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Molecular Cloning of Menaquinone Biosynthetic Genes of *Escherichia coli* K12

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Summary. A transducing phage carrying some of the genes (*men*) defining the early stages of menaquinone biosynthesis was isolated from a pool of recombinant lambda phages that had been constructed from R.*Hin*dIII digests of *E. coli* DNA and the corresponding insertion vector. The lesions of *menB* and *menC* mutants were complemented by the phage but *menD* mutants were transduced either at low frequencies or not at all. This indicates that the transducing phage contains functional *menB* and *menC* genes but that only part of the *menD* gene had been cloned. The phage (λG68) was accordingly designated *λmenCB(D)*. Studies with the transducing phage enabled earlier mapping data (Guest 1979) to be reinterpreted in favour of the gene order *nalA....menC..menB..menD....purF*. Restriction analyses established the presence of a bacterial DNA fragment (11.5 kb) linked by a R.*Hin*dIII target to the right arm of the λ genome but fused to the left arm of the vector. Hybridization studies confirmed that the cloned DNA was derived from a larger R.*Hin*dIII fragment (21 kb). A physical map of the *men* region was constructed and some flanking and overlapping fragments were identified.

Introduction

Under anaerobic conditions, *Escherichia coli* can derive energy to support growth on non-fermentable substrates such as glycerol, lactate, formate or hydrogen, by coupling their oxidation to the reduction of fumarate. This is mediated by an energy-generating electron transport chain, the fumarate reductase system (Haddock and Jones 1977; Kröger 1977, 1978). The reduction of fumarate also provides a source of succinate (succinyl-CoA) for biosynthetic purposes during anaerobic growth (Creaghan and Guest 1978) and it is involved in the oxidation of dihydroorotate and protoporphyrinogen in anaerobic pyrimidine and porphyrin synthesis (Andrews et al. 1977; Jacobs and Jacobs 1978).

Menaquinone (vitamin K_2; MK) is an obligatory electron carrier in the energy-yielding fumarate reduction pathway and mutants blocked in menaquinone biosynthesis have been detected by their inability to use fumarate as terminal electron acceptor (Lambden and Guest 1976; Guest 1977, 1979). In *E. coli* the pathway proposed for the biosynthesis of menaquinone (MK) from chorismate involves 2-succinylbenzoate (SB), 1,4-dihydroxy-2-naphthoate (DHN) and demethylmenaquinone (DMK) thus:

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Chorismate $\xrightarrow{menC,D}$ SB $\xrightarrow{menB}$ DHN $\xrightarrow{menA}$ DMK ⟶ MK.

Menaquinone mutants have been characterized by their response to exogenous SB and DHN (*menC* and *menD*) or DHN alone (*menB*) during anaerobic growth on glycerol or lactate plus fumarate media (Guest 1977, 1979) and by the accumulation of SB (*menB*) or DHN (*menA*) in the corresponding cultures (Young 1975; Shineberg and Young 1976). The *menB,C* and *D* genes form a cluster at 48.5 min in the *E. coli* linkage map, whereas the *menA* gene is located at 88 min (Bachmann and Low 1980). It is possible that two classes of DHN-requiring mutant may exist amongst the *menB* mutants because the conversion of SB to DHN in *Mycobacterium phlei* and *Micrococcus luteus* involves two enzymes, succinylbenzoyl-CoA synthetase and naphthoate synthase, with 2-succinylbenzoyl-CoA as the intermediate-(Meganathan and Bentley 1979; Meganathan et al. 1980). Mutants of *Bacillus subtilis* blocked in each of these steps have also been isolated recently (R. Bentley and H. Tabor, personal communication).

In previous studies on biochemical and genetic aspects of the fumarate reductase system of *E. coli,* use was made of artificially-constructed lambda transducing phages, *λfrdA* and *λfnr* (Cole and Guest 1978, 1980a and b; Shaw and Guest 1981). In the present paper we report the isolation and characterization of a recombinant lambda transducing phage, *λmenCB(D)*, which contains functional *menB* and *menC* genes and part of the *menD* gene.

Methods

Bacteriophages. Populations of recombinant lambda phages, constructed by in vitro ligation of R.*Hin*dIII or R.*Eco*RI fragments of *E. coli* DNA into the corresponding vectors, were kindly provided by Dr. N.E. Murray (λNM540 and λNM781 derivatives) or prepared according to Borck et al. (1976) for the λNM761 and λNM816 derivatives. The characteristics of the R.*Hin*dIII vectors, λNM540 (*srIλ1–2Δ shn3*$^+$ *att*$^+$ *imm21 nin5 shnλ6°*) and λNM761, and the R.*Eco*RI vectors, λNM781 and λNM816, have been described previously (Wilson and Murray 1979; Murray et al. 1977).

Bacteria. The *men* mutants are listed in Table 1. They were isolated in PL2024 (*gal trpA trpR ic1R rpsL*) and JRG911, a *nalA* derivative of PL2024 (Guest 1979). The original designations for strains JRG954 and JRG961 have been revised as a result of the present work. The *menC16* mutant (JRG960) is a 2-succinylbenzoate-requiring derivative of JRG911; it was not included in earlier studies and has been assigned to the *menC* class in the present investigation. Strain CR63 (SuI$^+$, λ^R) was the source of bacterial DNA for cloning and C600 was routinely used for phage propagation and assay.

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Table 1. Menaquinone biosynthesis mutants of *E. coli*

Strain	Relevant genotype
JRG860	*menC3*
JRG862	*menC1*
JRG863	*menC2*
JRG915	*menC4 nalA*
JRG916	*menD5 nalA*
JRG917	*menD6 nalA*
JRG918	*menD7 nalA*
JRG952	*menC8 nalA*
JRG953	*menC9 nalA*
JRG954	*menB18*[a] *menD17* [b] *nalA* (formerly *menB10 nalA*)
JRG955	*menD11*[b] *nalA*
JRG956	*menD12*[b] *nalA*
JRG959	*menD13*[b] *nalA*
JRG960	*menC16 nalA*
JRG961	*men-14*[c] *nalA* (formerly *menC14 nalA*)
JRG962	*menB15*[a] *nalA*
JRG1203	*menD17*[b] *nalA* ($MenB^+$ revertant of JRG954)
JRG1205	*menB18*[a] *nalA* ($MenD^+$ revertant of JRG954)

[a] The DHN-requiring mutants are designated *menB* even though more than one class with this phenotype could exist

[b] Mutations causing an SB-requirement and complementing *menC* mutants are grouped with the *menD* mutants

[c] The *men-14* strain may be a *menC menD* double mutant or deletion strain or a polar *menC* or *menD* mutant

Media. Rich media for routine subculturing and for the propagation of phages have been described previously (Cole and Guest 1980a). The minimal media used for selecting Men^+ transductants contained lactate or glycerol plus fumarate (LF or GF), as described by Guest (1977). Anaerobic incubation was under H_2 plus CO_2 (95:5 by volume) at 37° C, or 30° C when using temperature-sensitive phages. The media were supplemented with disodium 2-succinylbenzoate (25 μM) or 1,4-dihydroxy-2-naphthoate (4 μM) when required for the growth of specific mutants. Samples of these intermediates were kindly provided by Dr. R. Bentley.

General Techniques. The methods of Murray et al. (1973) were used for the preparation of plating cells and phage stocks and for phage titration and other general procedures. Transductions were performed according to Cole and Guest (1980a) using anaerobic selection on LF or GF medium.

DNA Preparation and Analysis. Details of the methods and materials used for the preparation of phage and bacterial DNA, restriction endonuclease digestion and agarose gel electrophoresis have been described by Shaw and Guest (1981) and by Cole and Guest (1980a) for electron microscopic heteroduplex analysis, DNA-labelling and DNA:DNA hybridization.

Results

Isolation of λmen Transducing Phages. Several independent recombinant phage pools, constructed from R.*Hin*dIII digests of DNA from *E. coli* and from the integration-proficient insertion vector, λNM540, were screened for *λmenC* transducing phages. The recipient was JRG863 (*menC2*) and Men^+ transductants were selected anaerobically on lactate plus fumarate medium. Transductant colonies were obtained with only one of the phage pools, at a low frequency (3×10^{-6} per p.f.u.). The transductants were purified by streaking to single colonies and *λmen* transducing phages were released from lysogens by uv-induction and then isolated as single plaques under non-selective conditions. One phage line, designated λG68, was retained for further study.

Table 2. Transduction and complementation of *men* mutants by *λmen* (λG68)

Recipient *men* allele	Transduction frequency[a]	Complementation by *λmenC* prophage[b]
menC1, C2, C3, C4, C8, C9, C16	$1 \times 10^{-3} - 8 \times 10^{-3}$	+
menB15, B18	$1 \times 10^{-3} - 1 \times 10^{-4}$	+
menD5, D7, D11, D12, D17	$1 \times 10^{-5} - 3 \times 10^{-6}$	–
menD6, D13	$< 3 \times 10^{-9}$	–
men-10 (JRG954), *men-14* (JRG961)	6×10^{-6}	–

[a] The range is expressed in terms of the number of Men^+ transductants per plaqueforming unit arising anaerobically on lactate plus fumarate medium

[b] Positive complementation denotes that a Men^+ phenotype is conferred on the corresponding mutant by lysogenisation with *λmen* (λG68)

No Men^+ transduction was observed with populations of recombinant phages produced with the R.*Hin*dIII and R.*Eco*RI replacement vectors, λNM761, λNM781 and λNM816, using several recipients, JRG860 (*menC3*), JRG863 (*menC2*), JRG917 (*menD6*) and JRG955 (*menD11*).

Genetic Characterization of λmen Phages. Eighteen *men* mutants were used as recipients in quantitative transduction tests with the *λmen* phage, λG68 (Table 2). All the *menB* and *menC* mutants were transduced at the highest frequencies ($\geqq 1 \times 10^{-4}$ per p.f.u.). Most of the *menD* mutants were also transduced, albeit at lower frequencies, but no transduction was detectable with two of the *menD* mutants. The growth phenotypes of lysogenic derivatives prepared under nonselective conditions indicated that the *menB* and *menC* lesions are complemented by *λmen* prophages (Table 2). By contrast, lysogenic derivatives of all the *menD* mutants remained dependent on exogenous 2-succinylbenzoate for anaerobic growth on LF and GF media. These results can be interpreted in terms of the content of *men* genes in λG68. It appears that intact *menB* and *menC* genes have been cloned together with their promoter(s), because they are expressed from the transducing prophage. By contrast, only the part of the *menD* gene containing the lesions of the five *menD* mutants that are transduced at low frequency (presumably by general homologous recombination), but not the region affected in the two nontransducible mutants, appears to have been cloned. It is not known why the transduction frequencies observed for the *menB* and *menC* mutants are so low (in absolute terms) when their lesions are complemented by λG68, but it could be due to the anaerobic selective conditions that were used.

The strain containing the *menC16* mutation (JRG960) was assigned to the *menC* class because of its positive nutritional response to SB and its high transducibility and complementation by λG68. Another SB-requiring mutant (JRG961, *men-14*) was previously assigned to the *men*C class because it gave complete but no abortive P1-mediated transductants in crosses with another *menC* mutant (Guest 1979). It was not complemented by λG68 and it also differed from other *menC* mutants in its low frequency of transduction (Table 2). It therefore appears that the lesion in this strain affects expression of both the *menC* and *menD* genes. This could be due to mutations in both genes, a deletion affecting the two genes, or, if these genes are expressed from the same promoter, a polar mutation in the proximal gene

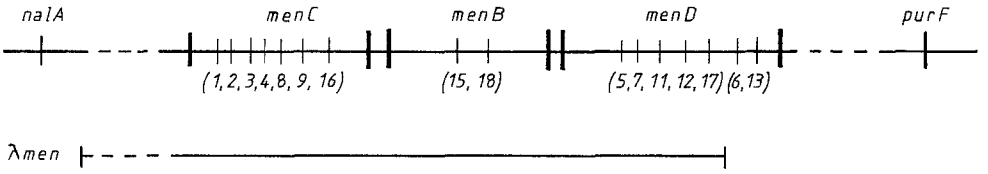

Fig. 1. Map of the *E. coli* chromosome (48–49 min, not to scale) showing the order of the *men* genes and some of the corresponding alleles based on studies with λG68(λ*men*) and previous work (Guest 1979). The extent of the segment of bacterial DNA cloned in λG68 is indicated but its orientation relative to the λ genome is not known

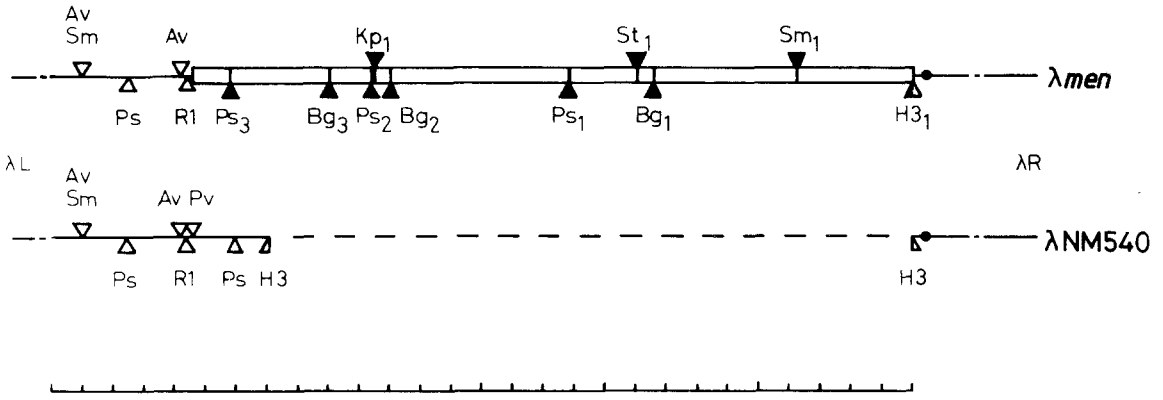

Fig. 2. Restriction map of the bacterial fragment cloned in λG68 (λ*men*). Details of the region near the junction between the left arm of the vector and the bacterial DNA are shown and also a map of the vector (λNM540) for comparison. The map is drawn to scale over the range of sites shown: each division on the scale corresponds to 0.5 kb. The orientation of the inserted DNA (double lines) relative to the flanking lambda arms (single lines) is indicated but the orientation of the inserted DNA relative to the bacterial chromosome is not known. The symbols for the restriction endonucleases are: Av, *Ava*I; Bg, *Bgl*II; H3, *Hind*III; Kp, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*I; RI, *Eco*RI; Sm, *Sma*I; St, *Sst*I. No sites for R.*Bam*HI, R.*Sal*I or R.*Xho*I were detected. Additional targets for R.*Ava*I and R.*Pvu*I in the cloned region were detected but their positions were not defined. The λ attachment site is denoted thus: ●

(*menC* or *menD*). In each case, the postulated *menD* lesion should be situated in the region of *menD* that is included in λG68, in order to explain the transducibility of the *men-14* strain.

The DHN-requiring mutant JRG954 (formerly designated *menB10*) was transduced at a much lower rate than JRG962 (*menB15*). This proved to be due to mutations in both the *menB* and *menD* genes and they were separated as follows. Firstly, a revertant of JRG954 requiring SB (but not DHN) for anaerobic growth on lactate-fumarate medium was isolated and designated JRG1203 (*menD17*) because, like other *menD* mutants, it was transduced at a low frequency but not complemented by λG68 (Table 2). Secondly, λG68 lysogens of JRG954 required SB, consistent with the complementation of the *menB* lesion of a *menB menD* double mutant by a prophage *menB*$^+$ gene. Reversion to nutritional independence and subsequent curing of the lysogen yielded a DHN-requiring strain (JRG1205, *menB18*) that was transduced by λG68 at frequencies characteristic of a *menB* mutant. The complexity of strain JRG954 (*menB18 menD17* but formerly *menB10*) explains the failure of previous attempts to order *menB* and *menD* by three-factor P1-transduction crosses using JRG954 as the representative *menB* mutant (Guest 1979). The earlier findings can now be reinterpreted in the light of the results obtained with λG68 and they support the gene order: *nalA*.....*menC*..*menB*..*menD*.....*purF*. A map summarizing the relative positions of the *men* loci is shown in Fig. 1.

Physical Characterization of the men Region. The construction of a λ*men* transducing phage from R.*Hin*dIII digests of bacterial and vector DNAs should produce a recombinant phage genome with two R.*Hin*dIII targets flanking the cloned fragment. However, restriction studies showed that λG68 possesses only one R.*Hin*dIII site, located at the right-hand end of the inserted fragment (Fig. 2). The other end appeared to have been fused to the left arm of the vector with the loss of the corresponding target. This was confirmed by electron microscopic heteroduplex analysis of molecules formed between DNAs from λG68 and the vector, λNM540 (Fig. 3). In addition to a large single-stranded loop (11.49 ± 0.69 kb) corresponding to the bacterial insert of λG68, there was a small region of nonhomology (1.38 ± 0.21 kb) representing a segment of vector DNA that had been deleted from the transducing phage. Photographs of 17 independent molecules each accompanied by at least one φX174 marker (5.38 kb) were measured and the left (deleted) and right arms of the vector were identified by size (22.33 ± 1.22 kb and 16.25 ± 1.04 kb, respectively). The degree of uncertainty associated with estimating the size of the deleted segment of vector DNA made it difficult to determine the exact size of the bacterial fragment cloned in λG68. It was also difficult to decide whether the R.*Eco*RI target, situated at or near the left-hand end of the inserted DNA (11.5 kb from the R.*Hin*dIII site; Fig. 2) originated in the vector (*srI*λ1–2) or *E. coli* (which contains a 12.3 kb R.*Eco*RI – R.*Hin*dIII fragment that hybridises with λG68 DNA; Table 4). Consequently, the junction between vector and bacterial DNA was investigated using enzymes R.*Pvu*I, R.*Ava*I and R.*Sma*I, which have targets in the corresponding region of the vector (Daniels et al. 1980). Digestion with R.*Ava*I gave rise to identical fragments (1.65 kb) with λNM540 and λG68 indicating that the R.*Ava*I target just to the left of the critical R.*Eco*RI site is not deleted in λG68 (Fig. 2). Double digestion with R.*Sma*I and R.*Eco*RI generated identical 1.8 kb fragments with both

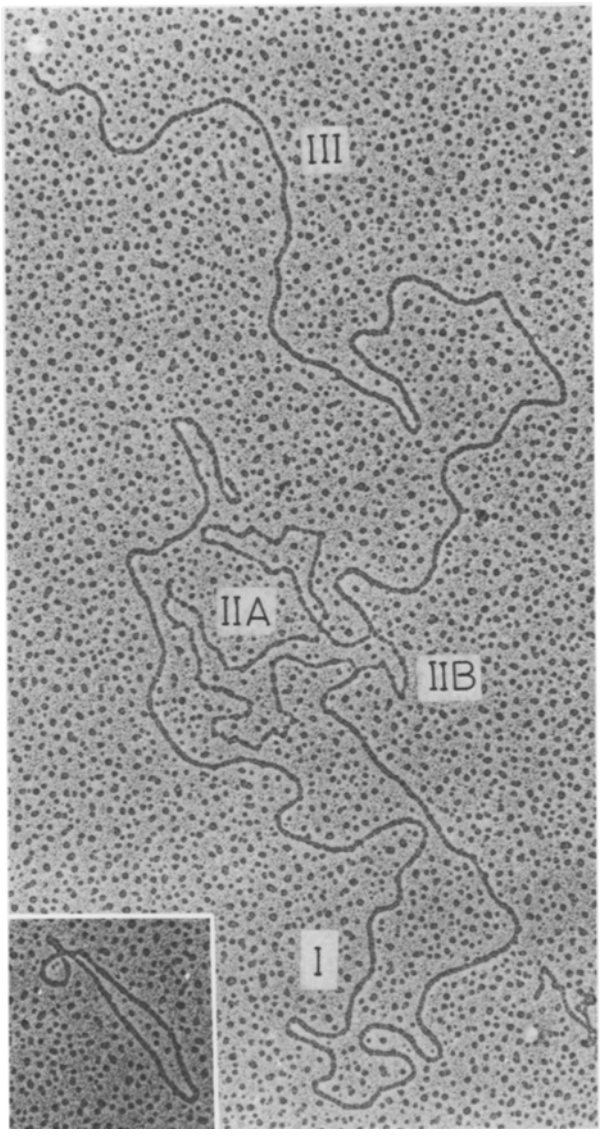

Fig. 3. Electron micrograph of a heteroduplex molecule formed between λG68 (*λmen*) and the insertion vector λNM540. The regions of homology correspond to the left(I) and right(III) arms of the vector. These flank the non-homologous single-stranded regions corresponding to the *men* region (IIA) and a segment of the left arm of the vector (IIB) that is deleted in λG68. Phage φX174 DNA (5.38 kb) was used as a length marker

phages, suggesting that the R.*Eco*RI site originates in the vector rather than the inserted DNA. Digestion with R.*Sma*I plus R.*Pvu*I gave a 1.95 kb fragment with vector DNA but not with λG68, indicating that the R.*Pvu*I site in the critical region of the vector has been deleted in λG68. Thus it appears that the vector deletion extends leftwards from the R.*Hin*dIII cloning site to a point between the R.*Pvu*I and R.*Eco*RI targets and that the R.*Eco*RI target in λG68 originated in the vector (Fig. 2).

The positions of other restriction targets in the cloned fragment were located by quantitative restriction analysis using λG68 with λNM540 serving as the control (Fig. 2). Internal sites were detected for R.*Bgl*II (3), R.*Pst*I (3), R.*Kpn*I (1), R.*Sst*I (1) and R.*Sma*I (1) but not for R.*Bam*HI, R.*Sal*I or R.*Xho*I: their coordinates relative to the R.*Hin*dIII target are listed in Table 3. Internal sites for R.*Pvu*I and R.*Ava*I were also detected but the complexity of the digestion patterns precluded definition of their number and positions.

Table 3. Restriction targets in the *men* region of *E. coli* cloned in λG68

Restriction target	Coordinate (kb)[a]
*Hin*dIII, (=*shn*λ3)	0.00
SmaI_1, (*Ava*I_1)	1.90
BglII_1, *Bgl*II_2, *Bgl*II_3	4.24, 8.50, 9.50
SstI_1	4.50
PstI_1, *Pst*I_2, *Pst*I_3	5.60, 8.80, 11.10
KpnI_1	8.75
*Eco*RI (=*srI*λ1-2)	11.80

[a] Coordinates are given as distances (kb) from the R.*Hin*dIII target at the right-hand end of the bacterial insert in λG68 (*λmen*) as illustrated in Fig. 2

Table 4. Restriction fragments of *E. coli* DNA hybridizing specifically with the bacterial DNA cloned in λG68(*λmen*)

R. endonuclease	Size of fragment (kb)
*Hin*dIII	21.0
*Eco*RI	14.1
*Hin*dIII plus *Eco*RI	12.3
*Bam*HI	26.5
*Bgl*II	1.0, 4.3, 7.0, 4.8[a], 5.4[a]
*Kpn*I	9.8, 20.0[a]
*Sal*I	14.0, 19.0[a]
*Xho*I	18.0
*Ava*I	1.6, 4.0, 5.1

[a] Denotes weak hybridization possibly due to products of partial digestion

Hybridization between restriction digests of *E. coli* DNA (after separation and transfer to nitrocellulose filters) and radioactively-labelled probes of λG68 and λNM540 DNAs was used to identify bacterial fragments possessing sequence homology with both the bacterial (*men*) and lambda regions of λG68. Homology with lambda DNA arises from the presence of cryptic prophages in *E. coli*. The sizes of the fragments hybridizing specifically with the *men* region are listed in Table 4. The results show that the *men* genes occur in a R.*Hin*dIII fragment, which would be too large (21 kb) to be cloned intact into the insertion vector used (maximum capacity 12 kb approx.). The entire cloned region also appears to be contained within large R.*Eco*RI, R.*Bam*HI, R.*Xho*I and probably R.*Sal*I fragments. The results with the R.*Hin*dIII plus R.*Eco*RI double digest suggest that there is an R.*Eco*RI target just outside the region cloned in λG68, 12.3 kb to the left to the R.*Hin*dIII site. Other fragments generated by R.*Bgl*II and R.*Ava*I correspond to internal and overlapping sequences and in a few cases their origins could be defined.

Discussion

Genetic studies with the recombinant lambda phage, λG68, showed that it carries most of the *menCBD* region of *E. coli* including the promoter(s) for the *menB* and *menC* genes, because *menB* and *menC* lesions were fully complemented by the transducing prophage. Mutations in the *menD* gene were not complemented although some could be transduced at a low frequency,

presumably by recombination in the *men* region. Consequently, the cloned segment of bacterial DNA appears to have one terminus in the *menD* gene, and λG68 can thus be designated a *λmenCB(D)* transducing phage. Previous studies on the *men* gene order were reinterpreted in favour of the order *nalA....menC..menB...menD...purF* as a result of the transducing activities of λG68.

Restriction studies established that the fragment of bacterial DNA cloned in λG68 is relatively large (11.5 kb) but not released by the corresponding restriction endonuclease, R.*Hin*dIII. It appeared that the formation of the recombinant phage genome had been accompanied by fusion of the bacterial DNA to the left arm of lambda with the loss of part of the vector arm including the R.*Hin*dIII site. Hybridization studies indicated that the cloned fragment is contained within large R.*Hin*dIII (21 kb) and R.*Eco*RI (14.1 kb) fragments. These are too large to be incorporated into the vectors used and this could explain why the λG68 genome appears to have arisen by a route involving the deletion of both bacterial and vector DNA.

The cloned fragment is large enough to encode several functions, in addition to the *men* gene products, but none of the neighbouring markers in the *E. coli* linkage map appears close enough to be transduced by λG68 (Bachmann and Low, 1980). The orientation of the bacterial fragment relative to the *E. coli* chromosome was not defined during this investigation. In order to exploit the potential of lambda-cloned genes for studying the biochemistry of menaquinone biosynthesis and the functional organization and expression of the *men* gene cluster, it would be useful to incorporate the entire *menD* gene. Attempts are currently being made to extend the transducing range of λG68 and to correlate the genetic and physical maps by subcloning specific fragments. It is expected that gene and gene-product amplification will facilitate studies on the early steps of menaquinone biosynthesis and lead to an understanding of the mechanisms regulating the biosynthesis of the electron carrier.

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