JOURNAL OF BACTERIOLOGY, Apr. 1974, p. 41-45

Vol. 118, No. 1
Printed in U.S.A.

Biosynthesis of Ubiquinone in *Escherichia coli* K-12: Biochemical and Genetic Characterization of a Mutant Unable to Convert Chorismate into 4-Hydroxybenzoate

J. LAWRENCE, G. B. COX, AND F. GIBSON

Department of Biochemistry, John Curtin School of Medical Research, The Institute of Advanced Studies, Australian National University, Canberra, A.C.T., Australia

Received for publication 5 December 1973

A mutant strain of *Escherichia coli* unable to carry out the first specific reaction of ubiquinone biosynthesis, that is the conversion of chorismate into 4-hydroxybenzoate, has been isolated. The gene concerned maps at about minute 79 on the *E. coli* chromosome and has been designated *ubiC*. This gene is probably the structural gene for chorismate lyase since cell extracts from a transductant strain carrying the *ubiC437* mutant allele are unable to convert chorismate into 4-hydroxybenzoate and growing cells of the mutant do not form appreciable quantities of ubiquinone unless 4-hydroxybenzoate is added to the growth medium.

In *Escherichia coli* the first specific precursor of ubiquinone, namely 4-hydroxybenzoate, is formed from chorismate (7), which is also the precursor of other aromatic compounds (Fig. 1). The enzyme converting chorismate into 4-hydroxybenzoate plus pyruvate has been partially purified and the trivial name chorismate lyase proposed (J. Lawrence, Ph.D. thesis, Australian National University, 1973). In at least one strain of *Aerobacter aerogenes* (1) and in cells of higher organisms (14, 15) tyrosine may serve as a source of 4-hydroxybenzoate probably with 4-hydroxyphenylpyruvate as an intermediate. Previous studies with mutants of *E. coli* K-12 in which ubiquinone biosynthesis is impaired have shown the accumulation of various phenolic and quinone precursors of ubiquinone (3, 19, 23-25). The present paper describes the biochemical and genetic characterization of a mutant strain of *E. coli* unable to convert chorismate into 4-hydroxybenzoate and showing the phenotype expected for a strain lacking chorismate lyase activity. Such a strain would be predicted to (i) require 4-hydroxybenzoate for growth on a medium containing succinate as the sole source of carbon (2), (ii) not accumulate any of the known precursors of ubiquinone, and (iii) not form ubiquinone unless supplied with 4-hydroxybenzoate.

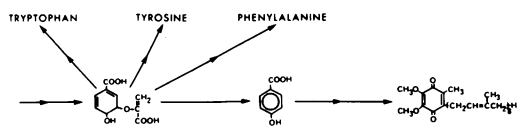

FIG. 1. *The first specific reaction of ubiquinone biosynthesis (the conversion of chorismate to 4-hydroxybenzoate) catalyzed by chorismate lyase and an outline of related metabolic pathways.*

MATERIALS AND METHODS

Bacterial strains. All strains used were derived from *E. coli* K-12. Cultures were maintained on nutrient agar and were subcultured monthly. The strains used and their relevant properties are set out in Table 1.

Media. The minimal medium used was a slight modification of that described by Monod et al. (12) and has been described previously (19). Agar and sterile solutions of the growth factor supplements were added to this medium as required. Solutions of tyrosine were sterilized by filtration. The nutrient broth used for genetic experiments was that described by Luria and Burrous (11).

Chemicals. All chemicals were obtained commercially with the exception of chorismic acid which was prepared as described previously (5).

Tests for growth response to 4-hydroxybenzoate. Tests for the requirement for 4-hydroxybenzoate on solid media were carried out on defined medium which included a relatively high concentration of 4-aminobenzoate. Mutants requiring 4-hydroxybenzoate generally respond to very low concentrations of this compound (2) and it is found that agar media often do not give clearly defined growth responses, presumably due to the presence of small amounts of 4-hydroxybenzoate. The early observations of Davis

TABLE 1. *Strains of E. coli used*

Strain	Relevant genetic loci[a]	Other information
AN244	*ubiC437*	Derived from strain AB259 after treatment with *N*-methyl-*N'*-nitrosoguanidine
AN245	*proA2, argE3, pheA1, tyrA4*	Derived from strain AN58 (9) by transduction
AN246	*proA2, pheA1, tyrA4, metA*$^-$	Derived from strain AN245 by transduction using PIkc grown on an *arg*$^+$ derivative of strain PA505MPE11 (23)
AN247	*proA2, pheA1, tyrA4, ubiC437*	Derived from AN246 by transduction using PIkc grown on AN244
AT2246	*leu-13, thr-13*	Hfr P10 (18)
AB2830	*aroC*$^-$	Pittard and Wallace (17)
AB3311	*metB*$^-$	Hfr Reeves

[a] The genetic nomenclature is that used by Taylor (20)

(4) that 4-aminobenzoate, at high concentrations, inhibited the growth of *E. coli* and that this inhibition could be reversed by 4-hydroxybenzoate, was explained recently by the observations of Hamilton and Cox (8). They found that 4-aminobenzoate probably acted as an inhibitor of ubiquinone biosynthesis by acting as a substrate for 4-hydroxybenzoate 3-octaprenyltransferase, thus inhibiting 3-octaprenyl 4-hydroxybenzoate synthesis. Therefore, the 4-hydroxybenzoate requirement of a multiple aromatic auxotroph on a succinate medium was tested on media containing a range of concentrations of 4-aminobenzoate. It was found that clear cut growth responses to 4-hydroxybenzoate were given by the mutant when 4-aminobenzoate was used at 50 μM, at which concentration the growth of the wild-type strain was not visibly affected. This concentration of 4-aminobenzoate was included in all solid media when testing for growth responses to 4-hydroxybenzoate. Not all samples of L-tyrosine were suitable for growth tests in liquid media, presumably due to the presence of 4-hydroxybenzoate.

Genetic techniques. The technique used for conjugation experiments was based on that described by Taylor and Thoman (21). Transduction experiments, using the generalized transducing phage P1kc, were carried out by the method of Pittard (16).

Streptomycin-resistant mutant. A spontaneous streptomycin-resistant mutant of strain AN244 was isolated after spreading 0.1 ml of an overnight culture on nutrient agar plates containing 0.2 mg of streptomycin sulfate per ml and incubating overnight at 37 C.

Growth of cells and preparation of cell-free extracts. Cultures were grown in minimal medium supplemented, as required, with the following growth factors: L-phenylalanine (0.2 mM), L-tryptophan (0.1 mM), L-tyrosine (0.2 mM), L-methionine (0.2 mM), L-arginine (0.8 mM), 2,3-dihydroxybenzoic acid (10 μM), and thiamine (0.02 μM). Glucose (37 mM) was used as carbon source. Cells were harvested in the early stationary phase of growth and were washed with cold 0.9% NaCl. Each gram (wet weight) of cells was suspended in 3 ml of cold 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0) and disrupted in a French press at 20,000 psi. The cell extracts were then centrifuged at 25,000 × *g* for 20 min at 4 C, and the supernatants were used as the cell-free extract. Proteins were estimated by using Folin reagent by the method of Lowry et al. (10).

Chorismate lyase activity. The activity of chorismate lyase was determined by using 0.5 ml of cell-free extract (about 15 mg of protein) in a reaction mixture consisting of the following: chorismic acid (2 μM), L-tryptophan (50 μM) and Tris-hydrochloride buffer (pH 8, 50 mM) in a final volume of 1 ml. Two controls were included with each determination. The first consisted of the reaction mixture as above with chorismic acid omitted, and the second, without enzyme preparation, was necessary because of the instability of chorismate which decomposes to form 4-hydroxybenzoate (6). The reaction mixtures and controls were incubated at 37 C and the reactions terminated by the addition of 0.5 ml of sodium acetate buffer (0.2 M, pH 4.0). The control tubes were then pooled and the samples were extracted with 5 ml of diethylether which, at pH 4.0, quantitatively extracts 4-hydroxybenzoate while leaving most of the remaining chorismic acid in the aqueous phase (I. G. Young, personal communication). The ether was dried over anhydrous sodium sulfate, and spectra were measured from 230 to 360 nm. 4-Hydroxybenzoic acid in ether has an absorption maximum at 252 nm (ϵ = 16,400). In some experiments, small amounts of 2,3-dihydroxybenzoate were formed. The latter compound also has a peak of absorption in the 250-nm region (ϵ = 7,200 at 252 nm) but the formation of 2,3-dihydroxybenzoate could be readily observed by the presence of a second peak at 320 nm (ϵ = 3,340) and the appropriate correction made.

Spectral determinations. Fluorescence spectra were measured by using an Aminco-Bowman spectrophotofluorimeter, and ultraviolet absorption spectra were recorded on a model 15 Cary spectrophotometer.

Determination of ubiquinone. The ubiquinone contents of cells were determined spectrophotometrically, after extraction of the cells with acetone, as described previously (13).

RESULTS

Isolation of a strain carrying a mutation affecting 4-hydroxybenzoate synthesis. Dur-

ing a search for strains of *E. coli* unable to form ubiquinone (2), a strain was isolated which would not grow with malate as the sole source of carbon, but would grow on glucose. This strain did not form ubiquinone when grown on a glucose medium, unless 10^{-4} M 4-hydroxybenzoate was present, suggesting that the reaction affected in ubiquinone biosynthesis was the conversion of chorismate to 4-hydroxybenzoate. However, the strain did not give a growth response if 4-hydroxybenzoate was included in a malate or succinate medium. This indicated that, in addition to a mutation affecting ubiquinone biosynthesis, there was an additional mutation(s) affecting the utilization of succinate or malate present in the original mutant. The mutant strain was female and an uninterrupted mating experiment with a wild-type Hfr strain was carried out and recombinants, able to grow on a succinate medium containing 4-hydroxybenzoate, were selected. One of these recombinants able to grow on the succinate medium only in the presence of 4-hydroxybenzoate was isolated (strain AN244) and the mutation causing the requirement for 4-hydroxybenzoate was designated *ubiC437*.

Mapping of the ubiC mutation. Interrupted mating experiments were carried out to locate the approximate position of the *ubiC437* allele on the chromosome of *E. coli*. The results of experiments, in which the female strain AN244 was mated with the Hfr strain AB3311 and selection carried out for recombinants (*ubiC*$^+$) able to grow on succinate as carbon source, are shown in Fig. 2. The *ubiC*$^+$ allele was transferred at about 17 min after pair formation, indicating a location at about minute 80 on the *E. coli* chromosome. The Hfr strain AT2246 which has its origin of transfer at about minute 79 (18) and transfers its chromosome in an anticlockwise direction (opposite to AB3311), was then used in an interrupted mating experiment. Strain AT2246 did not transfer the *ubiC* gene as an early marker. A co-transduction test was then carried out with strain AN246 as recipient and strain AN244 as donor. Examination of *metA*$^+$ transductants obtained showed that 11% (9 out of 80 colonies tested) were also *ubiC*$^-$. The *ubiC* gene was therefore located near minute 79. One of the *ubiC*$^-$ transductant strains (AN247) was retained for further characterization.

Characterization of the ubiC mutant. Tests for chorismate lyase in the *ubiC*$^-$ transductant strain, AN247, and the parent strain, AN246, were carried out with the results shown in Fig. 3. Determinations of enzymic activities which convert chorismate along the minor pathways of

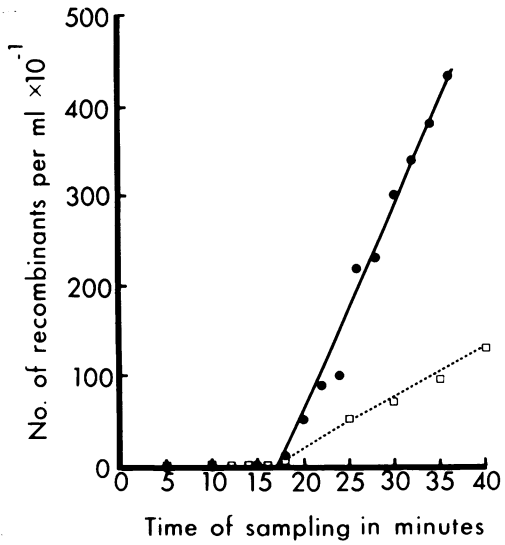

FIG. 2. *Kinetics of ubiC*$^+$ *recombinant formation in two separate experiments during matings between the Hfr strain AB3311 and the F*$^-$ *strain AN244.*

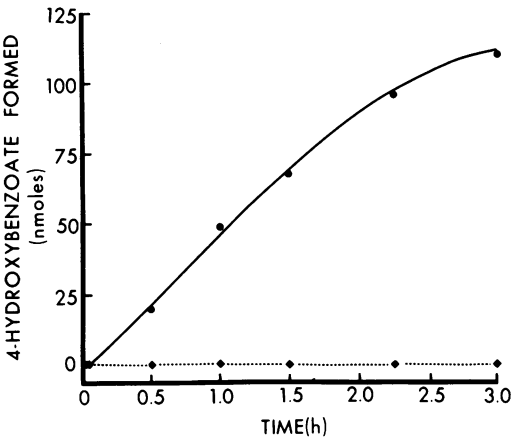

FIG. 3. *The formation of 4-hydroxybenzoate from chorismate by cell extracts of strain AN246* (●) *and strain AN247 (ubiC*$^-$) (◆). *Conditions as described in Materials and Methods.*

aromatic metabolism to 4-aminobenzoate (9) or 4-hydroxybenzoate need to be carried out by using cell extracts from strains with metabolic blocks preventing the metabolism of chorismate along the major pathways to the aromatic amino acids (see Fig. 1). In the present case, a strain (AN246) lacking both chorismate mutase-prephenate dehydratase and chorismate mutase-prephenate dehydrogenase activities was used and L-tryptophan was included in the reaction mixture to inhibit anthranilate synthetase. Strain AN247 (*ubiC*$^-$) showed no chorismate lyase activity while the parent strain

(AN246) formed 4-hydroxybenzoate from chorismate. The identity of the 4-hydroxybenzoate formed was confirmed by its fluorescence characteristics (activation, 300 nm; emission 346 nm; uncorrected), chromatography on cellulose and silica gel and the color given with *p*-nitraniline spray reagent (22). Strain AN247 (*ubiC*$^-$) gave a growth response to 4-hydroxybenzoate on succinate plates and the level of response in liquid medium was compared with that of an auxotrophic strain of *E. coli* (AB2830) unable to form chorismate. The *ubiC* mutant responded to the same levels of 4-hydroxybenzoate as did the multiple aromatic auxotroph (Fig. 4), in contrast to certain *ubiA* mutants which only respond to relatively high levels of 4-hydroxybenzoate (2, 23).

Cells of strain AN247 (*ubiC*$^-$) grown on a glucose-mineral salts medium in the absence of 4-hydroxybenzoate formed only 20 nmol of ubiquinone per g (wet weight) of cells compared with the 150 nmol formed by cells grown in the presence of 100 μM 4-hydroxybenzoate.

DISCUSSION

The data presented indicate that a mutation in a gene designated *ubiC* in *E. coli* K-12 results in the loss of chorismate lyase activity. This conclusion is based on enzymic studies and the requirement of 4-hydroxybenzoate for ubiquinone formation in a strain carrying the *ubiC437* allele. The small amount of ubiquinone formed by the *ubiC* mutant in the absence of added 4-hydroxybenzoate is probably due to the formation of traces of 4-hydroxybenzoate by the non-enzymic breakdown of chorismate which would be expected to accumulate in the strains used. The *ibiC* gene is therefore probably the structural gene for chorismate lyase.

The *ubiC* gene was shown by interrupted mating experiments with the Hfr strain AB3311 and transduction experiments, together with the fact that it was not transferred as an early marker by strain AT2466, to be located at about minute 79 on the *E. coli* chromosome. This is in the same region as the *ubiA* gene which codes for the enzyme, 4-hydroxybenzoate octaprenyltransferase (2, 23), which catalyzes the second specific reaction in ubiquinone biosynthesis. Whether the *ubiA* and *ubiC* genes are contiguous has yet to be determined. The locations, on the *E. coli* chromosome, of seven genes concerned with ubiquinone biosynthesis have now been determined (2, 3, 19, 23–25).

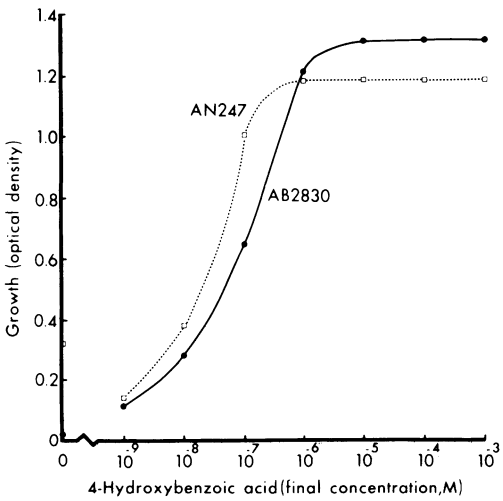

FIG. 4. *Growth responses of the multiple aromatic auxotroph AB2830 and AN247 (ubiC*$^-$*) to 4-hydroxybenzoate. The minimal medium contained succinate (20 mM) as carbon source and was supplemented with the mixture of growth factors used in the medium for the preparation of cell extracts (see Materials and Methods). Inocula, washed once in distilled water, were from 24-h nutrient agar slopes, and were added to give initial populations of about 10^6 cells per ml. Growth was measured after 20 h of incubation in shaken tubes at 37 C, by using a Spekker colorimeter with a neutral density filter.*

ACKNOWLEDGMENTS

We thank I. G. Young for helpful discussions and V. Frewin for technical assistance.

LITERATURE CITED

1. Cox, G. B., and F. Gibson. 1966. The role of shikimic acid in the biosynthesis of vitamin K_2. Biochem. J. **100**:1–6.
2. Cox, G. B., F. Gibson, and J. Pittard. 1968. Mutant strains of *Escherichia coli* K-12 unable to form ubiquinone. J. Bacteriol. **95**:1591–1598.
3. Cox, G. B., I. G. Young, L. M. McCann, and F. Gibson. 1969. Biosynthesis of ubiquinone in *Escherichia coli* K-12: location of genes affecting the metabolism of 3-octaprenyl-4-hydroxybenzoic acid and 2-octaprenylphenol. J. Bacteriol. **99**:450–458.
4. Davis, B. D. 1951. Inhibition of *Escherichia coli* by p-aminobenzoic acid and its reversal by p-hydroxybenzoic acid. J. Exp. Med. **94**:243–254.
5. Gibson, F. 1968. Chorismic acid. Biochem. Prep. **12**:94–97.
6. Gibson, F. 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. **90**:256–261.
7. Gibson, M. I., and F. Gibson. 1964. Preliminary studies on the isolation and metabolism of an intermediate in aromatic biosynthesis: chorismic acid. Biochem. J. **90**:248–256.
8. Hamilton, J. A., and G. B. Cox. 1971. Ubiquinone biosynthesis in *Escherichia coli* K-12. Accumulation of an octaprenol farnesylfarnesylgeraniol, by a multiple aromatic auxotroph. Biochem. J. **123**:435–443.
9. Huang, M., and F. Gibson. 1970. Biosynthesis of 4-aminobenzoate in *Escherichia coli*. J. Bacteriol. **102**:767–773.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

11. Luria, S. E., and J. W. Burrous. 1957. Hybridization between *E. coli* and shigella. J. Bacteriol. **74**:461-476.
12. Monod, J., S. Cohen-Bazire, and M. Cohn. 1951. Sur la biosynthese de la β-galactosidase (lactase) chez Escherichia coli. La specificite de l'induction. Biochim. Biophys. Acta **7**:585-599.
13. Newton, N. A., G. B. Cox, and F. Gibson. 1971. The function of menaquinone (vitamin K_2) in *Escherichia coli* K-12. Biochim. Biophys. Acta **244**:155-166.
14. Olson, R. E., G. H. Dialameh, R. Bentley, C. M. Springer, and V. G. Ramsey. 1965. Studies on coenzyme Q. Pattern of labelling in coenzyme Q_9 after administration of isotopic acetate and aromatic amino acids to rats. J. Biol. Chem. **240**:514-523.
15. Parson, W. W., and H. Rudney. 1964. The biosynthesis of the benzoquinone ring of ubiquinone from p-hydroxybenzaldehyde and p-hydroxybenzoic acid in rat kidney, *Azotobacter vinelandii*, and baker's yeast. Proc. Nat. Acad. Sci. U.S.A. **51**:444-450.
16. Pittard, J. 1965. Effect of integrated sex factor on transduction of chromosomal genes in *Escherichia coli*. J. Bacteriol. **89**:680-686.
17. Pittard, J., and B. J. Wallace. 1966. Distribution and function of genes concerned with aromatic biosynthesis in *Escherichia coli*. J. Bacteriol. **91**:1494-1508.
18. Schwartz, M. 1966. Location of the maltose A and B loci on the genetic map of *Escherichia coli*. J. Bacteriol. **92**:1083-1089.
19. Stroobant, P., I. G. Young, and F. Gibson. 1972. Mutants of *Escherichia coli* K-12 blocked in the final reaction of ubiquinone biosynthesis: characterization and genetic analysis. J. Bacteriol. **109**:134-139.
20. Taylor, A. L. 1970. Current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155-175.
21. Taylor, A. L., and M. S. Thoman. 1964. The genetic map of *Escherichia coli* K-12. Genetics **50**:659-677.
22. Young, I. G., F. Gibson, and C. G. Macdonald. 1969. Enzymic and non-enzymic transformations of chorismic acid and related cyclohexadienes. Biochim. Biophys. Acta **192**:62-72.
23. Young, I. G., R. A. Leppik, J. A. Hamilton, and F. Gibson. 1972. Biochemical and genetic studies on ubiquinone biosynthesis in *Escherichia coli* K-12: 4-Hydroxybenzoate octaprenyltransferase. J. Bacteriol. **110**:18-25.
24. Young, I. G., L. M. McCann, P. Stroobant, and F. Gibson. 1971. Characterization and genetic analysis of mutant strains of *Escherichia coli* K-12 accumulating the ubiquinone precursors 2-octaprenyl-6-methoxy-1,4 - benzoquinone and 2 - octaprenyl - 3 - methyl - 6-methoxy-1,4-benzoquinone. J. Bacteriol. **105**:769-778.
25. Young, I. G., P. Stroobant, C. G. Macdonald, and F. Gibson. 1973. Pathway for ubiquinone biosynthesis in *Escherichia coli* K-12: gene-enzyme relationships and intermediates. J. Bacteriol. **114**:42-52.