DEFECTS IN FORMATE HYDROGENLYASE IN NITRATE-NEGATIVE MUTANTS OF ESCHERICHIA COLI

Julia O'Hara and Clarke T. Gray

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire

and

Juan Puig and Francis Pichinoty

Laboratoire de Chimie Bacterienne, CNRS, Marseille, France

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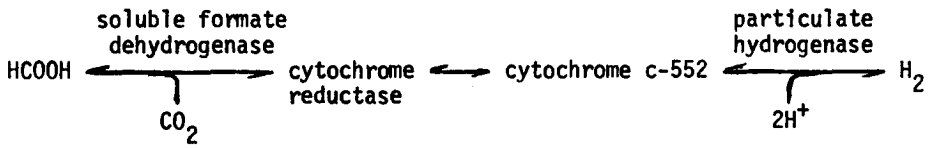
Recently, Piéchaud et al. (1967) reported the isolation of mutants of Escherichia coli K_{12} capable of anaerobic growth in the presence of $KClO_3$. These mutants did not reduce nitrate and were anaerogenic. Thus, the systems affected are nitrate reductase A, of which both nitrate and chlorate are substrates (Piéchaud et al., 1967), and formate hydrogenlyase. Both of these enzyme systems are complex, involve membrane bound components, and are not completely understood. Oxygen represses their formation but they are formed under anaerobic conditions where they play a role in anaerobic electron transport.

The present study was undertaken to increase our knowledge of the formate hydrogenlyase system by comparing the known or presumed components of this system in the parent and mutant strains. The model system which served as a guide in analyzing hydrogenlyase is that proposed by Gray and Gest (1965) for E. coli and other Enterobacteriaceae and, if cytochrome c_3 is substituted for c-552, corresponds with the hydrogenlyase system proposed for Desulfovibrio desulfuricans (Williams, Davidson and Peck, 1964).



Our results show that these mutants are characterized by two alterations in this system. Formate dehydrogenase (both soluble and particulate) was largely missing and excessive amounts of low-potential cytochrome c-552 were present.

Cytochrome c-552 levels were further investigated because of the proposed role of c-552 in nitrate reduction and because addition of nitrate to the growth medium stimulates production of this cytochrome in E. coli (Fujita and Sato, 1966b; Cole and Wimpenny, 1966). Addition of nitrate to the growth medium increased the amount of c-552 in the parent strain several fold as expected but lowered the level in the mutant. This observation supports the current idea that nitrate exercises a regulatory effect on the production of this cytochrome.

METHODS

Microorganisms: The CNRS parent $HfrP_{4X}$ strain of Escherichia coli K_{12} 303 (Chl-s) and its mutant, $HfrP_{4X}$ 376 (Chl-r), which is nitrate$^-$ and gas$^-$, were used. Another mutant, DMS 3-1, also nitrate$^-$ and gas-, independently derived from the Dartmouth strain of E. coli K_{12} using the method of Piéchaud et al. (1967) was used to confirm all experiments.

Culture conditions: The synthetic-mineral salts medium containing 0.2% glucose has been described (Gray et al., 1963). It was supplemented with 0.1% yeast extract for growth of the $HfrP_{4X}$ strains. Where indicated, 0.1% KNO_3 was added. Anaerobic cells were grown in 9 liter stationary stoppered bottles which were completely filled with medium and incubated at 37° C. Aerobic cells were grown in 2 liter baffled flasks containing 500 ml of medium on a platform shaker.

Preparation of cell suspensions and extracts: Cells were harvested in the late log phase by centrifugation, washed once with cold 0.01 M PO_4 buffer, pH 7.0, resuspended in buffer, and used as a cell suspension or for preparation of soluble and particulate fractions by a method modified from that of Fujita (1966). Cold cell suspensions were sonicated for 1 minute in a Branson Soni-

fier and centrifuged at 5000 x g for 10 minutes to remove whole cells and large debris. The supernatant fluid was then centrifuged at 105,000 x g for 3 hours. The resulting high speed supernatant fluid constituted the soluble fraction. The pellet, after gentle homogenization in buffer, was considered the particulate fraction. Protein determinations were made by the method of Lowry et al. (1951).

Cytochrome determinations: Difference spectra (hydrosulfite reduced vs. air or ferricyanide oxidized) were obtained using the Cary 14 spectrophotometer. The low potential cytochrome c-552 in the soluble fraction was determined by measuring the difference in absorbancy between the 552 mμ alpha peak and the trough at 538 mμ. The particle bound cytochrome b-559 was determined in the same fashion using the 559 mμ alpha peak and 540 trough as reference points. The extinction coefficients were those described by Fujita (1966).

Enzyme assays: Formate dehydrogenase was measured manometrically by observing the rate of CO_2 liberation from formate anaerobically with benzyl viologen as electron acceptor with the soluble enzyme and methylene blue as acceptor with the particulate fraction (Gest and Peck, 1955). The determinations were made with preparations obtained from anaerobically grown cells. Formate and succinate oxidation were determined by conventional manometric techniques in suspensions of whole cells grown aerobically (Gray et al., 1966).

RESULTS

The formate dehydrogenase activity in soluble and particulate fractions derived from anaerobically grown parent and mutant strains is shown in Table I. Both forms of the enzyme are very low in the mutant 376. The other enzyme component of hydrogenlyase, particulate hydrogenase, was found to be normal in this mutant, confirming the results of Piéchaud et al. (1967).

Since the particulate form of formate dehydrogenase is believed to play a role in aerobic formate oxidation, the ability of aerobic cells to oxidize formate was measured. The mutant also lacks the ability to oxidize formate in this manner. Succinoxidase, however, which resembles formate oxidase in

TABLE I

FORMATE DEHYDROGENASE ACTIVITY IN PARENT AND MUTANT STRAINS OF ESCHERICHIA COLI K_{12} Hfr P_{4x}

	Anaerobic Extracts		Aerobic Cells	
	Formate Dehydrogenase*			
	Soluble	Particulate	Formate Oxidase**	Succinate Oxidase**
303(Parent)	5.5	117.2	94.4	47.9
376(Mutant)	0.2	12.7	2.7	47.3

*µl CO_2 evolved/mg protein/hour **µl O_2 uptake/mg protein/hour

being membrane bound, involving cytochrome b-559, and not requiring pyridine nucleotide, was present in the mutant.

Figure 1 compares difference spectra of anaerobically grown whole cell suspensions of parent and mutant. The parent has a typical anaerobic E. coli spectrum, being dominated by b-559, with a broadness in the alpha region representing the c-552 content. The mutant pattern is dominated by the c-552 spectrum in both the alpha and beta regions. The latter spectrum shows very little evidence of b-559 contribution, although it will be shown that this mutant does produce b-559 anaerobically.

The amounts of c-552 and b-559 were measured in soluble and particulate fractions, respectively. Relevant data is given in Table II. Mutant 376 contains about 10 fold higher soluble concentrations of c-552 than its parent. Cytochrome b-559 is difficult to estimate in this mutant due to the large concentrations of c-552 residual in the pellet fraction. The mutant DMS 3-1, which was similar to mutant 376 in having high concentrations of c-552 in the soluble fraction, did not have this large amount of c-552 associated with its pellet. Thus the b-559 spectrum was not masked, and gave a value of 340 µµmoles cytochrome/ mg protein. It may be that the main difference between these two mutants is a higher total cytochrome c-552 content in the mutant 376. The value for b-559 in the 376 mutant, given in the table, should be con-

sidered minimal, and probably does not reflect an actual deficiency in the b-559 content.

Table II also shows the effect of nitrate on cytochrome levels. Addition of KNO_3 to the growth medium increases c-552 in the parent several fold, but decreases the mutant level to approximately that of its parent. Cytochrome b-559 also shows a nitrate effect and the mutant levels calculated are equivalent to those of the parent. This is further evidence that mutant 376 does not lack the ability to synthesize b-559 anaerobically.

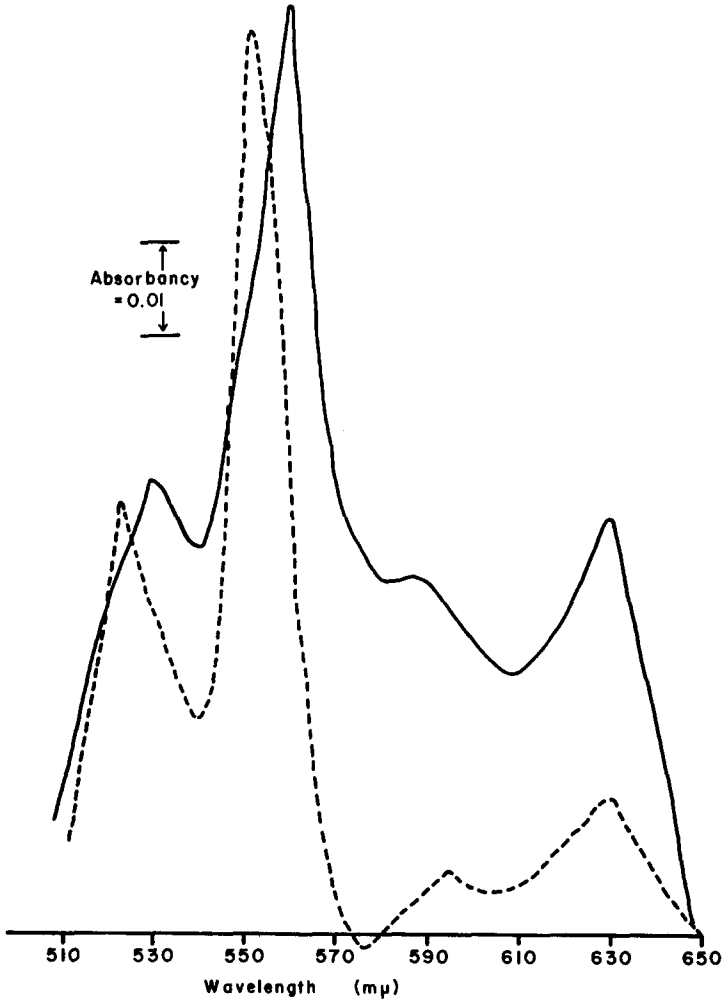


Figure 1. Difference spectra (reduced-oxidized) of anaerobically grown whole cell suspensions of mutant 376 (broken line) and its parent 303 (solid line). Protein concentration of each suspension was 9.8 mg/ml.

TABLE II

CYTOCHROME CONTENT OF EXTRACTS FROM ANAEROBICALLY GROWN PARENT AND MUTANT STRAINS OF *E. COLI* K_{12} Hfr P_{4x}

Growth Medium	Cytochrome c-552* (Soluble) 303 (Parent)	376 (Mutant)	Cytochrome b-559* (Particulate) 303 (Parent)	376 (Mutant)
Synthetic	16	154	338	150**
Synthetic +KNO_3	70	66	474	568

* μμmoles cytochrome/ mg protein
** approximated from shoulder height (see text)

DISCUSSION

Our results permit two conclusions about the nature of these mutants. First, that neither the nitrate nor the gas negative characteristic is due to a deficiency in c-552 content, which is, in fact, excessive. The negative effect of nitrate on the mutant which cannot reduce it, remains to be explained. Second, the anaerogenic character is due to the missing soluble formate dehydrogenase activity. The fact that the particulate form is also missing suggests that there is only one formate dehydrogenase enzyme, which has different properties and functions depending upon its localization.

Azoulay *et al.* (1967) in their investigations into the nature of the lesion in these mutants have proposed that a change in one species of membrane particle could cause the observed pleiotropic effect. They found that nitrate reductase activity in the parent strain sedimented with a particulate fraction which was absent in the mutant strain. Current studies on the formate dehydrogenase-bearing particle should help to clarify the relationships between the activities impaired by this mutation.

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