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# SHORT COMMUNICATION

## Generation of a Membrane Potential by One of Two Independent Pathways for Nitrite Reduction by *Escherichia coli*

By N. R. POPE AND J. A. COLE*

*Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.*

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A set of four isogenic *Escherichia coli* strains has been constructed in which all possible combinations of NADH- and formate-dependent nitrite reductases are active or inactive. Each pathway can be inactivated genetically without a corresponding loss in the other activity: the two pathways are therefore biochemically independent. The generation of a membrane potential during nitrite reduction by formate has been demonstrated using an ion-selective electrode specific for a lipophilic cation. The observed energy conservation results, at least in part, from the ability of formate dehydrogenase in *E. coli* to pump protons.

### INTRODUCTION

Anaerobic cultures of *Escherichia coli* K12 can use either NADH or formate to reduce nitrite rapidly to ammonia. The NADH-dependent enzyme has been extensively purified (Coleman *et al.*, 1978; Jackson *et al.*, 1981). It is a soluble, cytoplasmic protein with sirohaem, FAD and non-haem iron–sulphur clusters as its prosthetic groups: no useful energy is conserved during nitrite reduction by NADH (Cole & Brown, 1980).

The formate-dependent pathway is undetectable in many strains and contributes only about 20% to the total rate of nitrite reduction by others (Abou-Jaoudé *et al.*, 1977). The formate dehydrogenase involved in nitrate reduction by *E. coli* acts as a proton pump, so energy is conserved during nitrate reduction by formate (Jones, 1980). If the same formate dehydrogenase is involved in nitrite reduction by formate (Abou-Jaoudé *et al.*, 1979), it can be predicted that energy would also be conserved during this process. We now report the isolation and characterization of a set of four isogenic strains of *E. coli* in which all combinations of the NADH- and formate-dependent pathways for nitrite reduction are either active or inactive. They have been used to determine whether the two pathways for nitrite reduction are biochemically independent and whether energy is conserved during nitrite reduction by formate.

### METHODS

*Strains. Escherichia coli* strain W3102 was obtained from M. G. Marinus. It requires cysteine for growth on minimal medium because it carries Tn5 inserted into the *cysG* gene. The product of the *cysG* gene is believed to be required for the synthesis of sirohaem which is a prosthetic group of both sulphite reductase (EC 1.8.1.2) and the NADH-dependent nitrite reductase (EC 1.6.6.4) (Siegel *et al.*, 1973; Jackson *et al.*, 1981; Cole *et al.*, 1980).

The prototrophic strain CGSC4315 was obtained from B. Bachmann (*E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn., U.S.A.) and the following set of isogenic mutants was constructed with this strain. Strain CB401 is a spontaneous mutant resistant to 10 mM-sodium chlorate

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during anaerobic growth. The *cysG*::Tn5 lesion was transferred from strain W3102 to CGSC4315 by bacteriophage P1-mediated transduction (Lennox, 1955) and selection for resistance to 25 μg kanamycin $ml^{-1}$: strain CB402 was a purified single-colony isolate with the phenotype $Kan^R Cys^- Nir^-$ (Cole *et al.*, 1980). Strain CB403 was constructed in a similar manner but with CB401 as the transduction recipient: the phenotype of CB403 is $Kan^R Cys^- Nir^- Chl^R$.

*Growth conditions and preparation of extracts.* Bacteria were grown in static 2 l conical flasks filled with half-strength nutrient broth (Oxoid) in minimal salts which contained 4·5 g $KH_2PO_4$, 10·5 g $K_2HPO_4$, 1 g $(NH_4)_2SO_4$, 0·5 g sodium citrate, 0·1 g $MgSO_4.7H_2O$, 1 μmol sodium selenate, 1 μmol ammonium molybdate and 1 ml sulphur-free trace metal solution (Cole *et al.*, 1974) per litre distilled water. The autoclaved medium was supplemented to 5 mM with sterile sodium nitrite and to 0·2% (w/v) with glucose before inoculation. After 12 h at 37 °C, bacteria were harvested by centrifugation for 5 min at 11000 ***g*** and washed once in 50 mM-potassium phosphate pH 7·5 (phosphate buffer). Soluble extracts were prepared as described by Newman & Cole (1978).

*Enzyme assays.* Rates of nitrite reduction by washed bacteria were assayed by incubating 5 to 20 mg dry wt bacteria at 37 °C with 5 ml 1·2 mM-$NaNO_2$ and either 40 mM-glucose or 40 mM-formate in phosphate buffer. The concentration of nitrite in 50 μl samples taken at 2 to 5 min intervals was determined (Cole *et al.*, 1974). Rates of nitrite reduction by bacteria incubated in the absence of glucose or formate were subtracted from the rate with added substrate to give the formate- or glucose-dependent rate of nitrite reduction.

NADH-dependent nitrite reductase activity of soluble bacterial extracts was assayed as described by Coleman *et al.* (1978).

Each set of assays was repeated with between three and six independent cultures: typical variations between experiments were less than 20%.

*Uptake and release of butyltriphenylphosphonium by EDTA-treated bacteria.* Lipophilic cations such as butyltriphenylphosphonium ($BTPP^+$) can be driven through the bacterial membrane by a membrane potential: the extent of the uptake is related to the magnitude of the membrane potential ($\Delta\psi$) by the equation

$$\Delta\psi = \frac{RT}{nF} \ln\left(\frac{[BTPP^+]_{In}}{[BTPP^+]_{Out}}\right)$$

(McCarthy *et al.*, 1981). Uptake by many Gram-negative bacteria only occurs when their permeability has been increased by washing them with mild chelating agents. Bacterial suspensions were therefore incubated with 5 mM-EDTA for 5 min, other details being exactly as described by Padan *et al.* (1976). The EDTA-treated bacteria were washed and resuspended in 450 mM-sucrose, 3 mM-$MgCl_2$ in 50 mM-sodium phosphate pH 7·5 (reaction buffer).

An ion-selective electrode was used to detect changes in the extracellular $BTPP^+$ concentration. The recording equipment, electrodes and reaction chamber were as described by Alefounder *et al.* (1981) for their nitrate electrode. The electrode was modified to detect $BTPP^+$ by using a poly(vinyl chloride) membrane containing triphenylboron as ion exchanger (Kamo *et al.*, 1979). The electrode was filled with 1 mM-$BTPP^+$ bromide in 100 mM-NaCl (McCarthy *et al.*, 1981). The electrode vessel contained 10 ml of reaction buffer at 37 °C with $O_2$-free $N_2$ blowing over the surface. The electrode was calibrated by the stepwise addition of 10 μl portions of 1 mM-$BTPP^+$. The cell suspension (2 ml) was then added and subsequent additions were from stock solutions of 5 mM-carbonyl cyanide trifluoromethoxyphenylhydrazone (FCCP) or 1 M solutions of other reagents.

## RESULTS AND DISCUSSION

### *Rates of NADH- and formate-dependent nitrite reduction*

Glucose-dependent rates of nitrite reduction by strains CGSC4315 (wild-type), CB401 (*chl-35*), CB402 (*cysG*::Tn5) and CB403 (*chl-35 cysG*::Tn5) were 66·3, 68·6, 23·1 and 1·7 nmol nitrite reduced $min^{-1}$ (mg dry wt)$^{-1}$, respectively. Corresponding formate-dependent rates were 49·2, <0·5, 33·1 and <0·5 nmol $min^{-1}$ (mg dry wt)$^{-1}$ and NADH-dependent activities were 205, 537, <10 and <10 nmol NADH oxidized $min^{-1}$ (mg protein)$^{-1}$. Thus, although the loss of the formate-dependent activity in strain CB401 was more than compensated by increase in the NADH-dependent activity, there was no similar increase in the formate-dependent activity in strain CB402. Although neither the formate- nor the NADH-dependent activity could be detected in strain CB403, this double mutant retained about 5% of the wild-type rate of nitrite reduction by glucose. We assume this to be due to a minor pathway for nitrite reduction, possibly to the pyruvate-dependent activity described by Pascal

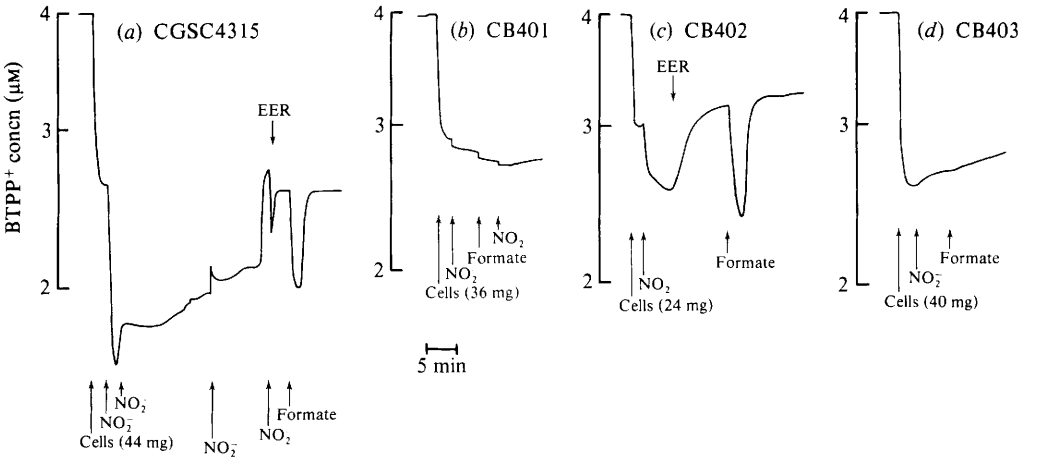


Fig. 1. Requirement for formate and formate-nitrite oxidoreductase activity for the generation of a membrane potential during nitrite reduction by *E. coli*. The electrode was calibrated by the sequential addition of 10 μl portions of 1 mM-$BTPP^+$ to 10 ml of reaction buffer at 37 °C in the electrode vessel. Subsequent additions are indicated on the figure [all formate additions were 2 μmol; nitrite additions were 3 μmol in (*a*), (*b*) and (*c*) and 2 μmol in (*d*)]. The strains and genotypes are (*a*) CGSC4315 (wild-type); (*b*) CB401 (*chl-35*); (*c*) CB402 (*cysG*::Tn5); (*d*) CB403 (*chl-35 cysG*::Tn5). (EER indicates exhaustion of endogenous reserves.)

*et al.* (1981). The simplest interpretation of these results is that the NADH- and formate-dependent pathways for nitrite reduction are biochemically independent. The possibility remained, however, that the apoprotein for the NADH-dependent enzyme is synthesized by strain CB402 and that this protein is a component of the formate-nitrite oxidoreductase. We are attempting to isolate mutants defective in the structural gene for this enzyme so that this possibility can be eliminated.

### *Generation of a membrane potential during nitrite reduction*

When nitrite was added to suspensions of EDTA-washed bacteria incubated in the electrode vessel with formate and $BTPP^+$, the concentration of $BTPP^+$ in the reaction buffer decreased rapidly as the cation moved into the bacteria in response to the generation of a $\Delta\psi$. This $BTPP^+$ was released as soon as the added nitrite had been reduced, or when the $\Delta\psi$ collapsed in response to the addition of FCCP. No changes in $BTPP^+$ concentration were detected when nitrite was added after the addition of FCCP. Identical results were obtained with either the wild-type strain or with strain CB402 in which NADH-dependent nitrite reductase was inactive; potentials developed were calculated to be in the range 120 to 160 mV (inside negative).

It was possible that the $\Delta\psi$ generated during nitrite reduction was due to the oxidation of unidentified endogenous substrates other than formate. This possibility was eliminated by incubating suspensions of all four strains with $BTPP^+$ and excess nitrite until no further changes in the extracellular $BTPP^+$ concentration were detected. Addition of formate then resulted in a rapid decrease in extracellular $BTPP^+$ concentration as a $\Delta\psi$ was developed by strains CGSC4315 and CB402 (Fig. 1*a*, *c*). This response to the addition of formate was not detected with strains CB401 and CB403 in which formate-nitrite oxidoreductase was inactive (Fig. 1*b*, *d*).

These combined results establish that a $\Delta\psi$ is developed during nitrite reduction by formate. Although formate is the anion of a weak acid, the failure of strains CB401 and CB403 to generate a $\Delta\psi$ during nitrite reduction was not due to the uncoupling effect of formic acid because $BTPP^+$ uptake was readily detected when strains CGSC4315 and

CB402 were incubated with excess formate (data not shown). Similarly, the possibility that the $\Delta\psi$ was due to hydrolysis of ATP generated by substrate level phosphorylation during the oxidation of endogenous substrates by nitrite has been eliminated in three ways. Thus, although oxidation of NADH by nitrite would generate $NAD^+$ which could then oxidize endogenous substrates, similar $\Delta\psi$s were generated during nitrite reduction by strains CGSC4315 and CB402 in which the NADH-dependent pathway was inactive. Conversely, no $\Delta\psi$ was developed by strain CB401 in which the NADH-dependent pathway was active but the formate-dependent pathway inactive. Thirdly, $BTPP^+$ uptake was readily demonstrated on addition of formate to bacteria which had been depleted of endogenous substrates during incubation with excess nitrite (Fig. 1).

$BTPP^+$ which had moved into the bacteria in response to the addition of nitrite or formate was always released immediately FCCP was added. We therefore conclude that energy is conserved by a chemiosmotic mechanism during nitrite reduction to ammonia by formate. This is consistent with the suggestion that formate dehydrogenase of *E. coli* can act as a proton pump, and with the large decrease in free energy during the reaction.

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