Volume 279, number 2, 205–209 FEBS 09406 February 1991

ADONIS 001457939100129Z

The *nirSTBM* region coding for cytochrome cd_1-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins

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Received 4 December 1990

Genes for respiratory nitrite reduction (denitrification) of *Pseudomonas stutzeri* are clustered within 7 kbp. A 4.6-kbp *Hind* III–*Kpn* I fragment carrying *nirS*, the structural gene for cytochrome cd_1, was sequenced. An open reading frame immediately downstream of *nirS* codes for a 22.8-kDa protein with four heme *c*-binding motifs. Mutagenesis of this gene causes an apparent defect in electron donation to cytochrome cd_1. Following this ORF are the structural genes for cytochrome c_{551}, cytochrome c_{553}, and ORF5 that codes for a 11.9-kDa monoheme protein. All cytochromes have a signal sequence for protein export.

Nitrite respiration; Cytochrome cd_1; Cytochrome c_{551}; Cytochrome c_{553}; Tetraheme protein; Signal peptide; *Pseudomonas stutzeri*

1. INTRODUCTION

Pseudomonas stutzeri [1] and *Pseudomonas aeruginosa* [2] synthesize cytochrome cd_1 as respiratory nitrite reductase (ferrocytochrome c_{551}:oxygen oxidoreductase; EC 1.9.3.2) for their denitrification system [3,4]. The structural gene, *nirS*, for cytochrome cd_1 of *P. stutzeri* is vicinal to genes for nitrous oxide respiration, which are part of the overall denitrification process [5]. The primary structure of *nirS* from *P. aeruginosa* was recently elucidated [6]. Contiguous to *nirS* is the gene coding for cytochrome c_{551} [7,8], the *in vitro* electron donor for cytochrome cd_1 (in certain systems interchangeable with azurin). Here we report the substantially extended nucleotide sequence of the *nirS* region from *P. stutzeri* with the primary structure of five heme proteins and their respective precursor forms, and establish a gene order different from that of *P. aeruginosa*.

2. MATERIALS AND METHODS

The *nirS* region of *P. stutzeri* strain ZoBell (formerly *P. perfectomarina* ATCC 14405) was cloned from a lambda gt11 expression library [5]. Generation of subclones from cosmids, DNA sequencing, and other details of recombinant DNA work are described elsewhere [5,9,10]. Cytochrome cd_1, cytochrome c_{551} and cytochrome c_{553} were purified from anaerobically, nitrate-grown cells [4]. The conditions of Iso-dalt electrophoresis were as in [11]. Iron was determined by atomic absorption spectroscopy. Molecular masses were estimated by gel filtration, by pore-gradient electrophoresis and by SDS-PAGE. Manual and automated Edman degradations were used to determine the N-terminal sequences of Cm-cytochrome cd_1 and peptides [12].

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Abbreviations: ORF(s), open reading frame(s); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

3. RESULTS

3.1. *Gene map, sequencing strategy, open reading frames*

Fig. 1 shows the restriction map of the 4.6-kbp *Hind* III–*Kpn* I fragment and the sequencing strategy. Both directions were sequenced by the dideoxy-chain termination method; GC-rich regions were verified with dITP. The sequenced DNA region revealed five ORFs in the same transcriptional direction (Fig. 1), which showed a codon preference typical of *Pseudomonas* genes. Three ORFs could be assigned to known cytochromes.

3.2. *Sequence of the* nirS *region*

The nucleotide sequence and the derived gene products of the *nirSTBM* region are shown in Fig. 2. The first ORF codes for the structural gene of cytochrome cd_1. The *nirS* gene product carries a presequence of 26 amino acids, 14 of which are positionally identical to those of the *P. aeruginosa* nitrite reductase. Overall, the two proteins have 56.4% identity and 13.6% conser-

vative replacements when aligned by the algorithm of [13]. Fig. 3A shows an alignment of the N-terminal regions which have the greatest divergence due to insertions adjacent to the heme *c*-binding site without equivalent in the other protein; the binding site itself, however, is highly conserved. Of 14 histidine residues in cytochrome cd_1 11 are invariant. Histidine (besides methionine) coordinates heme *c* and is a likely candidate also for the axial ligand of the d_1 heme. His-167 resides within the most conserved neighbourhood; other conserved regions are around the histidine residues 261, 311, 353, and 362.

The *nirS* gene is followed by ORF2, assigned to the *nirT* gene whose mutagenesis leads to a Nir^- phenotype [4]. Its derived gene product carries four heme *c*-binding motifs. These motifs show an apparent pairwise arrangement (Fig. 2), yet there is no conspicuous sequence similarity between the N-terminal and C-terminal domains. A screening of the MIPSX data bank (Martinsried Institute for Protein Sequence Data, release 17) by the FASTA program [14] did not reveal significant matches with other multiheme cytochromes including that of the photosynthetic reaction center. In all four motifs the cysteines are spaced by two residues and not by four as found in the c_3-type cytochromes of the sulfate-reducing bacteria [15]. The motif starting with Cys-88 is unusual in having a proline residue [16]. No heme-binding motif with proline was found in the cytochrome entries of the SWISS-PROT Protein Sequence Databank (release 15). A *c*-type cytochrome with four heme groups within a single polypeptide chain has not been identified so far in any pseudomonad. The *nirT* product has a presumed leader sequence with a predicted cleavage site at Gly-28 [17] and a hydrophobic, potential membrane anchor of 17 amino acids directly following the N-terminus.

Immediately downstream of *nirT* the structural gene for the diheme cytochrome c_{552}, termed *nirB*, was found (Fig. 2). A comparison of the N-terminus of the purified protein [18] and the *nirB* gene product revealed a presequence of 23 amino acids (Table I). One of the two heme-binding sites of cytochrome c_{552} is unusual with tryptophan as a third residue spacing the thioether-forming cysteines. The derived amino acid sequence confirms this unique heme-binding motif of the cytochrome family and extends the C-terminus 21 residues beyond the previously reported, incomplete sequence of this protein [18]. The two uncertain amino acids Ser-245 and Asn-247 of that sequence were identified as arginine and histidine; for Asp-36 and Asn-206 the derived NirB sequence indicates alanine and aspartate, respectively.

The fourth ORF in row, assigned to *nirM*, codes for cytochrome c_{551}. The amino acid sequence of this cytochrome from *P. stutzeri* strain 221 [19] shows 11 replacements (5 of which are of conservative nature) versus the strain ZoBell used in this work (Fig. 2). Cytochrome c_{551} carries a signal peptide of 22 amino acids (Table I). The *nirM* gene is immediately followed by ORF5. This ORF is likely to code for a further monoheme *c*-type cytochrome. Its derived gene product is a hydrophilic protein with a canonical heme-binding motif and a predicted export signal sequence.

3.3. *Properties of derived gene products*

Properties of the derived gene products are summarized in Table I. The molecular mass of cytochrome cd_1 determined from iron analysis, SDS-PAGE, nondenaturing pore-gradient PAGE, and gel filtration, gave values of 35.3, 61.2, 135, and 119 kDa, respectively. Like the cytochrome cd_1 from other denitrifying bacteria the *P. stutzeri* protein is in solution a dimer, carrying pairs of heme *c* and d_1 groups. Its experimentally determined isoelectric point is 6.5 ± 0.25 (n = 3). Cytochrome cd_1 (see also [20]), cytochrome c_{551} and cytochrome c_{552} were localized in the periplasm by two-dimensional electrophoretic analysis of this compartment. Purified cytochrome c_{551} and cytochrome c_{552} separated in isoelectric focusing into several charge isomers. Their positions were identified on the gels by comparison with the purified components, and for cytochrome cd_1 also by immunoblotting (C. Siegel, B. Goos and W.G. Zumft, unpublished). In agreement with the periplasmic location of the three cytochromes is the presence of signal peptides (Table I). Mature cytochrome c_{552} has a calculated M_r of 29 413 Da including the two heme groups. A value of 25 800 Da was determined from sedimentation equilibrium [21]. The same method indicated 7660 Da for cytochrome c_{551} [22] versus 9180 Da from the composition.

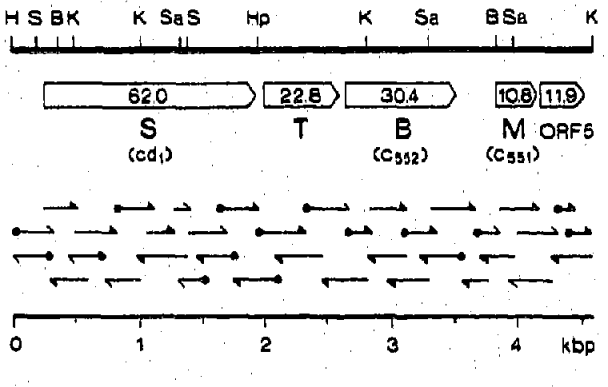

Fig. 1. Gene map, open reading frames, and sequencing strategy of the *nirS* region from *P. stutzeri*. Open arrows show the location and direction of transcription of the genes for *nirS* (cytochrome cd_1), *nirT* (tetraheme protein), *nirB* (cytochrome c_{552}), *nirM* (cytochrome c_{551}), and ORF5 (monoheme protein). Numbers indicate molecular masses of the precursor forms. Small arrows show the sequenced part of subclones. Arrows beginning with a dot identify clones sequenced with synthetic, others with M13 universal primers; filled-in arrowheads denote clones sequenced also with dITP. Physically mapped restriction sites are B, *Bgl* II; H, *Hind* III; Hp, *Hpa* I; K, *Kpn* I; S, *Sma* I; and Sa, *Sal* I.

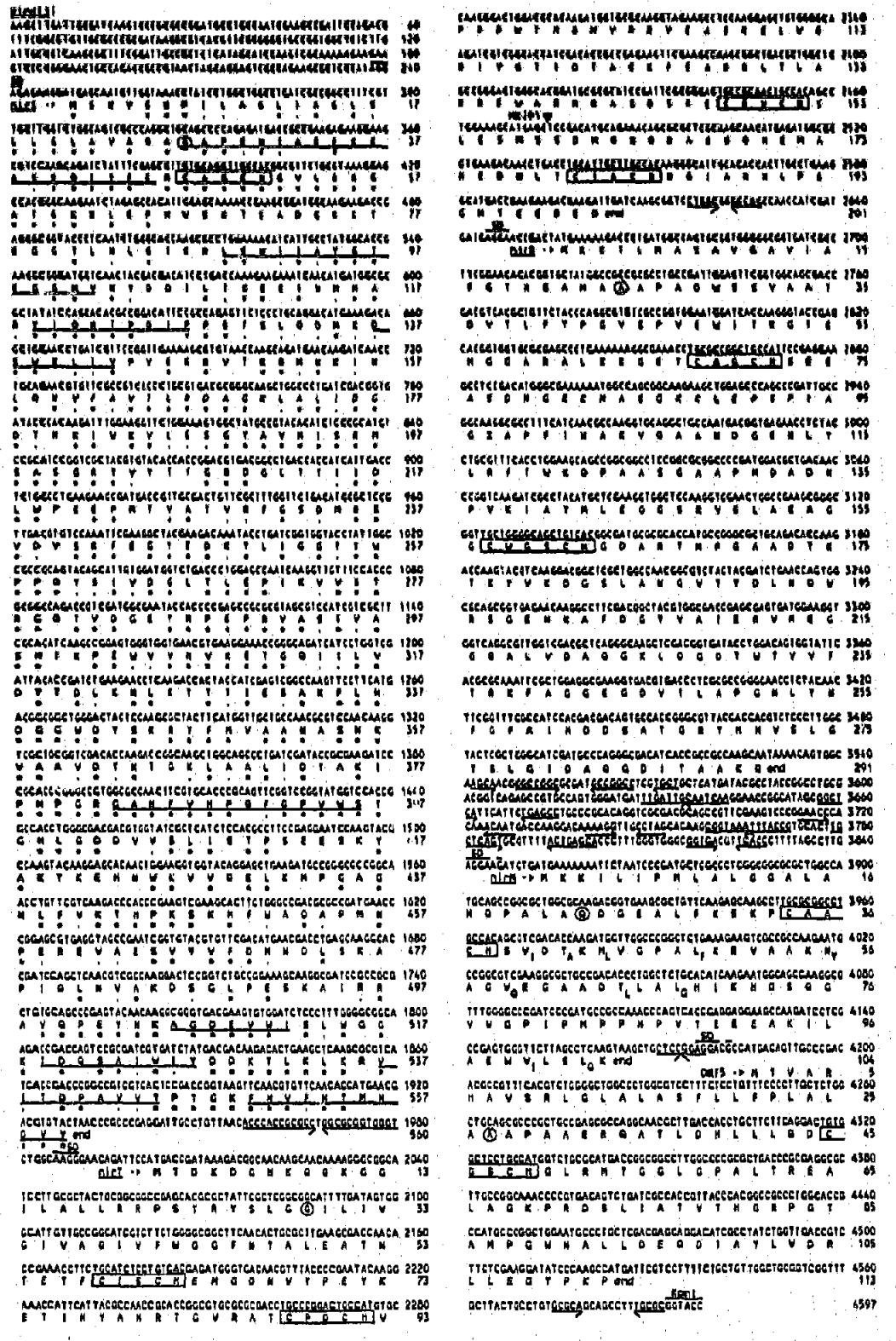

Fig. 2. Nucleotide and derived amino acid sequence of the *nirSTBM* genes and ORF5 of *P. stutzeri* ZoBell. Potential ribosome-binding sites (SD) are overlined. Stop codons are termed *end*. Inverted repeats are shown by opposing arrows. Underlined amino acids of the *nirS* product were determined by peptide sequencing. Asterisks denote identical, dots similar residues in comparison with the NirS protein from *P. aeruginosa* [6]; gaps for alignment are not shown. The triangle at nucleotide position 2474 of *nirT* indicates the Tn*5* insertion site of mutant MK201, which was determined by sequencing. The amino acids which differ in cytochrome c_{551} from *P. stutzeri* strain 221 [19] are shown as subscripts in the *nirM* sequence. Boxed residues indicate heme *c*-binding motifs. The N-terminal amino acid of a processed protein is circled. The nucleotide sequence data will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession no. X56813.

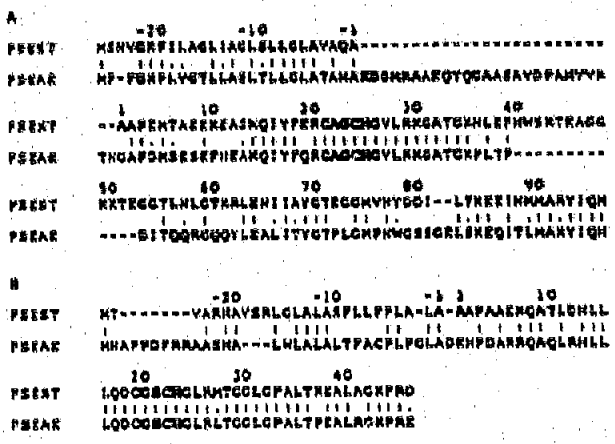

Fig. 3. Alignment of the N-terminal domains of (A) cytochrome cd_1 and (B) ORF5 from *P. stutzeri* (PSEST) and *P. aeruginosa* (PSEAE). Identical amino acids are shown by colons, conservative replacements by dots; heme c-binding sites and N-termini are printed in bold-face. Negative numbers label the signal sequence. Data for *P. aeruginosa* cytochrome cd_1 and ORF5 are from ref. 6 and 8, respectively.

4. DISCUSSION

The genes for nitrite respiration, *nir*, and the *nos* genes required for nitrous oxide respiration of *P. stutzeri* are closely linked. Of the former genes, *nirD* (presumably involved in heme d_1 synthesis or processing) and *nirS* have been previously identified. The two genes are separated by about 4 kbp [5]. Sequencing the *nirS* region towards *nirD* has revealed five ORFs all coding for heme proteins. The gene order of *P. stutzeri* with respect to cytochrome cd_1 and cytochrome c_{551} (cf. Fig. 1) is unexpectedly different from that found in *P. aeruginosa*, given the systematic relation between these bacterial species as members of the tight rRNA homology group I. The *nirS* gene of *P. aeruginosa* is followed by *nirM* [7,8] and ORF5. Arai et al. [8] reported 232 nontranslated nucleotides beyond the 3'-end of *nirM*. We found that this sequence harbors an ORF that overlaps four nucleotides with the preceding *nirM* gene. A potential ribosome-binding site is located −12 nucleotides upstream of the putative start codon. Translation of this region revealed that it corresponds to the ORF5 gene product of *P. stutzeri* showing 62.8% identity (Fig. 3B).

In *P. stutzeri* the genes *nirS* and *nirM*-ORF5 are separated by intercalation of the genes *nirT* and *nirB*. The *nirT* gene product of *P. stutzeri* has features of a novel tetraheme protein. Mutagenesis of this gene with the transposon Tn*5* leads to a Nir$^-$ phenotype (mutant strain MK201), yet catalytically active cytochrome cd_1 is still synthesized [4]. This is indicative of a defect in electron donation. As a result of the Tn*5* insertion, mutant MK201 also overproduces cytochrome c_{552}. The hydrophobic part at the mature N-terminus of the NirT protein might anchor it in the membrane, and allow electron flow between the respiratory chain and a periplasmic acceptor. Electrons could pass directly from NirT to the reductase or, as deduced from in vitro evidence, via cytochrome c_{551}. Given the proximity of *nirT* to *nirS*, it will depend on the transcriptional control exerted by the inverted repeat downstream of *nirS* with a free energy of formation of −126 kJ/mol [23], whether readthrough is possible and *nirT* expression will be regulated like *nirS*. Anaerobic regulation is indicated from the organizational feature that the *nirT* gene forms probably a transcriptional unit with the immediately following *nirB* gene. The *nirB* product (cytochrome c_{552}) is not found in aerobically grown cells [22].

Cytochrome c_{552} shows peroxidase activity on proteolytic modification [18]. The function of this component in vivo and of its acquired peroxidase activity is unknown. Like cytochrome cd_1 the synthesis of cytochrome c_{552} depends on anaerobic culture conditions and the concentration of this component increases with rising cellular nitrite reductase activity [22,24]. A role in nitrite reduction has been suggested. The location of the *nirB* gene next to a gene involved in electron

Table I

Properties of the derived products of the *nirSTBM* gene cluster and ORF5 of *P. stutzeri* strain ZoBell

Gene product	Residue number[a]	M_r[a,b]	Presequence[c]	pI[a,b]	Heme-*c* binding motifs
NirB	268	28 180	MKKTLMASAVGAV IAFGTHGAMA-A	4.93	CAGCH, CWGSCH
NirM	82	8 563	MKKILIPMLALG GALAMQPALA-Q	5.46	CAACH
NirS	534	59 532	MSNVGKPILAGLIA GLSLLGLAVAQA-A	6.43	CAGCH
NirT	173	19 738	MTDKDGNKQQKGGIL ALLRRPSTRYSLG-G[d]	6.32	CISCH, CPDCH CRNCH, CIACH
ORF5	87	9 202	MTVARHAVSRLGLA LASFLLFPLALA-A[e]	5.79	CGSCH

[a]Value for mature protein [b]M_r value without heme groups [c]Cleavage site indicated by a dash [d]Calculated value [e]Presequence not confirmed by N-terminal sequencing

donation to cytochrome cd_1, and perhaps within the same operon as *nirS*, adds further circumstantial evidence to this implication. Transposon mutants MK202 and MK206/207 which had lost cytochrome cd_1 or had only a low content also had a reduced cytochrome c_{552} level [4].

Unlike cytochrome cd_1, cytochrome c_{551} is not regulated anaerobically [22,25], questioning an exclusive role in nitrite reduction. The intergenic region of 320 nucleotides between *nirB* and *nirM* has multiple inverted repeats (Fig. 2) with the potential to form various transcriptional termination, attenuation or other regulatory structures. We assume from this that the genes for cytochrome cd_1 and cytochrome c_{551} of *P. stutzeri* will belong to separate operons.

Acknowledgements: We thank H. Cuypers for oligonucleotide synthesis and helpful advice, and A. Messerschmidt for support with the data bank search. C. Siegel and B. Goos provided the iso-dalt data. The work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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