bacteria at different growth stages were determined. Total RNA was isolated from bacteria at log phase. T_0, T_2, and T_4 (see Fig. 3 legend for definitions), and the start points of *sdh* transcripts were mapped by the primer extension technique. The results of these experiments (Fig. 5) suggest that the same transcriptional start is used at all growth stages tested. From this we conclude that the accumulation of SDH when growth ceases does not engage any promoter other than the previously defined σ^{43}-like promoter (19).

The SDH enzyme activity correlated in all samples with the amount of SDH protein as measured by rocket immunoelectrophoresis with Fp-specific antibodies (11) (data not shown).

Stability of *sdh* mRNA is growth stage dependent. A surprising finding in the previous experiments (Fig. 5) is that the steady-state level of *sdh* mRNA was about sixfold lower in stationary-phase cells than in exponentially growing cells. This result shows that the increase in SDH activity is not the result of increased levels of *sdh* mRNA.

The steady-state level of an mRNA is a function of the rate of transcriptional initiation and the rate of decay. We therefore measured the rate of decay of *sdh* transcripts in T_2 cells. The results of this experiment (Fig. 6) showed that at T_2, *sdh* mRNA decayed with a half-life of 0.4 min, compared with 2.6 min in exponentially growing cells. This sixfold decrease in *sdh* mRNA half-life at T_2 corresponded almost exactly to a sixfold reduction in the *sdh* mRNA steady-state level. The most straightforward interpretation of these findings is that the *sdh* operon is transcribed at the same rate in growing bacteria and in stationary-phase bacteria. In the latter cells, however, the half-life of *sdh* mRNA is reduced.

We thus seem to have a paradox regarding accumulation of the SDH enzyme in stationary-phase cells, where a differential increase in SDH enzyme activity and protein is accompanied by a decrease in *sdh* mRNA levels. We believe, however, that this paradox can easily be resolved when the properties of growing and stationary-phase cells are considered in relation to the stability of the membrane-bound SDH complex, as outlined in the Discussion.

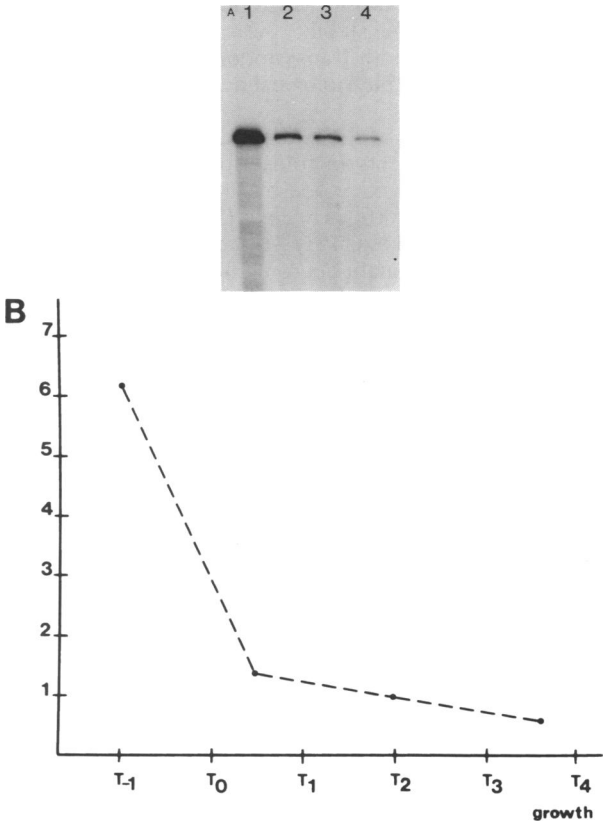


FIG. 5. (A) RNA was extracted from 3G18 grown in NSMP without glucose before and after the cells had entered stationary phase, and *sdh* mRNA was analyzed as described in the legend to Fig. 1A. Lanes 1, 2, 3, and 4 contain samples collected at T_{-1}, T_0, T_2, and T_4, respectively (10 μg of total RNA each). (B) Autoradiogram in panel A was analyzed by densitometric scanning, and the values obtained were plotted as in Fig. 1B.

DISCUSSION

The expression of the *B. subtilis sdh* operon is repressed by the presence of glucose (glucose repression) and affected by the stage of cell growth. In the present study, we determined the steady-state concentration of the polycistronic *sdh* transcript in growing cells in the presence and absence of glucose. Furthermore, we determined the transcriptional start and the steady-state level and rate of decay of the *sdh* mRNA at different growth stages in the absence of

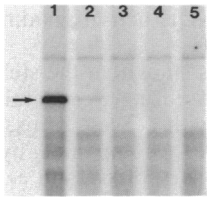


FIG. 6. Rate of decay of *sdh* mRNA in 3G18 growing in NSMP without glucose at T_2 was measured as described in the legend to Fig. 1A. Lanes 1, 2, 3, 4, and 5 contain samples collected 0, 1, 3, 5, and 8 min after rifamycin was added, respectively (50 μg of total RNA each). Due to the rapid decay of the *sdh* transcript at T_2, the half-life was estimated on only two time points. However, the estimate is based on data from several independent measurements.

glucose. Glucose repression was found to reflect transcriptional control. mRNA stability was found to vary with growth stage. Thus, both transcriptional and posttranscriptional events rule the physiological adaption of *sdh* expression.

In *Escherichia coli*, cyclic AMP and cyclic AMP receptor protein are key elements in transcriptional control of glucose- or catabolite-sensitive operons (4), whereas the nature of the catabolite effector(s) is unclear (15). Catabolite repression has been less extensively studied in *B. subtilis*. For example, for the gluconate operon (10), *amvE* (21), and *citB* (27), it has been shown that catabolite repression affects transcription, but to our knowledge, there are no data suggesting a plausible mechanism for this effect. From the present experiments we conclude that catabolite repression of the *sdh* operon can also be attributed to transcriptional control. mRNA stability is the same with and without glucose in the growth medium, while the level of *sdh* transcript correlates with the level of SDH protein. Thus, if there are any effects of glucose on translational efficiency, they must be marginal. Two lines of experiments argue against an *E. coli*-like repressor model explaining catabolite repression of the *sdh* operon. The region upstream of the core promoter could be removed without abolishing glucose repression. The presence of the transcriptional control region of the *sdh* operon on a high-copy-number plasmid did not alleviate catabolite repression. Thus, neither a *cis*-acting site upstream of the promoter nor a *trans*-acting repressor could be identified. Furthermore, analysis of the 5′ endpoint of the *sdh* mRNA did not indicate a switch of promoters as a result of glucose repression.

Mutations which render expression of the *B. subtilis amvE* gene resistant to glucose repression map at the nontranslated 5′ end of *amvE* mRNA (21). How these mutations exert their effect is not understood. Mutations which permit *B. subtilis* to sporulate in the presence of glucose map at different loci on the bacterial chromosome (26, 30). The mechanisms for glucose repression of sporulation and of vegetatively expressed genes are probably not identical (13, 31). The mechanism by which glucose affects *sdh* transcription is unknown. The present results, however, restrict the DNA target of such a control mechanism to the core promoter and the 5′ noncoding region of the *sdh* operon.

When a growing culture of *B. subtilis* approaches stationary phase, time T_0, a number of genes which are silent during exponential growth become active. Some of these temporally regulated genes, like *aprE*, are not essential for sporulation but are dependent on the products of *spo0* genes for activation (8, 25, 29). The *sdh* operon is active in *B. subtilis* under all growth conditions examined. In glucose-free medium, the SDH activity increases severalfold as the bacteria pass through T_0 and further. Clearly, growth phase-dependent modulation of SDH activity is different from temporal control of, e.g., *aprE*.

Our present results show that the steady-state level of *sdh* transcripts is highest during exponential growth and drops about sixfold as growth ceases. Thus, an increase in SDH activity seems to be accompanied by a decrease in *sdh* mRNA during stationary phase and early sporulation. This paradoxical relationship between SDH protein and *sdh* mRNA can be explained by the retardation and cessation of growth when bacteria have passed through T_0; i.e., the relative amount of SDH protein in the cell increases when the rate of synthesis exceeds the rate of dilution that is caused by cell division. However, such an explanation is reasonable only if the SDH protein is stable in relation to the rate of dilution. Another way to connect the accumulation of SDH protein to the decrease in *sdh* transcripts is to postulate an increase in the efficiency of translation. The latter explanation does not seem reasonable to us, namely to increase the efficiency of translation of a transcript while the stability of the same transcript decreases. Therefore, we prefer to attribute the accumulation of SDH protein in stationary phase and during onset of sporulation to lack of dilution of the protein.

One may argue that the observed decay of *sdh* mRNA only concerns the first gene of the *sdh* operon, *sdhC*, which encodes the anchor protein cytochrome b_{558}, while expression of the remaining operon is not or little affected. However, previous data have shown that all three gene products encoded by the *sdh* operon are found in equimolar amounts, and no excess of anchor protein is produced during growth (11, 12). Furthermore, analyzing the decay of the *sdh* transcript in the middle and at the 3′ end has revealed no evidence for such a notion (L. Melin, L. Rutberg, and A. von Gabain, manuscript in preparation).

In a recent study, we noticed that the amount of chloramphenicol acetyltransferase (CAT) encoded by a fusion gene containing the 5′ end of the *sdh* operon and the coding region of the *cat-86* gene increases significantly more than the SDH protein when bacteria enter stationary phase and sporulation (19). We extended this observation to the level of mRNA and found that the steady-state level of *cat* mRNA is not affected by growth stage (Melin et al., in preparation). This result indicates that the sequences contained in this hybrid transcript are not sufficient to mediate a loss of stability of the *cat-86* mRNA as growth ceases.

Growth rate-dependent regulation of mRNA stability has been previously identified in *E. coli* (22), and it has been found that the stability of certain transcripts is similarly affected when cells enter the stationary phase (22). Such an example is the *ompA* transcript. It will be interesting to see whether mRNA stability is a general means for controlling gene activity when cells enter stationary phase.

In the case of the *E. coli ompA* transcript, site-specific cleavages in the 5′ noncoding region seem to initiate degradation (18). The *sdh* transcript provides an attractive experimental system to search for such a rate-limiting step of mRNA degradation in *B. subtilis*.

ACKNOWLEDGMENTS

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