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Cloning and Expression in *Escherichia coli* of *sdhA*, the Structural Gene for Cytochrome b_{558} of the *Bacillus subtilis* Succinate Dehydrogenase Complex

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Bacillus subtilis **cytochrome b_{558} is a transmembrane protein which anchors succinate dehydrogenase (SDH) to the cytoplasmic membrane and is reduced by succinate. The structural gene for this cytochrome was cloned and expressed in *Escherichia coli*. Random *Bam*HI or *Bgl*II fragments of *B. subtilis* 168 DNA were cloned in the *Bam*HI site of plasmid pHV32. The derived plasmids were used to transform *B. subtilis* SDH mutants to chloramphenicol resistance by integration of the plasmid via DNA homology. Of some 3,000 transformants tested, 6 were SDH positive and had pHV32 integrated close to the *sdh* operon. Two plasmids, pKIM2 and pKIM4, with an insert of *B. subtilis* DNA of 5.7 and 3.4 kilobases, respectively, were generated by transforming *E. coli* with DNA from the SDH-positive transformants after cleavage with *Eco*RI or *Bgl*II and ligation. In *E. coli* carrying either of the two plasmids, about 4% of total membrane protein was *B. subtilis* cytochrome b_{558}. *E. coli* (pKIM2) also contained antigen which reacted with antibodies specific for the flavoprotein and the iron-sulfur protein subunit of *B. subtilis* SDH. Enzymatically active, membrane-bound *B. subtilis* SDH could not be demonstrated in *E. coli* (pKIM2). The *B. subtilis* DNA insert in pKIM2 could transform *B. subtilis sdhA* (cytochrome b_{558}), *sdhB* (flavoprotein), and *sdhC* (iron-sulfur protein) mutants to the wild type. The results suggest that pKIM2 carries the whole *B. subtilis sdh* operon. The data confirm the gene order and the proposed direction of transcription of the *B. subtilis sdh* operon. Most likely the *sdh* genes in *E. coli*(pKIM2) are controlled by their natural promoter.**

Bacillus subtilis succinate dehydrogenase (SDH; EC 1.3.99.1; succinate: [acceptor] oxidoreductase) is a membrane-bound enzyme complex containing three unequal subunits, a flavoprotein (Fp; M_r, 65,000 [65K]), an iron-sulfur protein (Ip; M_r, 28K) and a cytochrome b_{558} (cyt*b*; M_r, 19K). (10). Cyt*b* is a transmembrane protein with Fp and Ip attached to its cytoplasmic side (13). In the absence of cyt*b*, Fp and Ip are made as soluble proteins and accumulate in the cytoplasm (12, 15). The membrane-bound SDH complex can only be isolated as an active, monodisperse complex after the cell membrane is disrupted with detergent (11). Attempts to purify sufficient amounts of the complex for detailed biochemical studies have met with little success. Limited information is therefore available about the structure of the subunits of the SDH complex and, accordingly, about the importance of various domains of the subunits for incorporation of prosthetic groups such as covalently bound flavin, iron-sulfur centers, and heme.

All mutations which abolish in vitro SDH activity map in one region, at 255°, on the *B. subtilis* genetic map (14). Data from transformation crosses and complementation studies suggest that the structural genes for the subunits of the SDH complex are organized in one operon which is probably transcribed in the order *sdhA*(cyt*b*), *sdhB*(Fp), *sdhC*(Ip) (18). SDH activity is regulated both at the transcriptional level and at the level of the enzyme (18, 25). A wealth of data is available concerning the effects of, e.g., various Krebs cycle intermediates on SDH activity, but essentially nothing is known about control of *sdh* at the level of the gene.

An obvious first approach to increasing our knowledge about the structure of the enzyme complex and its genetic regulation is to clone the *sdh* operon. Initial attempts to select directly for the *sdh* operon in various *B. subtilis* gene banks proved unsuccessful, and no closely linked genetic markers which can easily be selected are available.

Instead, we have used the plasmid insertion technique described by Niaudet et al. (22). With this technique, an *Escherichia coli* plasmid expressing a selectable marker in *B. subtilis* is inserted into a *B. subtilis* gene of interest through homologous recombination. The integrated plasmid can then be regenerated in *E. coli* together with flanking *B. subtilis* DNA by cleavage with appropriate restriction enzymes, ligation, and transformation of *E. coli* with selection for a plasmid marker. In this paper we describe the directed integration of plasmid pHV32 (22) adjacent to the *B. subtilis sdh* operon and the cloning and expression of the *sdhA* gene in *E. coli*.

MATERIALS AND METHODS

Bacteria and plasmids. The *B. subtilis* and *E. coli* strains and the plasmids used are listed in Table 1.

Media and growth of bacteria. *B. subtilis* strains were kept on Tryptose Blood Agar Base (TBAB; Difco Laboratories, Detroit, Mich.); the Sdh phenotype was checked on purification agar (PA) plates (2). Spizizen minimal medium (26) was used with required growth factors added at 20 mg liter^{-1}. Succinate minimal medium was Spizizen minimal medium in which glucose had been replaced by sodium succinate at 5 g liter^{-1}. *E. coli* strains were kept on LA plates (19). Liquid cultures were grown in L broth for preparation of chromosomal and plasmid DNA (*B. subtilis* and *E. coli*) and membranes (*E. coli*).

Antibiotics were added at the following concentrations: ampicillin, 35 to 50 mg liter^{-1}; chloramphenicol, 12.5 mg liter^{-1} (*E. coli*) or 5 mg liter^{-1} (*B. subtilis*).

In vitro DNA techniques. Preparation of chromosomal or plasmid DNA, agarose gel electrophoresis, and Southern

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TABLE 1. Bacteria and plasmids

Strains and plasmids	Genotype	Source or reference
B. subtilis		
168	*trpC2*	Our collection
3G18	*trpC2 ade met*	G. Venema
KA98012	*trpC2 sdhA12*	11
KA95078	*trpC2 sdhA78 ilvC1*	11
KA95083	*trpC2 sdhC83 ilvC1*	11
KA97101	*trpC2 sdhB101 leu-2*	11
KA97103	*trpC2 sdhC103 leu-2*	11
KA95115	*trpC2 sdh-115 ilvC1*	18
HS3A17	*trpC2 cit-17*	2
E. coli 5K	*hsdR hsdM thi thr rpsL lacZ*	L.-O. Hedén
Plasmids		
pHV14	*amp cat*	S. D. Ehrlich
pHV32	*amp tet cat*	22

blotting were performed as described by Maniatis et al. (19). Restriction enzymes and polynucleotide ligase were purchased from New England BioLabs, Beverly, Mass., and used as recommended by the manufacturer.

Transformation. *B. subtilis* competent cells were prepared as described by Arwert and Venema (1); the cells were stored in 10% glycerol at −80°C until used. Competent *E. coli* 5K cells were prepared by the method of Hanahan (7).

Absorption spectroscopy. Absorption difference spectra of membranes were recorded at room temperature on a Shimadzu UV-3000 spectrophotometer with the double-beam mode and a 1-nm slit. Cuvettes (1 ml) with a 1-cm light path were used. Membranes in the reference cuvette were oxidized with 1 mM $K_3Fe(CN)_6$, and those in the sample cuvette were reduced with a small amount of solid sodium dithionite.

Other methods. *E. coli* membranes were isolated from osmotically lysed spheroplasts, essentially as described by Kaback (16). The spheroplasts were obtained by lysozyme-EDTA treatment of cells grown aerobically in LB. The washed membranes were suspended in 50 mM Tris-hydrochloride (pH 8.0) to about 20 mg of protein ml^{-1}, frozen in liquid nitrogen, and stored at −80°C.

Heme was extracted with acidified acetone from delipidated membranes, and pyridine hemochromogens were determined as described by Rieske (23). The difference (reduced minus oxidized) extinction coefficient 20.7 mM^{-1} cm^{-1} for the wavelength pair 557 minus 541 nm (5) was used for the quantitative determination of protoheme IX. One-dimensional (rocket) immunoelectrophoresis in 1% agarose (SeaKem HGT; FMC Corp., Marine Colloids Div., Rockland, Maine) was done as described previously (12, 24). SDH enzyme activity in membranes was determined at 38°C as described previously (8), but bovine serum albumin was omitted and 2 mM KCN was added to the assay buffer. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the discontinuous buffer system of Neville was done as described previously (21). Protein was determined by the method of Lowry et al. (17) in the presence of 1.7% (wt/vol) SDS and with bovine serum albumin as standard.

RESULTS

Insertion of pHV32 at the *sdh* operon. The plasmid pHV32 is a derivative of pBR322 which carries part of pC194, including the *cat* (chloramphenicol acetyltransferase) gene. The *cat* gene is expressed in both *E. coli* and *B. subtilis*, but the plasmid can only replicate in the former host (22). After ligation of *B. subtilis* chromosomal DNA into pHV32, competent *B. subtilis* cells can be transformed to chloramphenicol resistance (Cm^r) by integrating the plasmid into the chromosome by homologous recombination (22).

Two well characterized *B. subtilis* Sdh mutants, KA95078 (*sdhA78*) and KA95083 (*sdhC83*), were transformed with pHV32 after *Bam*HI- or *Bgl*II-cleaved *B. subtilis* 168 DNA had been inserted into the unique *Bam*HI site of the plasmid. If an insert carries the wild-type allele of the respective *sdh* mutations, Sdh-positive clones should be found among the Cm^r transformants and the plasmid should be integrated close to the *sdh* operon.

A total of about 3,000 Cm^r transformants were screened for their Sdh phenotype on PA plates; 12 Sdh-positive clones were found. DNA was extracted from these clones and again used to transform KA95078 and KA95083, respectively, to Cm^r at nonsaturating concentrations. Cotransformation of *cat* and the wild-type *sdh* allele distinguishes between clones obtained by congression (or reversion of the *sdh* mutation) and the ones in which pHV32 had integrated close to the *sdh* operon. Six of the clones gave 20 to >90% cotransformation of *sdh* and *cat*.

Regeneration of integrated pHV32. DNA was extracted from the above six positive clones and restricted with *Bgl*II or *Eco*RI. *Bgl*II, for which there is no site in the plasmid, generates fragments in which pHV32 is flanked on both sides by *B. subtilis* DNA; these fragments were 10 to 15 kilobases (kb) (data not shown). *Eco*RI cuts within pBR322 and generates smaller fragments in which a deleted pBR322 replicon is linked to *B. subtilis* DNA at only one side. The restricted DNA was ligated at low concentrations (10 μg ml^{-1}) to increase intramolecular circle formation and to diminish the risk of inserting additional fragments. After ligation, *E. coli* 5K was transformed to ampicillin resistance (Amp^r) (*Eco*RI) or Cm^r (*Bgl*II). About 1,000 transformants per μg were obtained with the *Eco*RI fragments from five of the six clones; one strain gave no transformants. Only seven transformants from one strain were obtained with *Bgl*II fragments.

The plasmid contents of 10 clones within each set of *Eco*RI transformants were identical as judged by agarose gel electrophoresis, whereas for *Bgl*II the plasmid pattern varied among the transformants obtained from the same DNA sample (data not shown).

Plasmid DNA was prepared from one clone of each of the

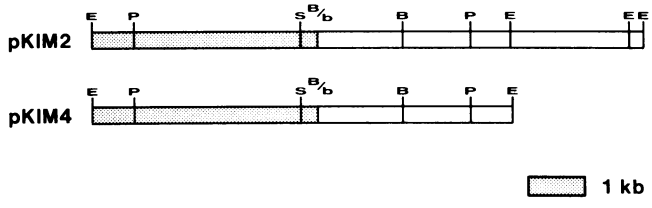

FIG. 1. Restriction maps of pKIM2 and pKIM4. Restriction maps were constructed by digesting plasmid DNA with one or two restriction enzymes and determining the size of the resulting fragments. Symbols: B, *Bam*HI; b, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sal*I. B/b denotes the *Sau*3A site generated in construction of the original pHV32 insertion plasmid from which pKIM2 and pKIM4 are derived. The shadowed area denotes the pBR322 part of the plasmid; all of the pC194 part of pHV32 has been lost.

TABLE 2. Insertional mapping of *B. subtilis sdh* genetic markers with pKIM30, a pHV32-pKIM2 chimera[a]

Recipient	Donor DNA	Sdh⁺/Cm^r
3G18 (wild type)	pKIM30	182/200
	pHV14	200/200
KA95115 (*sdh-115*)	pKIM30	69/208
	pHV14	7/208
KA95078 (*sdhA78*)	pKIM30	27/34
	pHV14	0/119
KA98012 (*sdhA12*)	pKIM30	44/81
	pHV14	0/200
KA97101 (*sdhB101*)	pKIM30	2/26
	pHV14	0/60
KA97103 (*sdhC103*)	pKIM30	5/17
	pHV14	0/46

[a] pKIM2 was partially cleaved with *Eco*RI and ligated to *Eco*RI-cleaved pHV32. A plasmid, pKIM30, carrying all of the *B. subtilis* DNA insert in pKIM2 except the 300-bp *Eco*RI fragment, was isolated after transformation of *E. coli* 5K and selection for Cm^r transformants. The Sdh phenotype of *B. subtilis* transformants obtained by transformation with pKIM30 or pHV14 was scored on PA plates. *B. subtilis* Sdh mutants transformed to Cm^r with pHV14, a pBR322-pC194 chimera which replicates in both *E. coli* and *B. subtilis*, served as controls for reversion of the *sdh* mutations.

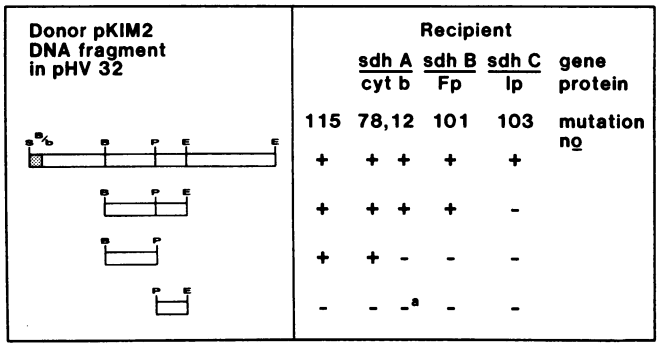

FIG. 2. Insertional mapping and marker rescue of *sdh* mutants. The pKIM2 donor DNA fragments were cloned in pHV32, and the resulting plasmids were used to transform Sdh-negative mutants carrying the indicated mutations to Cm^r. The Sdh phenotype of the transformants was then determined. Symbols for restriction enzymes are as in Fig. 1. The *sdh-115* mutation is thought to be in the *sdh* promoter region (18). Symbols: +, Sdh-positive Cm^r transformants obtained; −, no Sdh-positive Cm^r transformants obtained. a, Transformation of the Sdh-positive strain 3G18 with pHV32 carrying this *Pst*I-*Eco*RI fragment gave only Sdh-negative Cm^r transformants. All these transformants contained a membrane-bound cyt*b* but lacked both Fp and Ip antigens as tested by rocket immunoelectrophoresis. This result is expected from an integration by a Campbell-like mechanism of an internal fragment of the *sdh* operon carried on pHV32.

five *Eco*RI samples and from two *Bgl*II clones carrying the largest plasmids and therefore presumably the largest inserts of *B. subtilis* DNA. Since the *cat* gene is excised from the *Eco*RI-derived plasmids, the presence of the *sdh* operon or part of it cannot be tested as above by transformation and selection for Cm^r.

Instead, transformation of *B. subtilis* KA95078 with plasmid DNA was done by congression with chromosomal DNA, selecting for Ilv⁺ transformants, and testing their Sdh phenotype on PA plates, and with plasmid DNA only, with direct selection for Sdh-positive transformants on minimal succinate plates. Two *Eco*RI plasmids gave Sdh-positive transformants in both tests. They were named pKIM4 and pKIM7, respectively. One *Bgl*II plasmid was also positive and was named pKIM2; it originated from the same strain as did pKIM4. Preliminary experiments showed that pKIM4 and pKIM7 were similar. Only the former plasmid was used in further work. The original strain from which pKIM2 and pKIM4 were derived was made by using a *B. subtilis Bgl*II DNA fragment for insertion of pHV32.

Characterization of pKIM2 and pKIM4. Restriction maps were constructed for pKIM2 and pKIM4 after single and double digestion with a few selected restriction enzymes (Fig. 1). The *B. subtilis* DNA inserts in pKIM4 and pKIM2 are about 3.4 and about 5.7 kb, respectively. Although pKIM2 was derived from *Bgl*II-restricted DNA, it does not contain a *Bgl*II site. After the initial selection of pKIM2, the strain carrying this plasmid was kept on ampicillin plates and was no longer Cm^r. We have previously observed that selecting inserts from this region of the *B. subtilis* chromosome after *Bgl*II cleavage by pHV32 generates very unstable plasmids. However, the structure of pKIM2 as shown in Fig. 1 has proven stable on Amp selection. The heterogeneous plasmid pattern found for the *Bgl*II-derived plasmids originating from the same DNA sample also indicates that rearrangements have occurred in most of them.

Fragments of the *B. subtilis* DNA inserts in pKIM2 were subcloned into pHV32. The resulting plasmids were used for insertional mapping by transforming various Sdh mutants selecting for Cm^r and then scoring their Sdh phenotype on PA plates. The results obtained with the largest fragment

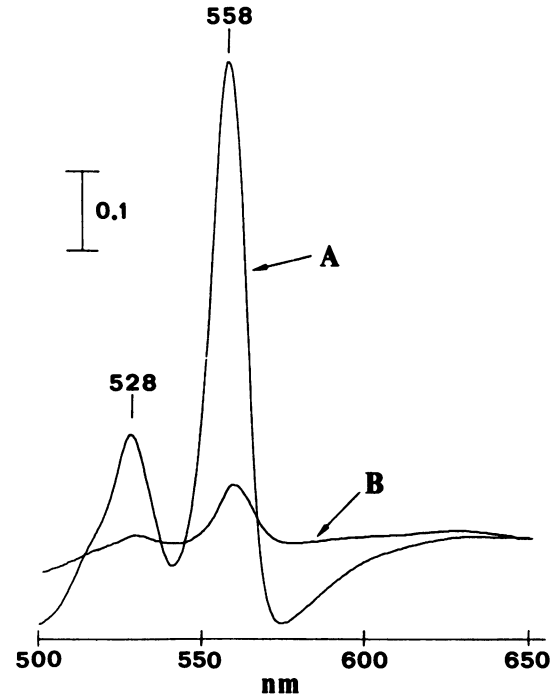

FIG. 3. Absorption difference spectra (reduced minus oxidized) of *E. coli* isolated crude membranes at room temperature. Spectrum A, *E. coli.* 5K(pKIM2) membranes, 4.9 mg of protein per ml, 11.8 nmol of protoheme IX per ml. Spectrum B, *E. coli* 5K(pKIM6) membranes, 5.5 mg of protein/ml, 2.3 nmol of protoheme IX/ml. Absorbance is shown on the vertical axis.

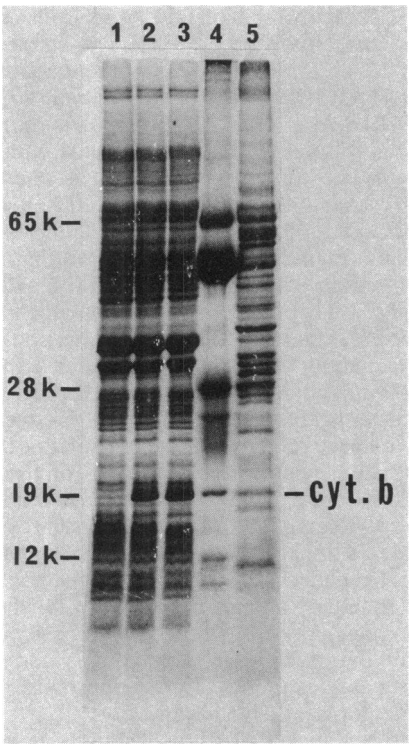

FIG. 4. SDS-polyacrylamide gradient gel electrophoresis of crude membranes isolated from *E. coli* control cells (lane 1) and *E. coli* cells expressing the cloned *B. subtilis sdhA* gene (lanes 2 and 3). Lanes: 1, *E. coli* 5K(pKIM6) membranes; 2, *E. coli* 5K(pKIM2) membranes; 3, *E. coli* 5K(pKIM4) membranes (about 35 μg of membrane protein of each *E. coli* preparation was loaded on the gel); 4, immunoprecipitated *B. subtilis* SDH-cyt*b* complex (10) (the fat protein band at M_r 50K and the diffuse band at about M_r 25K are the immunoglobulin subunits); 5, *B. subtilis* HS3A17 cytoplasmic membranes, 23 μg of protein. This strain produces relatively large amounts of the SDH-cyt*b* complex as a result of a defective 2-ketoglutarate dehydrogenase. The gradient of the gel was 10 to 15% (wt/vol) acrylamide–0.26 to 0.4% (wt/vol) bisacrylamide. The gel was stained for protein with Coomassie brilliant blue R.

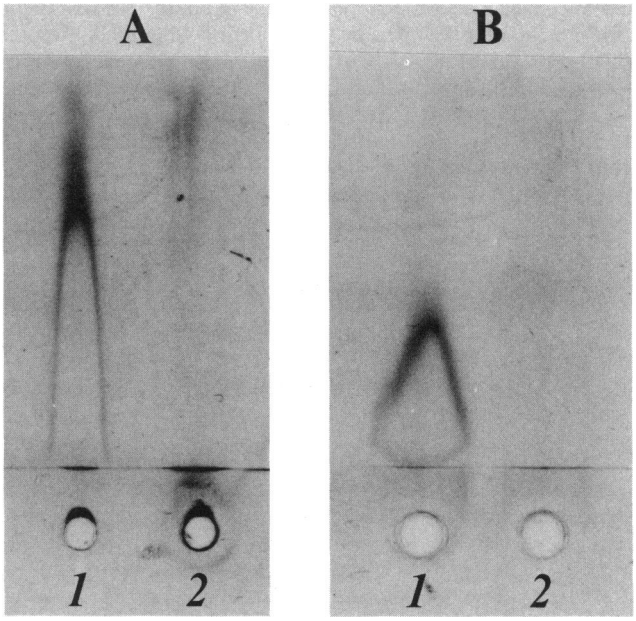

FIG. 5. Rocket immunoelectrophoresis of *E. coli* soluble cell fractions against *B. subtilis* Fp-specific (A) and Ip-specific (B) antisera. Well 1, *E. coli* 5K(pKIM2); well 2, *E. coli* 5K(pKIM6). Soluble fractions were prepared by sonication of spheroplasts in 24 mM sodium diethylbarbiturate buffer (pH 8.6) and subsequent centrifugation at 48,000 × *g* for 30 min at 4°C. The supernatant was withdrawn, and 5- to 20-μl samples were loaded on the gels: A1, 275 μg; A2, 610 μg; B1, 640 μg; B2, 700 μg of protein. The anode was at the top of the gels. The gels were stained with Coomassie brilliant blue R after electrophoresis.

tested are given in Table 2, and the results for all fragments are summarized in Fig. 2. From these data we conclude that pKIM4 carries all of *sdhA* and extends into *sdhB*, whereas pKIM2 may carry the whole of the *B. subtilis sdh* operon. Southern blots have verified that the inserts are of *B. subtilis* origin (data not shown).

Expression of the *B. subtilis sdhA* gene product cyt*b* in *E. coli*. Colonies of *E. coli* 5K carrying either pKIM2 or pKIM4 have a reddish color compared with the pale colonies of bacteria carrying pHV32 with inserts from other parts of the *B. subtilis* chromosome. This suggests that the reddish bacteria contain large amounts of cytochrome. Absorption difference spectra (reduced minus oxidized) were recorded for a crude membrane preparation from *E. coli* 5K(pKIM2) and from *E. coli* 5K carrying an *sdh*-negative plasmid, pKIM6 (carrying an insert of *B. subtilis* DNA from outside the *sdh* operon), derived from *Eco*RI-cleaved DNA as described above. Membranes from *E. coli* 5K(pKIM2) contain large amounts of cytochrome with absorption maxima at 528 and 558 nm (Fig. 3). The spectrum obtained was identical to that of cyt*b* of the *B. subtilis* SDH complex (9). The same results were obtained with *E. coli* 5K(pKIM4). The control membranes contained normal amounts of cytochrome for *E. coli* 5K, and the difference spectrum showed an absorption maximum at 560 nm (Fig. 3). Cytochrome was only detected in the membrane fraction of the cells. The membrane content of protoheme IX, the prosthetic group of *b*-type cytochromes, was six- to sevenfold higher in membranes from *E. coli* 5K(pKIM2) and *E. coli* 5K(pKIM4) compared with the control (Table 3).

Membranes from *E. coli* 5K(pKIM2) and *E. coli* 5K (pKIM4) contain large amounts of a polypeptide of M_r 19K, which migrates similarly to the *B. subtilis* apocytochrome b_{558} polypeptide in SDS-polyacrylamide gels (Fig. 4). From these results we conclude that the *B. subtilis sdhA* gene is carried by both pKIM2 and pKIM4 and that the gene is highly expressed in *E. coli* 5K.

***E. coli* 5K(pKIM2) contains *B. subtilis* Fp- and Ip-specific antigens.** The expression of *sdhA* from pKIM2 in *E. coli* 5K

TABLE 3. Properties of isolated crude membranes from *E. coli* 5K containing different plasmids with cloned *B. subtilis* DNA

Plasmid	Cyt*b* spectrum[a]	Polypeptide (M_r, 19K)[b]	Protoheme IX (nmol/mg of protein)	Cyt*b* (% of total membrane protein)[c]	SDH activity (μmol/min per mg of protein)
pKIM6	−	−	0.4	0	0.32
pKIM2	+	+	2.4	3.8	0.21
pKIM4	+	+	2.9	4.8	0.28

[a] Absorption (reduced minus oxidized) difference spectrum (Fig. 3).
[b] Determined by SDS-polyacrylamide gel electrophoresis (Fig. 4).
[c] Calculated on a molecular mass of 19 kD for apocytochrome b_{558} and assuming one heme group per cyt*b* polypeptide.

and the presence on the plasmid of genetic markers from *sdhB* and *sdhC* prompted us to determine whether *B. subtilis* SDH proteins were also expressed. Soluble cell fractions from *E. coli* 5K(pKIM2) and *E. coli* 5K(pKIM6) were tested for material cross-reacting with *B. subtilis* Fp- and Ip-specific antibodies, respectively, in immunoelectrophoresis (12). Both Fp and Ip antigens were found in the bacteria carrying pKIM2 but not in the other strain (Fig. 5). Triton X-100-solubilized membranes from *E. coli* 5K(pKIM2) or whole-cell extracts from bacteria carrying either pKIM4 or pKIM6 had no detectable *B. subtilis* Fp or Ip antigens. Condon and Owen (3) have shown that antibodies against *E. coli* SDH do not cross-react with the *B. subtilis* enzyme. The specific SDH activity in membranes from *E. coli* 5K(pKIM2) or *E. coli* 5K(pKIM4) was not increased above the background (Table 3).

DISCUSSION

Previous biochemical and genetic studies of the *B. subtilis* SDH complex have indicated that *sdhA* is the structural gene for apocytochrome b_{558}, one of the three subunits of the complex (11). However, we have not been able to demonstrate the presence of an altered cyt*b* subunit in *sdhA* mutants, leaving open the possibility that *sdhA* contains information for some factor required for correct membrane insertion and orientation of the cytochrome or addition of the prosthetic group, protoheme IX.

In the present work we have used the plasmid insertion-excision technique (22) to clone DNA from the *B. subtilis* *sdh* region in *E. coli*. In the original method, insertion of a plasmid into a specific region was monitored by measuring the loss of gene function. Here we have used a variant of the technique based on selecting integrated pHV32 which has transformed a mutant bacterium to wild type (in this case Sdh negative to Sdh positive). This variation is not trivial, since it allows for isolation of plasmids inserted close to any gene, irrespective of the presence or absence of adjacent genes with an easily selected phenotype. Also, the maximum distance between the inserted plasmid and the gene of interest is given by the size of the homologous bacterial DNA fragment in the plasmid used for integration.

Two plasmids regenerated in *E. coli* from *Eco*RI (pKIM4)- or *Bgl*II (pKIM2)-cleaved DNA from an Sdh-positive pHV32 transformant have been studied. DNA from both plasmids transforms all tested *sdhA* mutants to wild type. The transforming activity resides on a 2.1-kb *Bam*HI-*Eco*RI fragment. *E. coli* 5K strains carrying pKIM2 or pKIM4 form reddish colonies. Membranes from these strains contain substantial amounts of a cytochrome *b* indistinguishable from *B. subtilis* cyt*b*.

The results obtained show unequivocally that *sdhA* is carried on the 2.1-kb *Bam*HI-*Eco*RI fragment and that it is the structural gene for *B. subtilis* apocytochrome b_{558}. To the best of our knowledge, this is the first report of cloning and expression of a bacterial *b*-type cytochrome.

We calculate that in, e.g., *E. coli* 5K(pKIM2), at least every 10th cytoplasmic membrane protein is *B. subtilis* cyt*b*. *E. coli* strains can thus respond to an abnormal increase in a (most probably) nonfunctional apocytochrome by increased heme synthesis. This probably occurs by apocytochrome draining a small heme pool with subsequent activation of 5-aminolevulinic acid synthetase, which is known to be repressed by heme (6). Incorporation of heme in apocytochrome b_{558} does not seem to require any host-specific factor.

The sum molecular mass of the three subunits of the *B. subtilis* SDH complex is about 110 kilodaltons (kD), requiring a coding capacity of about 3 kb. The whole of *sdhA* is carried on a 2.1-kb *Bam*HI-*Eco*RI fragment common to both pKIM2 and pKIM4. On this fragment, about 900 base pairs from the *Eco*RI site, is a *Pst*I site. Transformation data show that this site is within the *sdhA* gene downstream of *sdhA78*. The reading frame for a 19-kD protein is about 500 base pairs, which makes it likely that part of *sdhB*, coding for the amino-terminal part of Fp, is also located on the *Pst*I-*Eco*RI fragment. This fragment also has transforming activity for the *sdhB101* marker. Compared with pKIM4, pKIM2 carries an additional 2.3 kb of *B. subtilis* DNA which contains at least part of *sdhB* and *sdhC*. This represents coding capacity for a protein of about 90 kD, slightly smaller than the sum of the molecular mass of Fp and Ip (93 kD). *E. coli* 5K(pKIM2) also produces antigen which reacts with Fp- and Ip-specific antibodies, respectively. With the reasonable assumption that the coding regions for the subunits of the *B. subtilis* SDH complex are not separated by extensive nontranslated regions, the whole of the *B. subtilis* *sdh* region can be accommodated within the *B. subtilis* DNA cloned in pKIM2. The nucleotide sequence of the *E. coli* *sdh* operon has been determined by Guest and co-workers (4, 28). Here the Fp and Ip coding regions are separated by 15 base pairs and are preceded by overlapping reading frames for two small, hydrophobic peptides thought to be important for anchoring SDH to the cytoplasmic membrane.

Membrane binding of all three subunits is required for SDH activity in *B. subtilis* strains (12). Only cyt*b* is membrane bound in *E. coli* 5K(pKIM2). Fp and Ip are exclusively located in the cytoplasm, and an active SDH complex could not be demonstrated. A trivial explanation for this may be that some change has occurred in *sdhB* or *sdhC* during the cloning procedure. More interesting is the possibility that addition of prosthetic groups and assembly and membrane binding of the *B. subtilis* SDH complex require host-specific factors not present in *E. coli*. We are presently examining these alternatives.

In *B. subtilis*, the *sdh* operon is thought to be transcribed from a promoter located upstream of *sdhA* (18); the present data are compatible with this model and the proposed order of transcription. The pHV32 plasmid from which pKIM2 and pKIM4 are derived was inserted into the *B. subtilis* chromosome via a *Bgl*II fragment inserted into the pBR322 *Bam*HI site, which was thus destroyed. The restriction maps of the plasmids (Fig. 1) show that the orientation of the *sdhA* gene (and *sdhB* and *sdhC*) is opposite to the direction of transcription of the pBR322 *tet* gene which harbors the *Bam*HI site. The promoters and transcripts of pBR322 have been extensively characterized (27). There is no transcript which is known to enter the *tet* gene through its terminal part, nor are any strong promoters known between *ori* and the end of the *tet* gene, which transcribe towards that gene. The *Bam*HI-*Eco*RI fragment which carries and expresses the *sdhA* gene is preceded by about 1.5 kb of *B. subtilis* DNA in pKIM2 and pKIM4. We have also cloned the *Bam*HI-*Eco*RI fragment in pUC8 and M13mp9 and found *sdhA* to be expressed in both constructions (unpublished results). Most likely, the *sdh* genes are transcribed from their natural promoter in pKIM2 and pKIM4. It is well established that promoters of *B. subtilis* origin are often efficiently utilized in *E. coli* (20).

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