Gene Organization for Nitric Oxide Reduction in *Alcaligenes faecalis* S-6

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*norB* and *norC* encoding the cytochrome *b*-containing subunit and the cytochrome *c*-containing subunit, respectively, of the nitric oxide reductase (NOR) in *Alcaligenes faecalis* S-6 were cloned and sequenced. Both NorB and NorC showed more than 40% sequence identity to the corresponding subunits of cytochrome *bc*-type NORs in other denitrifying bacteria. *norCB* was in a gene cluster containing seven other genes; these were named *drr, orf2, orf3, norE, norF, norQ*, and *norD* on the basis of their similarity with NOR systems in other bacteria. Potential FNR-binding sites were present in front of *norCB*, *norEF*, and/or *orf2/orf3*, suggesting that most of these genes are regulated simultaneously by an FNR-related protein. NorB and NorC proteins produced in the membrane fraction in *Escherichia coli* showed no enzyme activity, probably due to lack of NorQ and NorD, which appear to perform some essential function for activation of the NorB-NorC complex in the recombinant *E. coli*.

**Key words:** *Alcaligenes faecalis*; denitrification; nitric oxide reductase; cytochrome *bc* complex

*Alcaligenes faecalis* S-6 has a dissimilatory nitrate reduction system called denitrification, in which nitrate is sequentially converted to nitrite, nitric oxide, and finally to molecular nitrogen.1) The second step of this process is catalyzed by a copper-containing nitrite reductase (NIR), which converts nitrite to nitric oxide under anaerobic conditions.2) Pseudoazurin, a copper-containing redox protein (cupredoxin), functions as an electron donor to NIR in this reaction.3) We cloned the genes coding for NIR and pseudoazurin from *A. faecalis* S-6,4,5 and described the specific interaction and electron transfer between the two copper-containing proteins through site-directed mutagenesis of both proteins.6-8) On the other hand, genetic studies suggest that nitrite reduction and nitric oxide reduction are two distinct, but functionally dependent, pathways in denitrifying bacteria.9,10) Mutants carrying a (a) mutation(s) in genes responsible for nitrite reduction also showed reduced activities of nitric oxide reduction.11,12) For a better understanding of the phenotypic and functional couplings between nitrite and nitric oxide reduction, the organization of these genes and studies on structure-function relationship of these enzymes are required. We here report the cloning and sequencing of the genes encoding nitric oxide reductase (NOR) from *A. faecalis* S-6.

NORs were purified from membranes of the denitrifying bacteria *Pseudomonas stutzeri*,13) *Paracoccus denitrificans*,14,15) and *Achromobacter cycloclastes*.16) All these enzymes form a membrane-bound cytochrome *bc* complex consisting of a single cytochrome *b* subunit (38 kDa) and a single cytochrome *c* subunit (17 kDa). On the other hand, a fungus, *Fusarium oxysporum*, contains a cytochrome P-450-type NOR in the soluble form in the cytoplasm.17) *A. faecalis* S-6 appeared to have a membrane-bound, probably cytochrome *bc*-type, NOR, because significant nitric oxide-reducing activity was detected in the membrane fraction of anaerobically grown cells (data not shown). To clone the genes for NOR from *A. faecalis* S-6, we partially purified a cytochrome *c*-type protein from the membrane fraction by monitoring cytochrome *c* by heme staining.18) *A. faecalis* S-6 was grown anaerobically as described previously.4) The cells were harvested, suspended in 20 mM potassium phosphate buffer (pH 6.9) (buffer A) containing 0.5 mM PMSF, and then disrupted. Cell debris was removed by centrifugation at 3,000 g, and the resulting supernatant was centrifuged at 10,000 g. The precipitate (a membrane fraction) was suspended in buffer A containing 0.5% Triton X-100 and centrifuged again at 10,000 g. The supernatant (a Triton X-100-solubilized fraction) was then put on a DEAE toyopearl column (25×80 mm) equilibrated with buffer A. The adsorbed proteins were washed with 0.3 M NaCl in buffer A and then eluted with buffer A containing 1 M NaCl and 0.6% sodium cholate. When the eluate was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained for heme to detect *c*-type hemes, a protein of about 17 kDa gave a positive signal. This

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**Abbreviations:** NOR, nitric oxide reductase; NIR, nitrite reductase
size coincided well with the molecular sizes of cytochrome c subunits of NOR from other bacteria. The eluate was concentrated with a Centricon 10 (Millipore, Bedford, USA) and then put on a Superdex 75 column (Amersham-Pharmacia Biotech., Tokyo, Japan) equilibrated with buffer A containing 200 mM NaCl, 0.5% sodium cholate, and 0.5% sodium deoxycholate. Heme c-positive fractions were pooled and dialyzed against buffer A. The dialysate was put on a MonoQ column (Amersham-Pharmacia Biotech.), and the adsorbed proteins were eluted with a linear gradient of 0-1 mM NaCl in buffer A containing 0.2% sodium cholate. The heme c-positive protein of 17 kDa was detected in fractions eluted at about 0.8 mM NaCl (Fig. 1A). N-terminal amino acid sequencing of this protein established the sequence, AERLTGARNVFGGSIFFFAIFVGLTAHSYYMKTTSTDELLLAV, which shows 34% sequence identity to cytochrome c subunits of NORs from other denitrifying bacteria.20-24 (Fig. 1B).

To clone the nor genes encoding NOR of A. faecalis S-6, a mixed oligonucleotide (5'-TAGAAGAAGAAGCGNTAGAAGAVCCGGATGGCGT-AAGGTAAGCGTAAATAATATGCTTG-3'); S, V, and N represent C+G, A+C+G, and A+C+G+T, respectively) corresponding to amino acids 18-37 (IFFFAIFVGLTAHSYYMK) of the determined sequence was synthesized and used as a probe for Southern hybridization. When chromosomal DNA of A. faecalis S-6 was digested with BamHI and Southern-blotted with the 32P-labeled oligonucleotide, a single signal of 2.7 kb was detected. This fragment was cloned into pUC19 by colony hybridization using the same probe, and the resulting plasmid was designated pNOR101. A 2.5-kb and a 4.3-kb HindIII fragment were also cloned in pUC19 with both distal portions of the 2.7-kb BamHI fragment as the hybridization probes, and the resultant plasmids containing fragment each were named pNOR102 and pNOR103, respectively (See Fig. 2). Thus a DNA fragment containing nor in a total of 6.8 kb was cloned.

The nucleotide sequence of the 6.8-kb DNA fragment was analyzed and has been deposited in the DDBJ, EMBL, and GeneBank DNA databases under the accession number AB031072. The sequence analysis found nine open reading frames (ORFs 1-9, named dnr, orf2, orf3, norE, norF, norC, norB, norQ, and norD, on the basis of their amino acid sequence similarity; see below). The N-terminal amino acid sequence of the cytochrome c-type protein corresponded to ORF6. This ORF encodes a 150 amino acid protein, showing 40% sequence identity to cytochrome c subunits of NORs of other denitrifying bacteria.20-24 We thus concluded that ORF6 represented the cytochrome c-containing subunit.

![Fig. 1. SDS-PAGE and N-Terminal Amino Acid Sequences of the A. faecalis S-6 Cytochrome c-Type Protein.](image-url)

A. SDS-PAGE of cytochrome c-type protein (arrowhead) partially purified from the membrane fraction of A. faecalis S-6 (lanes 2 and 4) and bovine cytochrome c as a control (lanes 1 and 3). Lanes 1 and 2; heme staining, lanes 3 and 4; Cooamassie brilliant blue (CBB) staining. B. Alignment of the N-terminal amino acid sequences of the A. faecalis S-6 cytochrome c-type protein and cytochrome c subunits from Pseudomonas sp. G-179,20 P. denitrificans,21 R. sphaeroides,22 P. aeruginosa,23 and P. stutzeri.22 Bold letters indicate the identical amino acids. C. SDS-PAGE of the insoluble fraction of cell-extract prepared form E. coli JM105 (lanes 1 and 3) and E. coli JM105 (pNOR201) (lanes 2 and 4). Lanes 1 and 2; heme staining, lanes 3 and 4; CBB staining.
Fig. 2. Restriction Map of the Cloned 6.8 kb Fragment and the Open Reading Frames.

A. The region on each of the plasmids, pNOR101, pNOR102, pNOR103 and pNOR201, is shown. The genes orf2, orf3, norE, norF, norC, norB, and norQ encode 157, 51, 187, 91, 150, 448, and 270 amino-acid proteins, respectively. A possible p-independent transcriptional terminator in front of norQ is shown by opposing arrows. The putative ribosomal-binding sites and the translational start codons are underlined and boxed, respectively. B. Possible FNR-binding sites between orf3 and norE (a) and in front of norC (b) are shown in bold letters, together with the consensus sequence. The sequences in front of the NIR (nor) and pseudoazurin ( paz) genes are also aligned.

(NorC) of NOR of A. faecalis S-6. Just downstream of norC, ORF7 of 448 amino acids is present. It shows 40% identity in amino acid sequence to cytochrome b subunits of NORs in other bacteria, indicating that ORF7 represents the cytochrome b-containing subunit (NorB) of NOR. Two ORFs showing significant similarity to NorQ and NorD, accessory components in NIR or NOR systems in other denitrifying bacteria, were present downstream of NorCB. In all the microorganisms close linkage between norCB and norD was found, suggesting the possible involvement of norD gene in NorCB function. Although norQ is found between norCB and norD in some organisms, not found in other organisms (Fig. 3). In P. aeruginosa, and P. stutzeri, for example, a norQ homologue is located in or close to the nir gene cluster. This suggests that norQ or its homologue is involved in both nitrite and nitric oxide reduction. Upstream of norCB, two genes encoding proteins similar to NorE and NorF in other bacteria were present. The gene organization of norCB and norEF is the same as in Pseudomonas sp. G-179 and P. aeruginosa; norEF is upstream of norCB in these bacteria. Further upstream, are three genes, dnr, orf2, and orf3. The gene named dnr shows high similarity in amino acid sequence to a member of the FNR family, an O2-regulated transcription factor. As shown in Fig. 3, the gene organizations of the nor clusters differ from each other. However, all the genes necessary for denitrification are clustered at a locus on their chromosomes. Such an arrangement of the gene clusters could facilitate functional coupling in gene expression. The arrangement of the nor genes of A. faecalis is most similar to that of Pseudomonas sp. G-179, which suggests that the nor gene clusters in both microorganisms have been transferred from a common ancestor.

A potential binding site for FNR is found upstream of norC (Fig. 2). Furthermore, downstream of norB is present an inverted repeat sequence, which could serve as a p-independent transcriptional terminator. Therefore, norC and norB are probably transcribed from a single mRNA, although transcriptional units for the nor genes have not been identified at present. It is interesting to note that neither the FNR box nor any typical promoter sequence is present in the intervening sequence between norB and norQ. This may suggest that norQD is under the control of
the promoter of norCB and their expression is regulated through termination or anti-termination events via the possible stem-loop structure of the transcript. In any case, Northern blot and primer extension analyses are necessary to understand the transcriptional control of nor genes. Because norEF and/or orf23 are also preceded by a sequence similar to the consensus sequence for FNR binding (Fig. 2), and because the genes encoding NIR and pseudoazurin are preceded by a similar sequence, it is likely that expression of most of the genes required for both nitrite and nitric oxide reduction is regulated in a coordinate and concerted way by FNR-related proteins in *A. faecalis* S-6, as in *P. denitrificans*.28

The norCB genes were expressed in *Escherichia coli* JM105 harboring the expression plasmid pNOR201, which was constructed as follows. Introducing an *XbaI* site just upstream of norC, PCR was done using a synthetic oligonucleotide (5′-CTT-TCTAGAATGAGGAGTAGCATAATGGGAC-GAACGCC-3′; underline indicates an *XbaI* site and bold letters indicate the translational start codon of norC) and the M4 universal primer, and pNOR101 as the template. The amplified DNA fragment was digested with *XbaI* and *HindIII*, and the resulting fragment was ligated with the *HindIII*-SalI fragment from pNOR102 and inserted into pUC18 digested with *XbaI* and SalI (See Fig. 2A) to give pNOR201. The norCB sequence was under the control of the lac promoter. Upon induction by IPTG, *E. coli* harboring pNOR201 produced NorC (17 kDa) and NorB (38 kDa) in the membrane fraction, as found by Coomassie brilliant blue staining of SDS-PAGE (Fig. 1C). NorC was also detected by heme staining, indicating that it contained a c-type heme. However, our repeated attempts to detect NOR activity in the membrane fraction failed (data not shown), probably because some other gene products encoded by the flanking regions of norCB are required for the activation of the enzyme. We assume that *norQ* is required for the functional expression of norCB in *A. faecalis* S-6, since deletion of *norQ* in *P. stutzeri* results in the loss of enzyme activities of both nitrite and nitric oxide reduction in vivo.29 *norQ* appears to serve as a transcriptional regulator for both *nit* and *nor*. In addition, *norD* seems to be required for functional expression of NorCB because a mutation in *norD* in *P. aeruginosa* was reported to actually cause loss of viability of the strain under denitrifying conditions.30 The close linkage between norD and norCB supports the idea that NorD is indispensable for the NOR function. On the other hand, norEF is not perhaps necessary for expression of NOR in an active form, since deletion of *norOP*, homologues of *norEF*, caused no effects on all the denitrification enzymes including NOR but just lowered energy conservation in anaerobic respiration in *P. aeruginosa*.30 At present, we suppose that the failure to express the enzyme activity of the NorCB proteins is due to the absence of the proteins NorQ and NorD in the recombinant cells. These two accessory proteins supposedly mature NOR in a post-translational manner.

Recently, four genes, *fmrA*, *drrD*, *drrS*, and *drrE*, encoding FNR-like proteins were cloned from *P. stutzeri*.31 Of these proteins, only FmrA, a genuine FNR-type regulator, has cysteine residues at its N-terminal region, complexing a [4Fe-4S] cluster of redox-active FNR-type regulators, while other homologues lacking cysteine residues serve as transcriptional regulators for the expression of the genes involved in each denitrification step. Since the *A. faecalis* FNR-homologue *drr* found in this study lacks its 5′-terminal portion coding for the corresponding cysteine residues, we currently cannot define into which type of FNR this Drr is classified. However, we speculate that the cloned Drr could regulate gene expression of nor and possibly *nit*, because DrrD encoded close to the nor gene cluster in *P. stutzeri*32 is a key regulator for denitrification by selective activation of the genes for NIR and NOR. Further cloning of the nor flanking regions and characterization of the gene products are necessary to elucidate the mechanism for NIR/NOR expression in
the denitrification process.

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