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Reconstitution of Succinate Dehydrogenase in *Bacillus subtilis* by Protoplast Fusion

LARS HEDERSTEDT,* KERSTIN MAGNUSSON, AND LARS RUTBERG
Department of Bacteriology, Karolinska institutet, S-104 01 Stockholm, Sweden

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Bacillus subtilis succinate dehydrogenase (SDH) is composed of two unequal subunits designated Fp (M_r, 65,000) and Ip (M_r, 28,000). The enzyme is structurally and functionally complexed to cytochrome b_{558} (M_r, 19,000) in the membrane. A total of 21 *B. subtilis* SDH-negative mutants were isolated. The mutants fall into five phenotypic classes with respect to the presence and localization of the subunits of the SDH-cytochrome b_{558} complex. One class contains mutants with an inactive membrane-bound complex. Membrane-bound enzymatically active SDH could be reconstituted in fused protoplasts of selected pairs of SDH-negative mutants. Most likely reconstitution is due to the assembly of preformed subunits in the fused cells. On the basis of the reconstitution data, the mutants tested could be divided into three complementation groups. The combined data of the present and previous work indicate that the complementation groups correspond to the structural genes for the three subunits of the membrane-bound SDH-cytochrome b_{558} complex. A total of 31 SDH-negative mutants of *B. subtilis* have now been characterized. The respective mutations all map in the *citF* locus at 255° on the *B. subtilis* chromosomal map. In the present paper, we have revised the nomenclature for the genetics of SDH in *B. subtilis*. All mutations which give an SDH-negative phenotype will be called *sdh* followed by an isolation number. The designation *citF* will be omitted, and the *citF* locus will be divided into three genes: *sdhA*, *sdhB*, and *sdhC*. Mutations in *sdhA* affect cytochrome b_{558}, mutations in *sdhB* affect Fp, and mutations in *sdhC* affect Ip.

Succinate dehydrogenase [SDH; EC 1.3.99.1, succinate:(acceptor) oxidoreductase] is a membrane-bound enzyme found in aerobic cells. We have previously purified an SDH complex from *Bacillus subtilis* cytoplasmic membranes which contains equimolar amounts of three subunits, a flavoprotein (Fp—M_r, 65,000), a M_r-28,000 polypeptide tentatively called iron-protein (Ip), and cytochrome b_{558} (M_r, 19,000) (9, 10). Fp and Ip constitute SDH proper, whereas cytochrome b_{558} serves as a specific membrane-binding site for the enzyme. The cytochrome is also reduced by succinate (9).

The biosynthesis and membrane-binding of SDH in *B. subtilis* have been investigated in a heme mutant (11, 14). Fp and Ip are synthesized in the cytoplasm as soluble enzymatically inactive precursors, which later bind to cytochrome b_{558} in the membrane to form the SDH complex. The assembly of the enzymatically active SDH complex from cytoplasmic Fp and Ip subunits does not require protein synthesis (11).

B. subtilis strains which lack SDH enzyme activity carry mutations in the *citF* locus. The designation *cit* was originally used for mutants defective in the citric acid cycle (19, 21). In a previous study of nine SDH-negative mutants, all were found either to lack at least one of the subunits of the SDH-cytochrome b_{558} complex or to be unable to bind SDH to the membrane (10). Mutants which lack the cytochrome b_{558} chromophore accumulate enzymatically inactive Fp and Ip in the cytoplasm. The above nine mutants do not represent all (theoretically) possible SDH-negative phenotypes. We also do not know whether or not the subunits made by the mutants are functional.

In the present work, we isolated and characterized additional SDH-negative mutants of *B. subtilis*. The respective mutations all map in the *citF* locus as determined by transformation. Reconstitution of active SDH from individual subunits was obtained by polyethylene glycol (PEG)-induced fusion of protoplasts from selected pairs of mutants. From the results of these experiments, the SDH-negative mutants tested could be assigned to three complementation groups. Mutants which belong to one complementation group have their respective mutations clustered within the *citF* locus. The finding of three complementation groups within the *citF* locus makes it necessary to revise the terminolo-

gy for the genetics of SDH in *B. subtilis*. In the future, mutations which give an SDH-negative phenotype will be called *sdh* followed by an isolation number, and the designation *citF* will be replaced by *sdh* (4).

MATERIALS AND METHODS

Bacteria. The *B. subtilis* strains used are listed in Table 1.

Media and growth of bacteria. The bacteria were kept on tryptic blood agar base plates (Difco Laboratories, Detroit, Mich.). The SDH-negative phenotype was checked by streaking on purification medium (PA; 1). Minimal medium was prepared as described by Spizizen (25) with the addition of 10 μM $MnCl_2$. Required growth factors were added at 20 mg/liter. Min-CH is minimal medium with 5 g of casein hydrolysate (Difco) per liter. Nutrient sporulation medium (NSMP) was prepared as described by Fortnagel and Freese (7). Membrane and cytoplasmic fractions for immunological analysis were prepared from bacteria grown in Min-CH. Cytochrome spectra were determined on membranes from bacteria grown in NSMP as described previously (11). For the large-scale preparation of membranes for gel filtration, bacteria were grown in Min-CH in a 10-liter fermentor as recently described (9). The radioactive labeling of bacteria with L-^{14}C-amino acid mixture was done as described previously (11).

Preparation of membrane and cytoplasm for immunochemical analysis. Cells from a 100-ml culture at an absorbancy at 600 nm (A_{600}) of 0.7 to 0.9 were suspended in 0.4 ml of 0.1 M sodium phosphate buffer (pH 8.0)–0.5 mM phenylmethylsulfonyl fluoride–0.7 mg of lysozyme per ml–25 μg of DNase and RNase per ml–12 mM $MgSO_4$. The cells were lysed by incubation at 37°C for 1 h. The lysate was centrifuged at 48,000 × *g* for 30 min at 4°C. The supernatant was transferred to a new centrifuge tube and centrifuged at 48,000 × *g* for 1 h at 4°C. The top two-thirds of the supernatant, which contained cytoplasm, was withdrawn and stored at −80°C. The pellet obtained after centrifugation of the lysate, which contained membranes, was washed twice in 0.1 M sodium phosphate buffer (pH 8.0) and then homogenized in 0.3 ml of the same buffer and stored at −80°C.

Isolation of SDH-negative mutants. Mutagenesis was performed essentially as described by Ito and Spizizen (17). Strain 168 was grown in NSMP at 30°C with shaking for 48 h. The resulting spores were washed twice with 0.5 M potassium phosphate buffer (pH 7.0). The spores were activated by incubating about 10^8 spores per ml at 70°C for 15 min. Ethyl methane sulfonate (EMS; Sigma Chemical Co., St. Louis, Mo.) was then added to a final concentration of 0.45 M, and the suspension was incubated with shaking at 30°C for 15 h. The treated spores were washed five times with 0.05 M sodium-potassium phosphate buffer (pH 7.0), and dilutions were plated on PA plates. Yellow colonies (indicating acid excretion) were restreaked on PA plates. SDH-negative mutants were preliminarily identified by SDH zymogram staining of lysed bacterial colonies by a replica technique (5).

Extracts, membrane and cytoplasm, were prepared from the mutants and assayed for SDH activity. The mutants were subsequently made isogenic by transforming KA20 with limiting amounts of mutant DNA to isoleucine-valine prototrophy. Among these prototrophs, SDH-negative mutants were identified by streaking on PA plates.

Transformation. Extraction of DNA, determination of DNA concentration, and two- and three-factor transformation crosses with SDH-negative mutants were made as described by Ohné et al. (19).

Gel filtration of Triton X-100–solubilized membranes. Gel exclusion chromatography on an Ultrogel AcA 34 (LKB, Sweden) column (1.6 by 88 cm) in 0.1% (vol/vol) Triton X-100–50 mM Tris-acetate buffer (pH 7.0) was performed at 4°C, essentially as described by Weiss and Kolb (27). Three milliliters of solubilized membrane in 6.7% (vol/vol) Triton X-100–50 mM Tris-acetate (pH 7.0) was loaded on the column. The flow rate was 7.5 ml/h, and 2.5-ml fractions were collected. The void volume (72.5 ml) and the internal volume (192 ml) were determined with dextran blue (Pharmacia Fine Chemicals, Uppsala, Sweden) and riboflavin, respectively. The apparent Stokes radius (R_s) of the eluted proteins was calculated after the column had been calibrated with the following soluble proteins which do not bind Triton X-100: β-galactosidase (*Escherichia coli*, Sigma)—R_s, 6.8 nm; catalase—R_s, 5.2 nm; hemoglobin (beef)—R_s, 3.1 nm; and ovalbumin—R_s, 2.7 nm. The elution of cytochromes from the column was followed by measuring the absorption at 414 nm.

Protoplast fusion. The procedure for PEG-promoted fusion of protoplasts was based on the method of Schaeffer et al. (23). SDH-negative mutants were grown in hypertonic medium, NSMP with 0.5 M sucrose, which has been found to give more stable protoplasts (24). An overnight culture grown at 32°C in hypertonic medium was used to inoculate hypertonic medium (2× 300 ml) in 1,600-ml indentated Fernbach flasks to an A_{600} of 0.2. The bacteria were grown at 32°C in a New Brunswick G 25 incubator at 200 rotations per min. At an A_{600} of 0.7 to 0.8, the bacteria were harvested by centrifugation at 6,000 × *g* for 10 min at 15°C and washed once with 20 mM $MgCl_2$–0.5 M sucrose–20 mM maleate buffer (pH 6.5) (SMM). Protoplasts were prepared by suspending the bacteria in 12.5 ml of SMM containing 0.13 mg of lysozyme (3× crystallized, Sigma) per ml and 1% (wt/vol) bovine serum albumin (BSA) followed by incubation at 42°C with gentle agitation for 45 min. Less than 0.1% osmotically stable colony-forming units remained after this treatment. A 5-ml protoplast suspension of each mutant was mixed and centrifuged at 4,000 × *g* for 15 min at room temperature. To the protoplast pellet was added 0.65 ml of 1% (wt/vol) BSA in SMM. A 1-ml amount of the suspended protoplast mixture was then mixed with 5 ml of cold 50% (wt/vol) PEG (molecular weight, 6,000; Merck Sharp & Dohme, West Point, Pa.) in SMM. After 1.5 min of incubation at 0°C, 54 ml of 1% (wt/vol) BSA in 10 times-diluted Luria broth without glucose in SMM was added. The suspension was then incubated at 37°C for 1 h with gentle agitation, during which time the protoplast fusion is completed (8). Controls, i.e., each mutant fused with itself, were performed at the same protoplast density and under the same conditions as the mixed fusions. The fused protoplasts were centrifuged at 7,000 × *g* for 10 min at 15°C. The protoplasts were osmotically disrupted by suspending the pellet in 20 ml of 10 mM $MgSO_4$–

TABLE 1. Bacteria

Strain	Genotype	Former designation of *sdh* mutation	Source or reference
168	*trpC2*		J.A. Hoch
BR102	*trpC2 hisB*		J. Spizizen
KA20	*trpC2 leu-2 ilvC1*		this laboratory
KA98002	*sdh-2 trpC2*	*citF2*	Ohné et al. (19)
KA97002	*sdh-2 trpC2 leu-2*	*citF2*	Ohné et al. (19)
KA95002	*sdh-2 trpC2 ilvC1*	*citF2*	this work
KA98008	*sdh-8 trpC2*	*citF8*	Ohné et al. (19)
KA97008	*sdh-8 trpC2 leu-2*	*citF8*	Ohné et al. (19)
KA98011	*sdh-11 trpC2*	*citF11*	Ohné et al. (19)
KA97011	*sdh-11 trpC2 leu-2*	*citF11*	Ohné et al. (19)
KA98012	*sdh-12 trpC2*	*citF12*	Ohné et al. (19)
KA97012	*sdh-12 trpC2 leu-2*	*citF12*	Ohné et al. (19)
KA98042	*sdh-42 trpC2*	*citF42*	Ohné et al. (19)
KA97042	*sdh-42 trpC2 leu-2*	*citF42*	Ohné et al. (19)
KA98044	*sdh-44 trpC2*	*citF44*	Ohné et al. (19)
KA97044	*sdh-44 trpC2 leu-2*	*citF44*	Ohné et al. (19)
KA98069	*sdh-69 trpC2*	*citF69*	Ohné et al. (19)
KA97069	*sdh-69 trpC2 leu-2*	*citF69*	Ohné et al. (19)
KA95069	*sdh-69 trpC2 ilvC1*	*citF69*	this work
KA98078	*sdh-78 trpC2*	*citF78*	Ohné et al. (19)
KA97078	*sdh-78 trpC2 leu-2*	*citF78*	Ohné et al. (19)
KA95078	*sdh-78 trpC2 ilvC1*	*citF78*	this work
KA98083	*sdh-83 trpC2*	*citF83*	Ohné et al. (19)
KA97083	*sdh-83 trpC2 leu-2*	*citF83*	Ohné et al. (19)
KA95083	*sdh-83 trpC2 ilvC1*	*citF83*	this work
KA98101	*sdh-101 trpC2*		this work
KA97101	*sdh-101 trpC2 leu-2*		this work
KA98103	*sdh-103 trpC2*		this work
KA97103	*sdh-103 trpC2 leu-2*		this work
KA97107	*sdh-107 trpC2 leu-2*		this work
KA94109	*sdh-109 ilvB2*	*citF109*	Fortnagel & Freese (7)
KA97110	*sdh-110 trpC2 leu-2*		this work
KA97111	*sdh-111 trpC2 leu-2*		this work
KA97113	*sdh-113 trpC2 leu-2*		this work
KA98116	*sdh-116 trpC2*		this work
KA97116	*sdh-116 trpC2 leu-2*		this work
KA95116	*sdh-116 trpC2 ilvC1*		this work
KA97118	*sdh-118 trpC2 leu-2*		this work
KA97119	*sdh-119 trpC2 leu-2*		this work
KA97121	*sdh-121 trpC2 leu-2*		this work
KA97122	*sdh-122 trpC2 leu-2*		this work
KA97123	*sdh-123 trpC2 leu-2*		this work
KA98124	*sdh-124 trpC2*		this work
KA97124	*sdh-124 trpC2 leu-2*		this work
KA97129	*sdh-129 trpC2 leu-2*		this work
KA97134	*sdh-134 trpC2 leu-2*		this work
KA97141	*sdh-141 trpC2 leu-2*		this work
KA97144	*sdh-144 trpC2 leu-2*		this work
KA97147	*sdh-147 trpC2 leu-2*		this work
KA97153	*sdh-153 trpC2 leu-2*		this work
KA97161	*sdh-161 trpC2 leu-2*		this work
KA97162	*sdh-162 trpC2 leu-2*		this work

50 mM potassium phosphate buffer (pH 8.0)–10 μg of DNase per ml–10 μg of RNase per ml. The lysed protoplasts were incubated at 37°C for 15 min, and 0.4 M sodium EDTA (pH 8.0) was added to a final concentration of 15 mM. Two minutes later, 1M $MgSO_4$ was added to a final concentration of 40 mM. Whole cells were removed by centrifugation at 5,000 × *g* for 15 min at 4°C. Membranes were then isolated by

centrifugation at 48,200 × *g* for 30 min at 4°C. The pellets were washed twice with 24 mM sodium diethylbarbiturate buffer (pH 8.6) and finally suspended in 0.15 ml of the same buffer. The membrane preparations, which contained about 10 mg of protein per ml, were stored at −20°C until the next day, when their SDH activity was determined as described below.

Determination of protoplast lysis. The amount of lysis in the protoplast fusion experiments was calculated by comparing the malate dehydrogenase (MDH) (EC 1.1.1.37) activity in supernatants from protoplasts centrifuged at 7,000 × *g* for 10 min at 15°C with the activity in the supernatant from similarly centrifuged osmotically lysed protoplasts. The latter sample was taken to represent 100% lysis. MDH was determined as the oxaloacetate-dependent oxidation of NADH (28). Both types of supernatants were centrifuged at 48,000 × *g* for 30 min at 4°C before MDH activity was measured in the supernatant. This centrifugation step removed membrane fragments containing NADH-oxidase activity, which disturbs the MDH assay.

Other methods. The following methods used in this work have recently been described in detail: determination of protein concentrations, determination of SDH enzyme activity, autoradiography, Triton X-100 solubilization of membranes, immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10), SDH zymogram staining of immunoprecipitates (20), rocket immunoelectrophoresis (14), preparation of SDH-specific antisera, SDS-PAGE-crossed immunoelectrophoresis (SDS-PAGE-CIE), preparation of membranes for immunoprecipitation and spectroscopy, determination of radioactivity in polypeptides from SDS-PAGE gels by scintillation counting (11), and difference absorption spectroscopy of membranes (9).

RESULTS

Isolation and characterization of SDH-negative mutants. About 170 acid-excreting mutants were isolated from EMS-treated spores plated on PA plates. These mutants were screened for SDH activity by the zymogram technique of Edwards et al. (5); 26 mutants lacked SDH activity. The SDH-negative phenotype was confirmed by measuring enzyme activity in extracts prepared from each mutant. Five of the mutants reverted to the wild type at a high frequency and were discarded.

Membranes and soluble fractions were prepared from each of the above 21 mutants. The contents of Fp and Ip subunits in each fraction were analyzed by rocket immunoelectrophoresis, using subunit-specific antibodies. The apparent molecular weight of the antigens detected was determined by SDS-PAGE-CIE as recently described (11). The cytochrome b_{558} contents in the membranes were determined by difference absorption spectroscopy. Based on the results of these experiments, the mutants could be divided into five classes (Table 2). Mutants belonging to classes 2, 3, 4, or 5 represent SDH-negative phenotypes which have been found previously. In class 3 mutants, which lack the M_r-65,000 Fp antigen, the Ip antigen was generally found to be fragmented. An intact M_r-28,000 Ip antigen could, however, be detected in protoplasts disrupted by boiling in the presence of SDS, which minimizes proteolysis (manuscript in preparation).

Characterization of membrane-bound inactive SDH complex. Class 1 mutants, which contain all three subunits of the SDH complex in the membrane, represent a phenotype not previously found. The structure and stoichiometry of Fp, Ip, and cytochrome b_{558} in the membrane were

TABLE 2. Presence and localization of the subunits of the SDH-cytochrome b_{558} complex in *B. subtilis* SDH-negative mutants

Class	*sdh-* mutation[a]	Relevant phenotype		
		M_r 65,000 Fp-antigen	M_r 28,000 Ip-antigen	cytochrome b_{558} chromophore
1	*101, 124, 129, 141, 144*	membrane	membrane	membrane
2	*103, 118, 123, (83)*	cytoplasm	—[b]	membrane
3	*107, 110, 111, 113, 119, 121, 122, 147, 153, 161, 162, (2, 8, 11, 69)*	—	cytoplasm or —	membrane
4	*116,*[c] *134, (12)*	cytoplasm	cytoplasm	membrane
5	*109, (42, 44, 78)*	cytoplasm	cytoplasm	—

[a] Bacteria carrying mutations given within parentheses have been described previously (11, 19).

[b] —, No antigen could be detected in either fraction after the cells had been hypotonically lysed at 37°C for 1 h (see the text).

[c] Membranes from bacteria containing the *sdh-116* mutation have a decreased content of cytochrome b_{558}.

studied in strain KA97124 (which carries the *sdh-124* mutation). Membranes from KA97124 contained wild-type amounts of dithionite-reducible cytochrome with an absorption maximum at 560 nm. In contrast, the amount of Fp subunit was only 25 to 50% of that found in the wild type as determined by rocket immunoelectrophoresis of Triton X-100–solubilized membranes. The reduced amount of Fp is not a result of inefficient solubilization with Triton X-100 as the same results were obtained in SDS-PAGE-CIE of whole membranes.

SDH is solubilized with Triton X-100 from wild-type membranes as an SDH-cytochrome b_{558} complex (9, 10). This complex was monodisperse, and all cytochrome b_{558} was associated with the complex as determined by gel filtration in the presence of detergent (Fig. 1A; L. Hederstedt, thesis, Karolinska institutet, Stockholm, Sweden, 1981), i.e., cytochrome b_{558}, SDH activity, and the Fp subunit eluted together as a single peak from the column. In the mutant KA97124, as in the wild type, all of cytochromes *b* and *c* were solubilized with Triton X-100. The elution pattern of solubilized membranes from this mutant is shown in Fig. 1B. Some cytochrome b_{558} eluted with the Fp antigen at the position of the wild-type SDH complex (R_s of 7.0 nm), but most of the cytochrome *b* eluted at an R_s of 6.3 nm. The elution pattern of cytochrome *b* and *c* was verified by difference absorption spectroscopy of the column fractions. The cytoplasmic membrane of the class 1 mutant KA97124 thus contains an SDH-cytochrome b_{558} complex of wild-type size, but in contrast to the wild type, it contains an excess of cytochrome b_{558} relative to SDH.

Triton X-100–solubilized membranes of a class 3 mutant, KA98011 (*sdh-11*), were also analyzed by gel filtration. All of the solubilized cytochrome b_{558} of this mutant eluted at the same position as the excess cytochrome b_{558} of KA97124 (Fig. 1C).

In the mutant KA97124, the molar ratio of Fp/Ip/cytochrome b_{558} polypeptides in the solubilized complex was 0.8:1:1 as determined by SDS-PAGE of the immunoprecipitated, radioactively labeled SDH complex. The apparent molecular weights of the subunits were identical to those of the wild-type subunits.

Genetic mapping of isolated mutations. All previously isolated *B. subtilis* mutants which lack in vitro SDH enzyme activity carry mutations in the *sdh* region, formerly designated *citF* (see above), at 255° on the chromosomal map (13, 19). The respective mutations in the 21 newly isolated mutants were all linked to *ilvC1* by transformation (data not shown), and they were thus assumed to be located in the *sdh* region of the chromosome. Most *B. subtilis*

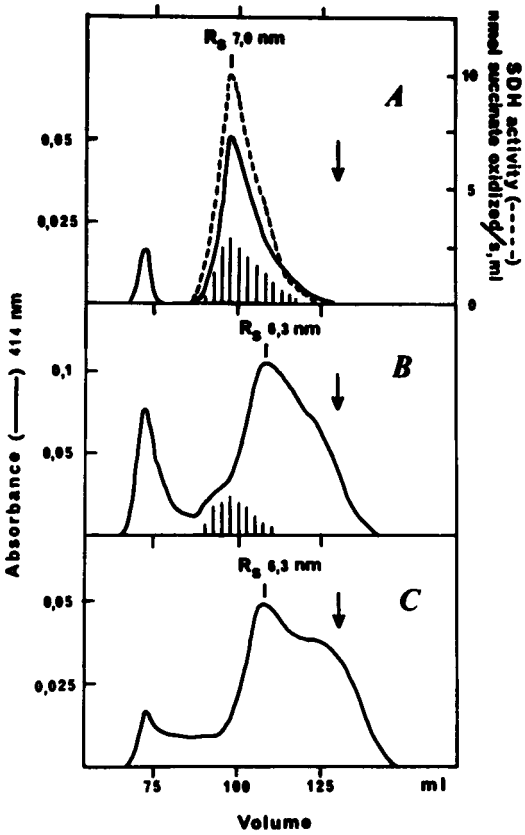

FIG. 1. Gel filtration of Triton X-100–solubilized membranes in the presence of 0.1% (vol/vol) Triton X-100 in all buffers. (A) BR102 (wild type), 9.3 mg of protein loaded onto the column. (B) KA97124 (*sdh-124*), 15.9 mg of protein loaded onto the column. (C) KA98011 (*sdh-11*), 9.6 mg of protein loaded onto the column. The vertical bars in (A) and (B) represent the relative amount of Fp subunit in the eluate determined by rocket immunoelectrophoresis (14). The arrows indicate the position at which excess Triton X-100 micelles, loaded onto the column together with the membrane proteins, are eluted. The A_{414} in the eluate at 95 and 110 ml in (A) and (B) and at 110 ml in (C) is mainly due to cytochrome b_{558}, whereas the absorbance at about 125 ml is mainly due to cytochromes c_{550} and c_{554}, as determined by difference absorption spectroscopy. The cytochrome *c* content in wild-type membranes from exponentially growing cells is low compared with that in SDH-negative mutants (9, 11), as also can be seen in the A_{414} profiles.

strains containing *sdh* mutations are poorly competent for transformation compared with bacteria containing regular auxotrophic markers (19). This fact, together with the lack of a system for the direct selection of SDH-positive transformants, makes genetic fine-mapping of *sdh* mutations by transformation extremely laborious. The theoretical considerations for three-factor

transformation crosses for mapping *sdh* mutations have been discussed before (19).

The relative position of three *sdh* mutations were determined. Two mutations, *sdh-101*, which gives the class 1 phenotype, and *sdh-103*, which results in the relatively rare class 2 phenotype, were mapped relative to the nine previously mapped *sdh* mutations in reciprocal three-factor transformation crosses with the linked *leu-2* mutation (Table 3). Mutation *sdh-116*, which gives a class 4 phenotype, was mapped relative to the *ilvC1* mutation and to four previously mapped *sdh* mutations (Table 4).

Reconstitution of SDH by protoplast fusion. When protoplasts from different strains are fused, both the cytoplasm and the cytoplasmic membrane become mosaic. Protoplast fusion (6, 15) was used to determine whether active SDH could be reconstituted from subunits made by different SDH-negative mutants. Selected pairs of SDH-negative mutants were protoplasted and fused as described above. The degree of lysis was monitored by measuring the leakage of the cytoplasmic enzyme MDH. Less than 20% lysis in the protoplasts of the mutants did not affect the qualitative results of the reconstitution.

The reconstitution of an active, membrane-bound, SDH complex was measured in two ways: (i) qualitatively by rocket immunoelectrophoresis of Triton X-100–solubilized membranes against Fp-specific antibodies followed by zymogram staining for SDH activity and (ii) quantitatively by measuring SDH enzyme activity in Triton X-100–solubilized membranes. As an example of a positive reconstitution, the results from a protoplast fusion experiment with KA98012 (*sdh-12*) and KA97083 (*sdh-83*) are shown in Fig. 2 and Table 5. No reconstitution of SDH activity was detected when PEG was omitted from the fusion protocol. Preliminary experiments indicated that the SDH-negative mutants fall into three groups with respect to the reconstitution of active SDH in fused protoplasts. Eleven well-characterized SDH-negative mutants were then fused with one mutant from each of the above three groups. The results of these experiments are summarized in Table 6.

All mutants that contain soluble Fp or Ip polypeptides or both gave reconstitution of active SDH when fused with two of the three mutants used as probes. In contrast, only one of the probe mutants gave an active SDH when fused with either KA97101 or KA97124, which both contain an inactive membrane-bound SDH-cytochrome b_{558} complex.

There was very little net protein synthesis in *B. subtilis* protoplasts incubated under our fusion conditions as measured by the incorporation of ^{3}H-labeled amino acids into acid-insoluble material (data not shown). Furthermore, active SDH was reconstituted also when the fusion was done in the presence of 200 μg of

TABLE 3. Three-factor transformation crosses

Donor *sdh-*	Recipient *sdh-*	Sdh$^+$/Leu$^+$ (%)[a]	Order implied
101	*78*	.6	*78—101—leu-2*
78	*101*	1.9	
101	*42*	.4	*42—101—leu-2*
42	*101*	1.8	
101	*44*	.5	*44—101—leu-2*
44	*101*	1.2	
101	*69*	.2	*69—101—leu-2*
69	*101*	.9	
101	*12*	1.9	*101— 12—leu-2*
12	*101*	.2	
101	*8*	.8	*101— 8—leu-2*
8	*101*	.2	
101	*11*	1.2	*11—101—leu-2*
11	*101*	1.6	
101	*2*	.5	*2—101—leu-2*
2	*101*	1.8	
101	*83*	1.4	*83—101—leu-2*
83	*101*	2.4	
103	*78*	1.9	*78—103—leu-2*
78	*103*	4.2	
103	*42*	2.6	*42—103—leu-2*
42	*103*	5.4	
103	*44*	2.7	*44—103—leu-2*
44	*103*	5.8	
103	*69*	«.1	*69—103—leu-2*
69	*103*	2.4	
103	*12*	2.2	*12—103—leu-2*
12	*103*	4.6	
103	*8*	2.8	*8—103—leu-2*
8	*103*	3.0	
103	*11*	.5	*11—103—leu-2*
11	*103*	3.1	
103	*2*	.6	*2—103—leu-2*
2	*103*	3.0	
103	*83*	.4	*103— 83—leu-2*
83	*103*	‹.1	

[a] Each value represents the average of four independent crosses. In each experiment at least 200 transformants were tested.

TABLE 4. Three-factor transformation crosses

Donor *sdh-*	Recipient *sdh-*	Sdh$^+$/Ilv$^+$ %	Order implied
116	*78*	not done	*78—116—ilvC1*
78	*116*	8.9	
116	*69*	4.9	*116— 69—ilvC1*
69	*116*	‹.3	
116	*2*	2.2	*116— 2—ilvC1*
2	*116*	.5	
116	*83*	4.1	*116— 83—ilvC1*
83	*116*	.1	

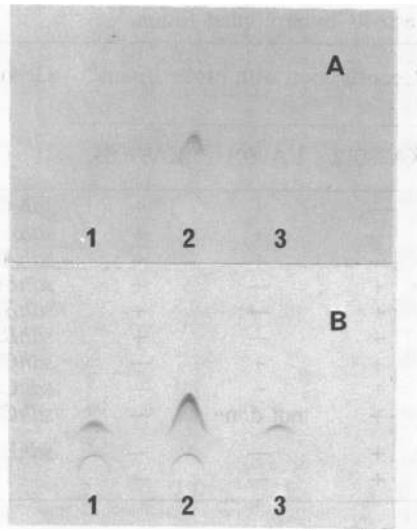

FIG. 2. Rocket immunoelectrophoresis of solubilized membranes from fused protoplasts run against Fp-specific antibodies. (A) Plate stained for SDH activity. (B) Plate stained for protein. Well 1, 146 μg of membrane protein from KA98012 (*sdh-12*) fused protoplasts. Well 2, 232 μg of membrane protein from KA98012 protoplasts fused with KA97083 protoplasts. (Each mutant contributes about equal amounts of membrane protein.) Well 3, 156 μg of membrane protein from KA97083 (*sdh-83*) fused protoplasts. Small protein-staining immunoprecipitates are seen both with solubilized membranes from hybrid protoplasts and from protoplasts of each mutant. The latter immunoprecipitates originate from cytoplasmic Fp subunits that have been trapped in the membrane preparation during the lysis of protoplasts (20). The Fp subunit itself did not exhibit SDH activity.

chloramphenicol per ml. This is in agreement with our previous finding that reconstitution of active membrane-bound SDH from soluble Fp and Ip subunits, in heme-starved cells after the resumption of heme synthesis, is independent of protein synthesis (11). Some SDH-negative mutants could not be tested by protoplast fusion due to a relatively high background SDH activity when they were grown under the conditions used for the fusion experiment.

DISCUSSION

The reconstitution of SDH from separate subunits has, to our knowledge, not been reported. Several facts may account for this (12). (i) No mild method is known by which active enzyme can be separated into Fp and Ip subunits. (ii) The non-heme iron acid-labile sulfur centers of SDH, designated S-1, S-2, and S-3 (particularly center S-3, which is believed to be located in the Ip subunit), are extremely sensitive to oxygen. An intact S-3 center seems to be required for the membrane binding of SDH in beef heart mitochondria. In addition, these centers may play a role in the association of Fp and Ip. (iii) The soluble Ip subunit, at least in *B. subtilis*, is very sensitive to proteolysis. (iv) Possible requirements for factors needed for the assembly, but not for the function, of SDH are unknown.

B. subtilis heme-deficient (11, 14) and SDH-negative mutants can serve as a source for separate SDH subunits, thus circumventing the problem of separating the subunits. However, as soon as the mutant cells have disintegrated, the problems of oxygen inactivation of iron-sulfur centers and the instability of the Ip subunit come into play. Cell fusion provides a means of avoiding these problems. The complementation of prophage mutants (22) and sporulation-deficient mutants (2, 3) has been demonstrated in fused *B. subtilis* protoplasts. In these cases, complementation is measured as the end result of a chain of complicated events leading to phage production or the formation of heat-resistant endospores. The extent to which genetic recombination contributes to these results is uncertain (15, 16).

The reconstitution of active SDH in fused protoplasts derived from SDH-negative mutants that is reported here must be due to genetic recombination or complementation or both. For our discussion of the reconstitution data, it is not important to distinguish between genetic complementation and complementation between preformed subunits of the SDH complex. Genetic recombination in hybrid protoplasts followed by transcription and translation of recombinant gene(s) cannot be a major mechanism for the reconstitution of SDH in our experiments for the following reasons: the efficiency of reconstitution is high (10 to 15%; Table 5); furthermore, very little, if any, net protein synthesis is required for reconstitution. A direct test of the frequency of genetic recombination is hardly possible due to the poor regeneration capacity of protoplasts from SDH-negative mutants. We conclude that complementation between preformed subunits is the most likely mechanism

TABLE 5.
SDH activity in membranes from fused protoplasts

Membrane	SDH activity[a]
KA98012 (*sdh-12*)	<0.3
KA97083 (*sdh-83*)	<0.3
KA98012/KA97083	2.2[b]
BR102 (wild-type)	37.0

[a] Nanomoles of succinate oxidized per minute per milligram of protein.

[b] Each mutant contributes about equal amounts of membrane protein.

TABLE 6. Reconstitution of SDH enzyme activity by protoplast fusion

Test strain	*sdh* mutation	Relevant phenotype of test strain[a]		Reconstitution with probe strains[b]			Genotype[c]
		Membrane	Cytoplasm	KA98012	KA98011	KA97083	
KA94109	*sdh-109*		Fp, Ip	—	+	+	*sdhA109*
KA98012	*sdh-12*	cyt b_{558}	Fp, Ip	—	+	+	*sdhA12*
KA97116	*sdh-116*	cyt b_{558}	Fp, Ip	—	+	+	*sdhA116*
KA97069	*sdh-69*	cyt b_{558}	Ip	+	—	+	*sdhB69*
KA98011	*sdh-11*	cyt b_{558}	Ip	+	—	+	*sdhB11*
KA97002	*sdh-2*	cyt b_{558}	Ip	+	—	+	*sdhB2*
KA97103	*sdh-103*	cyt b_{558}	Fp	+	+	—	*sdhC103*
KA97083	*sdh-83*	cyt b_{558}	Fp	+	+	—	*sdhC83*
KA97118	*sdh-118*	cyt b_{558}	Fp	+	not done	—	*sdhC118*
KA97101	*sdh-101*	cyt b_{558}, Fp, Ip		+	—	—	*sdhB101*
KA97124	*sdh-124*	cyt b_{558}, Fp, Ip		+	—	—	

[a] Data from Table 2.
[b] + = reconstitution, — = no reconstitution.
[c] Genotype derived from complementation and mutation mapping data.

for the reconstitution of active SDH in fused protoplasts.

From the data shown in Table 6, the SDH-negative mutants fall into three complementation groups, represented by the three probe mutants. Among these, KA97083 lacks the Ip subunit and has a soluble Fp subunit and a membrane-bound cytochrome b_{558}, KA98011 lacks Fp and has a soluble Ip subunit and a membrane-bound cytochrome b_{558}, and KA98012 contains all three subunits but is unable to bind Fp and Ip to the membrane. The last mutant can complement KA97083 and KA98011, but not KA94109, which lacks the membrane-bound cytochrome b_{558} chromophore. This fact strongly suggests that KA98012 has a defective cytochrome b_{558}. We can thus recognize two types of cytochrome b_{558} mutants. Both fail to membrane-bind SDH, and one also lacks the chromophore. In summary, each complementation group corresponds to a defect in one of the three subunits of the SDH-cytochrome b_{558} complex.

Mutations which give a subunit-specific defect are clustered (Fig. 3). This indicates that the three complementation groups correspond to three cistrons coding for the Fp, Ip, and cytochrome b_{558} subunits. The combined results from this and previously published work (10–12, 18–20) indicate that the *sdh* region of the chromosome contains at least three genes which we will call *sdhA*, *sdhB*, and *sdhC*. It is compatible with our data that these three genes represent the structural genes for the cytochrome b_{558}, Fp, and Ip polypeptides, respectively. We cannot yet exclude other possibilities, however, such as a processing enzyme required for the incorporation of covalently bound flavin or iron-sulfur centers. Our current concept of the organization of the *sdh* genes in *B. subtilis* is presented in Fig. 3.

Superficially, mutants KA97101 and KA97124 do not fit into any of the three complementation groups (Table 6). Both mutants have an inactive membrane-bound SDH-cytochrome b_{558} complex and no soluble Fp or Ip subunits. At least KA97124 has an excess of cytochrome b_{558} relative to Fp and Ip in the membrane. We have previously shown that there is little exchange between membrane-bound and soluble SDH subunits (11). Thus, KA97124 should only be able to complement a mutant with a defective cytochrome b_{558} and soluble functional Fp and Ip subunits. This is also the case. Both mutants probably have a defective Fp subunit, which is indicated by the position in the *sdh* loci of the

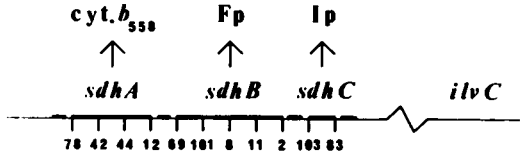

FIG. 3. Proposed genetic organization of the *sdh* genes in *B. subtilis*. The numbers refer to different mapped *sdh* mutations. The relative order of the *sdh* mutations is shown. The indicated position of mutation *sdhA12* is supported by reconstitution data and the phenotype of strains carrying the mutation, whereas genetic mapping data (19; Table 3) indicate a position between the *sdhB101* and *sdhB8* mutations. The *sdhA116* mutation, which is not included in the figure, maps between the *sdhA78* and *sdhB69* mutations (Table 4).

sdh-101 mutation of strain KA97101 and by the recent finding that the membrane-bound Fp subunit in strain KA97124 lacks covalently bound flavin (manuscript in preparation).

Triton X-100–solubilized *B. subtilis* SDH complex with a R_s of 7 nm (Fig. 1A) is very similar to Triton X-100–solubilized *Neurospora crassa* succinate-ubiquinone reductase (complex II; R_s of 6.7 nm) (27) and *Vibrio succinogenes* fumarate reductase (R_s of 6.25 nm) (26). *N. crassa* complex II contains three unequal polypeptides, M_r, 72,000, 28,000, and 14,000. The two largest polypeptides are the *N. crassa* SDH Fp and Ip subunits, whereas the 14,000-molecular-weight polypeptide is a cytochrome b_{559}. Detergent-solubilized *Vibrio succinogenes* fumarate reductase (the enzyme catalyzes the reverse reaction to that of SDH) contains three different subunits: M_r, 79,000 and 31,000, which are present in equimolar amounts, and a M_r-25,000 cytochrome *b* polypeptide present in twice the molar amount of the two larger subunits. Both the *N. crassa* complex II and the *V. succinogenes* fumarate reductase complex are monomeric in micellar concentrations of Triton X-100 and bind approximately one detergent micelle. Cytochrome *b* is the detergent-binding polypeptide in both complexes. By analogy, the *B. subtilis* SDH-cytochrome b_{558} complex seems to be monomeric in Triton X-100 and to bind detergent. The data presented in Fig. 1 and the finding that the Fp subunit does not bind detergent (L. Hederstedt, thesis) strongly indicate that the cytochrome b_{558} is the detergent-binding, hydrophobic residue also in the *B. subtilis* SDH complex.

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