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Ferrous iron dependent nitric oxide production in nitrate reducing cultures of *Escherichia coli*

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Abstract. L-Lactate-driven ferric and nitrate reduction was studied in *Escherichia coli* E4. Ferric iron reduction activity in *E. coli* E4 was found to be constitutive. Contrary to nitrate, ferric iron could not be used as electron acceptor for growth. "Ferric iron reductase" activity of 9 nmol Fe^{2+} mg^{-1} protein min^{-1} could not be inhibited by inhibitors for the respiratory chain, like Rotenone, Quinacrine, Actinomycin A, or potassium cyanide. Active cells and L-lactate were required for ferric iron reduction. The L-lactate-driven nitrate respiration in *E. coli* E4 leading to the production of nitrite, was reduced to about 20% of its maximum activity with 5 mM ferric iron, or to about 50% in presence of 5 mM ferrous iron. The inhibition was caused by nitric oxide formed by a purely chemical reduction of nitrite by ferrous iron. Nitric oxide was further chemically reduced by ferrous iron to nitrous oxide. With electron paramagnetic resonance spectroscopy, the presence of a free [Fe^{2+}-NO] complex was shown. In presence of ferrous or ferric iron and L-lactate, nitrate was anaerobically converted to nitric oxide and nitrous oxide by the combined action of *E. coli* E4 and chemical reduction reactions (chemodenitrification).

Key words: Chemodenitrification – Nitric oxide – Nitrous oxide – Ferric iron reduction – Ferrous iron oxidation – Nitrate reduction – Nitrite reduction – *Escherichia coli*

Many bacteria have the capacity to reduce iron under conditions in which it would not be spontaneously reduced. Several enzymatic mechanisms can account for bacterial ferric iron reduction. Enzymatic iron reduction may serve either as an electron sink for excess reductant (Jones et al. 1984) or it may act as a terminal acceptor in an electron transfer chain (Short and Blakemore 1986). The iron reductase systems in *Spirillum itersonii* (Daily and Lascelles 1977), *Staphylococcus aureus* (Lascelles and Burke 1978) and *Pseudomonas aeruginosa* (Cox 1980) were studied in cell free extracts and the effects of respiratory inhibitors suggested that reduced components of the electron transfer chain that precede cytochrome *b* or *c* serve as a reductant for ferric iron. An induction of two ferri reductase systems was observed in *Pseudomonas* sp. strain 200 (Arnold et al. 1986). At high oxygen concentrations iron reduction occurred via an abbreviated electron transfer chain. Low oxygen tension (< 0.01 atm) gave rise to the induction of alternative respiratory pathways, and iron reduction was accelerated six- to eightfold, although the increased activity apparently was uncoupled from oxidative phosphorylation.

Iron reduction can be inhibited by oxygen and nitrate. This inhibition can either be predicted based on thermodynamic relationship of the chemical reactions involved (Zehnder and Stumm 1988), or be the result of a direct action of nitrate on the process of ferric iron reduction. Ottow (1970) proposed that in facultative anaerobic bacteria nitrate reductase can transfer electrons to either ferric iron or nitrate. Lascelles and Burke (1978) found ferric iron to be reduced independently of nitrate at a level in the electron transfer chain preceding cytochrome *b* and nitrite inhibited iron reduction. Based on studies with *Pseudomonas* sp. Obuekwe et al. (1981) suggested that the inhibitory effect of nitrate was due to ferrous iron oxidation by nitrite. Direct evidence for the oxidation of ferrous iron was obtained by Williams and Poole (1987) with the addition of nitrite to ferric iron reducing cultures of *Escherichia coli* K12. The chemical oxidation of ferrous iron to ferric iron with nitrite results in nitric oxide formation. Such a reaction has actually been reported for an in vitro system by Komatsu et al. (1978). For in vivo systems this reaction is poorly understood and is not yet well quantified (Ghiorse 1988).

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In the following the influence of nitrate on the L-lactate-driven ferric iron reduction in *E. coli* E4 was investigated. Considerable amounts of nitric oxide were formed during the concurrent reduction of nitrate and ferric iron. Nitric oxide was found to be inhibitory for lactate oxidation and concomitant nitrate reduction in *E. coli* E 4.

Materials and methods

Organism

Escherichia coli E4 (NCTC 9002) used in this study was obtained from the culture collection of the Department of Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands. The organism was maintained on yeast extract-glucose agar slants, containing (g/l): yeast extract (2.5), glucose (5) and agar (12). The agar slants were stored at 4°C and subcultured every 2 months.

Cultivation

Escherichia coli E4 was cultivated in demineralised water (pH = 7.2) containing (g/l): Na-L-lactate (5.6), NH_4Cl (0.82), $MgSO_4 \cdot 7H_2O$ (0.1), K_2HPO_4 (3) and KH_2PO_4 (2). To 1 l of this culture medium 1 ml of a combined trace element and vitamin solution was added as was described previously (Brons and Zehnder 1990). The pH was set at 7.2 with HCl.

Escherichia coli E4 was grown at 30°C in continuous culture using a 1 l working volume chemostat (Applikon B.V., Schiedam, NL) which was sterilized and recultivated ever 30–35 volume changes to prevent the selection of spontaneous mutants. The growth rate of *E. coli* E4 was fixed at 0.10 h^{-1}. The pH was monitored with a steam sterilisable glass electrode (Ingold, Van Oortmessen B.V., Den Haag, NL) connected to a pH meter and titrator (Radiometer, Instrumenthandel Zuid-Holland B.V., Den Haag, NL). The pH was maintained at 7.2 by the automatic addition of sterile 3 M KOH. Sterile air was passed through the medium at a rate of 1 litre per litre working volume per minute. The impeller speed was set at 600 r.p.m.

Batch experiments

Steady state cultures of *E. coli* E4 were harvested and centrifuged in portions of 50 ml at 10.000 *g* for 10 minutes. Pellets were washed in a 50 mM Tris-HCl buffer (pH = 7.2) centrifuged again, resuspended in 50 ml of the same buffer and supplemented with 1 ml/l each of a trace element and vitamin solution. Batch experiments with 50 ml resuspended whole cells of *E. coli* E4 (12 mg protein) were carried out in anaerobic argon flushed vials (headspace 72 ml) sealed with a rubber stopper, on an orbital incubator in a temperature controlled room at 30 ± 1°C.

Experiments for chemodenitrification were done anaerobically under argon in 50 ml of a 50 mM Tris-HCl buffer. The pH was adjusted with either HCl or NaOH. If necessary, KNO_3, KNO_2, $FeCl_3$, $FeCl_2$ or Na-L-lactate were added as well.

Preparation of cell free extracts

Steady state cultures of *E. coli* E4 were harvested and centrifuged for 10 min at 10.000 *g*. Pellets were washed in a 50 mM Tris-HCl buffer (pH = 7.2; 4°C), centrifuged again and resuspended in 2 ml of the same buffer. Ultrasonic disruption of the cells was achieved by treatment with a Branson model B12 sonifier (Marius Instrumtens B.V., Utrecht, NL) applying 36 W for 3 min at intervals of 1 min while cooling one ice. The crude extracts were centrifuged at 10.000 *g* for 15 min at 4°C to remove the cell debris. The supernatant was used as a cell free extract and was stored on ice until further use.

Oxygen respiration

Oxygen respiration was monitored at 30°C in the thermostatically controlled reaction vessel of a Yellow Springs Instruments model 53 biological oxygen monitor equipped with a polarographic oxygen probe (Tamson B.V., Zoetermeer, NL). The oxygen uptake rate of 0.3 ml (0.24–0.36 mg protein) cell free extract was determined in 3 ml final volume of an air saturated Tris-HCl buffer (pH = 7.2; 50 mM). The endogenous oxygen uptake rate was subtracted from the respiration rate observed in the presence of 10 mM L-lactate.

Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance spectroscopy of whole cell incubations was done with a Bruker model ER 200 D EPR spectrometer (Bruker Spectrospin N.V., Wormer, NL). The microwave frequency was measured with a Systron Donner model 1292 A frequency counter (Intechmij B.V., Diemen, NL). The magnetic field was modulated with a frequency of 100 kHz and an amplitude 8 Gauss. The microwave power was 5 mW. Cooling was done with a helium-flow cryostat (Lundin and Aasa 1972). The detection temperature was 20 K.

High-spin (S = 5/2) ferric iron was quantified by comparing the amplitude at $g = 4.3$ with that of a standard solution of 5 mM Fe^{3+} in 50 mM Tris-HCl, pH 7.2. Signals from ferrous-NO complex (S = 3/2) were quantified by comparing amplitudes with that from a standard solution of 5 mM Fe^{2+} in 50 mM Tris-HCl, pH 7.2, reacted with NO(g). The spin concentration of the iron nitrosyl in the latter standard solution was determined by double integration with respect to the spectrum from 5 mM Fe^{3+}, with correction for different Boltzmann distributions and transition probabilities (Aasa and Vänngård 1975).

Chemical and biochemical determinations

Whole cells were removed by centrifugation at 10.000 *g* for 5 min. Nitrate was measured with alkaline salicilate, nitrite with acid sulfonilamide and ammonium after direct Nesslerization according to Deutsche Einheitsverfahren (1989). Autoxidation of iron was prevented by acidification to pH 2 with HCl prior to centrifugation. Ferrous iron was measured as a magenta Fe^{2+}-complex. For this purpose [3-(4-Phenyl-2-pyridyl)-5-(phenylsulfonic acid)-1,2,4-triazine sodium salt] was used a chelating agent and the complex analyzed spectrophotometrically at 565 nm. This compound is commercially available as Ferrospectral II. For ferric iron determinations, 5 mM ammonium thioglycollate had been added to the 1 mM Ferrospectral II solution in order to convert all iron into the ferrous form. Ferric iron reduction activity was assayed anaerobically at 30°C in a Beckman model 25 recording spectrophotometer (Beckman Instruments Nederland B.V., Mijdrecht, NL). The assay was carried out in a 1-cm argon flushed cuvette sealed with a rubber stopper. The reaction mixture, final volume 1 ml in Tris-HCl (50 mM; pH = 7.2), comprised 10 mM Na-L-lactate, 1 mM $FeCl_3 \cdot H_2O$, and 0.1 mM Ferrospectral II. The reaction was started by the addition of 100 µl (80–120 µg protein) cell free extract.

Nitric oxide, nitrous oxide, and carbon dioxide were measured gaschromatographically using a Packard Becker model 417 GC (Chrompack B.V., Middelburg, NL) equipped with a Poropak Q (60–80 mesh) column (600 cm × 0.3 cm internal diameter). Carrier

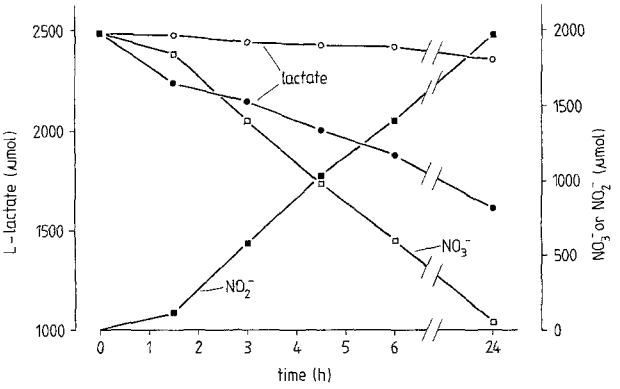

Fig. 1. L-Lactate utilisation by anaerobic non-growing batch cultures of *Escherichia coli* E4 in presence (●) and absence (○) of nitrate. Nitrate consumption (□) and concomitant nitrite (■) production are also given. Absolute amounts are reported for 50 ml medium. All data are means of two independent experiments

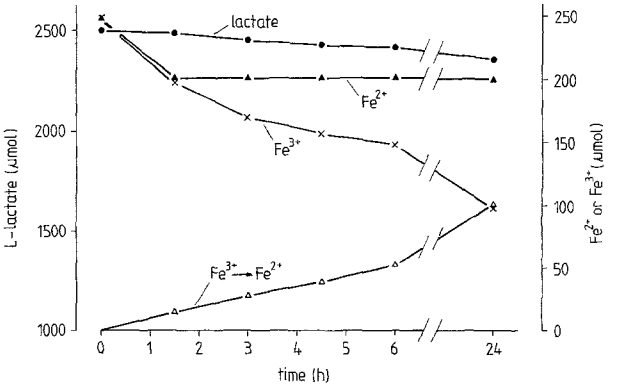

Fig. 2. L-Lactate consumption (●) by anaerobic non-growing batch cultures of *Escherichia coli* E4 after addition of either $FeCl_2$ or $FeCl_3$. "Uptake" of Fe^{2+} (▲) and conversion of Fe^{3+} (X) to Fe^{2+} (△) are also given. Concentrations and statistics as in Fig. 1

gas was argon at a flow rate of 30 ml/min (60 psi). Termal conductivity detector and oven were kept at 100° C. Organic acids were analyzed by HPLC with a LKB model 2158 Uvicord SD detector (Pharmacia LKB Biotechnology B.V., Woerden, NL) equipped with a 30 cm × 3/8 inch internal diameter organic acids column (Chrompack B.V., Middelburg, NL) which was operated at 65° C and 80 bar and protected by a guard column (anion exchanger). The detection wavelength was 206 nm, elution rate of 10 mM H_2SO_4 0.8 ml/min, and injection volume 20 µl. Pyruvate was quantified as described by Katsuki et al. (1971). Volatile alcohols were determined on a Packard Becker model 417 GC (Chrompack B.V., Middelburg, NL) using a Sil5CB column (10 m × 0.53 mm internal diameter). Flowrate 10 ml N_2/min, oven 60° C, flame ionization detector (FID) 150° C, injection part 140° C. Volatile fatty acids were analyzed on a Varian model 2400 GC (Varian Benelux B.V., Amsterdam, NL) equipped with a Chromosorb 101 glass column (200 cm × 1/8 inch internal diameter). Flowrate of formic acid saturated N_2 was 30 ml/min. Oven 190° C, FID-detector 240° C, injection port 170° C.

For protein determinations of cell free extracts and whole cells a modified Lowry method (De Moss and Bard 1957) with bovine serum albumin as a standard was used. Whole cells were boiled 10 min in 0.5 M NaOH previous to protein determination. Glycogen was quantified after extraction and acid hydrolysis as glucose by the anthrone reaction (Hanson and Phillips 1981).

Chemicals

Sodium L-lactate was from I. T. Baker B.V., Deventer, NL. Growth media constituents, ammonium thioglycollate and Ferrospectral II were from E. Merck Nederland B.V., Amsterdam, NL. Yeast extract and agar used in slants were from Difco (Brunschwig Chemie B.V., Amsterdam, NL). Rotenone, antimycin A, KCN and bovine serum albumin were obtained from Sigma (Brunschwig Chemie B.V., Amsterdam, NL). Quinacrine was purchased from Aldrich Chemie B.V., Brussels, Belgium. Nitric oxide (chemically pure) was from Matheson Gas Products (Hoek Loos B.V., Schiedam, NL).

Results

Nitrate reduction

Escherichia coli cells grown aerobically at a dilution rate of 0.1 h^{-1} show nitrate and nitrite reductase activity (Brons and Zehnder 1990). Cells washed in Tris-buffer started to reduce nitrate to nitrite by oxidizing L-lactate after a lag of 90 min (Fig. 1). Only minor amounts of nitrite were further reduced to ammonium (50 µmol within 24 h, not shown), despite the fact that nitrite reductase was present in cell free extracts at an activity of 80 nmol NH_4^+ mg^{-1} protein min^{-1}. Most of the lactate was converted to acetate (1150 µmol) and intracellular glycogen (215 µmol as glucose). In the absence of nitrate or nitrite some lactate (150 µmol) was converted to pyruvate (60 µmol), acetate (30 µmol), carbon dioxide (30 µmol), and intracellular glycogen (20 µmol) after 24 h. The protein content increased from 12 mg to 15 mg in presence of nitrate and from 12 mg to 12.5 mg in its absence within 24 h. This slight increase is due to the lack of essential nutrients such as sulphur and phosphorus in the first case and in addition nitrogen in the second case.

Iron reduction

In presence of 50 mM Tris-buffer both 5 mM Fe^{3+} and Fe^{2+} remained in solution at pH 7.2. L-Lactate could not be used by *E. coli* E4 to a significant extent as electron donor for the reduction of Fe^{3+}. Within 24 h only 40% of Fe^{3+} was reduced to Fe^{2+} (Fig. 2) and the decrease in L-lactate was the same whether Fe^{3+}, Fe^{2+} or no iron at all was added to the incubation mixture (Fig. 1 and 2). Higher Fe^{3+} concentrations (10 and 20 mM) did not increase CO_2 or acetate production from lactate (not shown). After 24 h of incubation, the HCl extractable iron of the biomass (15 mg protein) increased from 14.4 µmol to 62.2 µmol. This cellular iron accumulation may explain the drop of Fe^{2+} from 250 µmol to 200 µmol in the experiments shown in Fig. 2.

To obtain indications where a possible ferri reductase is located in the respiratory chain, experiments were done with some specific inhibitors. These inhibitors acted on NADH-dehydrogenase (1 mM Rotenone; Boogerd et al. 1980), flavinedehydrogenase (1 mM Quinacrine; Wolfe 1975), cytochrome *b* (10 µg/ml Actinomycin A; Boogerd et al. 1980), and terminal cytochrome oxidase (1 mM potassium cyanide). None of these inhibitors had any

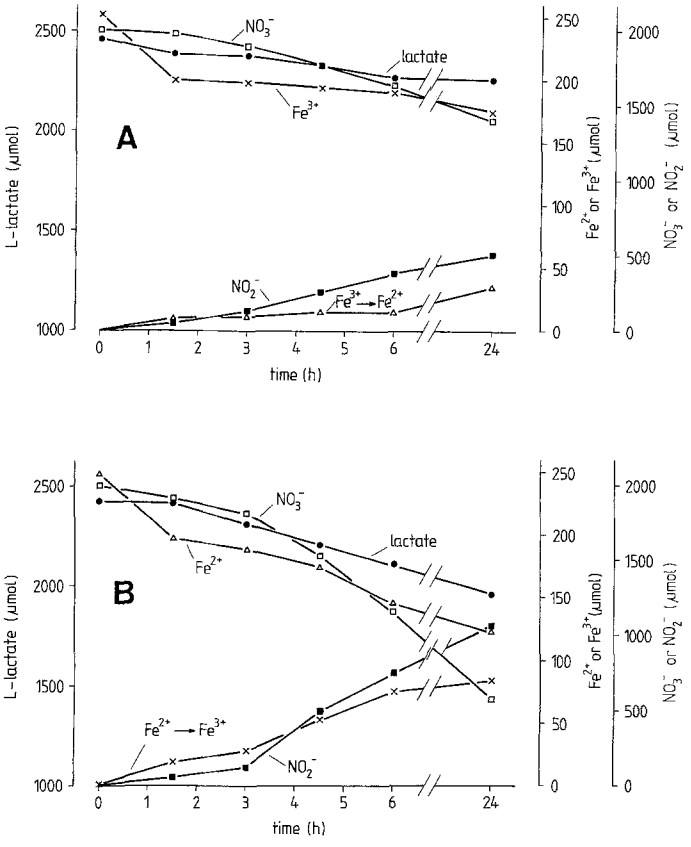

Fig. 3. **A** L-Lactate consumption (●), nitrate conversion (□) to nitrite (■) and Fe^{3+} reduction (X) to Fe^{2+} (△) by anaerobic non-growing batch cultures of *Escherichia coli* E4 after addition of $FeCl_2$. **B** Same as in **A** but after addition of $FeCl_3$. Concentrations and statistics as in Fig. 1

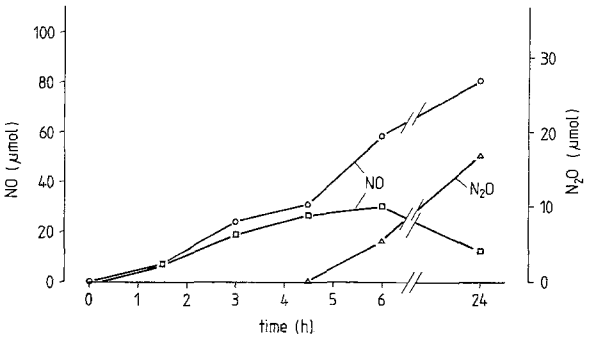

Fig. 4. Time course of nitric oxide and nitrous oxide production in anaerobic non-growing batch cultures of *Escherichia coli* E4. Symbols referring to nitric oxide, (○) when 250 µmol $FeCl_3$ was added or (□) when 250 µmol $FeCl_2$ were added. (△): N_2O production in response to the additon of 250 µmol $FeCl_2$. Concentrations and statistics as in Fig. 1

effect on the rate of Fe^{3+} reduction in cell free extracts. This rate was actually linearly proportional to the amount of extract present. The constant specific rate was 9 nmol $Fe^{2+} \cdot mg^{-1}$ protein $\cdot min^{-1}$. However, oxygen respiration was clearly reduced to various degrees by these chemicals in cell free extracts. Rotenone inhibited oxygen respiration by 13%, Quinacrine by 40%, Actinomycin A by 52%, and potassium cyanide by 84%.

Iron reduction in presence of nitrate

Fe^{3+} seems to inhibit L-lactate oxidation with nitrate as electron acceptor. But also the rate of Fe^{3+} reduction is diminished in presence of nitrate (Fig. 3A). Fe^{2+} inhibits nitrate reduction with lactate but to a lesser extent than Fe^{3+} (Fig. 3B). Interestingly, in both cases with Fe^{3+} and Fe^{2+} more nitrate is reduced than nitrite formed, namely 420 µmol versus 350 µmol with Fe^{3+} and 850 µmol versus 700 µmol with Fe^{2+} after 6 h of incubation (Fig. 3A, B). This loss of nitrogen could be explained in three ways: (*i*) nitrate was assimilated for growth, (*ii*) nitrite was converted to ammonium, or (*iii*) nitrite was transformed to something else than ammonium. Almost no growth has been observed in batches with either iron ions. Ammonium was only present in very small amounts (40 µmol) and could not account for the whole difference. The only other possible nitrogen compounds were N_2O and NO. In fact both were found (Fig. 4). Only NO when Fe^{3+} was present, and NO and N_2O with Fe^{2+}. NO could account for the lacking 60 µmols of nitrogen in incubations with Fe^{3+}. However, NO and N_2O could only make up for one fourth of the missing nitrogen in the batch cultures with Fe^{2+}. Electron paramagnetic resonance (EPR) showed the presence of an [Fe^{2+}-NO] complex at concentrations of about 135 µmol (Fig. 5). This is enough to explain the missing nitrogen. Data in Fig. 5 confirm also the observation that in absence of nitrate a considerable amount of Fe^{3+} is converted to the EPR invisible Fe^{2+} (Fig. 2) and that this conversion is strongly inhibited when nitrate was added (Fig. 3A). [Fe^{3+}-NO] complexes are apparently not formed. Interestingly, the rate of Fe^{3+} reduction was not affected by the presence of nitrate in cell free extract (not shown).

Nitric oxide

In the range between 0 and 5 mM Fe^{3+} added, NO production increased as the amount of added Fe^{3+} increased (Fig. 6). At higher Fe^{3+} concentration, the rate of NO increased only slightly. Parallel to the higher production of free NO, nitrite formation and lactate consumption is reduced (Fig. 6) indicating an inhibitory effect of NO. The addition of NO to cell suspensions reduced in fact the lactate-driven nitrate respiration rates by 50% already at a NO concentration of 85 µM (Fig. 7). The reduction of NO_2^- to NO and further to N_2O (Moraghan and Buresh 1977) by the simultaneous oxidation of Fe^{2+} is probably a purely chemical process (Table 1) and is referred to in the literature as chemodenitrification (Tiedje 1988). In the system investigated here the role of *E. coli* E4 is to provide continuously NO_2^- and Fe^{2+} by reducing NO_3^- and Fe^{3+} with lactate.

The chemodenitrification in our system is pH-dependent (Knowles 1981). The apparent rate constants for the

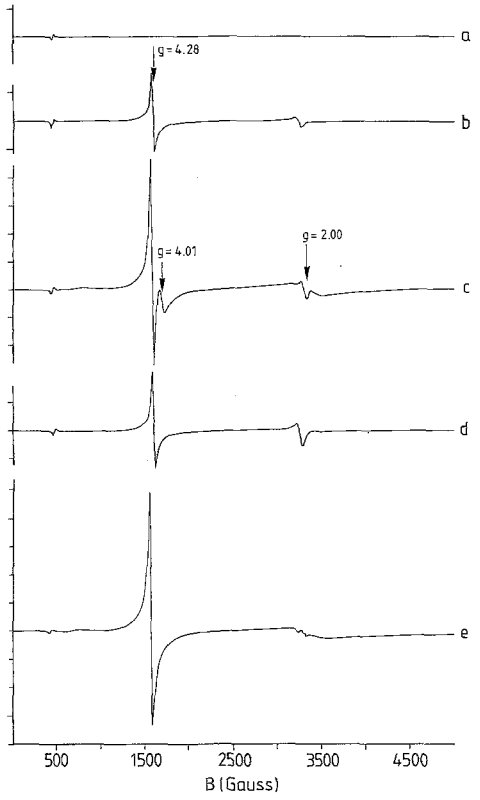

Fig. 5. EPR spectra detected after 6 h in anaerobic non-growing batch cultures of *Escherichia coli* E4 (12 mg protein; 50 mM L-lactate as an energy source). (*a*): control experiment (*E. coli* E4, L-lactate and 2 mmol NO_3^-). (*b*): 250 µmol Fe^{2+} were added, signal at $g = 4.28$ represent 0.57 mM Fe^{3+}. (*c*): 250 µmol Fe^{2+} and 2 mmol NO_3^- were added, signal at $g = 4.28$ is 1.5 mM Fe^{3+}, signals at $g = 4.01$ and $g = 2.00$ were quantified 2.7 mM [Fe^{2+}-NO]. (*d*) 250 µmolFe^{3+} was added, $g = 4.28$ represents 0.71 mM Fe^{3+}. (*e*): attenuation 0.5:250 µmol Fe^{3+} and 2 mmol NO_3^- were added, signal $g = 4.28$ is 4.8 mM Fe^{3+}. The microwave frequency was 9329 ± 3 MHz

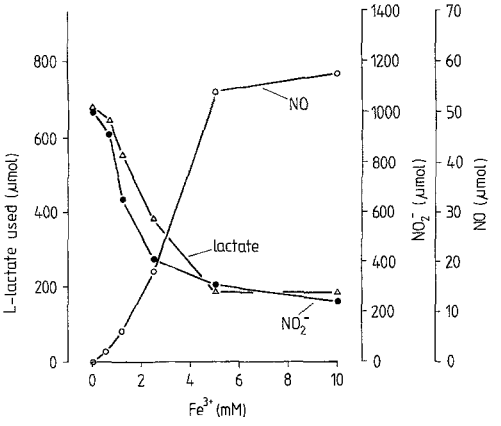

Fig. 6. Nitrite and nitric oxide formation and L-lactate uptake determined after 6 h in anaerobic non-growing batch cultures of *Escherichia coli* E4 as a function of initial ferric iron concentration. Concentrations of L-lactate, NO_2^-, and NO and statistics as in Fig. 1

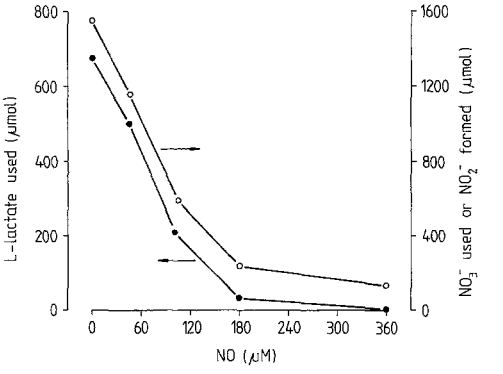

Fig. 7. Inhibition of L-lactate-driven nitrate respiration by nitric oxide in anaerobic non-growing batch cultures of *Escherichia coli* E4. Concentrations and statistics as in Fig. 1

initial NO formation in Tris-HCl buffer were, 2.0 h^{-1} at pH 4, 0.12 h^{-1} at pH 5, 0.05 h^{-1} at pH 6, 0.01 h^{-1} at pH 7 and $\ll 0.01$ h^{-1} at pH 8.

Discussion

Ferric iron is a suitable electron acceptor under anaerobic conditions (Zehnder and Stumm 1988). Experiments in this paper with *Escherichia coli* E4 have shown that this organism can reduce ferric iron with electrons from lactate but is not able to specifically couple iron reduction to the respiratory chain. This is in accordance with the findings of Williams and Poole (1987) that in *E. coli* K12 the respiratory chain is not involved in the reduction of ferric iron. Yet Short and Blakemore (1986) report a translocation of protons with ferric iron as terminal electron acceptor in *E. coli* CSII27, but no evidence was presented for energy conservation via this reaction. As a result of the presence of ferric iron some minor increase of cell yield was found by Jones et al. (1984) for a malate fermenting *Vibrio* sp., although ferric iron reduction was primarily associated with the diversion of the metabolism to energetically more favorable end products. Positive evidence for the direct coupling of dissimilatory iron reduction to growth was obtained with a hydrogen metabolizing *Pseudomonas* sp. (Balashova and Zavarzin 1979). Growth at the expense of acetate oxidation coupled to ferric iron reduction was recently reported for a strictly anaerobic gram-negative rod, isolated from fresh water sediments (Lovley and Phillips 1988).

Ferric iron inhibited nitrate reduction by *E. coli* (Fig. 3 A). This inhibition was not due to the competition for electrons by both electron acceptors but to the formation of a considerable amount of toxic nitric oxide. In these cultures nitric oxide is formed by a process which can schematically be represented by Fig. 8. Ferric iron is reduced to ferrous iron by compounds which are directly or indirectly reduced by lactate. Lactate cannot reduce ferric iron without an electron carrier in our system (Table 1). Ferrous iron then reduces nitrite by chemodenitrification (Tiedje 1988) to nitric oxide without the mediation of electron carriers or enzymes (Table 1). Nitric oxide can further react with ferrous iron by the same process to nitrous oxide. In case ferrous iron is added together with nitrate, in total more nitric oxide is formed

Table 1. Formation of nitric oxide, nitrous oxide, [Fe^{2+}-NO], and ferric iron after 6 h of incubation. Experiments were done at pH 7.2, in 50 mM Tris-HCl buffer containing 40 mM KNO_3 or KNO_2, 5 mM $FeCl_3$ or $FeCl_2$, and 50 mM Na-L-lactate and were indicated *Escherichia coli* E4 cells at a concentration of 12 mg protein per 50 ml. Nitric oxide, nitrous oxide, [Fe^{2+}-NO] and ferric iron are given in µmol produced in 50 ml reaction mixture

Reaction mixture	NO[a]	N_2O	[Fe^{2+}-NO]	Fe^{3+}
Fe^{3+}	–[b]	–	–	250[c]
Fe^{3+} + L-lactate	–	–	–	250
Fe^{2+}	–	–	–	–
NO_3^- + Fe^{2+}	–	–	–	–
NO_3^- + Fe^{2+} + *E. coli* E4 (without L-lactate)	–	–	–	–
NO_3^- + Fe^{2+} + *E. coli* E4 (autoclaved)[d]	–	–	–	–
NO_3^- + Fe^{2+} + *E. coli* E4 (plus L-lactate)	16	10	135	75
NO[e] + Fe^{2+} + *E. coli* E4 (plus L-lactate)	4	85	72	90
NO_3^- + Fe^{3+} + *E. coli* E4 (plus L-lactate)	59	–	–	241[f]
NO_2^- + Fe^{2+}	170	23	< 40[g]	200
NO[h] + Fe^{2+}	2	10	25	10

[a] NO and N_2O were quantified gaschromatographically, [Fe^{2+}-NO] with EPR, and Fe^{3+} either spectrophotometrically or with EPR
[b] Means not detected
[c] All data are means of at least two independent experiments
[d] *E. coli* E4 cells were autoclaved at 120° C for 20 min in the Tris-HCl buffer before sterile addition of KNO_3, $FeCl_2$, and L-lactate
[e] 150 µmol NO added
[f] Total amount of Fe^{3+} incl. the portion adsorbed to the cells
[g] [Fe^{2+}-NO] can only be detected with this method if the concentration of this complex is at least 20% of the Fe^{3+} concentration
[h] 35 µmol NO added

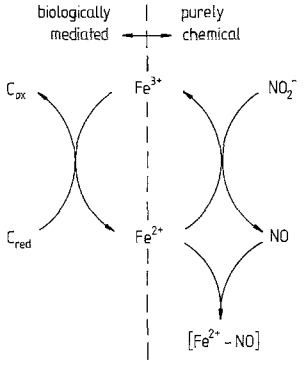

Fig. 8. Schematic represenation of the coupling of biologically mediated and purely chemical processes leading to the formation of free nitric oxide and the [Fe^{2+}-NO] complex from nitrite and ferrous iron. The biologically mediated nitrate reduction and the further purely chemical reduction of nitric to nitrous oxide are not included in this figure. C_{red} and C_{ox} means reduced and oxidized "coenzyme(s)" which can reduce ferric to ferrous iron

than with ferric iron. Most nitric oxide is subsequently bound to excess ferrous iron by the formation of a [Fe^{2+}-NO] complex. Ferric iron can be converted back to ferrous iron as long as the biological system is functioning.

Nitrate reduction is much stronger inhibited by ferric iron than by ferrous iron (Fig. 3). This is probably due to the higher free NO concentration in cultures supplemented with ferric iron (Fig. 4). When ferrous iron was added most nitric oxide was bound in the [Fe^{2+}-NO] complex. The data in Fig. 3 and Table 1 together with the scheme presented in Fig. 8 suggest that the purely chemical oxidation of ferrous iron by nitrite is much faster than the subsequent biologically mediated reduction of ferric iron by lactate. This difference keeps ferrous iron concentration low. As a consequence the [Fe^{2+}-NO] complex is not formed and nitric oxide is not removed. This mechanism suggest that the binding constant between free Fe^{2+} and NO is relatively low, since [Fe^{2+}-NO] does not act as an ultimate sink of either Fe^{2+} and NO. In addition, the iron in the [Fe^{2+}-NO] complex seems still to be able to become oxidized, thereby releasing NO or perhaps even N_2O. The production of NO is probably also responsible for the inhibition of ferric iron reduction by nitrate in *Staphylococcus aureus* (Lascelles and Burke 1978), *Pseudomonas* sp. (Obuekwe et al. 1981) and *E. coli* K12 (Williams and Poole 1987).

According to Muhoberac and Wharton (1980) nitric oxide toxicity is due to direct binding to heme iron. Stouthamer (1988) hypothesizes that the specific binding of nitric oxide to ferrous iron is a possible reason that during denitrification and dissimilatory nitrate reduction to ammonium free nitric oxide is not found or only measured in trace amounts. In fact some nitric oxide reductases are clearly heme proteins (Heiss et al. 1989; Carr and Ferguson 1990). Nitrite reduction by purified nitrite reductases from *Thiobacillus denitrificans* (Le Gall et al. 1979) and *Desulfovibrio desulfuricans* (Liu et al. 1980) occurs also via a heme-nitric oxide complex as intermediate. In both cases evidence for an enzyme-NO complex was obtained with the characteristic ^{14}N hyperfine pattern in the EPR signal in the $g = 2.0$ region. The spectrum for *E. coli* E4 does not exhibit this pattern in the $g = 2.0$ region (Fig. 5C). The observed spectrum is characteristic for a non-heme iron nitric oxide complex, suggesting that in our experiments nitric oxide is directly bound to the free ferrous iron. This could be confirmed with ferrous iron and nitric oxide in the absence of *E. coli* cells (Table 1). The binding of nitric oxide to heme iron has

been used recently to prove that nitric oxide can indeed be a free intermediate in denitrification (Goretski and Hollocher 1988; Kučera 1989).

The data presented in this paper can explain N_2O production by enterobacteria and are an indication for the mechanism leading to the change of N_2O in human breath before and after a meal containing nitrate and nitrite (Bleakley and Tiedje 1982; Smith 1982). The results also show that organisms, other than nitrifiers and denitrifiers, able to produce nitrate and to reduce ferric iron, can contribute to the loss of nitrogen to the atmosphere. It remains to further investigations to quantify the contribution of the reactions described in this paper for the nitric and nitrous oxide content of the atmosphere.

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