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Nucleotide Sequence of the Gene for Cytochrome b_{558} of the *Bacillus subtilis* Succinate Dehydrogenase Complex

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The nucleotide sequence was determined for the first part of the *Bacillus subtilis sdh* operon. An open reading frame corresponding to the structural gene, *sdhA*, for cytochrome b_{558} was identified. The predicted molecular weight of the cytochrome (excluding the N-terminal methionine) is 22,770. It is a very hydrophobic protein with five probable membrane-spanning segments. There is little homology between the *B. subtilis* cytochrome b_{558} and cytochrome *b* of mitochondrial complex III from different organisms or between cytochrome b_{558} and the hydrophobic *sdhC* and *sdhD* peptides of the *Escherichia coli sdh* operon. About 30 bases downstream of the *sdhA* stop codon, a new open reading frame starts. The nucleotide sequence predicts the presence of a typical flavin-binding peptide which identifies this reading frame as part of the *sdhB* gene. Seven bases upstream of the *sdhA* initiation codon ATG there is a typical *B. subtilis* ribosome binding site (free energy of interaction, −63 kJ), and further upstream, tentative sigma 55 and sigma 32 promoter sequences were found. The upstream region also contains two 12-base-long direct repeats; their significance is unknown.

Bacillus subtilis succinate dehydrogenase [SDH; EC 1.3.99.1; succinate: (acceptor) oxidoreductase] forms part of a membrane-bound enzyme complex containing three different subunits (11). The cytochrome b_{558} subunit is a strongly hydrophobic protein which is required for binding the two SDH subunits, flavoprotein and iron-sulfur protein, to the membrane. The two SDH subunits are located on the inside of the cytoplasmic membrane, whereas cytochrome b_{558} is a transmembrane protein (12) which is the primary acceptor of electrons from SDH after succinate oxidation (9).

Small hydrophobic peptides are also known or thought to be required for binding SDH to membranes in other procaryotes and in eucaryotes. Their role in electron flow during succinate oxidation is not as well defined as it is in *B. subtilis*, but there is evidence that they represent *b*-type cytochromes in the mammalian (8) and *Escherichia coli* enzyme complexes (2).

In *B. subtilis* the structural genes for cytochrome b_{558} (*sdhA*), flavoprotein (*sdhB*), and iron-sulfur protein (*sdhC*) are arranged in an operon which is transcribed in the same order (18). The *B. subtilis sdh* operon has recently been cloned in *E. coli* (7, 17). The *sdhA* gene and part of the *sdhB* gene are contained within a 2.1-kilobase *Bam*HI-*Eco*RI fragment. When cloned in plasmid pBR322, the *sdhA* gene is expressed and the cytochrome constitutes about 4% of the total membrane protein in the *E. coli* host.

The primary structures of the *b*-type cytochromes from complex III (ubiquinol-cytochrome *c* oxidoreductase) of human, bovine, mouse, *Saccharomyces cerevisiae*, and fungal mitochondria have been determined, as has that of the cytochrome *b* from the b_6-f complex of spinach chloroplasts. All of these *b* cytochromes show a strong degree of mutual sequence homology. They also contain several conserved histidine residues, four of which are thought to be the heme ligands (29). Hydropathy profiles (16) indicate that these cytochromes have five to nine membrane-spanning segments.

In comparison, very little is known about the structure of *b*-type cytochromes from procaryotic organisms. The *cytB* gene encoding cytochrome b_{561} (24) and the *cyd* locus containing the cytochrome b_{558} gene of the cytochrome *d* complex (5) of *E. coli* have recently been cloned. The genes for the bc_1 complex of *Rhodopseudomonas sphaeroides* have been isolated, and some partial nucleotide sequences have been reported (4). These cytochromes show a high degree of homology with the bc_1 complex of eucaryotic organisms.

In this paper we report the nucleotide sequence of the *B. subtilis sdhA* gene. The predicted amino acid sequence revealed a strongly hydrophobic protein of about 22 kilodaltons and suggested the presence of five membrane-spanning segments. The *sdhA* sequence shows little homology to previously sequenced *b*-type cytochrome genes.

MATERIALS AND METHODS

Plasmid. Plasmid pKIM4 is a pBR322 derivative carrying the entire *sdhA* gene, part of the *sdhB* gene, and the promoter region of the *sdh* operon of *B. subtilis* on a 2.1-kilobase *Bam*HI-*Eco*RI fragment (Fig. 1) (17). Plasmid DNA was prepared by standard methods, and the structure of the plasmid was verified by agarose gel electrophoresis of the DNA after it had been cleaved with appropriate restriction enzymes (19).

DNA sequencing. DNA sequencing was done by the dideoxy method (27) by using the M13 cloning system (20). The 2.1-kilobase *Bam*HI-*Eco*RI fragment from plasmid pKIM4 was cloned in vectors M13mp8 and M13mp9, and the *Bam*HI-*Pst*I and *Pst*I-*Eco*RI subfragments were subcloned in M13mp8. With these clones as templates, the sequence of about 600 bases around the *Pst*I site was determined with the universal M13 primer. New primers were then synthesized (15-mers) and used to extend the sequence, with the *Bam*HI-*Eco*RI fragment in M13mp8 and M13mp9 as templates. By

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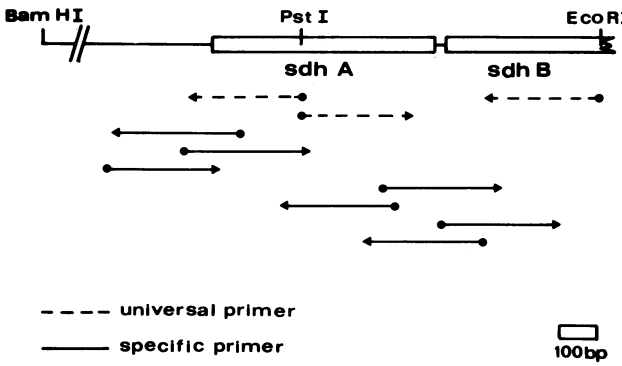

FIG. 1. Outline of strategy for sequencing the *sdhA* gene.

repeating this procedure, the sequences of both strands of the *sdhA* gene and adjacent regions were obtained. The sequenced region and the position of the primers are shown in Fig. 1.

RESULTS and DISCUSSION

Nucleotide sequence of the *sdhA* gene and adjacent regions. Mapping data from transformation crosses have shown that the *sdhA* gene contains a unique *Pst*I site about 830 base pairs (bp) from the *Eco*RI site in the 2.1-kilobase *Bam*HI-*Eco*RI fragment in plasmid pKIM4 (17). Preliminary sequence data for the region around this *Pst*I site revealed one open reading frame. With this information the sequence was extended and consolidated by using a strategy based on the synthesis of specific oligonucleotide primers (see Materials and Methods). The nucleotide sequence of a 997-bp segment including the *sdhA* gene is shown in Fig. 2. There is an open reading frame starting with an ATG codon at position 265 and ending with a TAA stop codon at position 871. The open reading frame codes for a protein with a predicted molecular weight of 22,770 (excluding the N-terminal methionine).

Seven base pairs upstream of the initiation codon ATG there is a potential ribosome binding site, -AGGGGGT-. The calculated free energy of interaction (29) of this sequence with the 3′ end of *B. subtilis* 16S rRNA (−15 kcal [−63kJ]) was similar to that reported for other *B. subtilis* ribosome binding sites (6).

B. subtilis contains several RNA polymerase holoenzymes with different sigma factors which recognize specific promoter sequences. In vegetative cells, RNA polymerase sigma 55 is the dominating holoenzyme (3, 23), and it recognizes the same consensus −35 (TTGACA) and −10 (TATAAT) sequences as does the *E. coli* RNA polymerase (23). A search for potential promoter structures upstream of the *sdhA* gene revealed a nearly perfect −35 sequence, TTGACG, starting at position 140 and followed 17 bp downstream by a −10 sequence, TAAAAT. Several *B. subtilis* sigma 55 promoters contain A+T-rich regions immediately upstream of the −35 sequence (3). Of the first 30 bases upstream of the proposed −35 sequence, 80% are adenine or thymine. We have previously described a pleiotropic mutation, *sdh-115*, which maps upstream of all known *sdhA* mutations and abolishes expression of the whole *sdh* operon (18). The *sdh-115* mutation has recently been located in the above −35 sequence, strengthening the suggestion that this sequence is part of an *sdh* promoter region (unpublished results). Screening of the *sdhA* upstream region for other promoter regions revealed two potential sigma 32 sequences starting at position 85 (AAATT-15 bp-TCAATTCGGA) and position 170 (AAATT-15 bp-TAAAGTGCTT). The latter sequence has the same consensus as that shared by the sigma 32 promoters of the *sprE* (30) and *spoVG* (13) genes. However, the fact that the *sdh-115* mutation is located outside both of the possible sigma 32 promoter regions suggests that they do not play a major role in expression of the *sdh* operon. Studies of *sdh*-specific transcripts should resolve these questions. No obvious sigma 37 or sigma 28 sequences were found in the *sdhA* upstream region.

The activity of the enzymes of the dicarboxylic acid part of the Krebs cycle are known to increase as *B. subtilis* enters postexponential growth, and the enzyme levels are also glucose repressible (26). This points to the possible existence of common regulatory mechanism(s) for the genes. We therefore compared the *sdhA* upstream region with the corresponding region of the *B. subtilis* fumarase gene, *citG* (22). The distance between the ATG initiation codon and the proposed sigma 55 promoter is 125 to 127 bp in both genes. Two areas of homology were found in the upstream regions of *sdhA* and *citG*. The sequence TTCTTCTGAAA matches in 9 of 11 positions, and GGAAAATTAT matches in 8 of 10 positions. The first region starts 223 bp upstream of the ATG initiation codon in the *sdhA* gene and 267 bp upstream in the *citG* gene. The second region of homology is located 153 bp upstream in *sdhA* and 46 bp upstream in *citG*. The significance of these homologies remains to be determined. In the upstream *sdhA* region there are also two 12-bp direct repeats, TCAAACAGGGGG, at coordinates 7 to 18 and 246 to 257. Their possible significance is also unknown.

After the *sdhA* stop codon TAA, a new open reading frame starts with ATG at position 907. Preliminary sequence data predicted the presence of a typical flavin-binding peptide which identified this reading frame as part of the *sdhB* gene. The positioning of the *sdhB* gene close to the end of the *sdhA* gene was expected from mapping data and is consistent with the existence of an *sdh* operon.

Predicted structure of cytochrome b_{558} and comparison with other *b*-type cytochromes. The calculated molecular weight of the 201 amino acid residues of cytochrome b_{558}, 22,770, is about 20% larger than the molecular weight of 19,000 estimated from gel electrophoresis (10). The cytochrome is very hydrophobic (polarity index, 31.4% [1]), and it is known that hydrophobic proteins may move aberrantly in electrophoresis. Although we cannot exclude the possibility that cytochrome b_{558} is posttranslationally processed, the predicted N-terminal part does not have the features of a typical signal sequence (21). The hydropathy profile for cytochrome b_{558} suggests that it has five membrane-spanning segments (Fig. 3). The average hydropathy index for these segments was 1.6 or higher, strengthening the idea that they are membrane-spanning rather than internal hydrophobic stretches (16). It has also been proposed that the 32-kilodalton cytochrome *b* from the spinach chloroplast b_6-f complex has five membrane-spanning segments, whereas eight to nine such segments are found in the 42-kilodalton cytochrome *b* of mitochondrial complex III (29). A comparison between these cytochromes and *B. subtilis* cytochrome b_{558} revealed little sequence homology.

Histidine residues are considered particularly important in the spinach chloroplast and in the complex III cytochrome *b* because it is thought that they serve as heme ligands (29). Electron-paramagnetic-resonance spectroscopy has indicated that histidine residues are the fifth and sixth axial ligands to heme iron in *B. subtilis* cytochrome b_{558} (L. Hederstedt and K. K. Andersson, J. Bacteriol., in press).

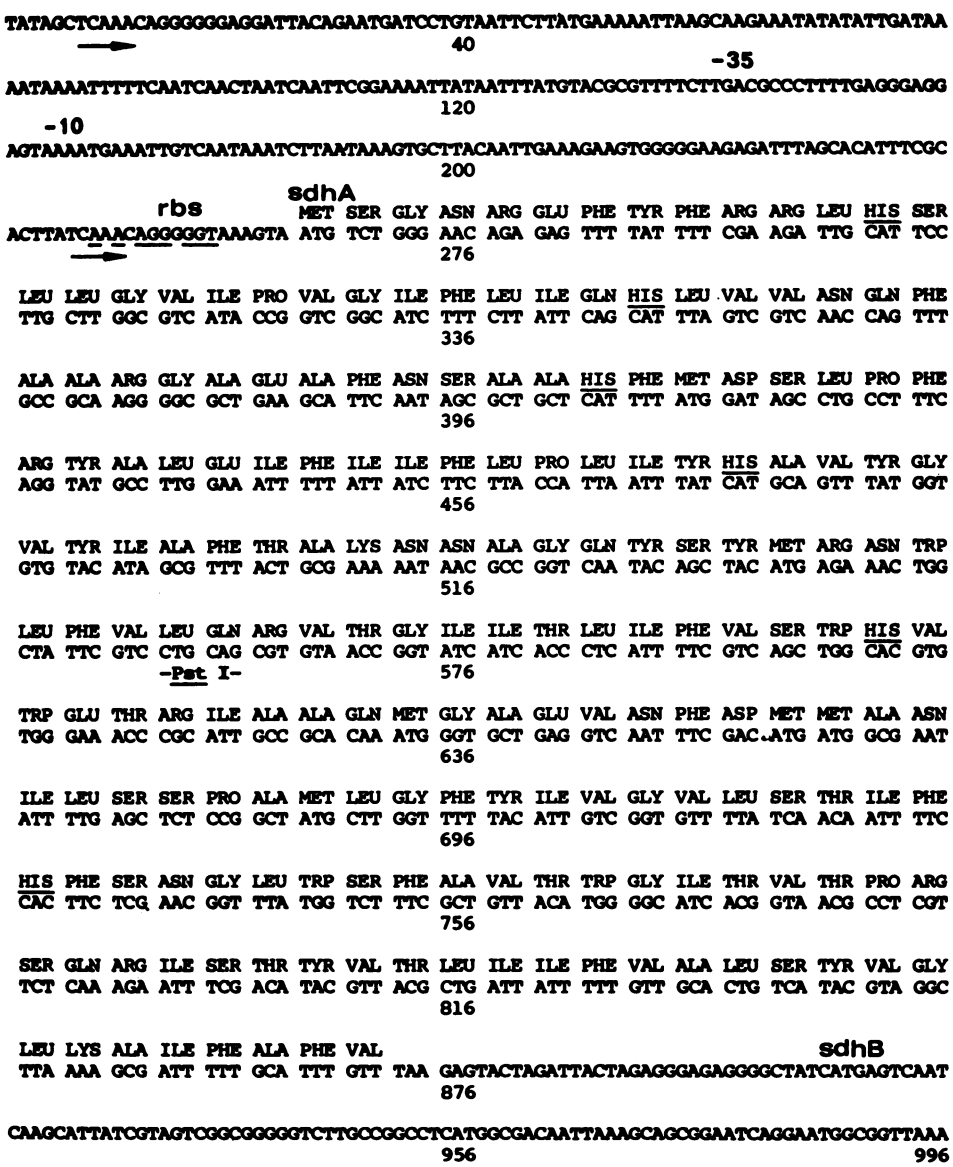

FIG. 2. Nucleotide sequence of the *sdhA* gene and adjacent regions. The initiation codons are denoted *sdhA* and *sdhB*, rbs marks the proposed ribosome binding site, and −35 and −10 indicate regions proposed to be involved in RNA polymerase (sigma 55) binding. The arrows mark the start of the 12-bp direct repeats. The histidine residues are underlined, and the position of the *Pst*I site is indicated.

There are six histidine residues in the cytochrome. It can only be speculated which of these residues may serve as heme ligands. A detailed comparison of the sequences around the histidine residues in cytochrome b_{558} with the corresponding sequences in the complex III *b*-type cytochromes revealed little homology. Some correspondence was found between the sequence around the histidine residue at position 13 in cytochrome b_{558} and the sequence (*Phe*-Arg-Arg-*Leu-His*-Ser-*Leu-Leu*-Gly-Val) around the histidine residue at position 183 in the *Aspergillus nidulans* cytochrome, but its significance is difficult to assess (14).

For *E. coli* SDH there is good evidence that the enzyme is bound to the cytoplasmic membrane via the hydrophobic peptides coded for by the *sdhC* and *sdhD* genes (2, 31). Both peptides are found associated with SDH immunoprecipitated from detergent-solubilized membranes. Furthermore, light absorbtion spectroscopy data indicate that they form a *b*-type cytochrome. *E. coli* cytochrome b_{556} is the product of the *cytA* gene, which maps close to the *sdh* locus (25). This cytochrome was purified in 1978, but its function was not known (15). Recently, the sequence of the 24 N-terminal amino acids of the cytochrome was determined and found to correspond to residues 4 to 27 predicted from the *sdhC* nucleotide sequence (Y. Anraku, personal communication). A comparison between the *sdhA* gene of *B. subtilis* and the *sdhC* and *sdhD* genes of *E. coli* disclosed no obvious homology between the nucleotide sequences. It is however notable that the amino acid sequence His. . .Gly occurs at three of six histidine residues in the *B. subtilis sdhA* peptide, and at two of three and one of two histidine residues in the *E. coli sdhC* and *sdhD* peptides, respectively (Table 1).

It appears that the *b*-type cytochromes of complex III and spinach chloroplast b_6 cytochrome form a family of closely related proteins (29), whereas the SDH-binding proteins,

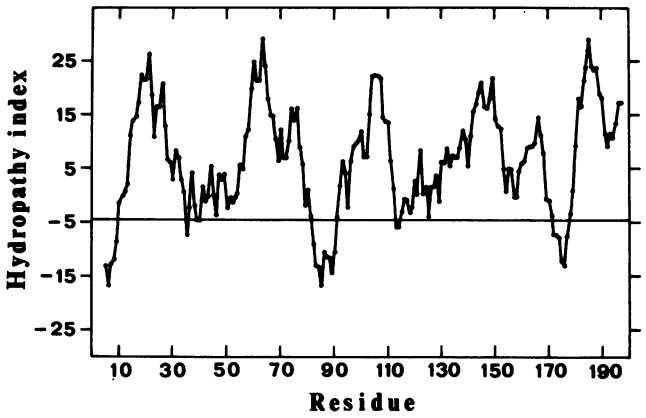

FIG. 3. Hydropathy profile of the predicted amino acid sequence of cytochrome b_{558} (span = 9 [16]). The amino-terminal methionine residue was excluded.

TABLE 1. Amino acid sequences around selected histidine residues in the *B. subtilis sdhA* peptide and the *E. coli sdhC* and *sdhD* peptides

Peptide	Histidine residue (no.)	Amino acid sequence
B. subtilis sdhA	13	Leu-*His*-Ser-Leu-Leu-*Gly*-Val-Ile
	70	Tyr-*His*-Ala-Val-Tyr-*Gly*-Val-Tyr
	155	Phe-*His*-Phe-Ser-Asn-*Gly*-Leu-Trp
E. coli sdhC	30	Leu-*His*-Arg-Val-Ser-*Gly*-Val-Ile
	84	Tyr-*His*-Val-Val-Val-*Gly*-Ile-Arg
E. coli sdhD	71	Ile-*His*-Ala-Trp-Ile-*Gly*-Met-Trp

cytochrome b_{558} in *B. subtilis* and the *E. coli sdhC* and *sdhD* peptides, are less related. This may reflect independent evolution of these proteins. Alternatively, the function of the major part of the SDH-binding proteins could simply be to provide a hydrophobic transmembrane structure; this might impose only weak constraints on the primary structure so that considerable sequence divergence is permitted.

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