Cloning and characterization of a nitrite reductase gene from *Alcaligenes faecalis* and its expression in *Escherichia coli*

MAKOTO NISHIYAMA,* JUNKO SUZUKI, MUTSUKO KUKIMOTO, TATSUYA OHNUKI, SUEHARU HORINOCHI and TERUHIKO BEPPU

Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

(Received 14 September 1992; revised 25 November 1992; accepted 7 December 1992)

The gene (*nir*) encoding the copper-containing nitrite reductase (NIR) of a denitrifying bacterium, *Alcaligenes faecalis* S-6, was cloned by a synthetic oligonucleotide-probing method. The nucleotide sequence of the cloned DNA fragment revealed the primary structure of the NIR precursor containing the N-terminal signal sequence for secretion. A nucleotide sequence, possibly recognized by a transcriptional regulator resembling FNR was found upstream of the structural gene. When the cloned gene was expressed in *Escherichia coli* under the control of the *lac* promoter at 37 °C, NIR was produced as large inclusion bodies and little activity was detected. When cultivation was at 20 °C, most of the NIR was detected in the soluble fraction and a significant portion of the protein was translocated into the periplasmic space, accompanied by removal of its signal sequence.

Introduction

The denitrifying bacterium *Alcaligenes faecalis* S-6 possesses an anaerobic nitrate respiration system. A copper-containing nitrite reductase (NIR) catalyses the reduction of NO₂⁻ to NO under anaerobic conditions (Kakutani *et al.*, 1981a, b). Pseudoazurin, a member of the cupredoxin family, also a copper-containing protein composed of 123 amino acids, is required as a direct electron donor for the reduction of NO₂⁻ by the enzyme (Kakutani *et al.*, 1981c; Hormel *et al.*, 1986). When reduced pseudoazurin and NIR are incubated under aerobic conditions, however, NIR catalyses the reduction of O₂ to H₂O₂, which inactivates this enzyme. Thus NIR and pseudoazurin form a unique electron transferring system between two copper-containing proteins. A similar system comprising the copper-containing NIR and pseudoazurin was reported in *Achromobacter cycloclastes* (Liu *et al.*, 1986).

Crystallographic analyses of pseudoazurin from *A. faecalis* S-6 have revealed a three-dimensional structure

consisting of a typical β -barrel containing a single type I copper atom and two additional C-terminal α -helices (Adman *et al.*, 1989; Petratos *et al.*, 1987). The gene encoding pseudoazurin precursor with an N-terminal signal sequence for secretion has been cloned and expressed in *Escherichia coli*, allowing production of correctly processed pseudoazurin in the periplasmic space (Yamamoto *et al.*, 1987). This expression system, with site-directed mutagenesis, has generated several mutant proteins. One of these mutants, with Pro-80 replaced by Ala at the position adjacent to two copper ligands, Cys-78 and His-81, shows a marked increase in the redox potential but no change in affinity for NIR (Nishiyama *et al.*, 1992). Further characterization of the interaction between pseudoazurin and NIR, requires information on NIR structure and function. NIR from *A. faecalis* S-6 has been crystallized and preliminary X-ray analysis done (Petratos *et al.*, 1986). Recent crystallographic analysis of *Ach. cycloclastes* NIR revealed a trimeric structure composed of three identical subunits and six copper atoms (Godden *et al.*, 1991). In this paper, we describe the cloning and nucleotide sequence of the *nir* gene from *A. faecalis* S-6 and its expression in *E. coli*. These results provide a base for site-directed mutagenesis of NIR and for a better understanding of the mechanism of electron transfer between the copper-containing proteins.

* Author for correspondence. Tel 03 3812 2111, ext. 5126; fax 03 3812 0544.

The sequence data reported in this paper has been submitted to DDBJ, EMBL and GenBank and have been assigned the accession number D13155.

Methods

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and pyroglutamate aminopeptidase were purchased from Takara Shuzo. [α - 32 P]dCTP and [γ - 32 P]ATP were from Amersham Japan. A kit for nucleotide sequencing by the M13-dideoxynucleotide method was obtained from United States Biochemicals. Oligonucleotides were synthesized by a Cyclone Plus DNA synthesizer (Milligen/Biosearch).

Bacterial strains and plasmids. *A. faecalis* S-6 (Kakutani *et al.*, 1981a) was used as the source for NIR and used as the DNA donor. *E. coli* JM105 [Δ (*lac-pro*) *thi strA endA sbc-15 hsdR4 F'* *traD36 proAB lac^r* *lacZAM15*] was used as a host for cloning and expression of the *nir* gene from *A. faecalis* S-6 and was used for M13 phage propagation. pUC19 (Yanisch-Perron *et al.*, 1985) was used for cloning and the expression of the *nir* gene. pNIR201 is a plasmid, obtained in this study, which contains the *nir* gene in a 2.4 kb *Pst*I fragment cloned in pUC19. pNIR501 is a plasmid which allows expression of the *nir* gene under the control of the *lac* promoter. It was constructed as follows. To introduce the *Hind*III site just upstream of the putative ribosome-binding site of the *nir* gene, polymerase chain reactions were carried out using synthetic oligonucleotides 5'CAAGCTTGTAAAGGAGAGT-ATCGATGGCC3', which corresponded to the translational initiation region, and 5'CATGAATTCGACCACCTTGGG3' including an *Eco*RI site within the coding sequence (see Fig. 2). Thermal cycling (94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min) was repeated 20 times. After digestion of the amplified DNA with *Hind*III and *Eco*RI, the resulting restriction fragment of about 250 bp and a 1.5 kb *Eco*RI-*Pst*I fragment from pNIR201 were ligated with pUC19 digested with *Hind*III and *Pst*I to produce pNIR501.

Gene manipulation. Total chromosomal DNA of *A. faecalis* was prepared by the method of Saito & Miura (1963). For cloning the *nir* gene, three oligonucleotides (probe 1, TAQGAQAAPATQTAQT-AQGT; probe 2, GAPCAPGAQTTQTAQGT; probe 3, AAPTA-QAAPAAPTAQGAPGC; P and Q represent G+A and C+T, respectively) were synthesized based on the partial amino acid sequences of NIR and were used as the probes for Southern hybridization (Southern, 1975) with the modifications as follows. Hybridization in 6 × NET (0.9 M-NaCl, 90 mM-Tris/HCl; pH 7.5, 6 mM-EDTA) containing 0.5% Nonidet P-40 was carried out at room temperature for 8–10 h and non-hybridized probes were washed out with 4 × SSC at an appropriate temperature (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0). Colony hybridization was carried out according to Grunstein & Hogness (1975) under the same hybridization conditions described above. Nucleotide sequencing was done by the dideoxy chain-termination method using M13 phages (Messing, 1983; Sanger *et al.*, 1977). All the restriction sites used for cloning on M13 RFI DNA were verified by determination of part of an overlapping sequence.

Purification of NIR from *A. faecalis* S-6. *A. faecalis* S-6 was precultured in 10 ml L-broth (Maniatis *et al.*, 1982) aerobically at 30 °C for 15 h. The cells were then transferred to 11 NABN medium containing 0.7 g K₂HPO₄, 0.3 g KH₂PO₄, 5 g KNO₃, 10 g bouillon (Kyokuto, Tokyo), 1 g yeast extract (Difco), 10 g CH₃COONa.3H₂O, 100 mg MgSO₄.7H₂O, 20 mg CaCl₂.2H₂O, 2 mg MnCl₂.4H₂O, 1 mg NaMoO₄.2H₂O, 1 mg CuSO₄.5H₂O, and 1 mg FeCl₃.6H₂O, pH 6.8, and were cultured anaerobically at 30 °C for 48 h. The cells were harvested by centrifugation, suspended in 60 ml 20 mM-potassium phosphate buffer (pH 6.2) containing 1 mM-PMSF (buffer A), and disrupted by ultrasonication (Branson Sonifier; cell disrupter model 200). After removal of the insoluble materials by centrifugation at 50 000 g for 1 h, 25 g ammonium sulphate was added to the supernatant (about 60% saturation). The mixture was stored at 4 °C

overnight. The precipitate, which was collected by centrifugation at 3000 g for 30 min, was dissolved in 5 ml water and dialysed against water overnight. After dialysis against 20 mM-potassium phosphate buffer (pH 5.3), insoluble materials were removed by centrifugation at 10 000 g for 20 min. The supernatant was dialysed against buffer A and the dialysate was applied to a DEAE-Toyopearl column (4.5 × 50 cm; Tosoh, Tokyo). The adsorbed proteins were eluted with buffer A containing 300 mM-KCl and again dialysed against the same buffer. The solution prepared in this way was applied to FPLC equipped with a MonoQ anion-exchanger column (5 × 50 mm; Pharmacia) and eluted with a linear gradient of KCl (150–300 mM) in buffer A. The active fractions eluting at about 220 mM-KCl were collected and concentrated by using a Centricon 30 filter (Amicon). The concentrated preparation was then applied to FPLC equipped with a Superose 12 gel filtration column (1 × 30 cm; Pharmacia) and active fractions were collected. The sample was applied once again to MonoQ FPLC in the same way, and the eluted active fractions were used as the purified preparation.

Amino acid sequencing of NIR. Cleavage of NIR with CNBr and isolation of the generated peptides were carried out as follows. The purified NIR (2 nmol) dissolved in 1 ml water was dialysed against 500 ml of 70% (v/v) formic acid. One milligram CNBr was added to the dialysate (1 ml) and incubated for 24 h. The mixture was then diluted with 9 vols water and lyophilized. The lyophilized sample, which was dissolved in 25 µl water containing 5% acetonitrile, was subjected to HPLC (Shimadzu, LC-4A, Kyoto) using an ODS-304-1251 reverse-phase column (4.6 × 250 mm; Senshu Chemicals, Tokyo). Elution with a linear gradient of acetonitrile (5–95%) gave more than five peptide peaks, of which the largest was collected and purified by rechromatography on the same column. This purified CNBr-cleaved peptide fragment was subjected to automated Edman degradation (Applied Biosystems, model 473A). For determination of the N-terminal amino acid sequence, 700 pmol NIR was applied to SDS-PAGE (Laemmli, 1970) and the proteins were electroblotted onto polyvinylidene difluoride membrane (Immobilon, Millipore) with a blotting apparatus (Sartorius, Sartoblot II-S). NIR proteins blotted onto the membrane were stained with amidoblack and each protein band was collected separately by cutting the sheets. After being washed with water, the sheets were subjected directly to automated Edman degradation. Similar analysis was carried out with the NIR preparation treated with 2.5 milliunits of pyroglutamate aminopeptidase in 50 µl 25 mM-potassium phosphate buffer (pH 7.0) containing 10 mM-DTT at 37 °C for 8 h.

Preparation of anti-NIR antiserum. The purified NIR preparation (0.1 mg) dissolved in 0.5 ml distilled water was mixed and homogenized with an equal volume of complete Freund's adjuvant and injected into a young male rabbit (2 kg) at multiple sites on the back. Two weeks later, a booster of 0.1 mg NIR was injected in the same way. After an additional two weeks, whole blood was collected. About 30 ml anti-NIR antiserum was obtained by centrifugation at 1500 g for 15 min. Western blotting with this antiserum was carried out by the method of Burnett (1981).

Expression of *nir* gene in *E. coli*. *E. coli* cells carrying pNIR501 were precultured in 10 ml 2 × YT medium (Yanisch-Perron *et al.*, 1985) containing 50 µg ampicillin ml⁻¹ at 30 °C for 12 h. A portion (0.1 ml) of the preculture was transferred to 10 ml of the same medium and cultured aerobically for 4 h at 37 °C, 26.5 °C, and 20 °C. Isopropyl- β -D-thiogalactopyranoside (IPTG) and CuSO₄.5H₂O were then added to give concentrations of 1 mM and 100 µM, respectively, and cultivation was continued for 10 h more. The cells were harvested, suspended in 0.5 ml 10 mM-Tris/HCl (pH 7.5), and disrupted by sonication. NIR activity was determined according to Kakutani *et al.* (1981b) and one unit of the enzyme activity was defined as the amount of enzyme which catalysed the reduction of 1 µmol NO₂⁻ min⁻¹. Proteins were determined

Fig. 1. Restriction maps of pNIR104 and pNIR201 containing the *nir* gene from *A. faecalis*, with sequencing strategy. pNIR104 contains a 5.0 kb *HindIII* fragment at the *HindIII* site of pUC19, and pNIR201 carries a 2.4 kb *PstI* fragment at the *PstI* site.

by the method of Bradford (1976). Cell fractionation of *E. coli* cells was performed according to the method of Cornelis (1982).

Results

Purification of NIR and determination of its partial amino acid sequences

Although we tried to purify the NIR from *A. faecalis* to homogeneity, the final preparation still showed two or three protein bands with molecular sizes from 36 to 37 kDa on SDS-PAGE. When the NIR preparation containing apparently three components was subjected to the automated amino acid sequencing, two major phenylhydantoinyl-amino acid signals were obtained in the ratio 2:1. From this result, two N-terminal amino acid sequences, KATAAE and AEIAAL, were determined. In addition, this sequencing analysis suggested the presence of a minor component with the N-terminal sequence RKATAA. When the same NIR preparation was treated with pyroglutamate aminopeptidase and was then subjected to the automated amino acid sequencing, another sequence, GAVRKA, was obtained together with the three amino acid sequences described above. Considering the nucleotide sequence of the *nir* gene determined as described below, we assume that the N-terminal amino acid of the intact NIR from *A. faecalis* is pyroglutamate and that the N-terminal part is processed by a protease to produce the observed heterogeneity.

We also cleaved the NIR preparation with CNBr and isolated a single peak by HPLC. The amino acid sequencing analysis of this peptide fragment gave an

internal amino acid sequence with 49 amino acids: VLPREGLHDGKGKALTYDKIYYGEQDFYVPRD ENGKYKKYEAPGDAYE.

Cloning and sequencing of the *nir* gene

For cloning of the *nir* gene from *A. faecalis*, three oligonucleotide probes were synthesized according to the amino acid sequence of the internal peptide fragment. When chromosomal DNA from *A. faecalis* digested with *HindIII* or *PstI* was Southern-blotted with the ³²P-labelled oligonucleotides, two restriction fragments of about 5.0 kb and 2.4 kb showed positive hybridization with probe 3. Each restriction fragment was purified from the agarose gel, ligated with pUC19 digested with *HindIII* or *PstI*, and introduced into *E. coli*. Screening of several hundred transformants by the method of colony hybridization with probe 3 yielded a single positive colony containing a plasmid with a 5.0 kb *HindIII* insert and two positive colonies carrying a plasmid with a 2.4 kb *PstI* insert in the same orientation. The plasmid containing the 5.0 kb *HindIII* fragment was designated pNIR104 and the plasmid containing the 2.4 kb *PstI* fragment was named pNIR201 (Fig. 1).

The nucleotide sequence of the 2.4 kb *PstI* fragment was as shown in Fig. 2. A single ORF which starts at ATG (nucleotide 751) and terminates at TAA (nucleotide 1879) was found. This ORF encodes a protein of 376 amino acids including the amino acid sequences identical to those determined by the Edman degradation. At its N-terminus, an amino acid sequence with two arginine residues followed by hydrophobic amino acids was present, which is a feature typical of the signal peptide in

Pst I	CTCCAGGCGCGGTTCTGTTCCGCCCTGCTCCACCGGAACTTTATTCCTCTTTCCGAACTCGAAACCGTGGTACGAATGGGTCCGCAATTTTCGATGA	100
Sau 3A I	GACCGGACCGGCTATCCTATATATGATCCCTGTTGGAGATCGAGGTAGTCTCATGCAACACACCGTACATGACCACTGGAGAGCGAGATCATCGAAC	200
Sau 3A I	ATCTGCGTCTGTTCTGATCCGAACTTGGGTTCAACCTGTTGACCTTGGCATGATCTACAGTATCGACATTTGGTGTGTCGCGGAACTTCGATATCAG	300
Eco R V	TATGACGACGACAAACCGCGGTTGCCCGGCTGCGGTTATCTGACTGATGCCCTTCCTCGAGCGCCGAATCTGTGGAAGGGGTTTCATGCACTCAATGTC	400
	GAACTGACCTACGAGCGCGGATGGATTCCGGAGATGGGATGCTGAAGTACAGGCGGATTGGAGATTAACTCCCGACGATATCGCATCATCGAA	500
Bal I	ACCAGCGAACAGGCACTATCTGTTGCTGCTTCACTTTTGGCCAAACCCCTCGGCTGAGCCCTTTGTCAGCGCAATAAAGCGGACAGACATCAAT	600
Sau 3A I	AACTAGCTTAGTATTATGATATTAAACGCTTAATATTACCAATTTTCTGTCACCTTGATCGCAGTCAAGGAACATCGAGTGCCTTTCTCTATAGT	700
SD	CGCATGACAGACAGAAATGCTCGGTCTATCTGTAAGGAGATTCGATGATCGGCAAGATCAATCACTCGCCCAACCATATTCGAGGAGCGCC	800
Cla I	MetAlaGluGlnMetGlnIleSerArgArgThrIleLeuAlaGlyAlaAla	
	CTGCTCGGCGCTTCGCGGCTTCTGGCAACCACTGCGCTGGGACAGGCGGCTCGGAGGCACTGCGGCGAAATACGAGCACTTCACGCG	900
	AlaLeuAlaGlyAlaLeuAlaProValLeuAlaThrThrSerAlaTrpGlyGlnGlyAlaValArgLysAlaThrAlaAlaGluIleAlaAlaLeuProArg	
Sph I	CGAAGGTGAGCTTGTGAGCCCTCTCTGTCATGCTCCATAGTCAGGTTCAGAGGCGGAGCCAGGTGTCGATTCACCATGTCATGTCGAGGAAA	1000
Eco R I	GlnLysValGluLeuValLysProProPheValHisAlaHisSerGlnValAlaGluGlyGlyProLysValValGluPheThrMetValIleGluGlu	
Cla I	AGAAGATGCTCATGATGATGCGGACCGAATTCACGCCATGCGATTCACGCGACCGCTGATGCTGTCATCAGGACGATTTACTCT	1100
	ysLysIleValIleAspPheAlaGlyThrGluValHisAlaMetAlaPheAsnGlyThrValProGlyProLeuMetValValHisGlnAspAspTyrLe	
Cla I	CGAAGTGCATCATCAACCTGAAACCAACCGCTGATGACATGATTCATGCGGCAACCGGTGATTCGCGCGCGCGGCTGACCGAAATC	1200
	uGluLeuThrLeuIleAsnProGluThrAsnThrLeuMetHisAsnIleAspPheHisAlaAlaThrGlyAlaLeuGlyGlyGlyLeuThrGluIle	
	AATCGGCGGAGAAAGACATCTGCTTCAAGCGGACCAAGCGCGCTCTTCCTTACCACTGCGACCTCCCGAATGTTCCCTGCGCATGCTAT	1300
	AsnProGlyGluLysThrIleLeuArgPheLysAlaThrLysProGlyValPheValTyrHisCysAlaProProGlyMetValProTrpHisValValS	
Sau 3A I	CGGCGATGAATGTCGATCAGGTGCTCGCGCGGAGGCTGCTGATGACCGCAAGCGCAAGCACTGACCTACGACAGATTTATTATGTCGCGAACA	1400
	erGlyMetAsnGlyAlaIleMetValLeuProArgGluGlyLeuHisAspGlyLysGlyLysAlaLeuThrTyrAspLysIleTyrTyrValGlyGluG	
	GGATTTCTATGATCCCGCGGAGAGAGGCAAAATACAGAAATACGAGGCGCGCGCGGCTTATGAGACACCGCTCAGGTCATGCGCACTCTCACT	1500
	nAspPheTyrValProArgAspGluAsnGlyLysLysLysTyrGluAlaProGlyAspAlaTyrGluAspThrValLysValMetArgThrLeuThr	
Sau 3A I	CCGACCATGTCGCTTCAACGCGCTTGGCGGCTGACTGCGGACAGGCGATGACGCGCGGCTTGGCGGAAATGTCGATGCTGCTCACTCGCAGG	1600
	ProThrHisValValPheAsnGlyAlaValGlyAlaLeuThrGlyAspLysAlaMetThrAlaAlaValGlyGluLysValLeuIleValHisSerGlnA	
Sau 3A I	CCAACCGGATACGAGACCACTGATCGCGGCGGATGGGATATGCTGCGGCGACCGCAAGTTCAATACGCGCGCGGATGCTGATCGAGAACTG	1700
	laAsnArgAspThrArgProHisLeuIleGlyGlyHisGlyAspTyrValTrpAlaThrGlyLysPheAsnThrProProAspValAspGlnGluThrTr	
Sau 3A I	GTTCACTGCGGCTGTCGCGCGGAGCGCTTCAACGCTTCAGGACCGCGCGCTTACGCTATGTAACCAATTCGATGCGGCTTTTGAATC	1800
	pPheIleProGlyGlyAlaAlaGlyAlaAlaPheTyrThrPheGlnGlnProGlyIleTyrAlaTyrValAsnHisAsnLeuIleGluAlaPheGluLeu	
Sau 3A I	GGCGCTGCGGCACTTCAAGGTCAAGGTGAAATGGAACGAGCTGATGACCTGCTTCCGACCATCTGCGACATTAAGCGAATGATGAGGCGC	1900
	GlyAlaAlaAlaHisPheLysValThrGlyGluTrpAsnAspAspLeuMetThrSerValLeuAlaProSerGlyThr	
	TTGCGCTCCGAGCGCTTTGGCAACCTGAGCGGCTCCAGTCAAAACGGAATCGGAACCGCTCTCTGCTCTGACGCAATTCACGCGGAACCGC	2000
Eco R V	TTGCGCATTCGCTGGAATGCTCTGACCATGCTTAGGATGAAGGCTGCGGCTGCACTGCTGCTCCCGACGCGATCGGTCGCTGATATCGGA	2100
Sau 3A I	TGAACGAGGCGAAAGGAAAGGTCCTGCGGAGCTTCCGCTGCGGCTTCCGCGGCTCTGCGGCGTCACTCGCGGATGATCTGCTGATGTCGT	2200
Sau 3A I	TGAGCTCGACGCGCAATGCGCGCGCTGCGCGCTGCTGCTGCGGAAATGATGCTTATCGCTCTGATACGATATCGGCGGATGCGGACTTT	2300
Pst I	CAGAGAACAAATACCGCTTGACGCGCCCAATGACGACCGCGACGCTGCGG	2351

Fig. 2. Nucleotide and deduced amino acid sequences of the *nir* gene of *A. faecalis*. A potential ribosome-binding site is marked as SD. Facing arrows indicate a putative FNR binding sequence.

secreted proteins (Inouye *et al.*, 1977). By amino acid sequencing analyses with the NIR mixtures, four N-terminal amino acid sequences had been obtained as described above. These sequences were found to start from Gln-34, Arg-38, Lys-39, and Ala-43 in the deduced sequence. By analogy with the signal sequences of other secreted proteins, we concluded that the peptide from Met-1 to Gly-33 serves as the signal sequence for secretion and that the N-terminal Gln-34 in the mature NIR is converted to pyroglutamate in *A. faecalis*. Three other N-terminal amino acid sequences presumably resulted from nonspecific proteolysis by an amino-peptidase present in *A. faecalis*.

Recently, Fenderson *et al.* (1991) chemically determined the primary structure of a similar copper-containing NIR from *Achromobacter cycloclastes* which

also requires a small copper-containing cupredoxin as an acceptor/donor of proton for the enzyme reaction. When the deduced amino acid sequence of the NIR from *A. faecalis* was compared with that from *Ach. cycloclastes*, 81% identity was observed (Fig. 3), which is higher than the amino acid identity (65%) observed between the cupredoxins from these organisms (Ambler, 1977).

A nucleotide sequence similar to that recognized by the transcription factor FNR, which controls the expression of various genes involved in the anaerobic metabolism of *E. coli* (Spiro & Guest, 1990), is found approximately 85 bp upstream of the putative translational initiation codon of the *nir* gene. A similar FNR-like sequence was also found upstream of the pseudo-azurin structural gene of this bacterium (Fig. 4).

Fig. 3. Comparison of amino acid sequences of NIRs. Identical amino acids are marked with asterisks (*). Ac, NIR of *Ach. cycloclastes*; Af, NIR from *A. faecalis*. The ligands for type I and type II copper atoms of the NIR from *Ach. cycloclastes* are indicated as I and II, respectively.

Expression of the *nir* gene in *E. coli*

To express the *nir* gene in *E. coli*, we constructed a plasmid, pNIR501, in which the *nir* gene including the Shine-Dalgarno sequence (Fig. 2) was located just downstream of the *lac* promoter. When *E. coli* transformants harbouring pNIR501 were cultured at 37 °C, the formation of inclusion bodies was observed irrespective of the presence or absence of IPTG (data not shown). The cell extracts were then analysed by SDS-PAGE and by Western blotting with antibody raised against NIR purified from *A. faecalis*. A large amount of the protein cross-reactive to the anti-NIR antibody was detected in the cells grown in both the presence and absence of IPTG. Its molecular mass (43 kDa) is close to that calculated from the deduced sequence of preNIR protein with the signal sequence (41 kDa). Fractionation of the cell extract revealed that the protein was localized mainly (more than 95%) in the insoluble fraction (Fig. 5). In accordance with this observation, very little NIR activity [0.19 U (mg protein)⁻¹] was detected in the

supernatant fraction of the cell extract (Table 1) and almost no activity in the insoluble fraction (data not shown). N-terminal sequencing of NIR in the insoluble fraction showed the sequence AEQMQIS, which corresponds to the N-terminal portion of the deduced amino acid sequence, lacking the first methionine residue at the N-terminus of the signal sequence.

We next examined a possible effect of lowering the cultivation temperature on the formation of inclusion bodies. When the same *E. coli* transformants were grown at 26.5 °C, the protein cross-reactive to the antibody was detected in the soluble fraction almost exclusively. Expression of the *nir* gene at 26.5 °C also depended on the presence of IPTG. NIR activity in the supernatant fraction of the crude cell extract of the *E. coli* transformant grown at 26.5 °C was 3.62 U (mg protein)⁻¹, which was 20 times higher than that from the cells grown at 37 °C (Table 1). The specific activity obtained from the *E. coli* transformant was approximately half that [6.5 U (mg protein)⁻¹] from *A. faecalis*. Although SDS-PAGE showed the presence of multiple cross-reactive proteins

paz TAACTTCACCTGTTTGGATCCAGATCAAGAGGTTGGCAGGCGACAGGTCTAAACCCCGTTACGTGGCGTG
nir TTTTGTGTACCTTGGATCGCAGTCAAGGAACATCGAAGTGCCTCTTCTATAGTCGGCATGACACAGCA
frdA Ec GCGCACTAAAAAATCGATCTCGTCAAAATTTCCGACCTTACATTTCGTTATACTAAATTAACAGTCAAT
frdA Pv ATACGGGAAAACCATTCATTCATATCAAAATTTCCGTACCTTACATTTCGTTATACTAAATTAACAGTCAAT
glpA AATGTTCAAAATGACCGCATGAAATCACGTTTCACTTTTCGAATTATGAGCGAATATGCGCGAAATCAAACA
dmsA TTTTGTCTCCCTTGGATACCGGAAATAATTACTCCTCACTTACACGTAAATACTACTTTTCGAGTGAAA
narG CCCATCATCACTCTTGGATCGTTATCAATTCACGCTGTTTCAGAGCGTTACCTTGCCCTTAAACATTAG
nirB TATAAAGGTGAATTTGGATTTACATCAATAAGCGGGTTGCTGAATCGTTAAGGTAGGCGGTAATAGAAAA

TTGAT----ATCAA

FNR consensus sequence

-10 region

Fig. 4. Alignment of FNR-binding sequences. Abbreviations: *paz*, pseudoazurin gene of *A. faecalis*; *nir*, *nir* gene of *A. faecalis*; *frdA Ec*, *frdA* gene encoding fumarate reductase of *E. coli*; *frdA Pv*, *frdA* gene encoding fumarate reductase of *Proteus vulgaris*; *glpA*, *glpA* gene encoding glycerol-3-phosphate dehydrogenase of *E. coli*; *dmsA*, *dmsA* gene encoding dimethylsulphoxide reductase of *E. coli*; *narG*, *narG* gene encoding nitrate reductase of *E. coli*; and *nirB*, *nirB* gene coding for nitrite reductase of *E. coli*. Nucleotide sequence and transcription mapping data are from: *paz* (Yamamoto *et al.*, 1987); *frdA Ec* (Eiglmeier *et al.*, 1989); *frdA Pv* (Eiglmeier *et al.*, 1989); *glpA* (Eiglmeier *et al.*, 1989); *dmsA* (Eiglmeier *et al.*, 1989); *narG* (Li & DeMoss, 1987), and *nirB* (Jayaraman *et al.*, 1988). FNR-binding sequences are boxed. Bold letters represent -10 regions of these genes. Transcriptional start points are underlined.

Fig. 5. SDS-PAGE and Western blotting with anti-NIR serum. SDS-PAGE (lanes 1–15), Western blotting with antiserum raised against NIR (lanes 16–29). Molecular markers (lane 1), crude extract prepared from *E. coli* JM105(pNIR501) cultured at 37 °C (lanes 2, 3, 16 and 17), that from *E. coli* JM105(pNIR501) grown in the presence of 1 mM-IPTG at 37 °C (lanes 4, 5, 18 and 19), that from *E. coli* JM105(pNIR501) grown in the presence of 1 mM-IPTG and 100 µM-CuSO₄ at 37 °C (lanes 6, 7, 20 and 21), that from *E. coli* JM105(pNIR501) grown at 26.5 °C (lanes 8, 9, 22 and 23), that from *E. coli* JM105(pNIR501) cultured in the presence of 1 mM-IPTG at 26.5 °C (lanes 10, 11, 24 and 25), that from *E. coli* JM105(pNIR501) cultured in the presence of 1 mM-IPTG and 100 µM-CuSO₄ at 26.5 °C (lanes 12, 13, 26 and 27), and from *E. coli* JM105 grown at 37 °C (lanes 14, 15, 28 and 29). Supernatants of the crude extracts, lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28, precipitates of the crude extracts, lanes 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29.

Table 1. Effect of cultivation temperature on NIR production by *E. coli* JM105 harbouring pNIR501

Plasmid	Cultivation temp. (°C)	Specific NIR activity* [units (mg protein) ⁻¹]
None	26.5	0.01
pNIR501	26.5	3.62
pNIR501	37.0	0.19

* Specific activity in the supernatant fraction obtained by centrifugation of the cell lysate.

with very similar molecular masses, the largest component had a molecular mass almost identical to that of the NIR produced as inclusion bodies at 37 °C. When

cells were cultivated at 20 °C, the cross-reactive proteins were detected not only in the cytoplasmic but also in the periplasmic fraction (Fig. 6). The molecular mass of the NIR protein in the periplasmic space was 37 kDa, which is identical to that of the mature NIR purified from *A. faecalis*. These results, suggest that a significant portion of the NIR produced at lower temperatures was translocated into the periplasmic space after processing of the signal sequence. We also examined the effect of copper in the culture medium on the NIR production at 26.5 °C, because copper was required for the efficient production of pseudoazurin in *E. coli* (Nishiyama *et al.*, 1992). However, no effect was observed at 37 °C or 26.5 °C (Fig. 5).

Fig. 6. Effect of temperature on localization of the NIR in *E. coli* cells carrying pNIR501, analysed by Western blotting with anti-NIR serum. After removal of the culture medium as extracellular fraction (E), the cells were fractionated into periplasmic (P), cytoplasmic (C), and insoluble (I) fractions. (a), *E. coli* JM105(pNIR501) cultured at 26.5 °C; (b), *E. coli* JM105(pNIR501) cultured at 20 °C.

Discussion

Cloning and sequencing of the *nir* gene revealed the deduced amino acid sequence of a subunit. NIR from *Achromobacter cycloclastes* was recently crystallized and its three dimensional structure has been already determined by X-ray crystallographic analysis at 2.3 Å resolution (Godden *et al.*, 1991). This analysis revealed that *Ach. cycloclastes* NIR is a trimer composed of three identical subunits. Three type I copper atoms occur in the complex (one in each subunit) while three type II copper atoms are bound in the spaces between the subunits. The significant identity in the sequence strongly suggests that NIR of *A. faecalis* also takes a similar trimeric form. We previously reported that *A. faecalis* NIR was a 110 kDa protein containing 4.5 mol copper atoms per mol enzyme (Kakutani, *et al.*, 1981*b*). In that work, we also estimated the molecular mass of a monomer to be 30 kDa. According to these values, we concluded that the NIR of *A. faecalis* consists of four identical subunits, each of which contains a single copper atom. However, it seems obvious from the present study that the former conclusion was wrong due to low estimates of both the copper contents and the subunit size. We here conclude that the NIR of *A. faecalis* is also composed of three identical subunits containing two copper atoms per monomer. In the *Ach. cycloclastes* NIR, four amino acid residues (His-95, Cys-136, His-145, and Met-150) serve as the ligands for the type I copper

with a distorted tetrahedral arrangement and three histidines, His-100, His-135, and His-306, and a water molecule are associated with the type II copper atom with a regular tetrahedral arrangement. All these residues are conserved in the sequence of the NIR of *A. faecalis* (Fig. 3). These residues are good candidates for site-directed mutagenesis studies to elucidate the mechanism of the catalytic reaction and of electron-transfer in copper-containing NIRs.

Although the expression plasmid pNIR501 was constructed to express the *nir* gene under the control of the *lac* promoter, large amounts of the NIR proteins were produced at 37 °C even in the absence of IPTG. We assume that the LacI repressor could recognize the operator sequence only at the lower affinity when the 5'-flanking region of the *nir* gene was located just downstream of it, and that the repressor protein may be partially inactivated at the higher temperature during a somewhat long period of cultivation (10 h). The de-repressed expression of the *nir* gene at the higher temperature may result from the combination of these two factors.

When the *nir* gene was expressed at 37 °C, the NIR protein was produced in the cell as enzymically inactive inclusion bodies. But at 26.5 °C, the protein was produced in soluble forms in the cytoplasmic fraction. Furthermore, at 20 °C NIR was partially secreted into the periplasmic space. It has been reported that human interferon- α 2, which forms inclusion bodies in *E. coli* at

37 °C, took a soluble form when the cells were grown at a lower temperature (Schein & Noteborn, 1988) and that the processing of subtilisin E of *Bacillus subtilis* fused to the *E. coli* OmpA signal peptide was greatly affected by the growth temperature during its secretion into the periplasm of *E. coli* (Ikemura *et al.*, 1987). These results support the idea that a lower rate of protein synthesis at a lower temperature may allow the NIR protein to be folded correctly in the cytoplasm and may facilitate the secretion to the periplasmic space.

The FNR protein is a transcription factor for a number of genes expressed only during anaerobic growth of *E. coli* cells (Spiro & Guest, 1990). In the genes positively regulated by FNR, the FNR-binding sequences are present about 25 bp upstream of the -10 consensus sequence, while the -35 consensus sequence is not found (Fig. 4). Such a feature is also found in the *nir* gene (TATAGT with 22 bp spacing to the putative FNR-binding site) (see Fig. 4). This is also true for the pseudoazurin gene of this organism (TTACGT with 31 bp spacing), which encodes the electron donor to NIR (Yamamoto *et al.*, 1987).

Since FNR is detected at the same concentration in the cells during either aerobic or anaerobic growth (Unden & Guest, 1985), this protein is assumed to take an active form only in the absence of oxygen to bind a specific sequence in the promoters of FNR-dependent genes. Our previous work revealed the necessity of anaerobic growth for the efficient production of both NIR and pseudoazurin in *A. faecalis* (Kakutani *et al.*, 1981a). We may assume that the FNR-like sequences located upstream of the *nir* and pseudoazurin structural genes are recognized by the FNR protein presumably present in *A. faecalis* and serve to enhance the transcription of these genes under anaerobic growth conditions.

E. coli possesses NADH-dependent NIR, which may account for the low NIR activity even in the untransformed *E. coli* cells (see Table 1); the expression of the *nirB* gene encoding the NIR is regulated not only by oxygen but also by nitrite (Cole, 1968; Jayaraman *et al.*, 1987). The production of *E. coli* NIR was stimulated by two- to threefold by the addition of nitrite. Although several studies to reveal the nucleotide sequence responsible for the nitrite induction of the *nirB* gene of *E. coli* have been carried out, it has not yet been identified precisely (Jayaraman *et al.*, 1988; Bell *et al.*, 1990). Similar induction of the NIR production by nitrite was also found in *A. faecalis* (Kakutani *et al.*, 1981a), suggesting the presence of a similar mechanism of induction by nitrite. However, comparison of the nucleotide sequences upstream of the *nir* gene from *E. coli* and *nirB* gene from *A. faecalis* does not lead to identification of the homologous sequence possibly responsible for the nitrite induction. Further studies to

reveal the mechanism for enhancing the expression of the *nir* and pseudoazurin genes are apparently required.

We thank K. Okamoto and H. Nagasawa (Department of Agricultural Chemistry, The University of Tokyo) for amino acid sequence determination of the NIR of *A. faecalis*.

References

- ADMAN, E. T., TURLEY, S., BRAMSON, R., PETRATOS, K., BANNER, D. V., TSERNOGLOU, D., BEPPU, T. & WATANABE, H. (1989). A 2.0-Å structure of the blue copper protein (cupredoxin) from *Alcaligenes faecalis* S-6. *Journal of Biological Chemistry* **264**, 87-99.
- AMBLER, R. P. (1977). The evolution of metalloenzymes, metalloproteins and related materials. In *Proceedings of a Symposium of the Inorganic Biochemistry Discussion group of the Chemical Society, University of Sussex*, pp. 100-118. Edited by G. S. Leigh. London: Symposium Press.
- BELL, A. I., COLE, J. A. & BUSBY, S. J. W. (1990). Molecular genetic analysis of an FNR-dependent anaerobically inducible *Escherichia coli* promoter. *Molecular Microbiology* **4**, 1753-1763.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- BURNETT, W. N. (1981). 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. *Analytical Biochemistry* **112**, 195-203.
- COLE, J. A. (1968). Cytochrome c_{552} and nitrite reductase in *Escherichia coli*. *Biochimica et Biophysica Acta* **162**, 356-368.
- CORNELIS, P., DIGNEFFE, C. & WILLEMOT, K. (1982). Cloning and expression of a *Bacillus coagulans* amylase gene in *Escherichia coli*. *Molecular and General Genetics* **186**, 507-511.
- EIGLMEIER, N., HONORÉ, N., IUCHI, S., LIN, E. C. C. & COLE, S. T. (1989). Molecular genetic analysis of FNR-dependent promoters. *Molecular Microbiology* **3**, 869-878.
- FENDERSON, E. F., KUMAR, S., ADMAN, E. T., LIU, M.-Y., PAYNE, W. J. & LEGALL, J. (1991). Amino acid sequence of nitrite reductase: a copper protein from *Achromobacter cycloclastes*. *Biochemistry* **30**, 7180-7185.
- GODDEN, J. W., TURLEY, S., TELLER, D. C., ADMAN, E. T., LIU, M.-Y., PAYNE, W. J. & LEGALL, J. (1991). The 2.3 angstrom X-ray structure of nitrite reductase from *Achromobacter cycloclastes*. *Science* **253**, 438-442.
- GRUNSTEIN, M. & HOGNESS, D. S. (1975). Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 248-254.
- HORMEL, S., ADMAN, E. T., WALSH, K. A., BEPPU, T. & TITANI, K. (1986). The amino acid sequence of the blue copper protein of *Alcaligenes faecalis*. *FEBS Letters* **197**, 301-304.
- INOUE, S., WANG, S.-S., SEKIZAWA, J., HALEGOUA, S. & INOUE, M. (1977). Amino acid sequence for the peptide extension on the prolipoprotein of the *Escherichia coli* outer membrane. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 1004-1008.
- IKEMURA, H., TAKAGI, H. & INOUE, M. (1987). Requirement of prosequence for the production of active subtilisin E in *Escherichia coli*. *Journal of Biological Chemistry* **262**, 7859-7864.
- JAYARAMAN, P.-S., PEAKMAN, T. C., BUSBY, S. J. W., QUINCEY, R. V. & COLE, J. A. (1987). Location and sequence of the promoter of the gene for NADH-dependent nitrite reductase of *Escherichia coli* and its regulation by oxygen, the Fnr protein and nitrite. *Journal of Molecular Biology* **196**, 781-788.
- JAYARAMAN, P.-S., GASTON, K. L., COLE, J. A. & BUSBY, S. J. W. (1988). The *nirB* promoter of *Escherichia coli*: location of nucleotide sequences essential for regulation by oxygen, the FNR protein and nitrite. *Molecular Microbiology* **2**, 527-530.
- KAKUTANI, T., BEPPU, T. & ARIMA, K. (1981a). Regulation of nitrite

- reductase in the denitrifying bacterium *Alcaligenes faecalis*. (1981). *Agricultural and Biological Chemistry* **45**, 23–28.
- KAKUTANI, T., WATANABE, H., ARIMA, K. & BEPPU, T. (1981*b*). Purification and properties of a copper-containing nitrite reductase from a denitrifying bacterium, *Alcaligenes faecalis*. *Journal of Biochemistry, Tokyo* **89**, 453–461.
- KAKUTANI, T., WATANABE, H., ARIMA, K. & BEPPU, T. (1981*c*). A blue protein as an inactivating factor for nitrite reductase from *Alcaligenes faecalis*. *Journal of Biochemistry, Tokyo* **87**, 463–472.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LI, S.-F. & DEMOSS, J. A. (1987). Promoter region of the *nar* operon of *Escherichia coli*: nucleotide sequence and transcription initiation signals. *Journal of Bacteriology* **187**, 4614–4620.
- LIU, M.-Y., LIU, M.-C., PAYNE, W. J. & LEGALL, J. (1986). Properties and electron transfer specificity of copper proteins from the denitrifier *Achromobacter cycloclastes*. *Journal of Bacteriology* **166**, 604–608.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MESSING, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* **101**, 20–78.
- NISHIYAMA, M., SUZUKI, J., OHNUKI, T., CHANG, H.-C., HORINOCHI, S., TURLEY, S., ADMAN, E. T. & BEPPU, T. (1992). Site-directed mutagenesis of pseudoazurin from *Alcaligenes faecalis* S-6; Pro80Ala mutant exhibits marked increase in reduction potential. *Protein Engineering* **5**, 177–184.
- PETRATOS, K., BEPPU, T., BANNER, D. V. & TSERNOGLOU, D. (1986). Preliminary characterization of crystals of nitrite reductase isolated from *Alcaligenes faecalis* strain S-6. *Journal of Molecular Biology* **190**, 135.
- PETRATOS, K., BANNER, D. V., BEPPU, T., WILKINSON, K. S. & TSERNOGLOU, D. (1987). The crystal structure of pseudoazurin from *Alcaligenes faecalis* S-6 determined at 2.9 Å resolution. *FEBS Letters* **218**, 209–214.
- SAITO, H. & MIURA, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochimica et Biophysica Acta* **72**, 619–629.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCHEIN, C. H. & NOTEBOEN, M. H. M. (1988). Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. *Bio/Technology* **6**, 291–294.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by agarose gel electrophoresis. *Journal of Molecular Biology* **98**, 513–517.
- SPIRO, S. & GUEST, J. R. (1990). FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. *FEMS Microbiology Reviews* **75**, 399–428.
- UNDEN, G. & GUEST, J. R. (1985). Isolation and characterization of the Fnr protein, the transcriptional regulator of anaerobic electron transport in *Escherichia coli*. *European Journal of Biochemistry* **146**, 193–199.
- YAMAMOTO, K., UOZUMI, T. & BEPPU, T. (1987). The blue copper protein gene of *Alcaligenes faecalis* S-6 directs secretion of blue copper protein from *Escherichia coli* cells. *Journal of Bacteriology* **169**, 5648–5652.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.