SHORT COMMUNICATION

A Mutant Defective in Electron Transfer to Nitrate in *Escherichia coli* K12

By VIOLAINE ORTH, MARC CHIPPAUX*
AND MARIE-CLAIRE PASCAL

Laboratoire de Chimie Bactérienne, C.N.R.S., 31 chemin Joseph Aiguier, 13274 Marseille
Cedex 2, France

(Received 21 September 1979)

A mutant of *Escherichia coli* K12 is described which is unable to reduce nitrate with a variety of physiological electron donors but which retains nitrate reductase activity with the artificial electron donor benzyl viologen. It is suggested that the affected gene, *chlI*, located close to *chlC*, encodes the cytochrome b_{NR} apoprotein.

INTRODUCTION

In *Escherichia coli*, the nitrate reductase is a membrane-bound enzyme which reduces nitrate with electrons derived from various physiological sources such as formate, lactate or NADH. This enzyme has been solubilized and purified and is a 32000 dalton molybdo-iron-sulphur protein which reacts with artificial electron donors such as benzyl viologen (Forget, 1974). It is now generally accepted that the native nitrate reductase is part of a functional complex involving two other proteins. One of these is a *b*-type cytochrome, called cytochrome b_{NR} , which is considered to be the physiological electron donor of the nitrate reductase (MacGregor, 1975). The biosynthesis of cytochrome b_{NR} and of nitrate reductase appears to be regulated coordinately because the concentration of both proteins increases several fold when bacteria are grown anaerobically in the presence of nitrate (Itagaki *et al.*, 1962). Moreover, this cytochrome might be involved in the regulation of nitrate reductase biosynthesis and might also be responsible for the attachment of the enzyme to the membrane (MacGregor, 1976). The third protein, which has not been identified, could also be involved in this attachment. The functional nitrate reductase complex is part of a membrane-bound electron transfer system involving other subunits and enzymes, such as formate, D-lactate and NADH dehydrogenases (Enoch & Lester, 1974; Wallace & Young, 1977; Sanchez-Crispin, 1977).

Many mutants have been reported to be unable to reduce nitrate but only those specifically affected in nitrate reduction lack the terminal enzyme coded for by the *chlC* gene (Puig *et al.*, 1969). This report describes the preliminary biochemical and genetic analysis of a nitrate reduction deficient mutant carrying an unaffected terminal enzyme.

METHODS

Strains and media. The strains used in this work are listed in Table 1. Medium A, used to test nitrate reduction, contained 0.4% (w/v) glucose, 0.2% (w/v) Bacto-peptone and 0.5% (w/v) KNO₃. Nitrite produced was detected according to the method of Show & De Moss (1968). Medium B, used to characterize nitrate reductase activity with benzyl viologen, was minimal medium (Abou-Jaoudé *et al.*, 1978) supplemented

Table 1. *Strains of Escherichia coli* K12 used

Strain	Sex	Genotype	Origin
LCB5	F ⁻	<i>his cysB trpC str</i>	KY496, Igarashi
LCB11	F ⁻	<i>thr-1 leu-6 lacY str tonA22 thi ana-1 nirC11</i>	Abou-Jaoudé
LCB155	F ⁻	<i>thr-1 leu-6 lacY str tonA22 thi-1 ana-1 nirC11 gal chlI155</i>	From LCB11, by NTG mutagenesis
LCB156	F ⁻	<i>thr-1 leu-6 lacY str tonA22 thi-1 ana-1 nirC11 gal chlI155 xyl</i>	From LCB155, by penicillin enrichment for bacteria unable to ferment xylose
LCB157	F ⁻	<i>lac-2 purB trpC pyrF his tyrA mal thi str chlI155</i>	From LCB459, by transduction with P1 propagated on LCB156 and selection for chlorate-resistance
LCB158	F ⁻	<i>lac-2 purB trpC pyrF his tyrA mal thi str chlC</i>	From LCB459, spontaneous chlorate-resistant derivative
LCB166	F ⁻	<i>lac-2 purB pyrF his tyrA mal thi str chlC</i>	From LCB158, by transduction to Trp ⁺
LCB459	F ⁻	<i>lac-2 purB trpC pyrF his tyrA mal thi str</i>	X459, Jacob
LCB468	HfrEC1	<i>(lac, pro XII)Δ Flac⁺ su⁺</i>	Beckwith
LCB915	HfrB7	<i>metB77 lam phx rel-54</i>	KA70, Wissman

with 0.1% (w/v) Bacto-peptone, 0.1% (w/v) yeast extract and 0.2% (w/v) glucose. Chlorate resistance was tested on agar plus rich medium supplemented with 0.2% (w/v) glucose and 10 mM-KClO₃.

Isolation of mutants. Mutants were isolated by a method described previously (Abou-Jaoudé *et al.*, 1978) except that nitrite was replaced by 1 mM-nitrate.

Assays. Nitrate reductase (benzyl viologen) activity was measured in cell-free extracts according to Pichinoty & Chippaux (1969). The determination of the rates of nitrate or nitrite reduction by whole cells with formate, D-lactate or ascorbate plus phenazine methosulphate (APM) has also been described previously (Abou-Jaoudé *et al.*, 1979). Activities are expressed as $\mu\text{mol nitrate reduced min}^{-1} (\text{mg dry wt})^{-1}$.

Growth was estimated turbidimetrically at 450 nm.

Genetic techniques. Mating and transduction experiments were performed according to Taylor & Thoman (1964) and Lennox (1955), respectively.

RESULTS

Mutant isolation

Anaerobic growth of *E. coli* in glucose minimal medium is independent of the presence of an exogenous electron acceptor because this organism is able to ferment sugars. However, *ana* strains are unable to grow anaerobically under these conditions and require the presence of nitrate or nitrite as electron acceptors. This phenotype has been exploited to isolate mutants no longer able to use nitrate as an electron acceptor but which exhibit a normal nitrate reductase activity when measured with benzyl viologen as electron donor. In order to prevent growth of the desired mutants on nitrite produced by wild-type bacteria during penicillin treatment in the presence of nitrate, the strain used, LCB11, was a *nir* derivative of an *ana* strain (Abou-Jaoudé *et al.*, 1978). After mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) according to Adelberg *et al.* (1965), cultures growing exponentially on nitrate under anaerobic conditions were treated with penicillin. Bacteria were then plated on to nutrient agar from which 7000 colonies were re-isolated and grown in tubes filled with minimal medium supplemented with 0.2% (w/v) glucose and 1 mM-nitrate. A 1 cm layer of sterile paraffin was added to ensure anaerobiosis. After incubation overnight, growth was estimated turbidimetrically and compared with that of strain LCB11 under the same conditions. Strains exhibiting very poor growth, or no growth at all, were subsequently cultured aerobically in glucose minimal medium and those unable to grow were discarded. The nitrate-reducing capacity of the remaining mutants was tested by measuring the quantity of nitrite which had accumulated after overnight growth in medium A. Only 18 strains did not produce nitrite normally. Among them, 5 strains unable to produce gas

from formate were eliminated because they were pleiotropically affected in nitrate reductase and formate dehydrogenase. The remaining 13 mutants which appeared to be specifically affected in nitrate reduction were tested for their nitrate reductase activity with benzyl viologen as electron donor. All but one were devoid of this activity and were considered to be defective in the *chlC* gene. The remaining strain, LCB155, which possessed high levels of nitrate reductase activity, was submitted to further analysis and the gene affected was tentatively designated *chlI*.

Characterization of the mutant LCB155

Enzymic activities. Nitrate reductase activity measured with benzyl viologen was enhanced fourfold in the mutant, but activities with formate, D-lactate and APM were only 8% of that of the wild-type. In both mutant and parental strains, 80% of the activity with benzyl viologen was found in the pellet after high-speed centrifugation of the crude extract for 150 min at 225000 g. The higher nitrate reductase activity in the mutant could well be due to the failure to accumulate nitrite which is highly toxic and inactivates many enzymes. A similar increase in nitrate reductase activity was observed when *E. coli* was grown anaerobically with nitrate in the presence of azide, an inhibitor of nitrate reductase (Chippaux & Pichinoty, 1970).

Cytochromes. Spectra of bacteria grown anaerobically with nitrate revealed that the cytochrome *b* concentration in the mutant was less than 50% that of the wild-type. The mutant was also apparently deficient in haem *d*, but because *d*-type cytochromes are not involved in electron transfer to nitrate, the absence of this cytochrome is likely to be due to a secondary effect of the lack of nitrite accumulation during growth.

Sensitivity to chlorate. Mutants isolated by the chlorate resistance method (Piéchaud *et al.*, 1967) either completely lack nitrate reductase activity or possess it at a very low level (Glaser & De Moss, 1972). Mutant LCB155 was also insensitive to chlorate during anaerobic growth, presumably because the nitrate reductase activity with physiological electron donors was insufficient to produce a lethal concentration of chlorite.

Identification test for *chlI* strains. In the process of isolation, the differentiation between strain LCB155 and the *chlC* mutants was based on the manometric determination of the nitrate reductase activity. For future mutant isolations as well as for genetic analysis, we developed a rapid differentiation test based on the fact that nitrate reductase is partially constitutive during anaerobic growth of *E. coli*. Each strain to be tested was grown in two test tubes containing 5 ml medium B; after overnight incubation at 37 °C, 0.1 ml 2% benzyl viologen and 0.1 ml 10 mM-sodium dithionite were added to one tube and 0.1 ml 0.1 M-sodium formate was added to the other. In each tube the reaction was started by the addition of 0.1 ml 1 M-sodium nitrate; after 10 min at room temperature the concentration of nitrite produced was measured. Under these conditions, the parental strain LCB11 produced nitrite in both tubes, strain LCB155 accumulated nitrite only in the tube with benzyl viologen, and *chlC* strains did not produce nitrite in either tube. These results indicated that *chlI* strains could readily be differentiated from either wild-type or *chlC* strains. However, the test could not readily differentiate *chlC* strains from a mutant carrying both the *chlC* and *chlI* mutations.

Genetic analysis

Different Hfr strains were mated with strain LCB156, a *xyl* derivative of LCB155. Unselected *chlI*⁺ recombinants were found in crosses with HfrB7 and HfrEC1, indicating that the mutation is located between the origins of transfer of these two Hfr strains. Furthermore, the *ana* character of these recombinants was closely linked with *chlI*. A transduction experiment with strain LCB156 *chlI ana* as donor and LCB5 *trpC* as recipient (Table 2) showed that *chlI* was 43% cotransducible with *trp*. Analysis of three-factor crosses located

Table 2. Analysis of *chlI* by three-factor transduction crosses

Donor	Recipient	Selected marker	Unselected markers	Transductants in class	
				No.	%
LCB156 (<i>chlI ana</i>)	LCB5 (<i>trpC</i>)	<i>trpC</i> ⁺	<i>ana chlI</i> ⁺	12	5.5
			<i>ana chlI</i>	81	37.5
			<i>ana</i> ⁺ <i>chlI</i>	5	2.3
			<i>ana</i> ⁺ <i>chlI</i> ⁺	118	54.6
LCB156 (<i>chlI</i>)	LCB158 (<i>chlC trpC</i>)	<i>trpC</i> ⁺	<i>chlI</i> ⁺ <i>chlC</i> ⁺	1	0.6
			<i>chlI chlC</i> ⁺	80	46.5
			* <i>chlI chlC</i>	91	53.0
			* <i>chlI</i> ⁺ <i>chlC</i>		
LCB166 (<i>chlC</i>)	LCB157 (<i>chlI trpC</i>)	<i>trpC</i> ⁺	<i>chlI</i> ⁺ <i>chlC</i> ⁺	1	0.27
			<i>chlI chlC</i> ⁺	200	54.5
			* <i>chlI chlC</i>	166	45.2
			* <i>chlI</i> ⁺ <i>chlC</i>		

* It was impossible to distinguish between these two recombinant classes.

chlI outside the *trp-ana* segment, the most probable gene order in the region being *trp-ana-chlI*.

The same gene order and a similar cotransduction frequency with *trp* has been reported for *chlC* (Casse *et al.*, 1976), so it appeared that genes *chlI* and *chlC* were very close to each other. To determine their relative order with respect to *trp*, strains carrying *chlC* or *chlI* together with the same *trpC* marker were prepared. In experiments in which strain LCB156 *chlI* was the donor and strain LCB158 *chlC* the recipient (Table 2), 0.6% of the selected *trp*⁺ recombinants had acquired the wild-type character. In the reciprocal cross, in which strain LCB166 *chlC* was the donor and strain LCB157 *chlI* the recipient, only 0.27% of the *trp*⁺ recombinants exhibit the wild-type phenotype. As already pointed out, *chlI chlC* and *chlI*⁺*chlC* classes of recombinants could not be distinguished from one another and so they cannot be taken into account in the three-point analysis. The most significant class is therefore the wild-type *chlC*⁺*chlI*⁺. The results indicate that both genes are very close to each other, but do not allow us to assign precisely their order relative to *trp*.

DISCUSSION

The method used for the isolation of the *chlI* mutant was based on the utilization of an *ana* mutant which is unable to reoxidize NADH produced anaerobically in the absence of an exogenous electron acceptor (Casse *et al.*, 1976). When nitrate is provided, electron transfer from NADH to nitrate takes place through the membrane-bound NADH dehydrogenase-nitrate reductase pathway and therefore allows the strain to grow. The fact that the *chlI* strain does not grow in these conditions indicates that this pathway is inoperative. However, the defect does not result from the loss of either the NADH dehydrogenase (because aerobic growth is unaffected) or the nitrate reductase enzymes (Young & Wallace, 1976).

NADH is not the only donor which can no longer supply electrons for nitrate reduction in the *chlI* strain; the activities measured with formate or with D-lactate are also strongly diminished. As the formate-nitrite and D-lactate-nitrite reductase activities were unchanged in the mutant, one can suppose that the mutation caused an alteration of a component common to all the electron transfer pathways leading to nitrate without affecting the nitrate reductase terminal enzyme itself. APM which supplies electrons at a relatively high redox potential ($E'_0 = +80$ mV) can be used for nitrate reduction (Sanchez-Crispin, 1977) but not for nitrite reduction (Abou-Jaoudé *et al.*, 1979) in *E. coli*. The point of entry of the electrons supplied by APM is probably at the level of, or just before, the cytochrome *b_{NR}*, and the nitrate reductase activity with this electron donor is also strongly reduced in the mutant. When wild-type strains are grown anaerobically in the presence of nitrate, at least two *b*-type

cytochromes are present in almost equal concentrations. One is specific for the formate dehydrogenase and the other is the cytochrome b_{NR} . The loss of 50% of the cytochrome b by the *chlI* mutant may be explained in two ways. If the concentration of every b -type cytochrome is reduced equally, one would then expect all activities involving these cytochromes to be affected. Such is not the case for the formate-nitrite reductase activity which requires the cytochrome b_{FDH} (Abou-Jaoudé *et al.*, 1979). The alternative possibility is that the mutant has lost only one of the b -type cytochromes: the only candidate is the cytochrome b_{NR} .

The results of the genetic analysis which locates *chlI* very close to *chlC* could lead one to suppose that the gene affected in the mutant is *chlC* itself. In this case, the alteration of the nitrate reductase could possibly prevent the linkage between the enzyme and its physiological donor cytochrome b_{NR} , without affecting the location of the enzyme in the membrane or its activity. Additionally one would have to explain the decrease in the amount of the b -type cytochromes. It is more likely, therefore, that *chlI* differs from *chlC* and encodes the cytochrome b_{NR} apoprotein. Similar nutritional factors regulate the synthesis of nitrate reductase and the cytochrome b_{NR} so one can propose that both the *chlC* and *chlI* genes are part of a common transcription unit. Experiments are in progress to determine whether this is correct.

We are grateful to Dr William W. Kay for helpful discussion of the manuscript.

REFERENCES

- ABOU-JAOUDE, A., PASCAL, M. C., CASSE, F. & CHIPPAUX, M. (1978). Isolation and phenotypes of mutants from *Escherichia coli* K12 defective in nitrite reductase activity. *FEMS Microbiology Letters* **3**, 235–239.
- ABOU-JAOUDE, A., PASCAL, M. C. & CHIPPAUX, M. (1979). Formate-nitrite reduction in *Escherichia coli* K12. II. Identification of components involved in the electron transfer. *European Journal of Biochemistry* **95**, 315–321.
- ADELBERG, E. A., MANDEL, M. & CHIN CHING CHEN, G. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788–795.
- CASSE, F., PASCAL, M. C., CHIPPAUX, M. & RATOUCHNIAK, J. (1976). Genetic analysis of mutants from *Escherichia coli* K12 unable to grow anaerobically without exogenous acceptor. *Molecular and General Genetics* **148**, 337–340.
- CHIPPAUX, M. & PICHINOTY, F. (1970). Les nitrate-réductases bactériennes. V. Induction de la biosynthèse de l'enzyme A par l'azoture. *Archiv für Mikrobiologie* **71**, 361–366.
- ENOCH, H. G. & LESTER, R. L. (1974). The role of a novel cytochrome b -containing nitrate reductase and quinone in the *in vitro* reconstruction of formate-nitrate reductase activity of *E. coli*. *Biochemical and Biophysical Research Communications* **61**, 1234–1241.
- FORGET, P. (1974). The bacterial nitrate reductases. Solubilization, purification and properties of the enzyme A of *Escherichia coli* K12. *European Journal of Biochemistry* **42**, 325–332.
- GLASER, J. H. & DE MOSS, J. A. (1972). Comparison of nitrate mutants of *Escherichia coli* selected by alternative procedures. *Molecular and General Genetics* **116**, 1–10.
- ITAGAKI, E., FUJITA, T. & SATO, R. (1962). Solubilization and properties of formate dehydrogenase and cytochrome b_1 from *Escherichia coli*. *Journal of Biochemistry* **52**, 131–141.
- LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.
- MACGREGOR, C. H. (1975). Anaerobic cytochrome b_1 in *Escherichia coli*: association with and regulation of nitrate reductase. *Journal of Bacteriology* **121**, 1111–1116.
- MACGREGOR, C. H. (1976). Biosynthesis of membrane bound nitrate reductase in *Escherichia coli*: evidence for a soluble precursor. *Journal of Bacteriology* **126**, 122–131.
- PICHINOTY, F. & CHIPPAUX, M. (1969). Recherche sur des mutants bactériens ayant perdu les activités catalytiques liées à la nitrate réductase A. III. Caractères biochimiques. *Annales de l'Institut Pasteur* **117**, 145–178.
- PIÉCHAUD, M., PUIG, J., PICHINOTY, F., AZOULAY, E. & LE MINOR, L. (1967). Mutations affectant la nitrate réductase A et d'autres enzymes bactériens d'oxydo-réduction. *Annales de l'Institut Pasteur* **112**, 24–37.
- PUIG, J., AZOULAY, E., PICHINOTY, F. & GENDRE, J. (1969). Genetic mapping of the *chlC* gene of nitrate reductase A system in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **35**, 659–662.
- SANCHEZ-CRISPIN, A. (1977). *Etude des systèmes respiratoires du nitrate et de l'oxygène dans les vésicules membranaires de Escherichia coli*. Thèse de Doctorat d'Etat, Université d'Aix Marseille II, France.
- SHOW, M. K. & DE MOSS, J. A. (1968). Localization and regulation of synthesis of nitrate reductase in *Escherichia coli*. *Journal of Bacteriology* **95**, 1305–1313.
- TAYLOR, A. L. & THOMAN, M. S. (1964). The genetic map of *E. coli* K12. *Genetics* **50**, 659–677.
- WALLACE, B. J. & YOUNG, I. G. (1977). Role of

quinones in electron transport to oxygen and nitrate in *Escherichia coli*. Studies with *ubi A⁻ men A⁻* double quinone mutant. *Biochimica et biophysica acta* **461**, 84–100.

YOUNG, I. G. & WALLACE, B. J. (1976). Mutations affecting the reduced nicotinamide adenine dinucleotide dehydrogenase complex of *Escherichia coli*. *Biochimica et biophysica acta* **449**, 376–385.