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Identification of *Bacillus subtilis men* Mutants Which Lack *o*-Succinylbenzoyl-Coenzyme A Synthetase and Dihydroxynaphthoate Synthase

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Menaquinone (vitamin K_2)-deficient mutants of *Bacillus subtilis*, whose growth requirement is satisfied by 1,4-dihydroxy-2-naphthoic acid but not by *o*-succinylbenzoic acid (OSB), have been analyzed for enzymatic defects. Complementation analysis of cell-free extracts of the mutants revealed that there are two groups, as already indicated by genetic analysis. The missing enzyme in each group was identified by complementation of the cell-free extracts with *o*-succinylbenzoyl-coenzyme A (CoA) synthetase and dihydroxynaphthoate synthase extracted from *Mycobacterium phlei*. Mutants found to lack dihydroxynaphthoate synthase, and which therefore complement with dihydroxynaphthoate synthase of *M. phlei*, were designated as *menB*; those lacking *o*-succinylbenzoyl-CoA synthetase, and therefore complementing with *o*-succinylbenzoyl-CoA synthetase, were designated as *menE*. The *menB* mutants RB413 (*men-325*) and RB415 (*men-329*), when incubated with [2,3-$^{14}C_2$]OSB, produced only the spirodilactone form of OSB in a reaction that was CoA and adenosine 5'-triphosphate dependent.

An important step in the biosynthesis of menaquinones (vitamin K_2) is the enzymatic conversion of the benzenoid compound, *o*-succinylbenzoic acid [OSB; 4-(2'-carboxyphenyl)-4-oxobutyric acid], to the naphthalenoid compound, 1,4-dihydroxy-2-naphthoic acid (DHNA). In this step, the bicyclic ring system makes its first appearance. The conversion is coenzyme A (CoA) and ATP dependent, and *o*-succinylbenzoyl-CoA is believed to be an intermediate (2, 6, 7). It has been established that two enzymes are required, and these enzymes are named *o*-succinylbenzoyl-CoA synthetase (OSB-CoA synthetase) and 1,4-dihydroxy-2-naphthoate synthase (DHNA synthase) (7-9). Thus, the overall conversion OSB → DHNA is represented as the sum of reactions 1 and 2.

$$\text{OSB} + \text{CoA} + \text{ATP} \xrightarrow{\text{OSB-CoA synthetase}} \text{OSB-CoA} + \text{AMP} + \text{PP} \quad 1$$

$$\text{OSB-CoA} \xrightarrow{\text{DHNA synthase}} \text{DHNA} + \text{CoA} \quad 2$$

The putative CoA intermediate appears to be very unstable and has not been isolated. When OSB-CoA synthetase reacts with OSB, CoA, and ATP (in the absence of DHNA synthase), the formation of the spirodilactone form of OSB (OSB-spirodilactone) has been repeatedly observed (7, 8); this is believed to be derived from a nonenzymatic breakdown of the OSB-CoA intermediate:

$$\text{OSB} + \text{CoA} + \text{ATP} \xrightarrow{\text{OSB-CoA synthetase}} \text{OSB-CoA} + \text{AMP} + \text{PP}$$

$$\text{OSB-CoA} \xrightarrow{\text{(nonenzymatic)}} \text{OSB-spirodilactone} + \text{CoA}$$

To obtain further information on the biochemistry of the OSB → DHNA conversion, we have undertaken an analysis of the enzyme defects in certain mutants of *Bacillus subtilis*. The methods for the isolation of these *men* mutants and their genetic analysis have been described by Taber et al. (12). The mutants studied here behave as a single nutritional group in responding to DHNA (or the vitamin K analog, menadione) but not to OSB. However, other properties associated with these *men* mutations suggest that they comprise two independent groups, distinguished by genetic separability and their phenotypes (12; H. W. Taber, E. Dellers, and L. Lombardo, manuscript in preparation). One group is able to cross-feed *menC,D* mutants, and the other group is unable to do so. Furthermore, the growth of the latter group is stimulated by very low concentrations of aminoglycoside antibiotics in the absence of menadione, OSB, or DHNA. The detailed biochemical investigation reported here shows that the mutants also belong to two groups defined by reactions 1 and 2.

MATERIALS AND METHODS

Strains and media. The parent strain (*men*$^+$) of *B. subtilis*, the mutant strains, and their genotypes are described in Table 1. The culture media in this work were as follows: tryptose blood agar base (TBAB; Difco Laboratories); Penassay broth plus 0.5% glucose (PBG; Penassay broth obtained from Difco); and Penassay broth plus 0.5% glucose plus 18 μM menadione bisulfite (PBGM). For plates and slants, the PBG and PBGM media were supplemented with 1.5% agar (Difco).

Growth conditions. For maintenance, all strains were first grown on PBGM medium supplemented with 1.5% agar at 37°C for 12 to 18 h; the slants were then stored at 4°C. Liquid cultures were grown at 37°C, using a New Brunswick gyratory shaker at 200 to 250 rpm. The *men*$^+$ *B. subtilis* (strain RB356) was first grown overnight in 50 ml of PBG medium; this culture was in turn inoculated into 1 liter of the same medium, and the cells were harvested (see below) when the absorbance at 540 nm (A_{540}) was in the range of 0.8 to 1.0.

For growth of the mutant strains, cells from a slant culture were streaked onto PBGM plates. After 2 days of growth at 37°C a small inoculum (one loop) was used to inoculate 2-ml portions of PBGM medium in 15-ml screw-cap tubes. These tubes were shaken vigorously (in a rotary shaker at 37°C) until growth was initiated, usually in 2 to 5 h depending on the size of the inoculum. These cultures were used to inoculate 50-ml portions of PBGM medium contained in 500-ml flasks; growth proceeded at 37°C, with shaking, until the cultures had A_{540} values between 0.7 and 0.8. Finally, the 50-ml cultures were used to inoculate 1-liter portions of medium (contained in 2.8-liter Fernbach flasks). Growth was continued until the A_{540} of the culture was in the range of 0.9 to 1.2.

With both *men*$^+$ and mutant cultures, the cells were harvested by centrifugation in a Sorvall HG-4 rotor at 4,500 rpm and 4°C for 15 min. The cells were washed with a 0.5 volume of 0.02 M potasium phosphate buffer, pH 6.9, recentrifuged, and then stored frozen at −20°C.

Preparation of cell-free extracts. Approximately 1 g (wet weight) of cells was suspended in 2 ml of 0.02 M potassium phosphate buffer, pH 6.9, containing 5 mM mercaptoethanol; a 2-mg portion of lysozyme was then added, and the mixture was incubated at 37°C for 30 min. The mixture was then frozen and kept at −20°C overnight to lyse the cells completely. It was then warmed to 30°C, and 20 μg each of DNase and RNase was added. After incubating for 5 min at 30°C, the mixture was centrifuged for 15 min at 12,000 rpm in a Sorvall RC-5B centrifuge, using the SA600 rotor. The supernatant from this centrifugation was used in all experiments.

TABLE 1. *B. subtilis* strains

Strain no.	*men* allele	Genotype
RB356	*men*$^+$	*leuA8 aroG932 ald trpC2*
RB388	*310*	*leuA8 men-310 ald trpC2*
RB397	*312*	*leuA8 men-312 ald trpC2*
RB413	*325*	*leuA8 men-325 trpC2*
RB415	*329*	*leuA8 men-329 trpC2*

Enzymes and enzyme assay. Lysozyme (salt-free, 14,577 U/mg), DNase (1,200 U/mg), and RNase (2,800 U/mg) were obtained from Worthington Biochemicals Corp. DHNA synthase and OSB-CoA synthetase were prepared as previously described (7) from *Mycobacterium phlei* in 0.02 M potassium phosphate buffer, pH 6.9. In this process the OSB-CoA synthetase is present in the supernatant from a protamine sulfate precipitation. For the present work, this enzyme was further purified by the addition of 3 N HCl to give an acid concentration of 0.1 N. After standing for 5 min, the acid solution was dialyzed for 4 h against 0.02 M potassium phosphate buffer, pH 6.9, which contained 5 mM mercaptoethanol and 20% glycerol. The dialyzed mixture was centrifuged to remove an inactive protein (Sorvall RC-5B centrifuge, SA600 rotor, 10 min, 10,000 rpm, 4°C). The clear supernatant was treated with solid ammonium sulfate to a concentration of 75%, and the precipitate which formed was removed by centrifugation (10,000 rpm, 10 min). The precipitate was then dissolved in buffer similar in composition to the dialysis buffer. This solution was used as the OSB-CoA synthetase material. The treatment described resulted in a 27-fold purification, and the material had a specific activity of 47 nmol of DHNA/30 min per mg of protein when assayed in conjunction with excess DHNA synthase (7). In all cases, DHNA formation was measured spectrophotofluorometrically, as previously described (6, 7).

Chemicals. OSB, OSB-spirodilactone, and DHNA were prepared as previously described (2, 6). [2,3-$^{14}C_2$]OSB was prepared from phthalic anhydride and [2,3-$^{14}C_2$]succinic acid (50.9 mCi/mmol, Amersham Corp.). The synthesized [2,3-$^{14}C_2$]OSB had a specific activity of 2.3 mCi/mmol. All solvents were redistilled before use. Menadione bisulfite was obtained from Sigma Chemical Co.

General. Protein determinations were carried out by the method of Bradford (1), using reagents and protein standard supplied by Bio-Rad Laboratories. The spectrophotofluorometer was the Aminco-Bowman instrument (catalog no. 4-8202). Radioactivity on thin-layer chromatograms was detected with a Packard radiochromatogram scanner, model 7201.

RESULTS

When extracts from *men*$^+$ *B. subtilis* (RB356) were examined for DHNA formation under the conditions used with *M. phlei* (6), very little of this metabolite accumulated. The *B. subtilis* enzymes were found to have a different pH optimum from those of *M. phlei* (Fig. 1). There was very little activity at pH 6.0 and 6.5, a slight increase at pH 7, and a sharp rise beginning at pH 7.5 (with *M. phlei*, the formation of DHNA was maximal at pH 6.9). At pH 8.2, the activity was proportional to protein concentration over the range of 5 to 20 mg, and the reaction rate was linear for up to 50 min. DHNA formation was dependent on the presence of CoA and ATP (data not shown). For routine assay of the *B. subtilis* enzymes, we used a 30-min incubation

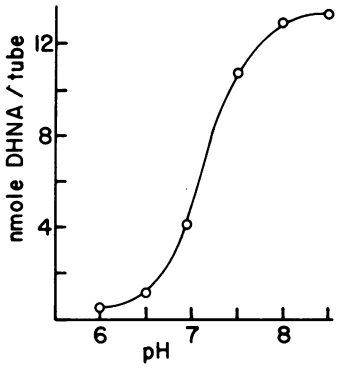

FIG. 1. *Effect of pH on the formation of DHNA by B. subtilis (men⁺). Routine assay conditions were used, as described in Materials and Methods, and each tube contained 8.4 mg of protein. The volume was made up to 3.0 ml with 0.1 M potassium phosphate buffer containing 5 mM mercaptoethanol, with various pH values as indicated.*

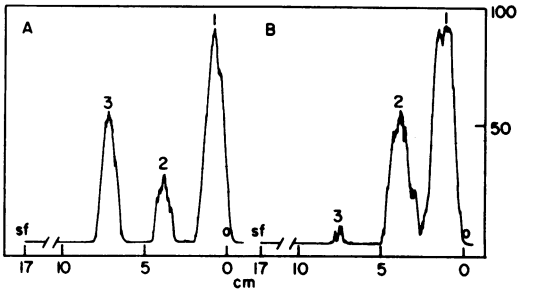

FIG. 2. *Formation of DHNA and spirodilactone by B. subtilis (men⁺) extracts. (A) The extract, 200 μl (7.8 mg of protein), and [2,3-$^{14}C_2$]OSB (approximately 100,000 cpm) were incubated under the usual assay conditions. (B) A similar incubation mixture was supplemented with 28 U of M. phlei DHNA synthase. After incubation, the reaction was terminated by the addition of 3 ml of acetone-HCl (100:1), and the products were extracted into 3 ml of benzene. The organic phase was withdrawn, mixed with approximately 100 μg each of OSB, DHNA, and spirodilactone, and evaporated to dryness in a rotary evaporator. The samples were redissolved in 500 μl of ethyl acetate and then spotted on Analtech Silica Gel GF thin-layer plates. They were developed in the following solvent: chloroform-ethyl acetate-formic acid (135:20:1.5). After development, the plates were scanned for radioactivity in the radiochromatogram scanner. The ordinate of the figure shows percentage of full-scale deflection; the instrument settings were: time constant, 10 s; range, 1 × 10⁵ cpm; speed, 1 cm/min; slit width, 2 mm. Abbreviations: O, origin; sf, solvent front. The peaks correspond with the position of standard samples and are identified by numbers: 1, OSB; 2, DHNA; 3, spirodilactone of OSB.*

at pH 8.2. In some experiments, combinations of *B. subtilis* and *M. phlei* enzymes were used at pH 8.2; under these conditions, the *M. phlei* enzymes had about 50% of their maximal activity, and in any case were present in excess.

Evidence for two enzyme activities in wild-type *B. subtilis*. Cell-free extracts of RB356 (*men*⁺) were incubated with [2,3-$^{14}C_2$]-OSB under the standard assay conditions just described, and the materials present in the solution were examined by thin-layer chromatography followed by scanning for the presence of radioactivity. In the scans for radioactivity, three prominent peaks were obtained (see Fig. 2A). Peak 1 (with R_f value of 0.05) was clearly unchanged OSB, whereas peaks 2 and 3 (with R_f values of 0.23 and 0.43, respectively) corresponded to DHNA and OSB-spirodilactone. Since previous studies with other microorganisms (7–9) suggested that spirodilactone formation is due to a deficiency of DHNA synthase in comparison with OSB-CoA synthetase, a DHNA synthase preparation from *M. phlei* was added to the standard incubation mixture. Under these conditions (see Fig. 2B) only two radioactive peaks were observed on scanning the thin-layer chromatograms; these peaks corresponded precisely to OSB and DHNA. It also proved possible to manipulate the product composition in the direction of increased spirodilactone synthesis and reduced DHNA formation. This was done by a mild acid treatment of the *B. subtilis* extract, using conditions previously determined to inactivate the DHNA synthase of *M. phlei* without changing the activity of OSB-CoA synthetase. The *B. subtilis* extracts on this acid treatment produced only the OSB-spirodilactone and no DHNA (Fig. 3A). If a preparation of DHNA synthase was added to the acid-treated extracts, DHNA production was restored (Fig. 3B).

DHNA formation by complementation of mutant extracts. Under conditions determined to be optimal for DHNA production by extracts of *men*⁺ *B. subtilis*, none of the mutant extracts formed DHNA. However, DHNA production was obtained when certain combinations of mutant extracts were examined (Table 2). Extracts of strains RB413 (*men-325*) and RB415 (*men-329*) produced DHNA in combination with extracts from either RB388 (*men-310*) or RB397 (*men-312*). The combinations RB413 (*men-325*) plus RB415 (*men-329*) and RB388 (*men-310*) plus RB397 (*men-312*), however, failed to yield DHNA. These results show that there are two complementation groups, which correlate with the genetic and phenotypic analyses of the mutants (11).

Identification of missing enzymes in the

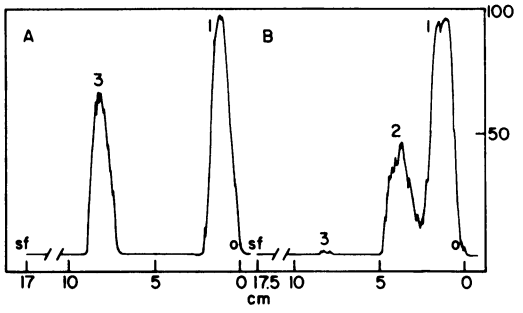

FIG. 3. *Effect of acid on enzyme activities of B. subtilis (men⁺) extracts. The extract, 400 μl (15.6 mg of protein), was placed in a test tube and treated with 3 N HCl to yield a final concentration of 0.1 N; the operation was carried out in an ice bath. After 1 min, 4.2 ml of 0.1 M potassium phosphate buffer, pH 8.2, containing 5 mM mercaptoethanol was added. After mixing, the contents of the tube were divided equally and added to two test tubes. Incubation was carried out in the presence of [2,3-$^{14}C_2$]OSB (total 100,000 cpm) under the usual assay conditions as described in the legend for Fig. 2. (A) Acid-treated extract; (B) acid-treated extract with addition of 28 U of M. phlei DHNA synthase. Extraction, thin-layer chromatography, and settings for scanning of radioactivity and identification of spots were the same as in Fig. 2. The ordinate of the figures shows the percentage of full-scale deflection.*

TABLE 2. *DHNA formation in in vitro complementation experiments*[a]

Source of protein		DHNA formed (nmol/30 min)
Extract 1	Extract 2	
RB388 *men-310*	RB397 *men-312*	0
RB413 *men-325*	RB388 *men-310*	7.1
RB413 *men-325*	RB397 *men-312*	11.0
RB413 *men-325*	RB415 *men-329*	0
RB415 *men-329*	RB388 *men-310*	6.9
RB415 *men-329*	RB397 *men-312*	8.8

[a] 200-μl portions of extract 1 and extract 2 were mixed in a total volume of 3 ml; the production of DHNA was estimated spectrophotofluorometrically. The amounts of protein in the extracts were as follows: RB388, 8.7 mg; RB397, 8.0 mg; RB413, 9.0 mg; and RB415, 8.7 mg. None of the extracts by itself produced DHNA.

mutants. To identify the missing enzyme in each mutant, a complementation analysis of the cell-free extracts was carried out, using preparations of OSB-CoA synthetase and DHNA synthase obtained from *M. phlei*. Extracts of mutants RB388 (*men-310*) and RB397 (*men-312*) complemented with the OSB-CoA synthetase preparation, and thus produced DHNA (Table 3). These extracts, however, did not produce DHNA in admixture with DHNA synthase.

TABLE 3. *Identification of enzyme defects in B. subtilis mutants*[a]

Source of extract	OSB-CoA synthetase[b]	DHNA synthase[c]	DHNA formed (nmol/30 min)
RB388 *men-310*	+	–[d]	5.2
RB388 *men-310*	–	+	0
RB397 *men-312*	+	–	6.6
RB397 *men-312*	–	+	0
RB413 *men-325*	+	–	0
RB413 *men-325*	–	+	18.4
RB415 *men-329*	+	–	0
RB415 *men-329*	–	+	15.7

[a] Concentration of protein in the extracts of mutants is the same as in Table 2.
[b] 18 U of *M. phlei* OSB-CoA synthetase.
[c] 28 U of *M. phlei* DHNA synthase.
[d] No addition of indicated enzyme.

Hence it can be concluded that RB388 (*men-310*) and RB397 (*men-312*) lack OSB-CoA synthetase but possess DHNA synthase. On the other hand, extracts from RB413 (*men-325*) and RB415 (*men-329*) produced DHNA when mixed with DHNA synthase but not with OSB-CoA synthetase. These two strains, therefore, possess OSB-CoA synthetase but not DHNA synthase. These results thus establish the biochemical basis for the grouping obtained by genetic analysis (11) and by enzymatic analysis of mixed extracts (Table 2).

When extracts from mutants RB413 (*men-325*) and RB415 (*men-329*) were complemented with *M. phlei* DHNA synthase (see Table 3), the DHNA production was much higher than that noted in Table 2 and in extracts of the wild-type organism. This is because *B. subtilis* has a lower level of DHNA synthase compared with OSB-CoA synthetase (see Fig. 2A for documentation). Hence, when a high level of DHNA synthase is added, all of the OSB-CoA formed is converted to DHNA rather than to spirodilactone. In fact, when the same quantity of DHNA synthase from *M. phlei* was added to *men*⁺ extracts, spirodilactone formation was completely suppressed (see Fig. 2B).

Formation of spirodilactone by strains RB413 (*men-325*) and RB415 (*men-329*). As already pointed out, denaturation of the DHNA synthase present in extracts of wild-type *B. subtilis* by acid treatment led only to the production of spirodilactone and no DHNA (see Fig. 3A). Hence mutants lacking the DHNA synthase should also accumulate only spirodilactone when incubated with [2,3-$^{14}C_2$]OSB. This expectation was fulfilled when extracts of the mutants RB413 (*men-325*) and RB415 (*men-329*) were examined.

DISCUSSION

Bacterial mutants deficient in menaquinone biosynthesis have been described for *Staphylococcus aureus* (10), *E. coli* (3–5, 13), and *B. subtilis* (11, 12). Young isolated two mutants of *E. coli* blocked in the conversion of OSB to DHNA (13). These mutants were designated *menB*; they were not subjected to detailed genetic analysis to distinguish more than one group. More recently, Guest has suggested that there might be two groups among the *menB* mutants of *E. coli* (4). The *S. aureus* mutants have not been analyzed either genetically or enzymatically.

The results reported here and in the accompanying publication (12) provide the first clear-cut demonstration, by biochemical as well as genetic means, for the existence of two classes of mutants within the *menB* group itself. Each class constitutes an enzymologically defined in vitro complementation group. Mutants with defects in the structural gene for DHNA synthase will henceforth be designated *menB*; strains RB413 (*men-325*) and RB415 (*men-329*) of *B. subtilis* are of this type. Strains carrying mutations in the structural gene for OSB-CoA synthetase, on the other hand, will be referred to as *menE* mutants; strains RB388 (*men-310*) and RB397 (*men-312*) of *B. subtilis* are of this type. These assignments are depicted in Fig. 4. The same designations will be used for *E. coli* mutants (J. R. Guest, personal communication).

The isolation of these two groups of mutants and the characterization of the enzyme defects support our earlier assertion that OSB-CoA is a transient intermediate in the conversion OSB → DHNA. The previous evidence for this intermediate was the isolation of two enzyme fractions from *M. phlei* extracts, the dependence of the reaction on the presence of both CoA and ATP, and the demonstrated conversion of ATP to AMP in the course of the reaction. The formation of the OSB-spirodilactone as the only product by extracts of the *menB* mutants supports the assumption that spirodilactone is a spontaneous degradation product of the very reactive OSB-CoA intermediate.

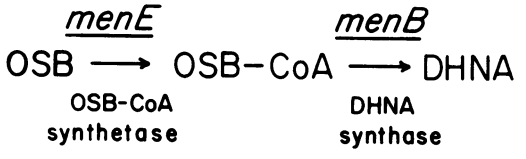

FIG. 4. *Identification of men mutants of B. subtilis.*

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