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Map Locations and Functions of *Salmonella typhimurium men* Genes

H. S. KWAN[1] AND E. L. BARRETT[2]*

Departments of Bacteriology[1] and Food Science and Technology,[2] University of California, Davis, California 95616

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Menaquinone (*men*) mutants of *Salmonella typhimurium* isolated on the basis of their inability to produce trimethylamine were characterized with respect to mutation site, the ability to cross-feed each other and be cross-fed by known *Escherichia coli men* mutants, and response to intermediates of the menaquinone biosynthetic pathway. Cross-feeding tests were based on the requirement of menaquinone for hydrogen sulfide production. Genotypes corresponding to the *menA*, *B*, *C*, *D*, and possibly *E* genes described in *E. coli* were all identified. Additional studies of deletions in the *menBCD* area revealed that this cluster lies between *ack*/*pta* and *glpT*, as in *E. coli*. The *ack* and *pta* mutants were also defective in the production of trimethylamine and failed to produce gas in the absence of added formate.

The biosynthesis of menaquinone (MK) in *Escherichia coli* involves at least five enzymatic steps, with chorismate as the initial substrate (2, 4, 5, 11, 13). Five genes, *menA*, *B*, *C*, *D*, and *E*, and four biosynthetic intermediates, *o*-succinylbenzoate (OSB), OSB-coenzyme A, 1,4-dihydroxy-2-naphthoate (DHNA), and demethylmenaquinone, have been identified, and the pathway (Fig. 1) has been proposed on the basis of feeding experiments with the biosynthetic intermediates (2, 4, 5, 11, 13). The *menB*, *C*, *D*, and *E* genes are clustered at 49 min in the *E. coli* linkage map, and *menA* is located at 88 min (1, 10). MK mutants are defective in anaerobic growth on glycerol with fumarate as electron acceptor, and, with the exception of *menE* mutants, which are typically "leaky," all were isolated on the basis of this characteristic (5, 10).

In contrast to the rather extensive studies of *E. coli* MK biosynthesis, there were no reports concerning MK biosynthesis in the closely related *Salmonella typhimurium* until we reported the isolation and characterization of *men* mutants in this organism as a class of mutants defective in trimethylamine *N*-oxide (TMAO) reduction (3, 7). We identified two classes of *men* mutants; one was fed by vitamins K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), K_3 (2-methyl-1,4-naphthoquinone), K_5 (2-methyl-4-amino-1-naphthol-HCl), and 1,4-naphthoquinone, and the other was fed by vitamin K_1 only. Mutants of the former class cotransduced at a frequency of 18 to 25% with *glpT* at 45.5 min, and those of the latter class cotransduced at a frequency of 54 to 62% with *glpK* at ca. 88 min (7). Both sites correlate closely with the two respective *E. coli men* gene loci. Unlike wild-type *S. typhimurium*, the *men* mutants were red (acidic) on MacConkey-glucose-TMAO (MGT) plates, could not produce H_2S from thiosulfate, and could not grow anaerobically on glycerol with either fumarate or TMAO as electron acceptor (3, 7), although two appeared to be leaky for H_2S production. To locate the *S. typhimurium men* genes more precisely and to see whether the *men* cluster at 46 min contained the same genes as have been identified in *E. coli*, we characterized a number of deletions in this area and studied the cross-feeding of MK intermediates among *S. typhimurium men* mutants and between strains of *S. typhimurium* and *E. coli*, using a system for cross-feeding based on the requirement of MK for H_2S production.

* Corresponding author.

S. typhimurium men mutants, when tested by streaking side by side on glycerol fumarate or glycerol TMAO plates (7), did not cross-feed each other. Similarly negative results were obtained in the studies of *men* mutants in *E. coli* (4, 5), although such cross-feeding studies were successful in *Bacillus subtilis* (12). However, we found that cross-feeding with respect to providing intermediates for H_2S production was easily detected. For assignment to respective complementation groups, triple sugar iron (TSI) agar tubes (Difco Laboratories) were stabbed with pairs of *men* mutant strains. Production of H_2S indicated cross-feeding between the pair of strains. A number of *men* mutants were so tested and found to comprise four cross-feeding groups. This cross-feeding test, however, did not indicate direction of feeding. To determine the direction of feeding between pairs of cross-feeding *men* mutants, we used TSI or peptone-iron agar (Difco) plates instead of tubes. Pairs of representative strains were stabbed diagonally into a thick TSI or peptone-iron plate in opposite directions to avoid direct contact of the cells. An example of such a test is shown in Fig. 2. The results are summarized in Table 1.

To identify which specific *men* mutant classes our mutants represented, we tested cross-feeding between *S. typhimurium* and known *men* mutants of *E. coli* for H_2S production. For these tests, we used a medium which contained all of the constituents of TSI except lactose and sucrose, the major sources of acid and gas production by *E. coli*, as acid and gas interfere with H_2S detection. Pairs of *E. coli* and *S. typhimurium men* mutants were stabbed into tubes of H_2S test medium, and H_2S production was observed after 24 to 48 h of incubation. Based on these results, we identifed TC94 as *menA*, TC99 as *menB*, and TC88 as *menD*. EB127 could be designated only as *menC* or *menE*. The leaky mutants, TC91 and EB115, could not be identified in these tests. The designations were further confirmed by plate cross-feeding tests, using the same medium (Table 1).

We also examined the response of the *men* mutants to three MK biosynthesis intermediates, OSB, OSB-spirodilactone, and DHNA, which were kindly provided by R. Bentley, University of Pittsburgh, Pittsburgh, Pa. OSB-spirodilactone is not a natural intermediate, but it can be converted to OSB-coenzyme A by the wild type (10). We tested the *men* mutants for H_2S production in TSI tubes, each supplemented with one of the three compounds. TC94 and EB93 did not respond to any of the supplements; TC99 and EB139

FIG. 1. MK biosynthesis in *E. coli*. Intermediates: OSB, *o*-succinylbenzoate; DHNA, 1,4-dihydrozy-2-naphthoate; DMK, demethylmenaquinone.

as well as leaky mutants TC91 and EB115 responded to DHNA but not to OSB or OSB-spirodilactone; and TC88, EB123, and EB127 responded to all three supplements. These results confirmed the cross-feeding results and further permitted the identification of EB127 as *menC* rather than *menE* (Fig. 1). We had expected that *menE* mutants would respond to OSB-spirodilactone as well as to DHNA. If so, then the results suggest that leaky mutants TC91 and EB115 are *menB* mutants. Although this may be the case for TC91, it seems unlikely that a Mu d insertion (EB115) could produce a leaky phenotype when nitrosoguanidine mutations in the same gene (TC99) do not. It seems more reasonable to conclude that EB115 has an insertion in *menE*, in which mutations are characteristically leaky (10), and that *menE* is required for efficient conversion of both OSB and OSB-spirodilactone into OSB-coenzyme A.

Cotransduction studies in our previous report (7) located TC94 and EB93 at 87 min on the chromosome near *glpK*, and located TC88, TC99, EB123, and EB139 at ca. 45 to 46 min near *glpT*. The *menBCDE* cluster in *E. coli* is between *glpT* and *ack/pta* (4, 5, 10). Initially we attempted to locate more precisely the *men* genes near *glpT* by means of P22 cotransduction with the Tn*10* insertion in TA3097 (*zei*::Tn*10*) obtained from G. F.-L. Ames, University of California, Berkeley. This insertion is between *ack/pta* and *glpT* (G. F.-L. Ames, personal communication). Transduction methods were as previously described (7). Phage prepared on TA3097 was used to transfer tetracycline resistance (Tet^r) to *men* mutants TC88 and TC99, *glpT* mutant EB55 (7), *pta* mutant TA3492 (from G. F.-L. Ames), and *ack* mutant TA3501 (also from G. F.-L. Ames). We found that the *ack* and *pta* mutants were, like the *men* mutants, red on MGT plates. Thus, cotransduction of Tet^r with Ack^+, Pta^+, or Men^+ could be scored as inheritance of white (wild-type) colony color on MGT. No cotransduction of Tet^r was found with *men* in TC88 (0 of 104 tested) or in TC99 (0 of 52), or with *glpT* (0 of 83). We found 80% (41 of 51) with *pta* and 50% (26 of 52) with *ack*.

To find out whether the *men* locus in *S. typhimurium* lies between *glpT* and *ack/pta*, we looked for Tn*10*-generated deletions which extended into *men*, *pta/ack*, or *glpT* by using a simultaneous selection-screening method for tetracycline sensitivity and acid reaction in the presence of TMAO. Our medium was a modification of the Maloy-Nunn medium for selection of tetracycline-sensitive derivatives of TET^r strains (9), which contained all of the constituents of the Maloy-Nunn medium together with the differential ingredients of MGT, i.e., 0.1% TMAO, 0.15% glucose, and MacConkey dye. Samples of stationary-phase nutrient broth culture of TA3097 were spread on this medium and incubated at 37°C anaerobically in a GasPak (BBL Microbiology Systems). Apparently because of the chelating agents in the medium, all of the colonies appeared pale purple. Nevertheless, the *men*, *ack*, and *pta* mutants were still identifiable as tiny colonies darker in color. Forty tiny, dark, tetracycline-sensitive deletions were isolated which were consistently red on MGT plates.

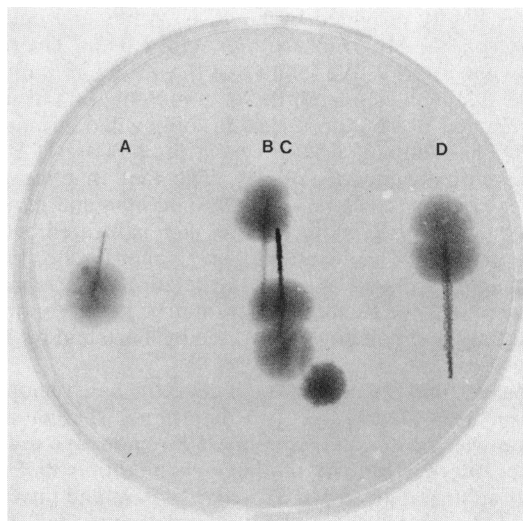

FIG. 2. Cross-feeding of *S. typhimurium* TC88 by TC94. Cells were stabbed diagonally into a thick peptone-iron plate, using opposite stab directions for neighboring inoculations to avoid direct cell contact. In this photograph, tan-yellow growth appears gray, and stabs blackened by FeS accumulation are black. Stabs A and B, TC94; stabs C and D, TC88.

We characterized the phenotypes of these deletion mutants, together with mutants containing deletions in this

TABLE 1. Cross-feeding among *men* mutants

Group[a]	Strain[b]	Cross-fed[c] by:									
		S. typhimurium					*E. coli*[d]				
		TA3512	TC88	EB127	TC99	TC94	PL2024 (WT)	JRG917 (*menD*)	JRG860 (*menC*)	JRG1205 (*menB*)	AN386 (*menA*)
I	TC88	−	NA	+	+	+	+	−	+	+	+
	EB123	−	−	+	+	+	ND	ND	ND	ND	ND
II	EB127	−	−	NA	+	+	+	−	−	+	+
III	TC99	−	−	−	NA	+	+	−	−	−	+
	EB139	−	−	−	−	+	ND	ND	ND	ND	ND
IV	TC94	−	−	−	−	NA	−	−	−	−	−
	EB93	−	−	−	−	−	ND	ND	ND	ND	ND
—	TA3512	−	−	−	−	+	ND	ND	ND	ND	ND

[a] Mutants were assigned to groups on the basis of feeding studies with TSI tubes (see the text).

[b] *S. typhimurium* TC strains (nitrosoguanidine-induced *men* mutations) and EB strains (Mu d insertions in *men* genes) were described previously (7). TA3512, which has a large deletion spanning *ack/pta*, was from G. F.-L. Ames. Group IV mutations cotransduced with *glpK* at 88 min; group I, II, and III mutations cotransduced with *glpT* at 45 min. Results for leaky mutants TC91 and EB115 are not shown because these results were ambiguous.

[c] Cross-feeding was detected as H_2S production in plates stabbed with pairs of strains as shown in Fig. 1. Symbols and abbreviations: +, H_2S produced; −, no H_2S produced; ND, not determined; NA, not applicable.

[d] *E. coli* strains were obtained from J. R. Guest. WT, Wild-type.

region as kindly provided by G. F.-L. Ames and deletions isolated from the Mu d1 insertion mutant EB138. The results are shown in Table 2. We found that the *ack* and *pta* mutants could be distinguished from the *men* mutants in TSI in that they produced H_2S but produced gas only when formate was added to the medium, whereas *men* mutants are H_2S^- and produce normal amounts of gas. The fact that we found deletions covering *ack/pta* and *men*, and *men* and *glpT*, but not *ack/pta* and *glpT*, indicates that *men* is located between *ack/pta* and *glpT*. This location of *men* genes on the chromosome is highly probable because the deletions of *ack/pta men glpT*$^+$ were isolated by three different procedures: spontaneous deletions and deletions generated by Tn*10* and by Mu d1 transpositions.

We have found the deletions covering the *men* region to be useful in cross-feeding tests to determine whether a *men* mutation was located in the *menBDCE* region. We used this basis for the assignment of the *men* mutation in EB127, which is an unstabilized Mu d1 fusion mutant and thus could not be used as donor to test cotransduction with *glpT*. EB127 cross-fed other *men* mutants but did not cross-feed TA3512; it was thus placed in the *men* region at 46 min on the linkage map.

In conclusion, we have shown that the *men* mutants isolated previously (3, 7) consist of at least four classes which are equivalent to *menA*, *menB*, *menC*, and *menD* mutants of *E. coli*. We developed a medium to isolate *ack/pta* and *men* deletions from TA3097 (*zei*::Tn*10*) and showed that the *menBDC/E* cluster in *S. typhimurium* is linked to *glpT* and is probably located between *ack/pta* and *glpT* at ca. 46 min. This map location is comparable to that of the *menBCDE* cluster reported in *E. coli*. We have also developed a cross-feeding test that can be used to characterize *men* mutants of both *S. typhimurium* and *E. coli*.

TABLE 2. Phenotypes[a] of deletions

Deletion strains	No. of strains	Presence (+) or absence (−) of the following mutation (identifying characteristic):		
		ack/pta (gas production)	*men* (H_2S production)	*glpT* (GLP utilization)
Spontaneous[b]				
TA3513	1	−	+	+
TA3512	1	−	−	+
Tn*10*-generated[c]				
EB160-166	7	+	−	+
EB167-192	26	+	−	−
EB193-194	2	−	+	+
EB195	1	−	−	+
Mu d1-generated[d]				
EB142	1	−	−	+

[a] *ack* and *pta* mutants (TA3501 and TA3492) (8) are both red on MGT plates, weak in H_2S production, and devoid of gas production when tested in TSI tubes. These mutants differ from *men* mutants in that *men* mutants produce gas. The *men* phenotype was identified as lack of H_2S production. *glpT* phenotype was identified as unable to grow on minimal medium (6) with α-glycerol phosphate (GLP) as carbon source.

[b] Strains obtained from G. F.-L. Ames (8).

[c] The isolation of these strains is described in the text.

[d] This strain was isolated as a temperature-resistant (growth at 42°C), ampicillin-sensitive derivative of EB94 [*men*::Mu d(Ap^r *lac*)*22*] as described previously (7).

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