Nitrite Reductase-deficient Mutants of *Escherichia coli* K12

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SUMMARY

Mutants of *Escherichia coli* K12 have been isolated which reduce nitrite 3 to 30% as rapidly as the wild-type. Activities of reduced nicotinamide adenine dinucleotide (NADH)-nitrite oxidoreductase were lower in cell-free extracts of these *nirA** mutants than in the wild-type. The mutants grew on minimal agar, and their sulphite reductase activity was the same as in the wild-type.

Double mutants deficient in both nitrite and sulphite reductases were constructed, as well as recombinants which had regained one or both activities following recombination with *Escherichia coli* Hfr Hayes. The inability to reduce sulphite was due to an altered *cysB*[†] gene. Suspensions of *nirA cysB*⁺ and *nirA cysB* bacteria reduced nitrite at similar rates, showing that sulphite reductase (which is a gratuitous nitrite reductase) contributes little to the rate of nitrite reduction *in vivo*. Cytochrome *c*₅₅₂ was synthesized by *nirA*⁺ *cysB*⁺ double recombinants but not by *nirA cysB* or *nirA cysB*⁺ bacteria. This data suggests that cytochrome *c*₅₅₂ is involved in nitrite reduction in *E. coli* either as a component of NADH-NO₂⁻ oxidoreductase, or as an electron carrier whose synthesis is affected by the *nir* gene.

INTRODUCTION

This paper describes the isolation and biochemical characteristics of mutants of *Escherichia coli* K12 which lack reduced nicotinamide adenine dinucleotide (NADH)-nitrite oxidoreductase activity (EC. 1.6.6.4). We have estimated the relative contributions of this enzyme, and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent sulphite reductase, to the rate of nitrite reduction *in vivo*.

The incentive to isolate nitrite reductase-deficient mutants arose from unsuccessful attempts in several laboratories to define the metabolic function of cytochrome *c*₅₅₂ in *Escherichia coli*. This cytochrome is synthesized during anaerobic growth with nitrate or nitrite (Gray, Wimpenny, Hughes & Ranlett, 1963; Fujita, 1966; Cole, 1968; Cole & Wimpenny, 1968) and maximum concentrations occur in bacterial extracts which reduce nitrite rapidly (Fujita & Sato, 1966; Cole, 1968). Although the cytochrome and nitrite reductase were separated by gel filtration, the cytochrome rich fractions did not stimulate nitrite reduction by the partially purified enzyme (Cole, 1968). The possibility arose, therefore, that these bacteria synthesize other enzymes capable of reducing nitrite. The properties of an alternative cytochrome *c*₅₅₂-dependent enzyme could be studied more easily in mutants deficient in the very active NADH-nitrite oxidoreductase. Conversely, cytochrome *c*₅₅₂-deficient mutants should lack the alternative nitrite reductase, but retain the NADH-dependent activity.

* *nirA* denotes nitrite reductase-deficient mutants, which show low activities of NADH-nitrite oxidoreductase when grown anaerobically in nutrient broth supplemented with 10 mM-sodium nitrite.

[†] *cysB* pleiotropic negative mutants of cysteine biosynthesis, lacking reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent sulphite reductase activity.

Table 1. *Bacterial strains*

Strain	Genotype	Origin/source
<i>Escherichia coli</i> K12	Prototroph	J. W. T. Wimpenny
<i>Escherichia coli</i> Hfr Hayes	<i>met</i>	NCIB* 10235
CB40	<i>nirA str</i>	<i>Escherichia coli</i> K12 (NG-induced)
CB51	<i>nirA met str</i>	CB40 (NG-induced)
FB2	<i>nirA met cysB thyA str</i>	FB17 (spontaneous mutant)
FB17	<i>nirA met cysB str</i>	CB51 (NG-induced)
FB22	<i>nirA met str</i>	FB17 × HfrH
FB52	<i>met str</i>	FB17 × HfrH
JM203	<i>F' trp⁺ cysB⁺ thr leu trp his arg thi</i> <i>pro recA str</i>	M. Jones-Mortimer

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Genotypic abbreviations are: *arg*, arginine; *cys*, cysteine; *his*, histidine; *leu*, leucine; *met*, methionine; *nir*, nitrite reductase; *pro*, proline; *rec*, recombination deficient; *str*, resistant to streptomycin; *thi*, thiamine; *thr*, threonine; *thy*, thymine; *trp*, tryptophan. NG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Sulphite reductase (NADPH-sulphite oxidoreductase, EC. 1.8.1.2) can also reduce nitrite (Kemp, Atkinson, Ehret & Lazzarini, 1963). The contribution of this enzyme *in vivo* was estimated by measuring the rate of nitrite reduction by a double mutant deficient in both sulphite and nitrite reductase.

METHODS

Bacteria and media. The bacterial strains used in this work and their origins are shown in Table 1.

Escherichia coli K12 was maintained and inocula prepared as described previously (Cole & Wimpenny, 1966). Bacteria for enzyme studies were grown without aeration in 2 l conical flasks filled with medium which contained (per l distilled water): peptone, 2 g; KH₂PO₄, 7 g; NH₄Cl, 0.05 g; trace salts (Cole & Wimpenny, 1966), 5 ml. The pH was adjusted to 7.4 before sterilization, and sterile glucose was added immediately before inoculation. The inoculum was 1% (v/v) of an overnight culture grown aerobically in nutrient broth (Oxoid CM1), and the initial concentration of glucose was 0.4% (w/v). Sterile 1.0 M-sodium nitrite was added aseptically where indicated. All incubations were at 30 °C unless otherwise stated.

The minimal medium in which nitrate was the only nitrogen compound contained (per l distilled water); KH₂PO₄, 7 g; KNO₃ 1 g; trace salts, 5 ml. The pH was adjusted to 7.4 with NaOH before, and sterile glucose added after sterilization.

Isolation of mutants. Mutants were induced in log phase cultures either by treating with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) as described by Adelberg, Mandel & Chen (1965). Surviving bacteria were centrifuged at 10000g for 10 min, resuspended in sterile 50 mM-phosphate, pH 7.4, and then recentrifuged. The pellet was resuspended in 10 ml sterile nutrient broth+0.4% (w/v) glucose and 10 mM-KNO₃. For this and subsequent steps, bacteria were cultured without aeration so that the synthesis of nitrate and nitrite reductases would be derepressed. After 5 h of incubation, 0.1 ml samples were transferred to the glucose-minimal salts medium in which nitrate was the only nitrogen compound. Penicillin G (200 µg/ml) was added after 5 h of incubation, and 12 h later 0.1 ml samples were either transferred to fresh tubes of nitrate-supplemented broth to repeat the enrichment cycle, or plated onto selective media for screening. Single colony isolates were tested for nitrate or nitrite reductase activity by growing them micro-aerobically in test tubes containing 5 ml nutrient broth+0.4% (w/v) glucose and 10 mM-KNO₃ or NaNO₂. After 20 h

of growth, a drop from each culture was mixed on a white tile with 1 drop (approximately 50 μ l) 1% (w/v) sulphanilamide in 1 N-HCl and one drop 0.02% (w/v) *N*-1-naphthyl ethylenediamine dihydrochloride. A deep red coloration indicated the presence of nitrite. Nitrite reductase mutants did not reduce nitrite significantly during growth. Potential mutants were purified by at least two successive single colony isolations on nutrient agar (NA).

The double mutant FB17, deficient in both nitrite and sulphite reductase activities, was isolated when a culture of CB51 (*Escherichia coli* K12 *nirA met*) was mutagenized with NG and treated with penicillin as described by Clowes & Hayes (1968a). Colonies of surviving bacteria were replicated from NA on to minimal agar (MA) + methionine, MA + methionine and cysteine and NA. All amino acid supplements were at a final concentration of 25 μ g/ml, unless otherwise stated. Colonies which grew on MA + methionine and cysteine and NA but not on MA + methionine were restreaked on to all three media to obtain well isolated colonies of a Cys⁻ Met⁻ phenotype. The cysteine mutants were further characterized by plating on to MA + methionine and either sulphate, sulphite, sulphide or cysteine. One mutant, FB17, grew on sulphide and cysteine, but not on sulphate or sulphite. It was therefore deficient in sulphite reductase.

FB52 (*nirA*⁺ *cys*⁺) and FB22 (*nirA* *cys*⁺) were isolated in an interrupted mating experiment in which Hfr Hayes was the wild-type donor and FB17 the recipient. Colonies of *cys*⁺ recombinants were picked and each restreaked on to NA + 200 μ g/ml streptomycin. A single colony from each plate was tested for nitrite reductase activity and plated on to MA and MA + methionine. Colonies formed on MA + methionine, but not on MA.

FB2 is a *thyA* derivative of FB17, isolated as a spontaneous mutant resistant to 5 μ g/ml trimethoprim (Roth, Falco, & Hitchings 1962; Clowes & Hayes, 1968b). Colonies that grew on MA + methionine, cysteine, trimethoprim and thymine (50 μ g/ml) were restreaked on to plates of the same medium. A well isolated colony which did not grow on MA + methionine and cysteine was further purified on MA + methionine, cysteine and thymine.

Stocks of all mutants were established by streaking bacteria from a single well-isolated colony on to two or more NA slants. After 24 h at 37 °C, the phenotype of one of these cultures was retested as described above, the other(s) were stored at 2 °C.

Preparation of bacterial extracts. Bacteria at the end of logarithmic growth were harvested by centrifugation at 15 000g for 10 min. The pellet was homogenized with 50 mM-phosphate buffer, pH 7.4, resedimented by centrifugation at 10 000g for 10 min, and suspended in 50 mM-phosphate buffer to a density of 30 to 60 mg protein/ml. Bacteria were broken either by three 20 s periods of sonication with an M.S.E. (London) sonicator (probe diam. 1.0 cm) operating at 20 000 Hz, or by passage through a Hughes (1951) press cooled to -30 °C. High-speed supernatant (HSS) and cell membrane (CM) fractions were prepared as described previously (Cole & Rittenberg, 1971).

Enzyme assays. Rates of nitrite reduction by unbroken bacteria were assayed in open test tubes (15 $\times$ 1.6 cm) which contained 5 ml 40 mM-glucose, 50 mM-phosphate, pH 7.4, 1 mM-nitrite and approximately 10 mg of bacterial protein. When the pH dependence of nitrite reduction was determined, 50 mM-tris-HCl buffer replaced the phosphate in the pH range 7.5 to 9.0. Tubes were incubated at 30 °C for 5 min and the concentration of nitrite then determined with 0.2 ml samples taken at 2 to 5 min intervals. Rates of nitrite reduction were constant for 15 to 30 min if the bacterial suspension was preincubated as described, and for periods up to 2 h if test tubes were replaced by Thunberg tubes filled with 95% N₂/5% CO₂. Nitrite was assayed colorimetrically by an adaptation of the method of Snell & Snell (1949). Samples (0.2 ml) were mixed with 8.8 ml 1% (w/v) sulphanilamide in 1 N-HCl and 1.0 ml 0.02% (w/v) *N*-1-naphthyl ethylene diamine dihydrochloride. After 20 min at

Fig. 1. Rates of nitrite reduction by washed suspensions of mutant and wild-type bacteria. Experimental details are given in the legend to Table 2.

18 to 22 °C, the absorption at 540 nm was determined. This was proportional to the quantity of nitrite added over the range 0 to 0.2 μmol. An internal standard was included with each batch of assays.

Rates of pyridine nucleotide oxidation by nitrite and sulphite were estimated spectrophotometrically, and nitrate reduction by cell membranes assayed with formate as electron donor (Cole, 1968; Cole & Wimpenny, 1968). The sulphite reductase activity of strains grown anaerobically in minimal medium + 125 μg/ml methionine but without sulphate (to derepress the enzymes for cysteine biosynthesis) was determined as described by Ellis (1964).

Cytochromes were identified and measured at 18 to 22 °C with a Cary 14 spectrophotometer (Cole & Rittenberg, 1971). Spectra of samples cooled with liquid nitrogen were recorded with an Acta V spectrophotometer (Beckman-RIIC Ltd, Glenrothes, Scotland). Two FH-02 cuvettes fitted with Perspex windows and metal spacers (0.6 cm light path) were used with VLT2 Low Temperature Units at the normal sample position. The temperature of the samples was monitored with a TEM 1C temperature controller (Beckman-RIIC Ltd).

RESULTS

Isolation of nitrite reductase-deficient mutants

Wild-type *Escherichia coli* did not grow on MA in which either nitrate or nitrite was the sole nitrogen source, even when plates were incubated for a week under hydrogen in an anaerobic jar. This is contrary to reports from other laboratories (Murray & Sanwal, 1963; Fujita & Sato, 1967). No simple selective medium was found which distinguished between wild-type bacteria and nitrite reductase-deficient mutants. Bacteria which survived mutagenesis and penicillin enrichment in minimal salts medium with nitrate as sole nitrogen source, were therefore plated on to NA for screening. Four thousand colonies were subsequently subcultured into nutrient broth + glucose and nitrite: after 48 h of growth, 40 of these had reduced little of the nitrite. When a hot wire loop was plunged into wild-type cultures, effervescence was observed due to formate-hydrogenlyase activity of the bacteria. No effervescence was observed from the 40 putative mutants. This is consistent with an earlier

Table 2. Nitrite reduction by mutant CB40 and wild-type bacteria

Bacteria were grown anaerobically with 1 g/l KNO_3 (for the estimation of nitrate reductase activity) or 0.1 g/l NaNO_2 . Rates of nitrite reduction by suspensions of washed bacteria were assayed in open test tubes which contained 50 mM-phosphate (pH 7.4), 40 mM-glucose, 1 mM- NaNO_2 and approximately 2 mg bacterial protein. Nitrate reductase activity was assayed by measuring the rate of formation of nitrite in tubes which contained 40 mM-formate, 50 mM-phosphate (pH 7.4), 5 mM- KNO_3 and approximately 2 mg membrane protein. Nitrite-dependent rates of pyridine nucleotide oxidation by HSS extracts were determined in open cuvettes which contained 0.1 mM-reduced pyridine nucleotide, 2 mM- NaNO_2 , 50 mM-phosphate (pH 7.4) and 0.1 to 2 mg bacterial protein. Nitrite was omitted from the reference cuvette.

Assay	Strain	
	CB40	Wild-type
NO_2^- reduction by glucose*	0.4	3.3
NADH oxidation by NO_2^- †	0.8	10.0
NADPH oxidation by NO_2^- †	0.1	0.1
NO_2^- formed from NO_3^- *	5.0	4.0

* $\mu\text{mol NO}_2^-$ reduced or formed/mg protein/h.

† $\mu\text{mol pyridine nucleotide oxidized/mg protein/h}$.

report that nitrite inhibits and represses the formation of formate hydrogen lyase. The 40 nitrite reductase-deficient isolates were wild-type with respect to nitrate reductase, and would not grow on NA + 20 mM-glucose and 10 mM- KClO_3 . Thirty-five of these cultures retained the mutant phenotype after 10 subcultures on NA.

Nitrite reductase activities of *nirA* mutants

The nitrite reductase activities of suspensions of the wild-type and CB40 bacteria were compared (Fig. 1). Although both reduced nitrite at a linear rate, the mutant was less active than the wild-type (0.4 μmol nitrite reduced/mg protein/h compared with 3.3 μmol /mg protein/h for the wild-type). Rates of nitrite reduction by the other mutants varied from 0.1 to 1.0 μmol /mg protein/h.

It seemed possible that the low nitrite reductase activity of CB40 batch cultures might be due to wild-type revertants selected during anaerobic growth with nitrite. However, this activity was reproducible and independent of the volume of the culture (and therefore independent of the length of time the hypothetical selective pressure was exerted). In one experiment, bacteria from a 10 l culture were diluted with sterile nutrient broth and plated on to NA to obtain well-isolated colonies. Eighty of these colonies were of the mutant phenotype; none were wild-type.

The pH optimum for nitrite reduction was 8.0 to 8.2 with both mutant and wild-type bacteria. We were therefore unable to establish that the residual activity of CB40 was due to a previously unrecognized enzyme with a different pH optimum.

Although the data from Fig. 1 gives an accurate estimate of the nitrite reductase activity of bacteria growing under laboratory conditions, it indicates neither the number of different pathways involved, nor the nature of the electron donor. Two nitrite reductase activities have previously been assayed in cell-free extracts. One, a sulphite reductase, requires NADPH and is a gratuitous nitrite reductase (Lazzarini & Atkinson, 1961; Kemp *et al.* 1963); the other is NADH-specific, and is most active in bacteria grown anaerobically with nitrate or nitrite (Fujita & Sato, 1966; Cole, 1968). Low activities of the NADPH-specific enzyme were detected in HSS extracts of the mutant and the wild-type (Table 2).

The reduction of one molecule of nitrite to ammonia requires three molecules of NADPH.

Table 3. *Nitrite and sulphite reductase activities of the double mutant FB17 and two cys⁺ recombinants*

The sulphite reductase activities were assayed with HSS extracts of bacteria grown aerobically with 125 μ g/ml methionine without sulphate or cysteine. Bacteria for nitrite reductase assays were grown anaerobically with 0.1 g/l NaNO₂. Details of these assays are given in the legend to Table 2.

Assay*	Strain and genotype		
	FB17 <i>nirA cysB</i>	FB22 <i>nirA cysB⁺</i>	FB52 <i>nirA⁺ cysB⁺</i>
NO ₂ ⁻ reduction by glucose	0.34	0.47	2.4
NADH oxidation by NO ₂ ⁻	0.85	0.54	10.7
S ²⁻ formed from SO ₃ ²⁻ and NADPH	< 0.001	0.08	0.09

* Units are μ mol/mg protein/h.

By comparing lines 1 and 3 of Table 2, it can be seen that the overall NADPH-dependent nitrite reductase is 100 times slower than the rate of nitrite reduction by unbroken bacteria. As all of the mutants grew as well as the wild-type on MA in which sulphate was the only sulphur compound, we conclude that they have normal sulphite reductase activities. In contrast, the NADH-specific activity was much reduced in the mutants (Table 2) and correlated with the rates of nitrite reduction by unbroken bacteria.

The synthesis of the NADH-specific enzyme appeared to be derepressed during anaerobic growth of the wild-type bacteria with nitrate or nitrite. The activity of this enzyme was five-fold greater in extracts of bacteria grown in the presence of these nitrogen compounds. In contrast, the nitrite reductase activity of CB40 was unaffected by the presence or absence of nitrite or nitrate.

Contribution of sulphite reductase to nitrite reduction in vivo

In order to assess the contribution of the non-specific sulphite reductase *in vivo* to the rate of nitrite reduction, double mutants were constructed deficient in both NADH-nitrite and NADPH-sulphite oxidoreductases. FB17, one such double mutant, was unable to grow when either sulphate or sulphite was the sulphur source, but grew on MA + sulphide or cysteine. In interrupted mating experiments in which Hfr Hayes was the wild-type donor and FB2, a thymine auxotroph of FB17, the recipient, *cys*⁺ recombinants were obtained over 30 min before *thy*⁺ recombinants. The *thyA* gene is located at min 55 on the *Escherichia coli* linkage map, close to the *cysCPHQ* genes and 30 min later than the pleiotropic *cysB* gene at min 25 (Taylor, 1970). Finally, in a sexduction experiment with JM203, an F' *cysB*⁺ strain of *Escherichia coli*, *cys*⁺ recombinants were obtained following episomal transfer. On the basis of these experiments it was concluded that FB17 carries a mutation in the *cysB* gene and lacks NADPH-sulphite oxidoreductase.

The nitrite reductase activities of FB17 are shown in the first column of Table 3, with similar data for recombinants isolated during an interrupted mating experiment with Hfr Hayes as the donor (columns 2 and 3). These strains have regained one or both of the reductase activities, and were isolated by selecting *cys*⁺ recombinants. The sulphite reductase activity was low in each strain even when bacteria were grown under optimal conditions for the derepression of enzymes for cysteine biosynthesis (in the presence of high concentrations of methionine, but not cysteine or sulphate). Once again the glucose- and NADH-supported rates of nitrite reduction for the mutants were only 15% of the wild-type. Only small changes

Fig. 2. Difference spectra of high-speed supernatant (HSS) extracts of *Escherichia coli* K12 FB17, FB22 and FB52. The bacterial extract (2.0 ml) was transferred to two cuvettes. A few grains of sodium dithionite were added to the test sample, and one drop (approximately 0.05 ml) of 10 mM-potassium ferricyanide was added to the control cuvette. Spectra were recorded 18 to 22 °C with a Cary 14 spectrophotometer. The length of the vertical bar represents an absorbancy increment of 0.01. The protein concentrations of the three HSS samples were 28.7 mg/ml (FB17), 22.7 mg/ml (FB22) and 25.4 mg/ml (FB52).

resulted from the change from *cysB* to *cysB*⁺. We conclude that NADPH-sulphite oxidoreductase contributes little to the rate of nitrite reduction *in vivo*.

Cytochrome spectra

FB17, FB22 and FB52 were broken with the Hughes (1951) press and centrifuged to give HSS extracts essentially free from membrane contamination.

HSS spectra from *nirA*⁺ bacteria were unlike those from *nirA* strains (Fig. 2). The wild-

type FB52 showed a symmetrical peak at 552 nm typical of cytochrome c_{552} in the absence of cytochrome b_1 . Spectra of *nirA* mutants were difficult to record because protein precipitated from the oxidized sample when ferricyanide was added. Furthermore, spectra below 540 nm were dominated by light absorption of the oxidized forms: the β absorption bands of the cytochrome(s) were therefore poorly defined. However further studies with freshly prepared cell-free extracts, and carefully controlled oxidation, gave spectra showing a broad band in the 556 to 560 nm region with a peak at approximately 558 nm.

The broad α absorption of FB17 and FB22 extracts might have been a composite peak due to low concentrations of both cytochromes b_1 and c_{552} . If this explanation were correct, two discrete α bands would be seen in spectra recorded at -190°C . At this temperature, wild-type HSS extracts gave a single peak at 550 nm corresponding to cytochrome c_{552} , but the mutants again gave a single symmetrical peak at just below 557 nm. All three strains gave membrane spectra indicating normal synthesis of cytochrome b_1 . We conclude therefore, that CB40 and its derivatives are defective in either the regulation or synthesis of cytochrome c_{552} .

DISCUSSION

We have shown that the mutant CB40 is deficient in NADH-nitrite oxidoreductase, the most active enzyme for catalysing nitrite reduction *in vivo* (Tables 2 and 3). This and similar mutants are able to grow on minimal agar without cysteine. They do not synthesize cytochrome c_{552} , but reduce nitrate as rapidly as the wild-type.

One might argue that CB40 is defective in a nitrite permease, rather than in the structural gene for nitrite reductase. A permease-deficient mutant would show an identical phenotype because extracellular nitrite would be unable to enter the bacteria to derepress nitrite reductase synthesis. It has previously been shown, however, that nitrite accumulates intracellularly when wild-type *Escherichia coli* is grown anaerobically with nitrate (Wimpenny & Warmsley, 1968). Nitrite permease mutants should therefore show Nir⁻ phenotype when grown in the presence of nitrite, but a Nir⁺ phenotype when grown with nitrate. This was not observed with CB40. Although it is unlikely, therefore, that this mutant is permease deficient, we are at present unable to exclude the alternative possibility that it is a regulatory mutant in which a super-repressor fails to bind the inducer, nitrite.

The enrichment cycle for mutant isolation was designed to kill wild-type bacteria which can assimilate nitrate. Amongst the survivors one might expect nitrite and nitrate reductase-deficient bacteria as well as mutants auxotrophic for various organic nitrogen compounds. The usefulness of this procedure was limited because the wild-type were themselves unable to grow with nitrate as sole nitrogen source. The fact that nitrite reductase mutants were isolated in three out of ten attempts suggests that a limited enrichment was achieved. This was possibly due to a sparing effect of nitrate on the endogenous pools of nitrogen compounds in the wild-type, but not in the mutants.

Experiments with double mutants deficient in both nitrite and sulphite reductase have shown that the NADPH-sulphite oxidoreductase contributes no more than 5% to the wild-type nitrite reductase activity (Table 3). The residual NADH-nitrite oxidoreductase activities of FB17 and FB22 were sufficient to account for the rates of nitrite reduction by unbroken bacteria, so it is unlikely that alternative enzymes exist which can reduce nitrite at a significant rate. A similar deduction was made from the pH optima of nitrite reduction by the wild-type and CB40.

The occurrence of bacteria with wild-type NADH-nitrite oxidoreductase activities amongst early *cys*⁺ recombinants from interrupted mating experiments with Hfr Hayes as

the donor shows that the *nirA* gene is located proximal or close to *cysB*. We are now developing selective media for the detection of other Nir mutants amongst large numbers of wild-type bacteria in order to map the gene(s) for nitrite reductase.

REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788–795.
- CLOWES, R. C. & HAYES, W. (1968*a*). *Experiments in Microbial Genetics*, pp. 17–21. Oxford and Edinburgh: Blackwell Scientific Publications.
- CLOWES, R. C. & HAYES, W. (1968*b*). *Experiments in Microbial Genetics*, pp. 38–39. Oxford and Edinburgh: Blackwell Scientific Publications.
- COLE, J. A. (1968). Cytochrome *c*₅₅₂ and nitrite reduction in *Escherichia coli*. *Biochimica et biophysica acta* **162**, 356–368.
- COLE, J. A. & RITTENBERG, S. C. (1971). A comparison of respiratory processes in *Spirillum volutans*, *Spirillum itersonii* and *Spirillum serpens*. *Journal of General Microbiology* **69**, 375–383.
- COLE, J. A. & WIMPENNY, J. W. T. (1966). The interrelationships of low redox potential cytochrome *c*₅₅₂ and hydrogenase in facultative anaerobes. *Biochimica et biophysica acta* **128**, 419–425.
- COLE, J. A. & WIMPENNY, J. W. T. (1968). Metabolic pathways for nitrate reduction in *Escherichia coli*. *Biochimica et biophysica acta* **162**, 39–48.
- ELLIS, R. J. (1964). A rapid assay for sulphite reductase. *Biochimica et biophysica acta* **85**, 335–338.
- FUJITA, T. (1966). Studies on soluble cytochromes in Enterobacteriaceae. I. Detection, purification and properties of cytochrome *c*₅₅₂ in anaerobically grown cells. *Journal of Biochemistry* **60**, 204–215.
- FUJITA, T. & SATO, R. (1966). Studies on soluble cytochromes in Enterobacteriaceae. IV. Possible involvement of cytochrome *c*₅₅₂ in anaerobic nitrite metabolism. *Journal of Biochemistry* **60**, 691–700.
- FUJITA, T. & SATO, R. (1967). Studies on soluble cytochromes in Enterobacteriaceae. V. Nitrite-dependent gas evolution in cells containing cytochrome *c*₅₅₂. *Journal of Biochemistry* **62**, 230–238.
- GRAY, C. T., WIMPENNY, J. W. T., HUGHES, D. E. & RANLETT, M. (1963). A soluble *c*-type cytochrome from anaerobically grown *Escherichia coli* and various enterobacteriaceae. *Biochimica et biophysica acta* **67**, 157–160.
- HUGHES, D. E. (1951). A press for disrupting bacteria and other microorganisms. *British Journal of Experimental Pathology* **32**, 97–109.
- KEMP, J. A., ATKINSON, D. E., EHRET, A. & LAZZARINI, R. A. (1963). Evidence for the identity of the nicotinamide adenine dinucleotide-specific sulphite and nitrite reductase of *Escherichia coli*. *Journal of Biological Chemistry* **238**, 2466–2471.
- LAZZARINI, R. A. & ATKINSON, D. E. (1961). A triphosphopyridine nucleotide-specific nitrite reductase from *Escherichia coli*. *Journal of Biological Chemistry* **236**, 3330–3335.
- MURRAY, E. D. & SANWAL, B. D. (1963). An immunological enquiry into the identity of assimilatory and dissimilatory nitrate reductase from *Escherichia coli*. *Canadian Journal of Microbiology* **9**, 781–790.
- ROTH, B., FALCO, E. A. & HITCHINGS, G. H. (1962). 5-Benzyl-2,4-diaminopyrimidines as antibacterial agents. I. Synthesis and antibacterial activity *in vivo*. *Journal of Medicinal and Pharmaceutical Chemistry* **5**, 1103–1123.
- SNELL, F. D. & SNELL, C. T. (1949). *Colorimetric Methods of Analysis*, 3rd edn, p. 804. New York: Van Nostrand.
- TAYLOR, A. L. (1970). Current linkage map of *Escherichia coli*. *Bacteriological Reviews* **34**, 155–175.
- WIMPENNY, J. W. T. & WARMSLEY, A. M. H. (1968). The effect of nitrate on Krebs cycle enzymes in various bacteria. *Biochimica et biophysica acta* **156**, 297–303.