Regulation of Nitrogenase Biosynthesis in *Klebsiella pneumoniae*: Effect of Nitrate

By SHERMAN S. M. HOM, H. HENNECKE and K. T. SHANMUGAM*

Plant Growth Laboratory and Department of Agronomy and Range Science, University of California, Davis, California 95616, U.S.A.

(Received 30 May 1979; revised 16 August 1979)

The rate of biosynthesis of nitrogenase polypeptides in *Klebsiella pneumoniae* was determined in a medium containing NaNO₃ or NaNO₂. Nitrogenase biosynthesis was completely repressed by NO₃⁻ in a mutant strain, strain SK-25, that is derepressed for nitrogenase biosynthesis in the presence of NH₄⁺. Chlorate-resistant mutants, derived from strain SK-25, that are defective in NO₃⁻ respiration produced nitrogenase in the presence of NO₃⁻. Strain SK-561, a chlorate-resistant derivative capable of NO₃⁻ respiration, produced no nitrogenase in the presence of NO₃⁻ or NO₂⁻. *Klebsiella pneumoniae* respired under anaerobic conditions utilizing either NO₃⁻ or NO₂⁻ as terminal electron acceptor. A mechanism for the control of nitrogenase biosynthesis is discussed involving the redox control of anaerobic enzyme systems.

**INTRODUCTION**

Nitrogenase utilizes large amounts of metabolic energy and reducing power during the reduction of N₂ to NH₄⁺ and as such is subject to several types of cellular control (Postgate, 1971; Brill, 1975). For example, nitrogenase biosynthesis in *Klebsiella pneumoniae* is repressed by NH₄⁺, a regulatory system believed to be mediated through metabolic intermediates generated from NH₄⁺ (Shanmugam et al., 1978). Nitrogenase biosynthesis is also controlled by O₂ (St John et al., 1974; Hill, 1976; Eady et al., 1978). Eady et al. (1978) observed that O₂ repressed biosynthesis of nitrogenase polypeptides. Studies on the regulation of nitrogenase biosynthesis by O₂ are hampered by the fact that O₂ irreversibly inactivates nitrogenase, a non-haem iron-sulphur-molybdenum containing protein (Eady et al., 1972).

In *K. pneumoniae*, nitrogenase activity was reported to be absent in medium containing NO₃⁻ (Tubb & Postgate, 1973). Since *K. pneumoniae* is capable of utilizing NO₃⁻ as a nitrogen source for growth, the effect of NO₃⁻ on nitrogenase activity can be explained through the NH₄⁺ control system. In this communication, we report results which show that NO₃⁻ and NO₂⁻ repress nitrogenase biosynthesis in mutant strains of *K. pneumoniae* that are derepressed for nitrogenase biosynthesis in the presence of NH₄⁺. A mechanism for NO₃⁻ and NO₂⁻ repression of nitrogenase biosynthesis is discussed in which NO₃⁻ and NO₂⁻ behave like O₂ to mediate a redox control over anaerobic enzyme systems.

**METHODS**

*Bacterial strains and growth.* *Klebsiella pneumoniae* wild-type strain M5A1, mutant strain SK-25, which is derepressed for nitrogenase biosynthesis in the presence of NH₄⁺ (Shanmugam et al., 1975), and its chlorate-resistant derivative strain SK-564, were described previously (strain SK-564 was previously...
designated as strain N-61) (Andersen & Shanmugam, 1977). Strains SK-561, SK-563 and SK-565 were isolated as spontaneous chlorate-resistant derivatives of strain SK-25. Culture media used (L-broth or sucrose minimal) were as described previously (Streicher et al., 1971).

Analytical methods. Nitrogenase activity in whole cells was determined using the acetylene reduction assay as described by Shanmugam et al. (1974). H₂ production by the cultures was monitored as described by Andersen & Shanmugam (1977). NH₄⁺ was determined using Nessler’s reagent (Ballantine, 1957). NO₃⁻ was estimated by the procedure described by Cataldo et al. (1975). NO₂⁻ was determined as described by Van’t Riet et al. (1968). Whole-cell protein was determined by the method of Drews (1965) using bovine serum albumin as a standard.

Enzyme assays. Formate hydrogenlyase activity was measured as described by Chippaux et al. (1977). Formate dehydrogenase-1 activity was determined using the procedure described by Pichinoty (1969a) with some modifications. The reaction mixture (2.0 ml) contained 0.01 M-sodium acetate, 0.88 mM-phenazine methosulphate, 0.68 mM-thiazolyl blue and 0.3 M-sodium phosphate buffer pH 6.5. Incubation was at room temperature under N₂, and the reaction was started by adding cell extract (0.35 to 0.85 mg protein). The rate of increase in absorbance at 560 nm, due to the reduction of thiazolyl blue, was measured spectrophotometrically. Formate dehydrogenase-2 activity was assayed as described by Chippaux et al. (1977) with minor modifications. The reaction mixture (2.0 ml) contained 0.01 M-sodium acetate, 6.5 mM-benzyl viologen and 0.3 M-sodium phosphate buffer pH 7.0. Incubation was at room temperature under N₂, and the reaction was initiated by adding cell extract (0.4 to 3.0 mg protein). The rate of increase in absorbance at 550 nm, due to the reduction of benzyl viologen, was measured spectrophotometrically. Hydrogenase activity was measured as described by Pichinoty (1969b).

Labelling of nitrogenase proteins. (i) Growth of cultures for labelling studies. Cultures for inoculation were grown aerobically in L-broth supplemented with 8.8 mM-sucrose and 6.8 mM-L-glutamine. When the culture reached an A₄₅₀ of 1.0, the cells were sedimented (2500 rev. min⁻¹, 10 min) at room temperature to remove the nutrients from the growth medium and resuspended in nitrogen-free minimal medium. The bacterial cell density was adjusted to an A₄₅₀ of 0.5 with sucrose minimal medium supplemented with 0.68 mM-L-glutamine and then transferred to a 71 ml serum bottle (Wheaton, no. 223746; Millville, N.J., U.S.A.). These cultures were evacuated and sparged four times with N₂. Acetylene was then added to a final concentration of 2% (v/v) so that nitrogenase activity, measured by ethylene formation, could be monitored. These standing cultures were incubated at room temperature. NaNO₃, NaNO₂, NaN₃ or water was added with a sterile 1.0 ml syringe at appropriate times (as indicated in individual experiments) after the onset of anaerobiosis. At various times during linear growth of the cultures, samples of the culture were withdrawn with a sterile syringe and used for labelling the proteins synthesized with ¹⁴C-labelled amino acids.

(ii) Whole-cell pulse labelling technique. To label the newly synthesized proteins, 2 ml samples of the culture were withdrawn and injected into rubber-stoppered thick-walled Pyrex centrifuge tubes (Dupont Instruments, 00119; prefilled with N₂ containing 5 pCi (185 kBq) of a mixture of ¹⁴C-labelled L-amino acids (New England Nuclear, Mass., U.S.A.; Amersham-Searle, Arlington Heights, Ill., U.S.A.). After 10 min incubation at 25°C, the samples were quickly chilled by plunging into ice to stop any further incorporation of radioactive amino acids. Two ml of chilled nitrogen-free sucrose minimal medium (chase medium) was added to each sample to dilute the labelled amino acids. The culture was centrifuged at 12000 g for 10 min at 4°C, and the cells were washed with 2.0 ml chase medium. The cell pellet was frozen quickly in an ethanol/dry ice bath and stored at −20°C until needed.

Polyacrylamide slab gel electrophoresis. Electrophoretic separation of proteins in polyacrylamide gels was performed essentially as described by Laemmli (1970). Samples for electrophoresis were prepared by adding 0.2 ml of solubilization solution [3·0% (w/v) sodium dodecyl sulphate (SDS; BDH), 5% (v/v) β-mercaptoethanol, 0.0025% (w/v) bromoresol purple, 20% (v/v) glycerol and 0.05 M-Tris buffer pH 7.0] and boiling for 2 min. After removing the residual particulate matter by centrifuging for 5 min, samples containing 100000 c.p.m. were added to each well of the two-layer slab gel system. The lower (resolving) gel consisted of 10% (w/v) bis-polyacrylamide, 0.4% (w/v) SDS and 0.375 M-Tris buffer pH 8.8, while the upper stacking gel consisted of 5.25% bis-polyacrylamide, 0.4% SDS and 0.125 M-Tris buffer pH 6.8. The reservoir buffer contained 0·025 M-Tris base, 0·192 M-glycine and 0·1% SDS. The slab gel was subjected to electrophoresis with a constant current of 10 mA through the upper gel and 15 mA through the lower gel. After electrophoresis, the protein bands in the slab gel were stained for a minimum of 1 h in a solution containing 0.04% (w/v) Coomassie brilliant blue R-250, 25% (v/v) propan-2-ol and 10% (v/v) acetic acid. Destaining was for 1 h in a solution containing 0.004% Coomassie brilliant blue R-250, 10% propan-2-ol and 10% acetic acid, followed by three washes (1 h each) with 10% acetic acid.

 Autoradiography. After drying the slab gel for 2 h at 60°C on to Whatman 3MM chromatography paper, a 5 × 7 in sheet of Kodak X-Omat X-ray film was placed over the dried gel and exposed for 12 h clamped
Effect of nitrate on nitrogenase biosynthesis

between two glass plates. The X-ray film was developed for 5 min in Kodak D-19 developer, washed in water (1 min), fixed in Kodak rapid X-ray fixer (1 min) and washed in running water (45 min). Each column of the autoradiograms was cut into strips and scanned at 580 nm at 150 cm h⁻¹ using an ISCO model UA-5 Absorbance Monitor (Instrumentation Specialties Co., Lincoln, Neb., U.S.A.) coupled to an ISCO Type 6 optical unit.

A typical experiment, showing the derepression kinetics of nitrogenase proteins under the conditions described above, is presented in Fig. 1. Figure 1(a) is a photograph of a Coomassie blue-stained gel, Fig. 1(b) is an autoradiogram of the same gel and Fig. 1(c) is a spectrophotometric scan of lane 8 of the autoradiogram. Nitrogenase contains two polypeptides and both polypeptides can be seen as one band (peak marked B in Fig. 1c). Throughout this paper, the nomenclature suggested by Hageman & Burris (1978) is used to define the components of the nitrogenase complex. According to this system, the term nitrogenase refers to the Mo–Fe protein (also known as nitrogenase component I). Nitrogenase reductase is used to define the Fe protein (component II).

Kennedy et al. (1976) demonstrated that, on electrophoresis in the presence of SDS from Koch-Light
Table 1. Effect of NO$_3^-$ or NH$_4^+$ on nitrogenase activity in chlorate-resistant mutant strains of Klebsiella pneumoniae

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Parent strain</th>
<th>Addition to medium*</th>
<th>NO$_3^-$ produced [μmol h$^{-1}$ (mg protein)$^{-1}$]</th>
<th>NO$_4^+$ produced [μmol h$^{-1}$ (mg protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5A1</td>
<td>Wild-type</td>
<td>None</td>
<td>2.61†</td>
<td>0.00†</td>
<td>0.00†</td>
</tr>
<tr>
<td>SK-25</td>
<td>Asm-, Gln-</td>
<td>Asm-1</td>
<td>2.93</td>
<td>0.00</td>
<td>3.00</td>
</tr>
<tr>
<td>SK-561</td>
<td>Asm-, Gln-, Chl$^R$</td>
<td>SK-25</td>
<td>2.50</td>
<td>0.00</td>
<td>1.51</td>
</tr>
<tr>
<td>SK-563</td>
<td>Asm-, Gln-, Chl$^R$</td>
<td>SK-25</td>
<td>1.56</td>
<td>1.83</td>
<td>0.83</td>
</tr>
<tr>
<td>SK-564</td>
<td>Asm-, Gln-, Chl$^R$</td>
<td>SK-25</td>
<td>1.40</td>
<td>2.31</td>
<td>1.33</td>
</tr>
<tr>
<td>SK-565</td>
<td>Asm-, Gln-, Chl$^R$</td>
<td>SK-25</td>
<td>2.04</td>
<td>2.65</td>
<td>2.02</td>
</tr>
<tr>
<td>SK-561</td>
<td>Asm-, Gln-, Chl$^R$</td>
<td>SK-25</td>
<td>2.50</td>
<td>0.00</td>
<td>1.98</td>
</tr>
</tbody>
</table>

ND, Not determined.
* Glutamine was present as the nitrogen source at 0.68 mM; NO$_3^-$ and NH$_4^+$ were added at 11.8 mM and 15 mM, respectively.
† For strain M5A1 glutamate replaced glutamine.

Nitrogenase separates into the two constituent polypeptides. Since SDS from BDH was used in the experiments described in this paper, both polypeptides of nitrogenase are seen as one band (peak marked B in Fig. 1c). Based on this scan, about 10% of the total protein synthesized was found to be nitrogenase. Other nif-specific proteins—nitrogenase reductase (peak C) and nifJ-encoded protein (120000 daltons; peak A; Roberts et al., 1978)—account for an additional 6 to 7%.

RESULTS

Effect of NO$_3^-$ on whole-cell nitrogenase activity

*Klebsiella pneumoniae* is capable of utilizing NO$_3^-$ as a nitrogen source for growth. The lack of nitrogenase activity in batch cultures of strain M5A1 reported earlier by Tubb & Postgate (1973) might have resulted in build-up of an excess of NH$_4^+$ through NO$_3^-$ assimilation. In confirmation of these results, we have found that strain M5A1 produced no detectable nitrogenase activity in a medium containing NaNO$_3$. However, no nitrogenase activity was detected in the presence of NO$_3^-$ in a mutant strain of *K. pneumoniae*, strain SK-25, that is derepressed for nitrogenase biosynthesis in the presence of NH$_4^+$ (Table 1). This raises the possibility that the lack of nitrogenase activity in strain SK-25 grown in the presence of NO$_3^-$ is due to other control systems besides NH$_4^+$ repression.

Several chlorate-resistant mutants were isolated from strain SK-25 and tested for their nitrogenase activity in the presence of NO$_3^-$, since mutants that are chlorate-resistant are known to have defects in their nitrate metabolism. All chlorate-resistant strains tested, except strain SK-561, showed nitrogenase activity in the presence of 11.8 mM NO$_3^-$. Properties of some of these strains are presented in Table 1. The chlorate-resistant strains SK-561, SK-563, SK-564 and SK-565 showed nitrogenase activity in the presence of NH$_4^+$, a property similar to their parent strain SK-25. Strains SK-563, SK-564 and SK-565 that are derepressed for nitrogenase biosynthesis in the presence of NO$_3^-$ produced no detectable NO$_2^-$ in the medium during anaerobic growth indicating that these strains have defects in their NO$_3^-$ respiratory pathways. One chlorate-resistant derivative, strain SK-561, in which nitrogenase activity was not observed in the presence of NO$_3^-$, produced considerable amounts of NO$_2^-$ during anaerobic growth. These results show that metabolism of NO$_3^-$ is likely to be a prerequisite before NO$_3^-$ affects nitrogenase activity in *K. pneumoniae*.

Repression of nitrogenase biosynthesis by NO$_3^-$

Our results show that strains such as M5A1, SK-25, SK-561 and other glutamate and glutamine auxotrophs described before (strains SK-24, SK-26, SK-27, SK-29 and SK-56;
Effect of nitrate on nitrogenase biosynthesis

Fig. 2. Effect of NO$_3^-$ on the rate of nitrogenase biosynthesis in strain SK-561. Cultures without NO$_3^-$ (○) or with 11.8 mM-NO$_3^-$ added at time 0 (□, ■) or at 1.5 h (△, ▲) were grown as described in Methods. (a) Rate of nitrogenase biosynthesis (○, □, △) and NO$_2^-$ production (■, ▲). (b) Rate of total protein synthesis and (inset) whole-cell nitrogenase activity (expressed as pmol C$_2$H$_4$ produced per culture).

Shanmugam et al., 1977) which have no detectable nitrogenase activity in the presence of NO$_3^-$ also produce considerable amounts of NO$_2^-$ in the medium. Since NO$_3^-$ inhibits nitrogenase activity in crude extracts of *Rhizobium japonicum* and *R. lupini* bacteroids (Kennedy et al., 1975) and *K. pneumoniae* (unpublished data), it is necessary to distinguish between repression of nitrogenase biosynthesis and inhibition of nitrogenase activity. Figure 2(a) shows that biosynthesis of nitrogenase polypeptides in the chlorate-resistant strain SK-561 was repressed by the addition of NaNO$_3$. Addition of NO$_3^-$ (11.8 mM) at the beginning of the experiment decreased the rate of nitrogenase biosynthesis. After 2.5 h, although nitrogenase biosynthesis could be detected (compared with the rate at 1.5 h), the rate of biosynthesis was less than 25% of the rate observed with the control. If NO$_3^-$ was added to the medium after the initiation of nitrogenase biosynthesis (1.5 h after the beginning of the experiment), nitrogenase biosynthesis continued at the same rate as the control for at least 1 h, and repression occurred approximately 2 h after addition of NO$_3^-$.

Although nitrogenase biosynthesis was completely repressed about 6.5 h after the beginning of the experiment, the cells continued to transport amino acids and synthesize other proteins (Fig. 2b). The repressive effect of NO$_3^-$ was coupled to the appearance of NO$_2^-$ and was probably specific for nitrogenase. Although NO$_2^-$ could be detected in the medium, the concentration was not sufficient to inhibit the activity of the preformed low concentrations of nitrogenase (inset, Fig. 2b), as determined by the amount of ethylene accumulated in the gas phase. At 10 h, nitrogenase activities [μmol ethylene produced h$^{-1}$ (mg protein)$^{-1}$] were 1.15, 0.001 and 0.004 for the derepressed (without NO$_3^-$) cultures and cultures with NO$_3^-$ added at time 0 and 1.5 h, respectively.

Repression of nitrogenase biosynthesis by NO$_2^-$

Since NO$_3^-$ was detected in the medium during the time of nitrogenase repression by NO$_3^-$, it is possible that the repressive effect may be mediated by NO$_2^-$, the product of NO$_3^-$ respiration, and not by NO$_3^-$ itself. To test this possibility, we investigated the effect of NO$_2^-$ on nitrogenase biosynthesis. As shown in Fig. 3, NO$_2^-$ also repressed nitrogenase biosynthesis when added to a culture of strain SK-561 (1.5 h after beginning the experiment).
Fig. 3. Effect of NO$_2^-$ on the rate of nitrogenase biosynthesis in strain SK-561. Whole-cell nitrogenase activity (○, ●), rate of nitrogenase synthesis (□, ■) and rate of total protein synthesis (△, ▲) were followed in cultures grown as described in Methods: (a) no NO$_2^-$ added; (b) 3.6 mM NO$_2^-$ added at 1.5 h.

At 3-6 mM NaNO$_2$ completely repressed nitrogenase biosynthesis (Fig. 3b); ethylene production by preformed enzyme was also inhibited (about 99%). Total protein synthesis was partially inhibited (about 35% of the control) by NO$_2^-$ at this concentration. Inhibition of protein synthesis was overcome after about 2 h although nitrogenase biosynthesis was still repressed. At a lower concentration of NaNO$_2$ (1.45 mM), a transient effect on nitrogenase biosynthesis was observed which lasted for less than 1 h. As soon as the NO$_2^-$ concentration decreased, due to cellular metabolism, repression by NO$_2^-$ was relieved.

**Stability of nitrogenase peptides in the presence of NO$_2^-$**

The inhibitory effect of NO$_2^-$ on nitrogenase activity raises the possibility that nitrogenase peptides that are produced are rapidly degraded in the presence of NO$_2^-$.

In order to test this possibility, a culture of strain SK-561, which is derepressed for nitrogenase synthesis, was labelled with $^{14}$C-labelled amino acids as described in Methods. After 10 min, the cells were washed free of the $^{14}$C-labelled amino acids and incubated anaerobically for 30 min in the sucrose minimal medium containing L-glutamine as the nitrogen source (0.68 mM). To these cultures, either NO$_2^-$ (3.6 mM) or chloramphenicol and rifampicin (50 and 100 µg ml$^{-1}$, respectively) were added. Samples were withdrawn from the cultures at different times and the ratios of nitrogenase to the total cell protein were determined as described in the Methods. In both conditions, no decrease in the ratio of nitrogenase to total cell protein was observed over a 3 h period indicating that the degradation of nitrogenase polypeptides is not enhanced in the presence of NO$_2^-$. 


Effect of nitrate on nitrogenase biosynthesis

Sodium nitrite is known to inhibit the activity of the formate hydrogenlyase system in *K. pneumoniae* (Shanmugam & Hennecke, 1979). The inhibitory effect of NO$_3^-$ on total protein synthesis may be mediated by the inhibition of energy production by NO$_2^-$ in the anaerobic cell. To test the possibility that the repression of nitrogenase biosynthesis by NO$_2^-$ is not mediated by the inhibition of total protein synthesis, we investigated the effect of azide, an inhibitor of cellular energy production, on nitrogenase biosynthesis. Azide at 1.5 mM inhibited total cellular protein synthesis by 90% (Fig. 4). Although the rate of biosynthesis of nitrogenase in the presence of azide (1.5 mM) was lower than the control, the maximum amount of nitrogenase synthesized either reached or exceeded the control levels. Azide also inhibited the acetylene reduction activity of nitrogenase by 99%. This experiment shows that although the rate of overall protein synthesis was drastically decreased in the presence of azide, nitrogenase was synthesized at maximal rates. At about 8 h after the beginning of the experiment, about 11% of the total protein being synthesized by the cell was nitrogenase.

**Effect of NO$_3^-$ on other anaerobic enzyme systems**

The addition of NO$_3^-$ to an anaerobic culture of enteric bacteria greatly decreases the activity of formate hydrogenlyase (Peck & Gest, 1957; Cole & Wimpenny, 1966; Ruiz-Herrera & Alvarez, 1972; Chippaux et al., 1977). Similar repression was also observed in the derivatives of *K. pneumoniae* strain M5A1 (Table 2). Although addition of NO$_3^-$ to the growth medium had very little effect on the activity of formate dehydrogenase-1, the activities of both formate dehydrogenase-2 and hydrogenase were greatly decreased in the strain SK-25 and its chlorate-resistant derivative, strain SK-561. Addition of NO$_3^-$ (11.8 mM) had very little effect on H$_2$ production by pre-induced formate hydrogenlyase enzyme system in strain SK-25 (results not presented). Both NO$_3^-$ (1.44 mM) and azide (1.5 mM) inhibited formate dehydrogenase-2 activity completely and formate dehydrogenase-1 and hydrogenase activities (produced in the absence of NO$_3^-$ in the growth medium) by 56% and 28%, respectively, in strain SK-25. Neither NO$_3^-$ nor azide significantly inhibited formate dehydrogenase-1 activity, produced in response to growth in the presence of NO$_3^-$. These experiments show that NO$_3^-$ represses the biosynthesis of nitrogenase as well as formate dehydrogenase-2 and hydrogenase.
Fig. 5. Effect of NO$_3^-$ or NO$_2^-$ on growth of strain M5A1 (wild-type). Cultures were grown anaerobically in a minimal salts medium with limiting glycerol (0.53%) and 15 mM-NH$_4^+$ (■). Other culture media were supplemented with either 11.8 mM-NO$_3^-$ (●) or 14.5 mM-NO$_2^-$ (▲).

Table 2. Effect of NO$_3^-$ on the synthesis of the enzymes of the formate hydrogenlyase pathway

Cultures were grown anaerobically, washed with 0.05 M-phosphate buffer pH 7.0 containing 10 mM-reduced glutathione, harvested and stored at −80°C until needed. Cells were lysed under a stream of argon with a French pressure cell operating at 140 MPa. Enzyme assays were performed as described in Methods. Formate dehydrogenase activities are expressed as μmol min$^{-1}$ (mg protein)$^{-1}$, hydrogenase activities as μmol H$_2$ consumed h$^{-1}$ (mg protein)$^{-1}$ and formate hydrogenlyase activities as μmol H$_2$ evolved h$^{-1}$ (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Addition(s) to growth medium*</th>
<th>Formate dehydrogenase-1 activity</th>
<th>Formate dehydrogenase-2 activity</th>
<th>Hydrogenase activity</th>
<th>Formate hydrogenlyase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-25</td>
<td>Gln</td>
<td>0.074</td>
<td>0.071</td>
<td>0.068</td>
<td>2.06</td>
</tr>
<tr>
<td>SK-25</td>
<td>Gln, NO$_3^-$</td>
<td>0.092</td>
<td>0.005</td>
<td>0.000</td>
<td>0.00</td>
</tr>
<tr>
<td>SK-561</td>
<td>Gln</td>
<td>0.038</td>
<td>0.074</td>
<td>0.105</td>
<td>2.82</td>
</tr>
<tr>
<td>SK-561</td>
<td>Gln, NO$_2^-$</td>
<td>0.078</td>
<td>0.006</td>
<td>0.002</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Glutamine (Gln) and NO$_3^-$ were present at 6.8 mM and 11.8 mM, respectively.

NO$_3^-$ and NO$_2^-$ as oxidants for anaerobic respiration

Repression of formate dehydrogenase-2 and hydrogenase by NO$_3^-$ in *Escherichia coli* is mediated through the anaerobic respiratory control system (Ruiz-Herrera & Alvarez, 1972). This raises the possibility that both NO$_3^-$ and NO$_2^-$ (which has a similar effect on nitrogenase) act as terminal electron acceptors for anaerobic respiration. As shown in Fig. 5, addition of NO$_2^-$ or NO$_3^-$ to a culture containing limiting amounts of glycerol increased the growth rate as well as the cell yield of strain M5A1. The generation time for strain M5A1 in the medium with no oxidant was about 3.75 h. Addition of NO$_3^-$ decreased the generation time to about 1.75 h while NO$_2^-$ decreased it to about 2.2 h. This experiment shows that *K. pneumoniae* can utilize both NO$_3^-$ and NO$_2^-$ as electron acceptors under anaerobic conditions.

DISCUSSION

Addition of O$_2$ to an anaerobic culture of enteric bacteria markedly alters the energy metabolism of the cell, from a fermentative pattern to that of aerobic respiratory metabolism. The enzymes responsible for production of ATP and reducing power under anaerobic conditions are repressed by O$_2$ while aerobic electron transport proteins are induced
Effect of nitrate on nitrogenase biosynthesis

(Haddock & Jones, 1977). Fermentative pathways are also repressed by NO_3^− (Payne, 1973; Stouthamer, 1976). On adding NO_3^−, anaerobic cells convert their metabolism to that of anaerobic respiration, a pathway of energy metabolism which is similar to aerobic electron transport with NO_3^− serving as the terminal electron acceptor.

Addition of NO_3^− to an anaerobic culture also represses the biosynthesis of the nitrogenase polypeptides (Fig. 2) and this includes not only nitrogenase but also nitrogenase reductase and the nifJ-encoded protein (120000 daltons). Chlorate-resistant mutant strains, e.g. SK-563 and SK-564, do not respire in the presence of NO_3^− and nitrogenase biosynthesis is not repressed by NO_3^− in these strains (Table 1). These results show that the NO_3^− itself is not the repressor, but that product(s) of NO_3^− metabolism play(s) an important role in the regulation of nitrogenase biosynthesis. Although NO_3^−, the product of NO_3^− respiration, inhibited enzymic activity, it had no effect on the stability of pre-formed nitrogenase protein.

Eady et al. (1978) observed that the t_4 for nitrogenase repression by NH_4^+ and O_2 was 11 to 12 min. In the experiments described in Fig. 2, NO_3^− showed a lag of up to 1 h and the t_4 for repression was about 1.5 h. This lag in the repression of nitrogenase by NO_3^− may be due to the requirement for induction of nitrate reductase which leads to the production of NO_2^−. It is possible that the repressive effect of NO_3^− may be mediated by NO_2^− or by other yet unknown intermediates of electron transport systems, since NO_3^− also represses the production of fermentative enzyme systems (which supply energy and reducing power to nitrogenase) in enteric bacteria (Stouthamer, 1976).

The addition of NO_2^− to the culture medium repressed nitrogenase biosynthesis without any lag in strain SK-561 (Fig. 3). NO_2^− repressed nitrogenase biosynthesis by about 50% in 1 h. NO_2^− was also metabolized by K. pneumoniae, and strain M5A1 utilized NO_2^− as an oxidant to support increased growth under anaerobic conditions, in a manner similar to NO_3^− respiration (Fig. 5). Kennedy & Postgate (1977) also reported that NO_2^− repressed the expression of K. pneumoniae nif in E. coli nar mutants. Cole (1978) recently reported that E. coli is capable of metabolizing NO_2^− under anaerobic conditions yielding NH_4^+ which can serve as nitrogen source for growth.

Although NO_3^− and NO_2^− can be converted to NH_4^+ and nitrogenase biosynthesis may be repressed by NH_4^+, this is not the case with strains SK-25 and SK-561, since both these strains produce nitrogenase even in the presence of externally added NH_4^+. Differences may exist between internally produced NH_4^+ (from NO_3^− or NO_2^−) and externally added NH_4^+. However, it has been observed with several strains, including strain SK-25 and its chlorate-resistant derivatives, that NH_4^+ produced from N_2 is excreted into the medium and nitrogenase was produced under conditions where NH_4^+ is exported (Andersen & Shanmugam, 1977).

The results discussed above show that NO_3^− plays a role in the regulation of nitrogenase biosynthesis and this effect is possibly mediated through two separate processes, one through the nitrogen metabolism and the other mediated through the control system operating on the oxidation-reduction (redox) pathways. Mutants that are derepressed for nitrogenase biosynthesis in the presence of NO_3^− and NO_2^− are currently being isolated. Studies with these mutants may lead to elucidation of the mechanism of redox control of nitrogenase biosynthesis.

We wish to thank R. C. Valentine for encouragement and support. This work was supported by grants from National Science Foundation (PCM 76–82766 and AER 77–07301). H. Hennecke was supported in part by the Deutsche Forschungsgemeinschaft.
REFERENCES


STOUTHAMER, A. H. (1976). *Biochemistry and...*
Effect of nitrate on nitrogenase biosynthesis

...genetics of nitrate reductase in bacteria. Advances in Microbial Physiology 14, 315-375.

