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Ubiquinone biosynthesis

Cloning of the genes coding for chorismate pyruvate-lyase and 4-hydroxybenzoate octaprenyl transferase from *Escherichia coli*

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Chorismate pyruvate-lyase activity was detected in extracts of *Escherichia coli*. 4-Hydroxybenzoate was identified as the product of the enzymatic reaction by chemical derivatization and GC-MS analysis. The *ubiC* gene, coding for the chorismate pyruvate-lyase, was cloned and sequenced. The molecular weight of the gene product was calculated as 18,776 Da and confirmed by expression of the protein in *E. coli* minicells. The *ubiA* gene, coding for the 4-hydroxybenzoate octaprenyl transferase, was identified by sequence homology and complementation of a *ubiA*⁻ strain. It is located directly downstream of *ubiC* in a typical operon structure.

Ubiquinone; Chorismate pyruvate-lyase; *ubiC*; 4-Hydroxybenzoate polyprenyl transferase; *ubiA*; *Escherichia coli*

1. INTRODUCTION

4-Hydroxybenzoate (PHB) is a key intermediate in ubiquinone biosynthesis [1,2]. In *E. coli*, PHB is thought to be formed by a chorismate pyruvate-lyase reaction [3,4]. A ubiquinone-deficient mutant strain (AN244) unable to carry out this reaction has been isolated [4]; the corresponding gene was termed *ubiC* and located on the *E. coli* genome by interrupted mating experiments. On the current *E. coli* linkage map [5], *ubiC* is assigned a position at approx. 92 min. An attempt to purify the enzyme has apparently been made (J. D. Lawrence, PhD thesis 1973; cited in [1]), but has not been published. The gene *ubiA*, coding for the PHB octaprenyl transferase which catalyses the next step in ubiquinone biosynthesis, has been mapped to the same chromosomal locus [6].

We have now cloned and sequenced the *ubiC* and *ubiA* genes coding for the chorismate pyruvate-lyase and the PHB octaprenyl transferase.

Abbreviations: PHB, 4-hydroxybenzoate; ORF, open reading frame; RBS, ribosome binding site.

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2. EXPERIMENTAL

2.1. *Bacteria, media and DNA manipulation*

E. coli AN244, AN385, and AN92 are described in [4,7,8], respectively. For differentiation between *ubi*⁻ and *ubi*⁺ phenotypes, cells were cultured on succinate minimal medium [9], solidified by purified agar for plant cell culture (Sigma) and containing 20 μM thiamin; for *ubiC*⁻ complementation tests, 125 μM 4-aminobenzoate was added [4]. For the measurement of enzyme activities, AN92 was cultivated in glucose minimal medium [8]. pBluescriptKSII+ [10] and *E. coli* XL1Blue were obtained from Stratagene, pTZ19R from Pharmacia, and pQE10 from Diagen. Cloning was carried out according to standard procedures [11]; the Sanger method was applied for sequencing of single-stranded templates [12]. Minicell experiments were done according to [13]. The expression of the fusion gene in pQE10 followed the manufacturer's instructions. DNA and protein sequence analysis was carried out using the HUSAR programme.

2.2. *Enzyme extraction and purification*

Cells (1.1 g) were suspended in 7.5 ml Tris-HCl buffer (0.05 M, pH 8.0) and disrupted with a Branson sonifier. After centrifugation (47,000 × *g*), the supernatant was passed through a Sephadex G-25 column and equilibrated with the same buffer.

For enzyme purification, strain XL1Blue harbouring pALMU1 was cultured in glucose minimal medium [8], containing tetracycline (10 μg/ml) and ampicillin (50 μg/ml), to an OD_{600} of 1.5. The extract (see above) was adjusted to pH 8.6 and subjected to chromatography on DEAE Sephacel (elution: 0–0.35 M NaCl in 0.05 M Tris-HCl, pH 8.6). Active fractions were combined, adjusted to pH 8.0 and applied to a Blue Sepharose column. After washing with 0.05 M Tris-HCl, pH 8.0, the enzyme was eluted with 1.5 M KCl in the same buffer. Active fractions were concentrated and gelchromatographed on Sephadex G-150 (elution: 0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl).

2.3. *Enzyme assay*

The assay for chorismate pyruvate-lyase contained in a final volume of 500 μl: chorismate (concentration see Table I), 50 mM Tris-HCl, and enzyme protein. After 10 min at 37°C, 300 μl NaAc (0.75 M, pH 4) and 100 nmol 3-hydroxybenzoate as internal standard were added. The mixture was extracted with 1 ml EtOAc. The organic layer was evaporated and the residue was analysed by HPLC (Multospher RP18 column; solvent MeOH/H_2O/HCOOH (300:693:7) detection 254 nm).

3. RESULTS

3.1. *Detection of chorismate pyruvate-lyase*

In crude enzyme extracts of *E. coli* AN92 [8], conversion of chorismate to PHB could be detected by an HPLC assay; strain AN92 is deficient of chorismate mutase activity, which otherwise interferes with the measurement of chorismate pyruvate-lyase [4]. Chorismate also decomposes non-enzymatically to PHB [4], but enzymatic conversion clearly exceeded chemical decomposition (Table I). The product of the enzymatical reaction was identified as PHB by GC-MS analysis after diazomethane conversion into both its monomethylated and dimethylated derivatives. The chorismate pyruvate-lyase reaction was not dependent on metal cofactors.

3.2. *Cloning of the* ubiC *gene*

Genomic DNA of the wild type *E. coli* strain MC4100 was partially digested with *Sau*3a, ligated into pBluescriptKSII+, and transformed into the *ubiC*⁻ strain AN244, which is unable to grow on succinate as the sole carbon source [4]. Complementation of the *ubiC*⁻ phenotype was achieved by a plasmid (named pALMU1) with a 3.6 kbp insert. Transformation of pALMU1 into AN92 yielded a 38-fold increase of the chorismate pyruvate-lyase activity (Table I).

Several fragments of the pALMU1 insert were subcloned and tested for complementation. The smallest of the tested fragments capable of restoring UbiC activity was the 1,860 bp insert in the plasmid pALMU3 (Fig. 1).

Table I

Chorismate pyruvate-lyase activity in crude extracts from *E. coli* AN92

Enzyme extract	PHB formation [pmol/mg protein/min]
(1) AN92, complete assay	172
(2) AN92, without chorismate	<1
(3) AN92, heat-denatured enzyme	6
(4) AN92::pBluescriptKSII+, complete assay	114
(5) AN92::pALMU1, complete assay	4,375

Incubations were carried out as described in Section 2. Chorismate concentrations were 53 γM (experiments No. 1 and 3) or 170 γM (experiments No. 4 and 5).

Sequence analysis of the pALMU1 insert (Fig. 2) showed two non-overlapping, closely spaced ORFs of 606 bp and 870 bp, respectively. The localization of this fragment at the expected position of *ubiC* within the *E. coli* genome is confirmed by partial overlapping with a DNA sequence containing the *plsB* locus [14]. Since a fragment containing only a part of the first, but the complete second ORF could not complement the *ubiC*⁻ strain, we concluded that the UbiC protein is encoded by the first ORF, for which no significant homologies to other proteins could be detected.

The presumptive translation start codon at bp 500, which would result in a protein of 18,776 Da, is preceeded by the Shine–Delgarno RBS at bp 488. Translation from another possible ATG start codon at bp 389, lacking a consensus RBS, should result in a protein of 23,073 Da. Translation of pALMU3 in *E. coli* minicells using [^{35}S]methionine showed after SDS gel electropho-

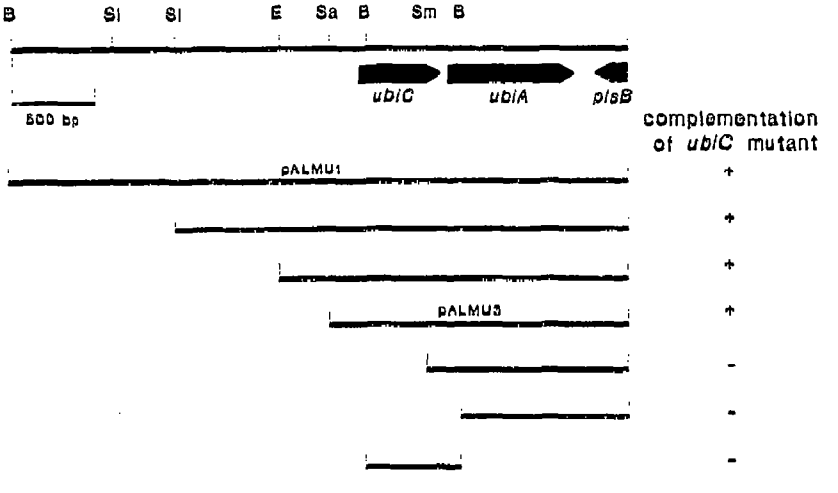

Fig. 1. Restriction map of the 3.6 kbp *Sau*3a fragment of genomic *E. coli* DNA, carrying the *ubiC* and *ubiA* genes. The ability of shortened DNA fragments to restore growth of the *ubiC*⁻ strain on succinate minimal medium is indicated by + or −. B, *Bam*HI; E, *Eco*RI; Sa, *Sac*I; Sl, *Sal*I; Sm, *Sma*I.

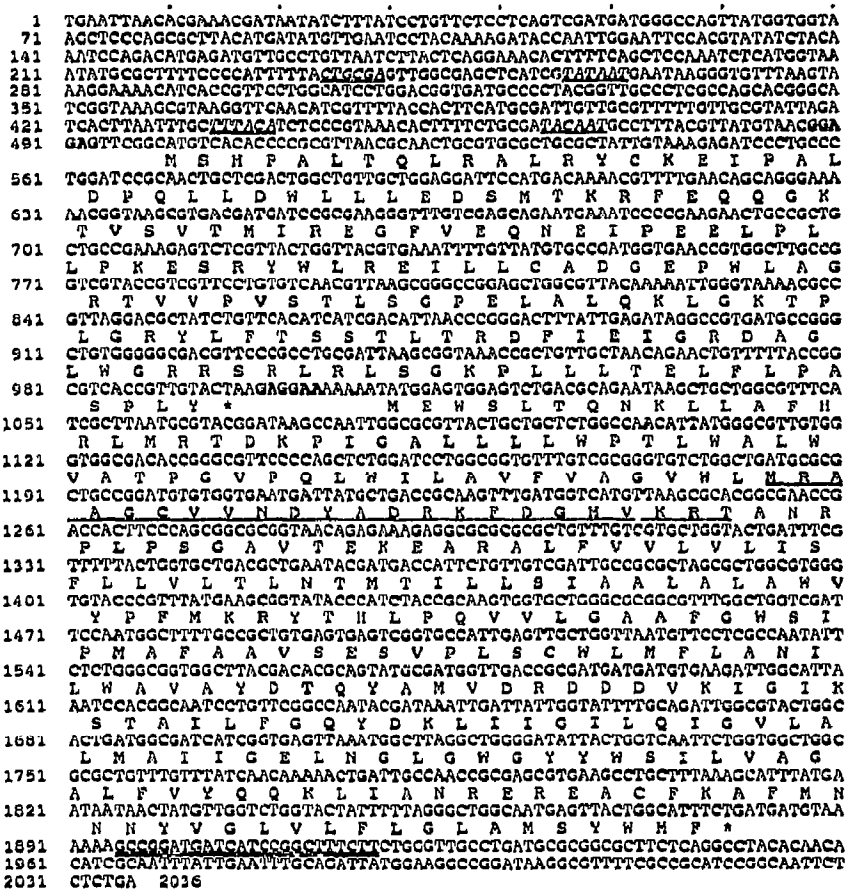

Fig. 2. Sequence of the *ubiC* and *ubiA* genes and proteins of *E. coli*. The RBS are set in bold letters, putative promotor sequences in underlined italics. The allylic pyrophosphate binding site is underlined and the terminator doubly underlined. The sequence has been submitted to the EMBL Data Library and given the accession number X66619.

resis a protein in the expected size of ca. 19 kDa (Fig. 3).

The *Bam*HI fragment from 563 to 1,153 bp, coding for 87% of the UbiC gene product, was ligated into pQE10 and overexpressed as a hexahistidyl fusion protein. The insoluble product could be dissolved in 8 M urea, and the hexahistidyl moiety allowed purification by Ni^{2+} affinity chromatography. The homogeneous protein showed the expected molecular weight of ca. 18 kDa in SDS gel electrophoresis and was used for antibody production in rabbit.

The intact native protein was expressed in *E. coli* XL1Blue transformed with pALMU1. It was purified approx. 100-fold by three chromatographic steps (see Section 2). A prominent band of the purified fraction showed a size of ca. 19 kDa in SDS gel electrophoresis and reacted with the antibody (data not shown).

A homology search of the translated second ORF, coding for a protein of 32,510 Da, in a protein sequence data base revealed on the amino acid level 35% identity and 61% similarity to the *COQ2A* gene product from *Saccharomyces cerevisiae* [15], the PHB polyprenyl transferase, which is essential in the biosynthetic pathway of ubiquinone in yeast. Both sequences include a highly conserved aspartate-rich region which has been proposed to be a binding site for allylic polyprenyl diphosphates [15]. Since this second ORF maps at the expected position of *ubiA* in the *E. coli* genome and shows a strong homology to the yeast enzyme with a similar catalytic function, we designated the gene *ubiA*. The identification was confirmed by complementation of the *ubiA*⁻ mutant strain AN385 with plasmids pALMU1 and pALMU3, both restoring growth of the bacteria on succinate minimal medium.

4. DISCUSSION

We have cloned and sequenced the structural genes *ubiC* and *ubiA* from *E. coli*. They were both identified by complementation of known mutants in each of the genes and, additionally, by determination of enzymatic activity for *ubiC* and sequence homology for *ubiA*. Both genes encode proteins involved in early steps of ubiquinone biosynthesis and are obviously organized as an operon. The presumed tranlation starts are preceeded by consensus RBS, and downstream of *ubiA* a GC-rich

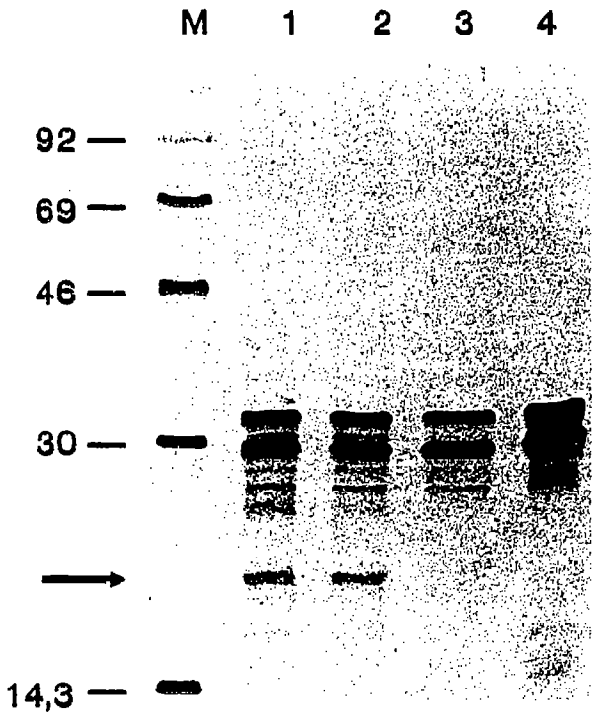

Fig. 3. Minicell expression of plasmids containing *ubiC* gene inserts. Lanes 1 and 2: pTZ19R with the ALMU3 insert in both orientations, respectively. Lane 3: as 1, with an internal deletion of the *Bam*HI fragment (bp 563–1153). Lane 4: pTZ19R vector control. M: molecular weight standard (in kDa). The arrow indicates the UbiC band at ca. 19 kDa. The expected UbiA gene product (ca. 32 kDa) cannot be separated from the plasmid encoded β-lactamase.

region with the ability to form a stem-loop structure followed by a poly-T sequence shows the typical features of a rho-independent terminator (bp 1,895–1,918). The promoter seems to be located in the region between bp 245 and the start codon, because a DNA fragment including only this upstream region and the structural genes showed gene expression independent of plasmid promoters in the minicell experiments. Consensus −35 and −10 promoter boxes are found in this sequence at bp 434 and 464, but with an unusually long spacing of 24 bp. A second possible promoter is located further upstream with a perfect −10 box at position 256, but exhibiting only weak homology to the −35 consensus.

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