Molec. gen. Genet. 160, 225–229 (1978)

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A Mutation Leading to the Total Lack of Nitrite Reductase Activity in *Escherichia coli* K 12

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Summary. Mutants of *E. coli*, completely devoid of nitrite reductase activity with glucose or formate as donor were studied. Biochemical analysis indicates that they are simultaneously affected in nitrate reductase, nitrite reductase, fumarate reductase and hydrogenase activities as well as in cytochrome c_{552} biosynthesis. The use of an antiserum specific for nitrate reductase shows that the nitrate reductase protein is probably missing. A single mutation is responsible for this phenotype: the gene affected, *nir* R, is located close to *tyr* R i.e. at 29 min on the chromosomal map.

Introduction

Unbroken cells of *E. coli* reduce nitrite using glucose or formate as an electron donor (Abou Jaoudé et al., 1977). In cell-free extracts, two nitrite reductase activities have been described. One is a sulfite reductase (EC 1.8. 1.2) which uses NADPH as a donor and displays a gratuitous nitrite reductase activity (Lazzarini and Atkinson, 1961). The second is NADH-specific (EC 1.6. 6.4) and is essentially found in cells grown anaerobically in the presence of nitrite (Kemp and Atkinson, 1966; Coleman et al., 1976). Under these cultural conditions, the cells have been reported to contain a low potential c-type cytochrome, cytochrome c_{552} (Gray et al., 1963). As this cytochrome can be reoxidized by nitrite it has been implicated in nitrite reduction but its metabolic function has not yet been defined.

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Abbreviations: BV, Benzyl-Viologen; NTG, N-methyl-N-nitro-N-nitrosoguanidine; NR, nitrate reductase; NIR, nitrite reductase; FR, fumarate reductase; HYD, hydrogenase; CYT c_{552}, cytochrome c_{552}

Until now, the only mutants known to be affected on nitrite reduction are the *nir* A mutants described by Cole and Ward (1973); they have lost simultaneously cytochrome c_{552} and 90% of the nitrite reductase activity. Gene *nir* A has been located at 28.5 min on the chromosome of *E. coli*, near *cys*. B.

We already described a method allowing the isolation from a *ana* strain (Casse et al., 1976) of mutants affected in the reduction of nitrite based on their inability to use nitrite as an electron acceptor for growth (Abou Jaoudé et al., 1978). These mutants were tentatively called *nir*. As expected, most of the *nir* mutants retained the ability to use nitrate as an electron acceptor for growth. Two of them however, nir_{22} and nir_{23}, lost the ability to use both nitrite and nitrate for growth. The purpose of this work was to study these mutants, to find out whether they miss other activities or enzymes and whether they carry a single mutation.

Material and Methods

a) Strains

The strains used in this work are listed in Table 1.

b) Media

Minimal medium (Davis and Mingioli, 1950) was supplemented with glucose (2 g/l). The concentration of the required aminoacids or bases was 40 mg/l. 5 L-fluorotyrosine was used at a concentration of 10^{-4} M.

c) Isolation of Mutants

Mutants CB 22 and CB 23 were isolated as described by Abou Jaoudé et al. (1978). To isolate revertants from strain CB 22, the bacteria were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine according to Adelberg et al. (1965); samples of surviving

0026-8925/78/0160/0225/$01.00

Table 1. List of strains

Number	Sex type	Genotype	Origin
CB5	F^-	*his, cys B, trp C, str*	K1496 Igarashi
CB22	F^-	thr_1, leu_6, *lac* Y_1, ana_1, *ton* A_1, *str, nir* R_{22}	CB 900 NTG
CB23	F^-	thr_1, leu_6, *lac* Y_1, ana_1, *ton* A_1, *str, nir* R_{23}	CB 900 NTG
CB37	F^-	*tyr* R_{366}, *trp* A_{9605}, his_{85}, ilv_{632}, tsx_{84}/λ^-	JP 2 144 A.J. Pittard
CB40	F^-	thr_1, leu_6, *lac* Y_1, *ton* A_2, *tyr* R_{366}, ana_1, *nir* R_{22}, *str*	CB 22 X $P_1$37 *tyr* R
CB106	F^-	*met* B_1, *trp* A_{43}, *lac* Y_1, *mal* A_1, str_{134}, *chl C, tre*	F. Casse
CB459	F^-	lac_2, *pur B, trp C, pyr F, his, tyr A, mal* B_1,/λ, *str*	F. Jacob
CB900	F^-	thr_1, leu_6, *lac* Y_1, *ton* A_1, ana_1, *str*	M.C. Pascal

bacteria were suspended in glucosed minimal medium. Nitrate was added at the final concentration of 10^{-3} M and the incubation was done anaerobically. When growth was visible after 24 h, bacteria were reisolated and tested for nitrate reductase.

d) Transduction

P_1 Kc-mediated transduction experiments were performed according to Lennox (1955).

e) Testing of Characters

The ability to reduce nitrate was detected by the accumulation of nitrite after overnight growth in rich medium supplemented with 10^{-2} M nitrate.

The *nir* character was tested in 10^{-3} M nitrite supplemented nutrient broth, conditions in which all the nitrite is eliminated after overnight growth of the wild type strains.

In both cases, nitrite was characterized by the Griess Isolway reaction.

f) Preparation of Bacterial Extracts

After overnight growth in the appropriate conditions, the bacteria were harvested by centrifugation, washed with—and resuspended in—0.016 M phosphate buffer pH 7.0. The cells were disrupted by passage through a French press and the extract was submitted 15 min to centrifugation at 30,000 × g. The supernatant of this centrifugation constituted the crude extract. High-speed supernatant was obtained after 2 h of centrifugation at 110,000 × g.

g) Enzymes Assays

Nitrate reductase, fumarate reductase, formate dehydrogenase and hydrogenase activities were estimated according to Pichinoty (1969_a, 1969_b). Hydrogenase activity was measured as described by Chippaux and Forget (1972).

Nitrite reductase activity and cytochrome spectra were estimated as previously described (Abou Jaoudé et al., 1978).

Proteins were determinated by the method of Lowry et al. (1951).

Results

1. Biochemical Study of the Mutants

As pointed out in the Introduction, mutants nir_{22} and nir_{23} are unable to use nitrite or nitrate as an electron acceptor for anaerobic growth in minimal medium plus glucose. Moreover, they can no longer reduce either nitrite or nitrate during growth in semi-synthetic medium. However, they can still grow aerobically in glucose minimal medium and keep on accumulating gas in Durham test tubes. Washed suspensions of the mutants were used for assays of nitrite reduction with glucose or formate as donor whereas cell-free extracts were assayed for nitrate reductase, fumarate reductase, hydrogenase and formate dehydrogenase. The results are summarized in Table 2. As can be seen, almost all nitrite reductase activity is lost. In addition, nitrate reductase, fumarate reductase and hydrogenase activities are simultaneously lost. Formate dehydrogenase activity is quite normal whereas formate hydrogenlyase is very low as a result of the quite undetectable hydrogenase activity.

Table 2. Specific activities

Strains	CB900	CB22	CB23
Nitrite reductase			
with formate	1.15	0	0
with glucose	4.7	0	0
Nitrate reductase	59	1	0.5
Fumarate reductase	43	0	0
Hydrogenase	8.7	<0.5	<0.5
Formate dehydrogenase	6.5	9.3	25.2

Activities are expressed in μmoles of substrate/h/mg of protein except for nitrite reductase activities which are expressed in μmoles/h/mg of cells (dry weight)

It was interesting to determine whether the proteins corresponding to these enzymes are absent or present in a inactive form. Utilization of an immunoprecipitation technique (Heidelberg and Kendall, 1935) indicates that the material able to give cross reactions with an anti-serum specific for the nitrate-reductase of *E. coli* was present in the crude extract of the mutant at a concentration ten to twenty times lower than the concentration of this material in the wild type strain. This result gives a good presumption of the absence of the protein nitrate-reductase in the mutant CB 22.

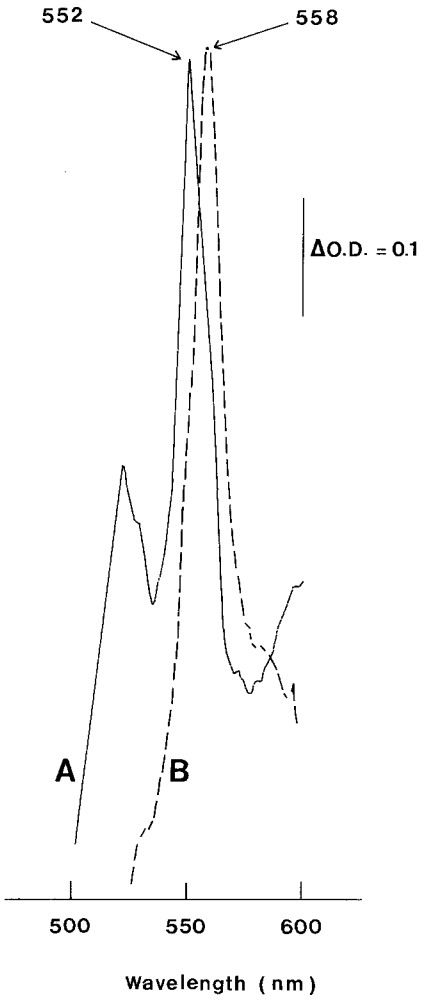

Fig. 1. Difference spectra of high-speed supernatant extracts of wild type strain CB900 (curve A) and mutant CB 22 (curve B). Spectrum of mutant CB 23, not shown on the figure, is similar to that of mutant CB 22. The sample in the test cuvette was reduced with sodium dithionite. The sample in the control cuvette was oxidized with potassium ferricyanide. The spectra were recorded at room temperature using a Beckman recording spectrophotometer

Both mutants being unable to reduce nitrite, it was important to determine whether they also lack cytochrome c_{552}. The cytochrome spectra of parental and mutant strains grown in the presence of nitrite are reported on Figure 1. As can be seen, high-speed supernatant spectrum of the parent shows a peak at 552 nm typical of cytochrome c_{552} and a shoulder at 558 nm due to small amount of cytochrome b_1 which has not been eliminated by the ultra-centrifugation. On the contrary, *HSS* spectra of the mutants display a single peak at 558 nm indicating that cytochrome c_{552} is absent. The presence of cytochrome b_1 in these mutants eliminates the possibility of a mutation in a gene involved in the biosynthesis of the heme, such as *hem* A, B or E.

Since the mutants do not require cysteine for growth it would seem that sulfite reductase, which is involved in cysteine biosynthesis, has not been affected. The contribution of this enzyme to the overall nitrite reductase activity must therefore be very low in the mutants and probably in the wild type as well. This biochemical study indicates that mutants CB 22 and CB 23 share the same phenotype, i.e.: NR^-, NIR^-, FR^-, HYD^-, CYT $c_{552}{}^-$. Such a phenotype can be explained either by different mutations each affecting one or more activities, or, alternatively, a single mutation. A genetic analysis was then performed to answer this question.

2. *Genetic Analysis of the Mutants*

As the *chl* C gene, which is considered as the structural gene for nitrate reductase, is 45% cotransducible with *trp* (Puig et al., 1969) preliminary experiments were performed to determine whether this gene was involved in the phenotype of the CB 22 and CB 23 mutants. P_1Kc mediated transduction experiments were done with strain CB 22 as donor and strain CB 5 as recipient. Analysis of the trp^+ transductants shows that only 1 to 3% of them had coinherited the inability to reduce nitrate. Furthermore the same transductants unable to reduce nitrate had simultaneously acquired the inability to reduce nitrite and to synthesize cytochrome c_{552}, i.e.: displayed the same phenotype as the donor strain CB 22. The low percentage of cotransduction with *trp* C and the pleiotropic phenotype of the transductants indicate that the *chl* C gene is not affected. This conclusion was fully confirmed in the transduction experiment where strain CB 22 was the donor and strain CB 106, *trp* A, *chl* C the recipient, Among the trp^+ selected transductants 60% are able to reduce nitrate indicating that strain CB 22 carries a *chl* C^+ allele.

Further experiments were done with strains carrying markers in the *trp* region. All transductants were tested for nitrate and nitrite reduction. When both characters were negative, cytochrome c_{552} was looked for by spectroscopy, and always found to be also missing; nir_{22} could be cotransduced with either *cys* B or *pyr* F. A three factors cross analysis (Table 3) reveals that none of the cys^+ or pyr^+ recombinants has simultaneously coinherited nir_{22} and *trp* C characters, indicating that these two markers are not on the same side relatively to *cys* B or *pyr* F.

More precise localization of the *nir* mutation was achieved using the *tyr* R marker (Camakaris and Pittard, 1973). Strain CB 40, carrying both the nir_{22} and *tyr* R markers was prepared by transduction using strain CB 37 as donor, strain CB 22 as recipient and

Table 3. Results of transduction experiments

Donor strain	Recipient strain	Selected marker	No. of transductants	Unselected markers	
				Type	Percent
CB 22	CB 5	*cys* B^+	274	*trp* C^- *nir*$^-$	20.4
				trp C^+ *nir*$^-$	0
				trp C^+ *nir*$^+$	19.3
				trp C^- *nir*$^+$	60.2
CB 22	CB 459	*pyr* F^+	170	*trp* C^- *nir*$^-$	1.2
				trp C^+ *nir*$^-$	0
				trp C^+ *nir*$^+$	21.8
				trp C^- *nir*$^+$	77.0
CB 40	CB 5	*cys* B^+	206	*nir*$^-$ *tyr* R^-	13.3
				nir$^+$ *tyr* R^-	9.3
				nir$^+$ *tyr* R^+	73.8
				nir$^-$ *tyr* R^+	3.4

selection on 5-fluoro-tyrosine plates. This strain CB 40 was used as donor in a transduction with strain CB 5 as recipient. Analysis of the *cys*$^+$ recombinants (Table 3) indicates that nir_{22} lies outside the *tyr* R-*cys* B segment. This is confirmed by the result of a transduction performed with strain CB 459 as recipient and strain CB 40 as donor: among the *tyr* R, *pyr*$^+$ selected transductants only 14% had coinherited the nir_{22} character. The most probable sequence in the *trp* region is therefore nir_{22}-*tyr* R-*pyr* F-*cys* B-*trp*. Similar experiments performed with strain CB 23 gave equivalent results and suggest that nir_{22} and nir_{23} are very closely linked.

3. Analysis of Revertant Strains

In order to demonstrate that the phenotype of CB 22 is due to a single mutation, it was necessary to select strains which had reverted for one character and to analyse these revertants for the other characters. Revertants able to utilize either nitrate or nitrite for anaerobic growth in glucose minimal medium could only be obtained after mutagenesis. All strains which had reverted for nitrate utilisation were found to have reverted simultaneously for nitrite utilisation. Two of them were then grown under the optimal conditions for induction of cytochrome c_{552} which was found at normal concentration in both revertants.

Discussion

We show here that mutants CB 22 and CB 23 are deficient in at least four activities: nitrate reductase, fumarate reductase, nitrite reductase and hydrogenase, and furthermore do not synthezise cytochrome c_{552}. Mutants partially defective in nitrite reductase activity had previously been reported (Cole and Ward, 1973; Abou Jaoudé et al., 1978) but they all had retained at least 10% of the wild type activity. Strains CB 22 and CB 23 are the only ones to be totally devoid of nitrite reductase activity, not taking into account the low and non specific activity displayed by sulfite reductase.

As this work was in progress we became aware of a paper by Lambden and Guest (1976) describing the *fnr* gene defined by mutations which lead to the loss of nitrate-reductase, fumarate-reductase and hydrogenase activities. In view of the genetic location of *nir* A and *fnr* (both genes precisely map at 28.5 min) a possible explanation for the phenotype of strain CB 22 would be the occurrence of two mutations, one affecting *fnr* and the other affecting *nir* A. If such were the case, one should have observed, even seldom, transductants bearing only part of the pleiotropic phenotype of strain CB 22 i.e.: having recovered some but not all characters. This was never found. Furthermore, strain CB 22 completely lacks nitrite reductase activity unlike all known *nir* A mutants. Finally, the reversion experiments very strongly suggest that a single mutation is responsible for the pleiotropic phenotype CB 22 or CB 23. Because of this pleiotropy we propose to name *nir* R the gene defined by nir_{22} and nir_{23}. Until the cytochrome c_{552} content and the nitrite-reductase activity of the *fnr* mutants are examined, one cannot tell if the genes designated *nir* R and *fnr* are the same or different. We suggest that they are in fact two designations for the same gene.

One could propose as do Lamden and Guest for *fnr* mutants that *nir* R mutants have a defect in some common component essential for electron transfer in the different systems. The lack of the nitrate-reductase protein itself makes this explanation very unlikely. On the other hand, the alteration of a structural component essential for the lost activities is not very likely either because it does not account for the lack of the cytochrome c_{552}, which is soluble.

At this stage of the discussion, it might be useful to recall some informations concerning the enzymes affected in *nir* R mutants. First they all are inducible: nitrate-reductase by nitrate (Pichinoty, 1966), fumarate-reductase by fumarate (Pichinoty, 1962), nitrite-reductase by nitrite (Kemp and Atkinson, 1966), hydrogenase by formate (Pascal, unpublished results) and cytochrome c_{552} by nitrite (Cole, 1968). Second, if no gene that can be considered as a structural gene for either nitrite-reductase or cytochrome c_{552} has been found to date, this is not the case for the other enzymes: *frd* A coding for fumarate-reductase, *chl* C coding for nitrate-reductase and *hyd* coding for

hydrogenase have been mapped on the chromosome of *E. coli* at 93 min, 26.5 min and 59 min, respectively (Spencer and Guest, 1973; Puig et al., 1969; Pascal et al., 1975). Third, the four enzymes are terminal enzymes involved in anaerobic electron transfer and the cytochrome c_{552} whose real function is not well understood has been implicated in nitrite reduction. In view of all these considerations it is not unreasonable to think that *nir* R could be a regulatory gene controlling the biosynthesis of nitrate-, fumarate- and nitrite reductase, of hydrogenase and cytochrome c_{552}.

Acknowledgments. We are greatly indebted to Dr. P. Forget for providing antiserum specific of nitrate reductase of *E. coli.*

References

Abou Jaoudé, A., Chippaux, M., Pascal, M.C., Casse, F.: Formate: a new electron donor for nitrite reduction in *Escherichia coli* K 12. Biochem. biophys. Res. Commun. **78**, 579–583 (1977)

Abou Jaoudé, A., Pascal, M.C., Casse, F., Chippaux, M.: Isolation and phenotypes of mutants from *Escherichia coli* K 12 defective in nitrite reductase activity. FEMS Letters (accepted for publication)

Adelberg, E.A., Mandel, M., Chein Ching Chen, G.: Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. biophys. Res. Commun. **18**, 788–795 (1965)

Bachman, B.J., Brooks Low, P., Taylor, A.L.: Recalibrated linkage map of *Escherichia coli* K 12. Bact. Rev. **40**, 116–167 (1976)

Camakaris, H., Pittard, J.: Regulation of tyrosine and phenylalanine biosynthesis in *Escherichia coli* K 12: Properties of the *tyr* R gene products. J. Bact. **115**, 1135–1144 (1973)

Casse, F., Pascal, M.C., Chippaux, M., Ratouchniak, J.: Genetic analysis of mutants from *Escherichia coli* K 12 unable to grow anaerobically without exogenous acceptor. Molec. gen. Genet. **148**, 337–340 (1976)

Chippaux, M., Forget, P.: Mesure de l'activité de l'hydrogénase. Biochimie **54**, 1217–1219 (1972)

Cole, J.: Cytochrome c_{552} and nitrite reduction in *Escherichia coli*: Biochim. biophys. Acta. (Amst.) **162**, 356–368 (1968)

Cole, J.A., Ward, F.B.: Nitrite reductase-deficient mutants of *Escherichia coli* K 12. J. gen. Microbiol. **76**, 21–29 (1973)

Coleman, K.J., Cornish-Bowden, A.J., Cole, J.A.: Purification and properties of nitrite reductase from *Escherichia coli* K 12. Proc. Soc. gen. Microbiol. **3**, 84 (1976)

Davis, B.D., Mingioli, E.S.: Mutants of *Escherichia coli* requiring methionine or vitamine B12. J. Bact. **60**, 17–28 (1950)

Gray, C.T., Wimpenny, J.W.T., Hughes, D.E., Ranlett, M.: A soluble c-type cytochrome from anaerobically grown *Escherichia coli* and various *entero-bacteriaceae*. Biochim. biophys. Acta (Amst.) **67**, 157–160 (1963)

Heidelberger, M., Kendall, F.E.: A quantitative theory of the precipiting reaction. III. The reaction between crystalline egg albumin and its homologous antibody. J. exp. Med. **62**, 697–720 (1935)

Kemp, J.D., Atkinson, D.E.: Nitrite reductase of *Escherichia coli* specific for reduced nicotinamide adenine dinucleotide. J. Bact. **92**, 628–634 (1966)

Lambden, P.R., Guest, J.R.: Mutants of *Escherichia coli* K 12 unable to use fumarate as an anaerobic electron acceptor. J. gen. Microbiol. **97**, 145–160 (1976)

Lazzarini, R.A., Atkinson, D.E.: A triphosphopyridine nucleotide-specific nitrite reductase from *Escherichia coli*. J. biol. Chem. **236**, 3330–3335 (1961)

Lennox, E.S.: Transduction of linked genetic characters of the host by bacteriophage P_1. Virology, **1**, 190–206 (1955)

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin reagent. J. biol. Chem. **193**, 265–275 (1951)

Pascal, M.C., Casse, F., Chippaux, M., Lepelletier, M.: Genetic analysis of mutants of *Escherichia coli* and *Salmonella typhimurium* deficient in hydrogenase activity. Molec. gen. Genet. **141**, 173–179 (1975)

Pichinoty, F.: Action répressive de l'oxygène sur la biosynthèse de la fumarique-reductase d'*Aerobacter aerogenes*. Experientia (Basel) **18**, 257–263 (1962)

Pichinoty, F.: Propriétés, régulation et fonction physiologique des nitrate-reductases bactériennes A et B. Enzymologia **26**, 176 (1966)

Pichinoty, F.: Recherche des activités formiate-oxydase, hydrogène lyase, hydrogénase et formiate déshydrogénase chez quelques *Entero-bacteriaceae*. Ann. Inst. Pasteur **117**, 3–15 (1969)

Pichinoty, F., Chippaux, M.: Recherches sur des mutants bactériens ayant perdu les activités catalytiques liées à la nitrate-réductase A. III. Caractères biochimiques. Ann. Inst. Pasteur **117**, 145–178 (1969)

Puig, J., Azoulay, E., Pichinoty, F., Gendre, J.: Genetic mapping of the *chl* C gene of nitrate reductase A system in *Escherichia coli* K 12. Biochem. biophys. Res. Commun. **35**, 659–662 (1969)

Spencer, M.E., Guest, J.R.: Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bact. **114**, 563–570 (1973)

Communicated by F. Gros

Received December 12, 1977