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Characterization of *Escherichia coli men* Mutants Defective in Conversion of *o*-Succinylbenzoate to 1,4-Dihydroxy-2-Naphthoate

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Four independent menaquinone (vitamin K_2)-deficient mutants of *Escherichia coli*, blocked in the conversion of *o*-succinylbenzoate (OSB) to 1,4-dihydroxy-2-naphthoate (DHNA), were found to represent two distinct classes. Enzymatic complementation was observed when a cell-free extract of one mutant was mixed with extracts of any of the remaining three mutants. The missing enzymes in the two classes were identified by in vitro complementation with preparations of OSB-coenzyme A (CoA) synthetase or DHNA synthase isolated from *Mycobacterium phlei*. Mutants lacking DHNA synthase (and therefore complementing with *M. phlei* DHNA synthase) were designated *menB*, and the mutant lacking OSB-CoA synthetase (and therefore complementing with *M. phlei* OSB-CoA synthetase) was designated *menE*. The *menB* mutants produced only the spirodilactone form of OSB when extracts were incubated with [2,3-$^{14}C_2$]OSB, ATP, and CoA; the OSB was unchanged on incubation with an extract from the *menE* mutant under these conditions. Experiments with strains lysogenized by a λ *men* transducing phage (λG68) and transduction studies with phage P1 indicated that the *menB* and *menE* genes form part of a cluster of four genes, controlling the early steps in menaquinone biosynthesis, located at 48.5 min in the *E. coli* linkage map. Evidence was obtained for the clockwise gene order *gyrA*. . . .*menC-E*-B-D*, where the asterisk denotes the uncertain position of *menE* relative to *menC* and *menB*. The transducing phage (λG68) contained functional *menB*, *menC*, and *menE* genes, but only part of the *menD* gene, and it was designated λ *menCEB(D)*.

Menaquinone (vitamin K_2) functions as an electron carrier in the respiratory chain of many microorganisms (for reviews see Taber [19] and Kröger [10]). In *Escherichia coli* it is an essential component of the energy-generating fumarate reductase system, and mutants blocked in menaquinone biosynthesis have been detected by their inability to grow anaerobically with fumarate as terminal electron acceptor (4, 5, 11). The overall pathway proposed for the biosynthesis of menaquinone from chorismate involves *o*-succinylbenzoate (OSB), 1,4-dihydroxy-2-naphthoate (DHNA), and demethylmenaquinone thus:

$$\text{chorismate} \xrightarrow{menC,D} \text{OSB} \xrightarrow{menB} \text{DHNA} \xrightarrow{menA} \text{DMK} \rightarrow \text{MK}$$

Mutants with lesions in this pathway have been characterized genetically and nutritionally by their response to OSB and DHNA (*menC* and *menD*) or DHNA alone (*menB*) and by the accumulation of OSB (*menB*) or DHNA (*menA*) in the corresponding cultures (4, 5, 18, 21). The *menB*, *C*, and *D* genes are clustered at 48.5 min in the *E. coli* linkage map, and the *menA* gene is at 88 min (1). The *menB* and *C* genes plus part of the *menD* gene have also been isolated in an artificially constructed lambda transducing phage, λ *menCB(D)* or λG68, by Guest and Shaw (6).

Enzymological studies have shown that cell-free extracts of *E. coli*, *Micrococcus luteus*, *Mycobacterium phlei*, and *Bacillus subtilis* are able to catalyze the ATP- and coenzyme A (CoA)-dependent conversion of OSB to DHNA (3, 13, 15, 16). Furthermore, in the latter three organisms, two distinct enzymes are required,

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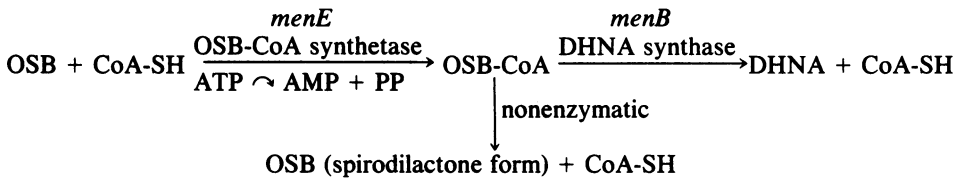
OSB-CoA synthetase and DHNA synthase. The two enzymes of *M. phlei* have been separated, and specific assays for them have been developed (13). A CoA derivative of OSB was postulated as an intermediate in the overall OSB → DHNA conversion (3). This intermediate has now been isolated and purified (8, 9); evidence that the CoA is located on the aromatic carboxyl, as originally postulated (3, 13), has also been obtained (9). The OSB-CoA compound is rather unstable and is transformed nonenzymatically to the spirodilactone derivative of OSB. Recently, DHNA-requiring mutants of *B. subtilis* were divided into two groups according to their enzymatic lesions and designated *menE* (lacking OSB-CoA synthetase) or *menB* (lacking DHNA synthase) (15). Hence, the formation of the naphthalenoid derivative DHNA from the benzenoid compound OSB can be represented more completely as follows:



This paper reports studies with four DHNA-requiring mutants of *E. coli* which indicate that the conversion of OSB to DHNA in this organism also requires the products of two genes (*menE* [OSB-CoA synthetase] and *menB* [DHNA synthase]). Experiments using the recombinant transducing phage (λG68) showed that the *menE* gene is linked to *menC*, *B*, and *D* and that the *menB* and *E* gene products are expressed from the transducing prophage.

MATERIALS AND METHODS

Bacteria, phages, and media. The characteristics of strains of *E. coli* K-12 are listed in Table 1. Two of the *men* mutants, AN209 (*menB404*) and AN213 (*menE408*), were kindly provided by I. G. Young. They were previously designated *menB* by their failure to synthesize menaquinone unless supplied with DHNA (21). The other *menB* mutants (JRG962 and JRG1205) were characterized by their DHNA-dependent anaerobic growth on lactate plus fumarate (LF) and glycerol plus fumarate (GF) media (5, 6). Phage λG68 is an artifically constructed λ *men* transducing phage, λ *menCB(D)* Δ(*srI*λ1-2) Δ(*spvuI*λ2-*shn*λ3) *att*$^+$ *imm21 nin5 shn*λ6°, recently characterized by Guest and Shaw (6). It contains an 11.5-kilobase fragment of *E. coli* DNA, derived from a large *Hin*dIII fragment, which is linked to the right arm of the vector at *shn*λ3 but fused to the left arm between *srI*λ2 and *spvuI*λ2. The phage carries intact and functional *menB* and *C* genes but only part of the *menD* gene. The preparation of lambda phage stocks and lambda lysogens and other general phage procedures were performed by the methods of Murray et al. (17). The LF and GF media and the conditions for anaerobic growth have been described previously (11). Pyridoxin (0.4 μg/ml) was added to minimal media to satisfy the previously unreported requirement for this compound of strain AN213.

Genetic techniques. Phage P1-mediated transduction was used in linkage studies to locate the *menB* mutation relative to the *purF* gene and also to establish the order of *menB* and *menC* relative to *gyrA* in three-factor crosses. The selective and diagnostic medium contained, as major substrates, glycerol plus fumarate (incubated anaerobically; *men*$^+$), DL-α-glycerophosphate (*glp*$^+$), and D-glucose (*pur*$^+$); and nutrient agar plus nalidixic acid (20 μg/ml) was used to select and identify nalidixic acid-resistant strains. Details of these procedures have been described previously (5).

Chemicals. OSB, OSB-spirodilactone, and DHNA were synthesized as previously described (12). [2,3-$^{14}C_2$]OSB was prepared from phthalic anhydride and [2,3-$^{14}C_2$]succinate (50.9 mCi/mmol; Amersham Corp., Arlington Heights, Ill.). After dilution, the synthesized radioactive OSB had a specific activity of 2.3 mCi/mmol. All solvents were glass distilled before use.

Cell-free extracts and enzyme assays. Stock cultures used for the preparation of enzyme extracts were grown in Trypticase soy broth medium (BBL Microbi-

TABLE 1. Strains of *E. coli* K-12

E. coli strain	Genotype	Source or reference
PL2024	*gal trpA trpR iclR rpsL*	(11)
JRG860	*menC3*	OSB-requiring mutant of PL2024 (5)
JRG863	*menC2*	OSB-requiring mutant of PL2024 (5)
JRG896	*purF54 trpR iclR*	(4)
JRG914	*menC3 gyrA*	OSB-requiring Nal^r derivative of PL2024 (5)
JRG955	*menD11 gyrA*	OSB-requiring Nal^r derivative of PL2024 (5)
JRG962	*menB15 gyrA*	DHNA-requiring mutant (5)
JRG1205	*menB18 gyrA*	DHNA-requiring mutant (6)
AN209	*menB404 metB*	Menaquinone-deficient mutant (21)
AN213	*menE408*[a] *metB pdx*	Menaquinone-deficient mutant (21)
Lin6	*glpT13 phoA relA1 tonA* T2^r	(7)

[a] Formerly *menB408* (21).

TABLE 2. Enzyme activities and in vitro complementation with extracts of *E. coli men* mutants

Source of primary extract	DHNA formation[a] with secondary extract:			
	None	JRG962 (*menB15*)	JRG1205 (*menB18*)	AN209 (*menB404*)
PL2024 (*men*$^+$)	7.7	—[b]	—	—
JRG962 (*menB15*)[c]	<0.05	—	—	—
JRG1205 (*menB18*)	<0.05	<0.05	—	—
AN209 (*menB404*)	<0.05	<0.05	0.2	—
AN213 (*menE408*)	0.49	6.4	6.4	6.1

[a] Incubations were carried out in a total volume of 3.0 ml for 30 min at 30°C. DHNA synthesis was assayed as described previously (15). The values given are nanomoles of DHNA produced per 30 min per tube. The amounts of protein in the individual extracts were: PL2024, 13.5 mg; JRG962, 15.1 mg; JRG1205, 12.6 mg; AN209, 14.8 mg; and AN213, 14.2 mg.

[b] —, None.

[c] The assignation of *men* mutations to the *B* or *E* class is based on the results in Tables 2 and 3.

ology systems, Cockeysville, Md.) as described earlier (14). The earlier methods were also used for large-scale growth, harvest, and extract preparation by use of the French press (14). The isolation and purification of OSB-CoA synthetase and DHNA synthase from *M. phlei* and the procedures for complementation assays have been described by Meganathan et al. (15). Protein was assayed by the method of Bradford (2).

RESULTS

Enzymological properties of *men* mutants. Four mutants, which had previously been assigned to the *menB* class on the basis of their failure to grow anaerobically on GF medium (*men-15*, *men-18*; references 5 and 6) or their failure to synthesize menaquinone unless supplied with DHNA (*men-404*, *men-408*; reference 21), were tested for the ability to catalyze the overall conversion of OSB to DHNA, using cell-free extracts. No activity was detected with three of the mutants, and the fourth (AN213, which contains the *men-408* mutation) exhibited only weak activity (Table 2). Under the same conditions, DHNA formation was readily observable, using cell-free extracts from a wild-type *E. coli* strain. When extracts from JRG962 (*men-15*) or from JRG1205 (*men-18*) or from AN209 (*men-404*) were mixed in pairwise combinations, no DHNA synthesis occurred. However, when an extract from AN213 (*men-408*) was mixed with any one of the three extracts just described, DHNA production was observed (Table 2). Hence, these studies provided enzymological confirmation of the existence of two distinct mutant classes. To identify the lesions of the two classes, in vitro complementation tests were carried out with partially purified extracts from *M. phlei* containing only one of the critical enzyme activities. The conversion of OSB to DHNA in an extract of AN213 (*men-408*) was promoted by adding OSB-CoA synthetase, whereas the activity was restored to extracts of the other mutants by adding DHNA synthase (Table 3). It is concluded that strain AN213 lacks OSB-CoA synthetase, and its mutation has been designated *menE408*. Likewise, strains JRG962, JRG1205, and AN209 must lack DHNA synthase due to mutations in the *menB* gene (*menB15*, *menB18*, and *menB404*).

Further evidence for the validity of these conclusions came from studies on the production of the spirodilactone form of OSB. Purified OSB-CoA synthetase from *M. phlei*, extracts of

TABLE 3. Identification of enzyme defects in *E. coli men* mutants and effects of lysogeny with λG68 (λ *men*$^+$)

E. coli strain	Genotype	DHNA formation[a] with:		
		No addition	Plus OSB-CoA synthetase[b]	Plus DHNA synthase[b]
PL2024	*men*$^+$	7.7[c]	8.8	6.7
JRG962	*menB15*	<0.05[c]	<0.05	8.3
JRG1205	*menB18*	<0.05[c]	<0.05	8.2
AN209	*menB404*	<0.05[c]	<0.05	9.3
AN213	*menE408*	0.49[c]	4.65	0.73
JRG962(λG68)	*menB15* (λ men$^+$)	1.5	1.7	10.0
JRG1205(λG68)	*menB18* (λ men$^+$)	1.2	1.7	10.0
AN209(λG68)	*menB404* (λ men$^+$)	0.5	1.0	10.3
AN213(λG68)	*menE408* (λ men$^+$)	3.7	8.6	3.4

[a] Expressed as nanomoles of DHNA produced per 30 min per tube; assay conditions were as described in Table 2, footnote *a*. The amounts of protein in extracts of lysogens were: JRG962(λG68), 12.8 mg; JRG1205(λG68), 13.6 mg; AN209(λG68), 12.2 mg; AN213(λG68), 13.9 mg. The amounts of protein in other extracts were as described in Table 2, footnote *a*.

[b] The combined activity of OSB-CoA synthetase and DNHA synthase, using the same amounts and under the same conditions as in the above assays, was 13 nmol/30 min.

[c] Results derived from Table 2.

several microorganisms in which DHNA synthase has been inactivated by brief exposure to acid, and *menB* mutants of *B. subtilis* are known to produce the spirodilactone form of OSB when incubated with OSB, CoA, and ATP (13, 15, 16). It appears that under these conditions, the relatively unstable OSB-CoA is formed, but undergoes a nonenzymatic lactonization with the elimination of CoA. Hence, an extract lacking DHNA synthase but containing OSB-CoA synthetase will produce only radioactive OSB spirodilactone on incubation with radioactive OSB; under similar conditions, the parent strain produces both spirodilactone and DHNA. An extract containing DHNA synthase but lacking OSB-CoA synthetase does not lactonize OSB under the same conditions. When this test was applied to extracts of the *E. coli* mutants, using [2,3-^{14}C]OSB as the substrate, thin-layer chromatography, followed by scanning for radioactivity, revealed the formation of OSB spirodilactone by extracts from JRG962 (*men-15*), JRG1205 (*men-18*), and AN209 (*men-404*); no spirodilactone formation occurred when an extract from AN213 was incubated under the same conditions. These observations reinforce the assignments made previously on the basis of the complementation assays.

Genetic studies with *men* mutants. Previous genetic studies have shown that two of the mutations, now designated *menB* (*menB15* and *menB18*), are located close to the *menC* and *menD* genes at 48.5 min on the *E. coli* linkage map, and the gene order *gyrA*. . . .*menC*. . . *menB*. .*menD*. . .*purF* has been indicated (5, 6). However, these conclusions were originally derived from experiments involving a mutant (JRG954, *men-10*), which was later found to contain two mutations (*menD17* and *menB18*). The two mutations now designated *menB404* and *menE408* were not mapped previously although they were shown not to be cotransduced with *glpK*, a gene which is close to the *menA* gene at 88 min (21). Consequently, it was of interest to confirm the location of the *menB* gene and determine whether the *menE* mutation is linked to the *menB*, *C*, and *D* genes.

Nutritional studies showed that AN209 (*menB404*) resembled other *menB* mutants in its inability to grow anaerobically on LF or GF media unless supplemented with DHNA. No cross-feeding was observed between any of the *menB* mutants nor between combinations of *menB*, *C*, and *D* mutants. In contrast, cross-feeding has been observed between *menB* and *menC* or *menD* mutants of *B. subtilis* (20). The *menE* mutant (AN213) grew on unsupplemented GF medium, indicating that the *menE408* mutation is sufficiently leaky to provide a growth-promoting supply of DHNA. This phenotype has seriously hampered attempts to define the precise location of *menE* by nutritional selection, which has been successful with other *men* mutants.

(i) Transduction with phage P1. The results of representative phage P1-mediated two-factor and three-factor crosses are summarized in Table 4. Crosses 1 to 4 show that the *menB* mutations are cotransducible with *purF*, *gyrA*, and *glpT* at frequencies similar to those observed previously with *menC* and *D* mutants (4, 5). In the three-factor analysis (Table 4, crosses 5 to 8), the inheritance of the unselected *gyrA* marker by *men*$^+$ transductants was used to deduce the order of the *menB* and *C* genes. Using a *menB* donor, the *men-gyrA* linkage was normal (17% in cross 8 compared with 14 and 16% in crosses 1 and 3), whereas it was much reduced in the reciprocal cross (4%, cross 7), indicating the gene order: *gyrA*. . . .*menC*. . *menB*. These results confirm that the *menB* gene is located in the *men* region at 48.5 min, clockwise from the *menC* gene.

(ii) Transduction with λG68 (λ *men*). Strain

TABLE 4. Transduction studies with *menB* mutants

Cross	Donor	Recipient	Selection	Transductants per 10^7 PFU	No. scored	Unselected donor marked	Cotransduction frequency (%)
1	JRG896 (*purF*)	JRG962 (*menB15 gyrA*)	*menB*$^+$	60	92	*purF*	14
						gyrA$^+$	14
2	JRG896 (*purF*)	AN209 (*menB404*)	*menB*$^+$	150	54	*purF*	9
3	Lin6 (*glpT*)	JRG1205 (*menB18 gyrA*)	*menB*$^+$	200	108	*gyrA*$^+$	16
						glpT	23
4	JRG1205 (*menB18 gyrA*)	Lin6 (*glpT*)	*glpT*$^+$	12	92	*menB*	24
5	JRG1205 (*menB18 gyrA*)	JRG1205 (*menB18 gyrA*)	*men*$^+$	<0.1	—[a]	—	—
6	JRG860 (*menC3 gyrA*$^+$)	JRG914 (*menC3 gyrA*)	*men*$^+$	<0.1	—	—	—
7	JRG860 (*menC3 gyrA*$^+$)	JRG1205 (*menB18 gyrA*)	*men*$^+$	0.7	108	*gyrA*$^+$	4
8	JRG1205 (*menB18 gyrA*)	JRG860 (*menC3 gyrA*$^+$)	*men*$^+$	0.6	102	*gyrA*	17

[a] —, None.

AN209 (*menB404*) was transduced by λG68, an artificially constructed λ *men* phage which has been shown to carry the *menB* and *menC* genes but only part of the *menD* gene (6). The frequency of *men*$^+$ transduction (10^{-3}/PFU) was similar to those observed with other *menB* and *menC* mutants (10^{-3} to 10^{-4}/PFU; reference 6). These frequencies, although relatively low, indicate that the *menB* (and *menC*) mutations are complemented by λG68, because *menD* mutants are not complemented and are transduced at much lower frequencies (10^{-5} to 10^{-6}/PFU) or not at all (6). In this connection, *recA* derivatives of strains carrying the *menB18*, *menB404*, and *menC2* mutations were transduced at normal frequencies (10^{-3}/PFU), but a *menD11 recA* strain was not transduced ($<10^{-9}$ transductants per PFU). Furthermore, lysogenic derivatives of *menB* and *C* mutants, prepared under nonselective conditions, exhibited a Men$^+$ nutritional phenotype. These results confirm that intact and functional *menB* and *menC* genes are cloned in the λ *men* transducing phage.

Enzymological studies with λG68 lysogens and the location of *menE*. The Men$^+$ phenotypes of the λG68 lysogenic derivatives of the three DHNA-requiring mutants designated *menB* were confirmed in enzymological studies (Table 3). In each case, lysogeny resulted in a significant increase in the ability to catalyze the overall conversion of OSB to DHNA. This indicates that active DHNA synthase is expressed from the λG68 prophage. The activities, especially for the *menB404* (λ *men*$^+$) strain, were low compared with wild-type *E. coli*, and this appeared to be due to limiting DHNA synthase because full activity was restored by adding an excess of this enzyme (Table 3). The reason for this deficiency is not known. The lowest activity was comparable to that found in the *menE* mutant (AN213), and in both cases, this was sufficient to support growth under anaerobic conditions with fumarate as electron acceptor.

The presence of a λG68 prophage in AN213, the *menE* mutant, also produced a substantial increase in the capacity to convert OSB to DHNA (Table 3). This indicates that the *menE* gene is located close to the *menB*, *C*, and *D* genes and that it is cloned together with its promoter in λG68.

DISCUSSION

It has been shown that a group of four *E. coli* mutants, blocked in the conversion of OSB to DHNA in menaquinone biosynthesis, contains two classes. These results provide the first clear-cut demonstration that in *E. coli* the conversion of the benzenoid compound OSB to the naphthalenoid compound DHNA requires the participation of two enzymes. The *menB* designation has been retained for mutants deficient in DHNA synthase (JRG962, *menB15*; JRG1205, *menB18*; and AN209, *menB404*), and the single mutant lacking OSB-CoA synthetase (AN213, *menE408*) has been assigned the *menE* designation. The results establish the following basic similarity in the biochemical conversion of OSB to DHNA in four species, *E. coli*, *B. subtilis*, *M. phlei*, and *M. luteus* (3, 13, 15, 16):

$$\text{OSB} + \text{CoA-SH} + \text{ATP} \xrightarrow[\text{OSB-CoA synthetase}]{menE} \text{OSB-CoA} + \text{AMP} + \text{PP} \xrightarrow[\text{DHNA synthase}]{menB} \text{DHNA} + \text{CoA-SH}$$

These genes defining the early stages of menaquinone biosynthesis in *E. coli* are clustered at min 48.5 on the linkage map, and all of the evidence favors the clockwise order *menC-B-D*. It was not possible to define the position of *menE* within this cluster, except insofar as it is not outside the *menD* gene. The corresponding genes in *B. subtilis* are also clustered, but a different order has been proposed: *menE*-(*C*,*D*)-*B* (20).

At present, the transcriptional organization of the *E. coli men* genes is not known. However, the fact that the *menB*, *C*, and *E* genes are expressed from a λ *men* transducing phage which contains only part of the *menD* gene indicates that the three former genes have been cloned, together with their promoter(s). Phage λG68 may thus be designated λ *menC*$\overset{*}{E}$*B*(*D*), where the asterisk denotes that the position of *menE* relative to the adjacent genes is uncertain, and the parentheses indicate that only part of the *menD* gene is cloned in the 11.5-kilobase segment of bacterial DNA.

It appears that a relatively low level of menaquinone biosynthesis enzyme activity is sufficient to provide adequate levels of menaquinone for anaerobic growth with fumarate as electron acceptor. Strains AN213 and AN209 (λG68) both grew normally despite having only approximately 6% of wild-type OSB to DHNA conversion activity (Tables 2 and 3). It should be noted that AN213 was originally isolated fortuitously during a search for ubiquinone mutants (21). Possibly, the wild-type organism synthesizes more menaquinone than is actually needed for growth under these conditions. It has been shown that strains of *B. subtilis*, producing only 10% of their normal level of menaquinone, show growth and respiration although sporulation is

impaired; this is particularly surprising since this organism, unlike aerobically grown *E. coli*, does not have access to ubiquinone carriers (19).

Further work is clearly necessary to resolve the transcriptional organization of the *men* genes and also to amplify and identify the gene products. It is anticipated that this will facilitate studies on the route of OSB biosynthesis and help to elucidate factors regulating the biosynthesis of the electron carrier.

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