An isochorismate hydroxymutase isogene in *Escherichia coli*

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Abstract The pivotal step in enterobactin and menaquinone biosynthesis is the conversion of chorismate to isochorismate. Circumstantial evidence pointed to *Escherichia coli* isochorismate hydroxymutase isogenes being responsbible for this conversion. While the gene involved in enterobactin synthesis (*entC*) was known, the corresponding gene for menaquinone biosynthesis (*menF*) was not but has now been identified and sequenced. The amino acid sequence of MenF is 23.5% identical and 57.8% similar to that of EntC.

Key words: Menaquinone (vitamin K_2) biosynthesis; Isochorismate hydroxymutase; Isochorismate synthase; *entC*; *menF*; *Escherichia coli*

1. Introduction

(+)-*trans*-isochorismic acid (i.e. *trans*-3-[(1-carboxyethenyl)-oxy]-2-hydroxy-4,6-cyclohexadiene-1-carboxylic acid) is an important metabolite in the final stages of the shikimate pathway [1]. Isochorismic acid is a structural isomer of chorismic acid. Both isomers play a role as metabolic branch points for the post-chorismate pathways. The interconversion of both isomers (Fig. 1) is catalyzed by isochorismate hydroxymutase (i.e. isochorismate synthase, EC 5.4.99.6). This enzyme has been detected in protein extracts of plants [2,3] and microorganisms [4,5]. The gene (*entC*) encoding isochorismate hydroxymutase in *Escherichia coli* is part of the enterobactin gene cluster, which is iron-regulated [6,7].

Isochorismate, however, is also a precursor of the menaquinones [8,9] (Fig. 1). The biosynthesis of menaquinones starts with the conversion of isochorismate to 2-succinyl-6-hydroxy-2.4-cyclohexadiene-1-carboxylate (SHCHC), catalyzed by SHCHC synthase, MenD, and to *o*-succinylbenzoate (OSB), catalyzed by OSB synthase, MenC [10,11]. *men*D has recently been sequenced [10]. We have found a homology to entC in the 5′ upstream region of *men*D. Amplification by PCR of this region revealed the presence of an open reading frame which was cloned, sequenced and named *men*F. The gene exhibits a significant homology and similarity to *ent*C. Selective mutation of *ent*C and of both *ent*C and *men*F and complementation of the *ent*C deletion showed that *men*F is involved in menaquinone biosynthesis.

2. Materials and methods

2.1. Materials

E. coli strains MC4100 [12], XL1 Blue [13] and Y1089 [14] have already been described in the literature. Bacteria were grown in LB medium [14].

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2.2. Molecular biological techniques

Competent *E. coli* cells were prepared as previously described [15]. Chromosomal DNA from *E. coli* MC 4100 was isolated by the method of Kohlbrecher et al. [15]. Other DNA manipulations were based on standard procedures [14]. Polymerase chain reactions (PCR) were performed as previously described [17].

2.3. Construction of pCR1–1

The DNA upstream of *men*D was amplified by a published procedure [18] using oligonucleotides homologous to pSK(−) [19] [(+)B1:5′CAAGGCGATTAAGTGGGTAACGCCAG3′, (+)B2:5′CCCAGTCACGACGTTGTAAAACGACG3′] or homologous to *men*D [(−)G2:5′GGCCAGGATATAATCGCACGAARTTGC3′ and (−)G1:5′AATTCGCTTTGTTGTAATGAGAGATAGCC 3′]. As a template for PCR genomic DNA (*E. coli* MC 4100) was used after digestion with *Bgl*II and ligation into the *Bam*HI site of pSK(−). In the first step, PCR oligonucleotides G2/B1 were used which gave a 2,2 kbp fragment. Reamplification with G1/B2 gave a slightly shorter fragment. The larger fragment (G2/B1) was cut (*Hin*DIII/*Eco*RI) and ligated into pSK(−) (The *Hin*dIII site in Fig. 2 is part of the vector pSK(−)). The resulting plasmid was named pCR1- 1. Sequencing of the *men*F gene was carried out on both strands.

2.4. Construction of E. coli PBB7 by mutation of entC in E. coli Y1089

pKS1-20 [17] harbouring entC was digested in the presence of *Pvu*II and *Eco*RV and religated. The shortened *ent*C (Δ 336 bp) was removed (*Xba*I) from the plasmid and inserted into the *Xba*I site of pMAK705 [20]. The resulting plasmid pKS1–705 was employed in the mutagenesis of genomic *ent*C in *E. coli* Y1089 following published procedures [20]. The mutant was named PBB7.

2.5. Construction of PBB8 by mutation of menF in E. coli PBB7

pMAK705 [20] harbouring *men*F with a deletion (Δ 363 bp) was constructed from two PCR-generated DNA fragments. The *men*F-specific oligonucleotides were:
(+)CD1:5′CTGCGAAGCTTCTACCAGAGT3′ and
(−)CR7:5′TTGTCCAGCTGACCTTCGGCGAC 3′ for one fragment and
(+)CR8:5′TACCGCAGCTGTACTCGCTCTGCG and
(−)K18:5′GGCATCAGATCTAAACGCTCACGCTC3′
for the other. Mismatches intended to introduce restriction sites are underlined. The fragment obtained with the first pair of oligonucleotides was cut with *Hin*DIII/*Pvu*II, whereas the fragment obtained with the second pair of oligonucleotides was cut using *Pvu*II/*Pst*I. The fragments were ligated into pMAK705 after digestion with *Hin*DIII/*Pst*I. The resulting plasmid pCR1–21 was employed in the mutagenesis of genomic *men*F in *E. coli* PBB7 following published procedures [20]. This gave *E. coli* PBB8.

2.6. Quantitative analysis of menaquinone (MK8)

3 g (wet weight) bacterial cells were refluxed in methanol (50 ml, 30 min) and the extract filtered. Extraction was repeated twice (40 ml methanol, 15 min) and the filtrates combined. The extract was mixed with a solution (300 ml, 3 M) of NaCl in H_2O and extracted with petrol ether (B.Pt. 40–60°C) (3 × 40 ml). The upper layer was collected, washed with water, dried (Na_2SO_4) and then evaporated. MK8 was quantatively determined by HPLC (ET 250/4 Nucleosil 100–5 C18, Macherey-Nagel, Düren, Germany; solvent THF (15%), MeOH (85%), flow 1 ml·min^{-1}; photometer $\lambda = 254$ nm; MK8 retention time 8.6 min).

2.7. Analysis of enterobactin

This was done on Chromazurol S agar plates as described [21].

SSDI 0014-5793(95)01436-5

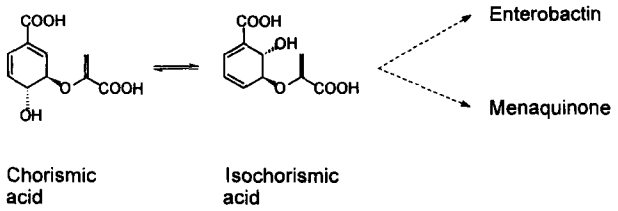

Fig. 1. Isochorismic acid as a metabolic branch point and its biosynthesis from chorismic acid catalyzed by isochorismate hydroxymutase (EntC or MenF).

3. Results

The 5′ end of *men*D was already known [10] and was found to be homologous to *ent*C. Therefore, RAGE-PCR (rapid amplification of genomic ends-PCR) [18] was employed to amplify the DNA strand upstream of *men*D. A PCR product (2.2 kb) was isolated and ligated into plasmid pSK(−). The resulting plasmid was named pCR1-1 (Fig. 2) and the insert sequenced. An open reading frame was found (Fig. 3) which overlapped the *men*D sequence published by Popp [10] by 114 base pairs. In contrast to Popp, however, we found two additional guanines in positions 637 and 729 (Fig. 3). The open reading frame encodes a protein of 37667.50 Da and exhibits 26.3% sequence identity at the DNA level, and 23.3% identity and 57.8% similarity at the amino acid level compared with *ent*C. Thus, it was reasonable to postulate another isochorismate hydroxymutase gene (*men*F) functionally related to menaquinone biosynthesis on account of its position next to menaquinone biosynthetic genes *men*D and *men*C [10,11]. The role of *men*F and *ent*C in menaquinone and enterobactin biosynthesis was investigated by selective mutation of *ent*C alone (this gave *E. coli* PBB7) and of both *ent*C and *men*F (this gave *E. coli* PBB8). Both genomic genes were replaced [20] by *men*F and *ent*C lacking either 336 bp (*ent*C) or 363 bp (*men*F), respectively. The deletion in *men*F is shown in Fig. 2.

In both cases, the proper positions of the resultant genomic deletions were verified by PCR in which oligonucleotides homologous to border regions of either *men*F or *ent*C were incubated with genomic DNA before (Y1089 and PBB7), and after (PBB7 and PBB8), the replacement reaction. In both cases (PBB7 and PBB8), the proper position of the deletions was evident from the appearance of shortened DNA fragments of the expected size.

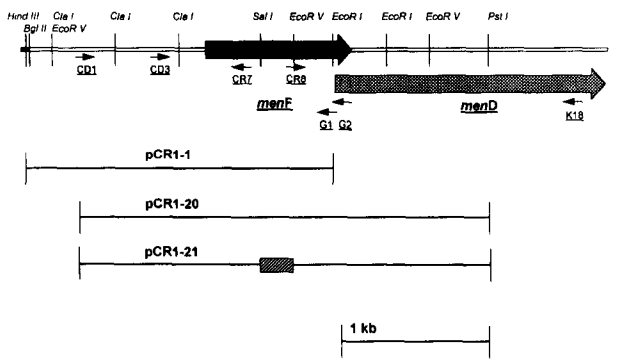

Fig. 2. Restriction sites, oligonucleotide-binding sites in the *men*F/*men*D gene region and inserts of plasmids pCR1-1, pCR1-20 and pCR1-21. The box in pCR1-21 indicates the deletion in *men*F.

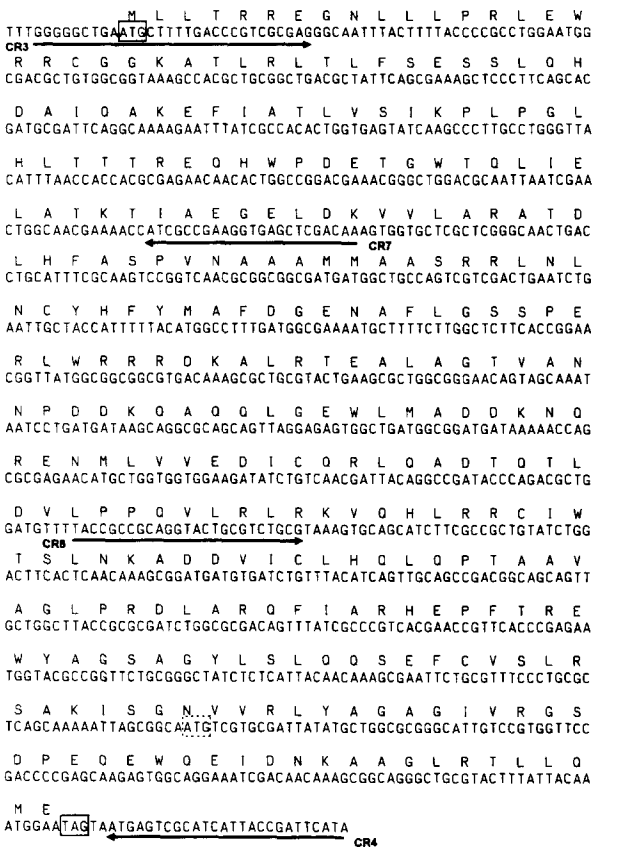

Fig. 3. DNA sequence (*men*F) and deduced amino acid sequence of a menaquinone-specific isochorismate hydroxymutase (MenF). The start codon of *men*D is boxed with a dotted line, the start and stop codon of *men*F with a solid line. Oligonucleotides (CR_n) mentioned in the text are indicated by arrows.

Mutants PBB7 and PBB8 grew aerobically and anaerobically although anaerobic growth of PBB8 ($menF^-$, $entC^-$) was retarded.

Enterobactin and menaquinone production was now determined in the parent strain (Y1089) and in its derived mutants (PBB7 and PBB8). The results are listed in Table 1. The strain lacking an intact *ent*C gene (PBB7) did not produce enterobactin. In contrast, menaquinone synthesis was observed. In the $entC^-/menF^-$ double mutant (PBB8), however, neither enterobactin nor menaquinone (MK8) were detectable. We conclude that *ent*C is responsible for enterobactin synthesis, whereas *men*F is involved in menaquinone formation.

Table 1
Enterobactin and menaquinone formation of *E. coli* Y1089 and its derived mutants (PBB7 and PBB8)

Strains	Y1089	PBB7 ($entC^-$)	PBB8 ($entC^-$, $menF^-$)
Enterobactin formation	Yes	n.d.	n.d.
Menaquinone (MK8) ($\mu g \cdot g^{-1}$ wet weight)	4.42	4.03	n.d.

Formation of enterobactin was checked on CAS agar plates. Menaquinone (MK8) production was determined after growth in liquid LB medium. Limit of detection $<0.2\ \mu g \cdot g^{-1}$ wet weight.
n.d. = not detectable.

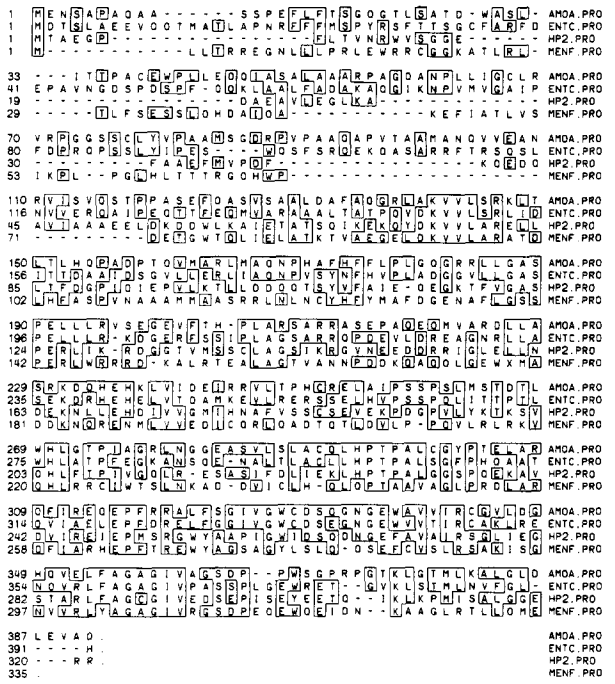

Fig. 4 Comparison between the inferred amino acid sequence of the *E. coli* menaquinone-specific isochorismate hydroxymutase (MenF) with that of the *E. coli* enterobactin-specific isochorismate hydroxymutase (EntC) and that of the *Amo*A protein of *A. hydrophila* and an open reading frame (HP2) found in *B. subtilis* (EMBL bank accession numbers M74538, M74182, M74183).

The function of *men*F as an isogene encoding isochorismate hydroxymutase was tested in the following experiment. The method in which a *men*F gene with a deletion was introduced into *E. coli* PBB7 (vide supra), lends itself to rescuing the replaced intact genomic *men*F gene [20] from the resultant PBB8. The plasmid (pCR1-20) (Fig. 2) carrying this gene was isolated. Its insert which is under control of the *lac* promotor covers the intact *men*F and additional upstream sequences (cf. Fig. 2) as confirmed by restriction site analysis and electrophoresis. When this plasmid was introduced into the enterobactin non-producing PBB7 (*ent*C$^-$) mutant, and enterobactin synthesis tested on CAS agar plates, restored enterobactin synthesis showed that *men*F (and its upstream sequences?) are able to cure the isochorismate deficiency. Complementation by pCR1-1, however, was not observed.

Plasmid pCR1-20 (Fig. 2) was also used to repeat the sequencing of *men*F. Thus, the sequence of *men*F given in Fig. 3 is based on a PCR-generated sequence and a genomic *men*F gene.

4. Discussion

Menaquinone (vitamin K_2) is detectable in *E. coli* after aerobic and anaerobic growth. Accumulation of menaquinone (MK8), however, is significantly stimulated in the absence of oxygen [22]. Menaquinone is an electron carrier involved in anaerobic ATP-generating redox reactions [23–25]. It also plays a role in the anaerobic biosynthesis of pyrimidines, porphyrins and succinyl CoA [26–28]. Thus, in bacteria only anaerobic functions of menaquinone are known.

By contrast, enterobactin, a phenolate siderophor, is essential under aerobic growth conditions, because in the presence of oxygen, iron occurs in the environment as the highly insoluble $Fe(OH)_3$. After acquisition by the microbial cell, the intracellular iron binds to the ferric uptake regulatory protein (Fur) which negatively regulates the enterobactin gene cluster [6]. It follows that menaquinone and enterobactin are required under completely different physiological conditions. Both compounds, however, are ultimately derived from isochorismic acid [5,7–9]. It is, therefore, reasonable that two isochorismate hydroxymutase genes exist in *E. coli* which are functionally and spatially associated with their respective menaquinone and enterobactin genes but differently regulated.

Since iron acquisition by living cells is a common problem, it is not surprising that genes homologous to *ent*C and hence also to *men*F (cf. Fig. 5), are known in bacteria other than *E. coli*. Thus, amonabactin is a phenolate siderophor produced by *Aeromonas hydrophila* [29]. This microorganism contains the amoA gene which is homologous to *ent*C and *men*F (Fig. 4). An open reading frame (HP2) of hitherto unknown function and homologous to *men*F, *ent*C and *amo*A has also been detected in the *men* gene region of *Bacillus subtilis* (Fig. 4).

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