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Characterization of a Pleiotropic Succinate Dehydrogenase-negative Mutant of *Bacillus subtilis*

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A succinate dehydrogenase-negative mutant of *Bacillus subtilis* is described which lacks all three subunits of the membrane-bound succinate dehydrogenase complex: flavoprotein, iron protein, and cytochrome b_{558}. The corresponding mutation is revertible and it maps at one extreme of the *sdh* region. The results presented suggest that the structural genes for the subunits of the succinate dehydrogenase complex are part of one operon.

INTRODUCTION

The membrane-bound *Bacillus subtilis* succinate dehydrogenase complex [EC 1.3.99.1, succinate: (acceptor) oxidoreductase] contains equimolar amounts of three subunits: a M_r 65000 flavoprotein (Fp), a M_r 28000 iron protein (Ip) and cytochrome b_{558} (Hederstedt *et al.*, 1979). We have isolated and characterized about 30 *B. subtilis* succinate dehydrogenase-negative mutants. All these mutants contain at least one of the subunits of the succinate dehydrogenase (SDH) complex, and the respective mutations all map in the *sdh* region (formerly called *citF*) (Hederstedt *et al.*, 1982). Mapping data from transformation crosses with SDH-negative mutants together with the phenotypes of the mutants, as well as results from reconstitution of active SDH by protoplast fusion of SDH-negative mutants, suggest that the *sdh* region contains the structural genes for the subunits of the SDH complex in the order *sdhA*(cytochrome b_{558})–*sdhB*(Fp)–*sdhC*(Ip)–*ilvC1*–*leu-2* (Ohné *et al.*, 1973; Hederstedt & Rutberg, 1981; Hederstedt *et al.*, 1982). In the present paper we describe a pleiotropic SDH-negative mutant of *B. subtilis* which lacks all subunits of the SDH complex. The properties of this mutant suggest that the structural genes for cytochrome b_{558}, Fp, and Ip constitute an *sdh* operon.

METHODS

Bacteria. The *Bacillus subtilis* strains used are listed in Table 1. Mutation *sdh-115* was isolated from *B. subtilis* 168 spores mutagenized with ethyl methanesulphonate as described by Ito & Spizizen (1971). Strain KA95115 was constructed by transforming KA20 with *sdh-115* DNA with primary selection for leucine prototrophy. Strain KA120 was constructed as follows. About 10^8 *B. subtilis* 168 were plated on minimal medium containing 100 μg *S*-(2-aminoethyl)cysteine (Aec) ml^{-1}. Ten independent Aec-resistant mutants were isolated. DNA was extracted from these mutants and used to transform BR95 to isoleucine–valine prototrophy at limiting DNA concentration. About 200 transformants from each cross were tested for Aec resistance. In one cross about 30% of the transformants were Aec-resistant, whereas no resistant transformants were found in the other nine crosses. The *aec* mutation linked to *ilvC1* was assumed to be located in the *aecA* locus (Mattioli *et al.*, 1979) and was designated *aecA5*. One Aec-resistant isoleucine–valine prototroph from the above cross was kept and designated KA120.

Media. The bacteria were kept on Tryptic Blood Agar Base plates (Difco). The SDH phenotype was checked on Purification agar (Carls & Hanson, 1971) which differentiates between acid-excreting (e.g. SDH mutants) and non-excreting (wild-type) strains of *B. subtilis*. Spizizen's minimal medium was used (Spizizen, 1958). Required

Abbreviations: Aec, *S*-(2-aminoethyl)cysteine; SDH, succinate dehydrogenase; Fp, flavoprotein; Ip, iron protein.

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Table 1. *Bacteria*

Strain	Genotype	Source or reference
168	*trpC2*	J. A. Hoch
BR95	*trpC2 ilvC1 pheA1*	J. Spizizen
BR102	*trpC2 hisB*	J. Spizizen
KA20	*trpC2 leu-2 ilvC1*	This laboratory
KA111	*ilvC1 pheA1 metB5*	This laboratory
KA120	*trpC2 pheA1 aecA5*	This laboratory
KA98011	*sdhB11 trpC2*	Ohné *et al.* (1973)
KA98012	*sdhA12 trpC2*	Ohné *et al.* (1973)
KA98069	*sdhB69 trpC2*	Ohné *et al.* (1973)
KA95069	*sdhB69 trpC2 ilvC1*	Hederstedt *et al.* (1982)
KA98078	*sdhA78 trpC2*	Ohné *et al.* (1973)
KA95078	*sdhA78 trpC2 ilvC1*	Hederstedt *et al.* (1982)
KA93078	*sdhA78 aecA5*	This work
KA98083	*sdhC83 trpC2*	Ohné *et al.* (1973)
KA97083	*sdhC83 trpC2 leu-2*	Ohné *et al.* (1973)
KA95083	*sdhC83 trpC2 ilvC1*	Hederstedt *et al.* (1982)
KA98115	*sdh-115 trpC2*	This work
KA95115	*sdh-115 trpC2 ilvC1*	This work
CU1886	*argA2 pheA2 att*SPβ *citF*::SPβc2	Mackey & Zahler (1982)
CU2058	(*metB5*) *dal-1*(SPβc2)(SPβc2 *int*5 d*sup*3-1)	Lipsky *et al.* (1981)
CU2059	(*metB5*) *dal-1*(SPβc2)(SPβc2 *int*5 d*sup*44-1)	Lipsky *et al.* (1981)

amino acids were added at 20 mg l^{-1}. SDH-positive revertants of KA95115 were isolated from cultures grown with shaking at 37 °C in CY medium (minimal medium with the following supplements per litre: 5 g casein hydrolysate, 2·5 g yeast extract, and 20 mg of any amino acid required). CYG is CY medium with 5 g glucose added per litre.

Transformation. Extraction of DNA, determination of DNA concentration, and two- and three-factor transformation crosses were made as described by Ohné *et al.* (1973). Competent cells were prepared as described by Arwert & Venema (1973). Ilv$^+$ transformants were selected on minimal plates without isoleucine–valine and with glucose (5 g l^{-1}) as carbon and energy source. Aec-resistant transformants were selected on minimal medium containing glucose, required amino acids and 100 µg Aec ml^{-1}. Selection for Sdh$^+$ transformants in the cross shown in Table 2 was on minimal medium with required amino acids but with citrate plus glutamate (5 g each l^{-1}) as carbon and energy source instead of glucose. All SDH mutants of *B. subtilis* fail to grow on citrate plus glutamate probably partly due to an inability to synthesize aspartate.

Other methods. The following methods have recently been described in detail. Preparation and characterization of anti-SDH antisera and quantitation of SDH protein by rocket immunoelectrophoresis (Holmgren *et al.*, 1979; Hederstedt & Rutberg, 1980; Owen, 1981), reconstitution of active SDH by protoplast fusion of SDH-negative mutants (Hederstedt *et al.*, 1982), spectroscopic determination of cytochrome b_{558} (Hederstedt, 1980). SDH enzyme activity was measured by a modification (Hederstedt *et al.*, 1979) of the method of Ells (1959). Protein was determined by the Lowry method with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Phenotype of KA95115

Strain KA95115 which carries the mutation *sdh-115* lacks *in vitro* SDH enzyme activity, excretes acid and cannot use glutamate plus citrate as carbon and energy source.

Three methods were used to detect the individual subunits of the membrane-bound SDH complex: spectroscopy (cytochrome b_{558}), subunit specific antibody (against Fp and Ip), and reconstitution of SDH by protoplast fusion of pairs of SDH-negative mutants (Hederstedt, 1980; Hederstedt & Rutberg, 1980; Hederstedt *et al.*, 1982). In KA95115 we could not detect any cytochrome b_{558} chromophore. The strain does not contain any Fp or Ip subunits, or fragments thereof, that could be recognized by our subunit-specific antibodies. Active SDH was reconstituted when protoplasts from strains carrying either mutations *sdhA12* (cytochrome b_{558} defective), *sdhB11* (Fp defective), or *sdhC83* (Ip defective) were fused with one another (Hederstedt *et al.*, 1982). No reconstitution of SDH was obtained when KA95115 was fused with

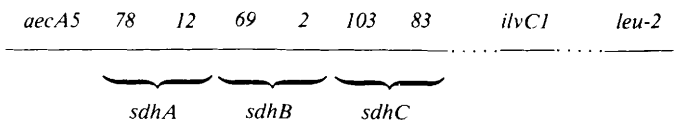

Fig. 1. Map of the *sdh* region in *Bacillus subtilis*.

Table 2. *Mapping of sdh-115 with three-factor transformation crosses*

At least 208 transformants were picked and tested for the non-selected marker.

Genotype		Selected	Recombinant		
Donor	Recipient	phenotype	class	%	Order implied
sdh-115	*sdhC83 ilvC1*	Ilv$^+$	Sdh$^+$ Ilv$^+$	9·4	*sdh-115 sdhC83 ilvC1*
sdhC83	*sdh-115 ilvC1*	Ilv$^+$	Sdh$^+$ Ilv$^+$	2·0	
sdh-115	*sdhB69 ilvC1*	Ilv$^+$	Sdh$^+$ Ilv$^+$	6·3	*sdh-115 sdhB69 ilvC1*
sdhB69	*sdh-115 ilvC1*	Ilv$^+$	Sdh$^+$ Ilv$^+$	2·7	
sdh-115	*sdhA78 ilvC1*	Ilv$^+$	Sdh$^+$ Ilv$^+$	2·9	*sdh-115 sdhA78 ilvC1*
sdhA78	*sdh-115 ilvC1*	Ilv$^+$	Sdh$^+$ Ilv$^+$	0·7	
*aecA5 sdhA78**	*sdh-115*	Sdh$^+$	Aec^R Sdh$^+$	91	*aecA5 sdh-115 sdhA78*
			Aec^S Sdh$^+$	9	
		Aec^R	Aec^R Sdh$^+$	53	
			Aec^R Sdh$^-$	47	

* This donor strain was isolated from the crosses shown in Table 3.

any of these strains. From these negative results we conclude that KA95115 does not contain any of the subunits of the SDH complex.

Mapping of sdh-115 by transformation

A number of *B. subtilis sdh* mutations have been mapped by three-factor transformation crosses with primary selection for the linked markers *ilvC1* or *leu-2* (Ohné *et al.*, 1973; Hederstedt *et al.*, 1982). An abbreviated map of the *sdh* region is shown in Fig. 1. In three-factor transformation crosses with primary selection for isoleucine–valine prototrophy *sdh-115* mapped outside *sdhA78* at one extreme of the *sdh* region (Table 2).

The *aecA* gene determines resistance to the lysine analogue Aec. Three-factor transformation crosses have established the order *aecA – ilvC – leu-8* (Mattioli *et al.*, 1979) and *aecA* has been placed between *sdh* (*citF*) and *ilvC* in the map of Henner & Hoch (1980). We have re-examined the position of *aecA* in three-factor transformation crosses with *ilvC* and *sdhA78*. The results of these crosses clearly give the order *aecA5 – sdhA78 – ilvC1* (Table 3) with about 70% co-transformation of *aecA5* and *sdhA78*.

To confirm the position of *sdh-115*, *aecA5* was used as an outside marker. In a two-factor cross with *aecA5* donor DNA and KA95115 as recipient, all Aec-resistant transformants tested (64/64) were SDH-positive. KA95115 was next transformed with *aecA5 sdhA78* donor DNA. SDH-positive or Aec-resistant transformants were selected and the distribution of the unselected marker was determined. The results of these crosses are compatible with the order *aecA5 – sdh-115 – sdhA78* (Table 2). For reasons not at present understood, we cannot transform a strain carrying *sdhA78* with *aecA5 sdh-115* donor DNA.

Mutation sdh-115 is not suppressed by suppressors sup-3 or sup-44

The pleiotropic nature of KA95115 may be due to a polar effect. Although the mechanism of extra-genic suppression is not well known in *B. subtilis* (Smith, 1982), we considered it of interest to determine if *sdh-115* was suppressed by either of the suppressors *sup-3* or *sup-44*. Mutation *sdh-115* was introduced into strain KA111 which carries the suppressor-sensitive mutation *metB5*. The bacteria were infected with high frequency transducing SPβ *sup-3* or *sup-44* lysates

Table 3. *Mapping of aecA5 with three-factor transformation crosses*

Donor	Recipient	Selected phenotype	Recombinant class	No. of colonies scored	Order implied
aecA5	*sdhA78 ilvC1*	Ilv$^+$	Aec^R Sdh$^+$	55	*aecA5 sdhA78 ilvC1*
			Aec^R Sdh$^-$	4	
			Aec^S Sdh$^+$	7	
			Aec^S Sdh$^-$	90	
		Aec^R	Ilv$^+$ Sdh$^+$	67	*aecA5 sdhA78 ilvC1*
			Ilv$^+$ Sdh$^-$	2	
			Ilv$^-$ Sdh$^+$	68	
			Ilv$^-$ Sdh$^-$	49	
aecA5 sdhA78	*ilvC1*	Ilv$^+$	Aec^R Sdh$^+$	7	*aecA5 sdhA78 ilvC1*
			Aec^R Sdh$^-$	31	
			Aec^S Sdh$^+$	96	
			Aec^S Sdh$^-$	23	
		Aec^R	Ilv$^+$ Sdh$^+$	5	*aecA5 sdhA78 ilvC1*
			Ilv$^+$ Sdh$^-$	52	
			Ilv$^-$ Sdh$^+$	58	
			Ilv$^-$ Sdh$^-$	41	

and methionine prototrophs were selected (Lipsky *et al.*, 1981). Five prototrophs were purified and the presence of the suppressor gene was verified by cross-streaking against phage SPO2 carrying the suppressor-sensitive mutation *susL244* (Yasunaka *et al.*, 1970). However, the suppressor-carrying strains were still SDH-negative.

Reversion of sdh-115

When KA95115 is grown in CY medium more rapidly growing wild-type revertants accumulate. Five independent revertants of KA95115 were isolated by plating KA95115 grown in CY medium on Purification agar. The amount of SDH enzyme activity in these revertants and in wild-type bacteria (strain BR102) grown in CY and CYG medium was measured. In all cases enzyme activity was proportional to the amount of enzyme protein measured by rocket immunoelectrophoresis of Triton X-100 solubilized SDH using anti-Fp antibody (Rutberg *et al.*, 1978; Hederstedt *et al.*, 1979; Owen, 1981). All revertants were sensitive to glucose repression of SDH although to various degrees.

Control of SDH

Synthesis of the three subunits of the SDH complex in *B. subtilis* must be carefully controlled and coordinated. Equimolar amounts of Fp, Ip, and cytochrome b_{558} are found in the complex and no, or very few, free subunits are present in wild-type cells (Hederstedt & Rutberg, 1980). SDH activity is repressed by glucose in *B. subtilis* (Hanson & Cox, 1967; Ohné, 1975) as it is in *Escherichia coli* (Ruiz-Herrera & Garcia, 1972; Takahashi, 1975). In *B. subtilis*, SDH enzyme activity and amount of enzyme protein show very good correlation. Furthermore, the synthesis of inactive subunits in SDH-negative *B. subtilis* mutants was repressed by glucose which shows that glucose affects the synthesis of enzyme protein (unpublished results). The extent of glucose repression can vary considerably for different genes: in *B. subtilis* grown in the presence of glycerol and glucose, glycerolphosphate dehydrogenase was repressed about 30-fold compared to its level in bacteria grown with only glycerol, whereas glycerol kinase was only repressed fivefold (Lindgren & Rutberg, 1974). The respective genes are closely linked, but they are probably in separate operons (Lindgren & Rutberg, 1976). Glucose represses all three subunits of the *B. subtilis* SDH complex equally. This would seem most easily accomplished if the respective genes were part of a single operon.

KA95115 lacks all subunits of the SDH complex. Mutation *sdh-115* maps outside all previously mapped mutations in *sdhA* the proposed structural gene for cytochrome b_{558}. There are two simple explanations for the pleiotropic effect of *sdh-115*. (i) It is a strong polar mutation in the *sdhA* gene or (ii) it is a mutation in a *sdh* promotor region. Although our present data do

not permit us to distinguish between these alternatives, they both require that *sdhA, sdhB* and *sdhC* form one operon which is transcribed in that order.

Mackey & Zahler (1982) have isolated an SDH-negative mutant of *B. subtilis* which has phage SPβ inserted into the *sdh* region. This mutant also lacks all subunits of the SDH complex (unpublished results). Although the exact point of insertion of the phage is not known this result supports the idea of an *sdh* operon.

Organization into operons of the genes coding for membrane-bound protein-complexes has been demonstrated for the *Escherichia coli* H^+-ATPase (*unc*) (Downie *et al.*, 1979; Gunsalus *et al.*, 1982) and the high affinity K^+ transport system (Rhoads *et al.*, 1978; Wieczorek & Altendorf, 1979). It may be worth noting that in both the proposed *B. subtilis sdh* operon and in the *E. coli unc* operon, the genes coding for the most hydrophobic subunits seem to be transcribed and translated first. This may be of importance, as recently suggested by Walker *et al.* (1982), for assembly of the complexes in the respective membranes.

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