Isolation, Sequencing, and Mutagenesis of the Gene Encoding Cytochrome c_{553i} of *Paracoccus denitrificans* and Characterization of the Mutant Strain

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The periplasmically located cytochrome c_{553i} of *Paracoccus denitrificans* was purified from cells grown aerobically on choline as the carbon source. The purified protein was digested with trypsin to obtain several protein fragments. The N-terminal regions of these fragments were sequenced. On the basis of one of these sequences, a mix of 17-mer oligonucleotides was synthesized. By using this mix as a probe, the structural gene encoding cytochrome c_{553i} (*cycB*) was isolated. The nucleotide sequence of this gene was determined from a genomic bank. The N-terminal region of the deduced amino acid sequence showed characteristics of a signal sequence. Based on the deduced amino acid sequence of the mature protein, the calculated molecular weight is 22,427. The gene encoding cytochrome c_{553i} was mutated by insertion of a kanamycin resistance gene. As a consequence of the mutation, cytochrome c_{553i} was absent from the periplasmic protein fraction. The mutation in *cycB* resulted in a decreased maximum specific growth rate on methanol, while the molecular growth yield was not affected. Growth on methylamine or succinate was not affected at all. Upstream of *cycB* the 3' part of an open reading frame (ORF1) was identified. The deduced amino acid sequence of this part of ORF1 showed homology with methanol dehydrogenases from *P. denitrificans* and *Methylobacterium extorquens* AM1. In addition, it showed homology with other quinoproteins like alcohol dehydrogenase from *Acetobacter aceti* and glucose dehydrogenase from both *Acinetobacter calcoaceticus* and *Escherichia coli*. Immediately downstream from *cycB*, the 5' part of another open reading frame (ORF2) was found. The deduced amino acid sequence of this part of ORF2 showed homology with the *moxJ* gene products from *P. denitrificans* and *M. extorquens* AM1.

Paracoccus denitrificans is a gram-negative, aerobic soil bacterium that is able to grow under a large variety of growth conditions. Depending on these conditions, different oxidases, reductases, electron carriers, and dehydrogenases can be synthesized. During aerobic growth, aa_3- and *o*-type oxidases are formed (45). In anaerobically grown cells, several inducible oxidoreductases are formed which enable the bacterium to use nitrate, nitrite, nitric oxide, or nitrous oxide as the terminal electron acceptor (13, 20, 47). *P. denitrificans* is able to synthesize up to at least 10 different *c*-type cytochromes (7–9). Some of them can be detected in the periplasm and the others in the cytoplasmic membrane. The cytochromes can be distinguished by their absorption maximum, redox midpoint potential, and molecular mass. *P. denitrificans* can grow methylotrophically on methylamine or methanol as the sole carbon and energy source. In the first step, these compounds are oxidized to formaldehyde by methylamine or methanol dehydrogenase (3, 5). During growth on methylamine or methanol, four periplasmically located cytochromes *c* are synthesized. Two cytochromes with estimated molecular masses of 22 kDa (c_{551i}) and 30 kDa (c_{553i}) are induced specifically during methylotrophic growth. The 15-kDa cytochrome c_{550} is constitutively present, and the 45-kDa cytochrome *c* is a peroxidase (21).

Both methylamine dehydrogenase and methanol dehydrogenase are located in the periplasm and consist of two identical small and two identical large subunits (22, 23, 25). Methylamine dehydrogenase contains a pyrroloquino-

linequinone (PQQ)-like cofactor that is covalently linked to the enzyme. During growth on methylamine, an additional electron carrier, the blue copper protein amicyanin is induced. Amicyanin mediates the flow of electrons from methylamine dehydrogenase to *c*-type cytochromes (24, 44).

Methanol dehydrogenase is a quinoprotein that contains a noncovalently bound PQQ (4, 18). Genes encoding the subunits of methanol dehydrogenase of *P. denitrificans* have been isolated (22, 42). *moxF*, encoding the large subunit, and *moxI*, encoding the small subunit, are located closely linked together with two additional genes, *moxJ* and *moxG*, in the gene order *moxF-moxJ-moxG-moxI*. The gene product of *moxJ* is suggested to be a chaparoninlike protein. The *moxG* gene encodes a 17.7-kDa *c*-type cytochrome. Recently, Long and Anthony (31) demonstrated by in vitro experiments with purified periplasmic cytochromes *c* and methanol dehydrogenase that cytochrome c_{551i} is the electron acceptor for methanol dehydrogenase. Van Spanning et al. (42) reached the same conclusion from growth experiments using a mutant lacking cytochrome c_{551i}, which showed unimpaired growth on methylamine and no growth on methanol. In a previous in vitro study by others, it was suggested that cytochrome c_{553i} was the electron acceptor for methanol dehydrogenase (14), but above-mentioned results disprove this suggestion. Consequently, the function of this *c*-type cytochrome in the electron transport chain of *P. denitrificans* is not clear at the moment.

This report describes the purification of the periplasmically located cytochrome c_{553i}, isolation and mutagenesis of the gene encoding this cytochrome, and the characterization

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacteria		
Paracoccus denitrificans		
Pd 1235	Rif[r], enhanced conjugation frequencies, m[−]	17
Pd 6121	Pd 1235 *cycB*::Km[r]	This study
Escherichia coli		
S17-1	Sm[r] *pro* r[−] m[+] RP4-2 integrated (Tc::Mu) (Km::Tn7)	39
JM101	*supE thi* Δ(*lac-proAB*) F' (*traD36 proAB lacI*[q] *lacZΔM15*)	34
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F' (*traD36 proAB lacI*[q] *lacZΔM15*)	37
Plasmids		
pUC4K	Km[r] (Tn*903*)	46
pWR20	*cycB*, pUC13 derivative	This study
pWR30	*cycB*::Km[r], pWR20 derivative	This study
pGRPd1	*oriV* (ColE1) Amp[r] *oriT* Sm[r] (Tn*1831*)	35
pRTd6121	*oriV* (ColE1) Amp[r] *oriT* Sm[r] *cycB*::Km[r]	This study

[a] Rif, rifampin; Sm, streptomycin; Km, kanamycin; Amp, ampicillin.

of the mutant strain. The possible physiological function of cytochrome c_{553i} in the respiratory chain is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids are described in Table 1. *P. denitrificans* and *Escherichia coli* were routinely grown on brain heart infusion broth (GIBCO, Life Technologies LTD, Paisley, United Kingdom). Antibiotics, when added, were used at final concentrations of 40 μg of rifampin per ml, 25 μg of kanamycin per ml, 25 μg of streptomycin per ml, and 100 μg of ampicillin per ml.

For large-scale purification of cytochrome c_{553i}, wild-type *P. denitrificans*, Pd 1235, was grown in an aerobic, choline-limited chemostat culture at 30°C and a controlled pH of 7, at a dilution rate of 0.15. Choline chloride (30 mM) was used as the carbon source in a mineral salts medium described by Chang and Morris (10) supplemented with a trace element solution described by Lawford et al. (30) and 0.02% yeast extract. To investigate the growth characteristics of the parent and the mutant strains, we used the same medium as described above to perform both batch and continuous cultures. In batch cultures, 50 mM methylamine, 25 mM glucose, or 25 mM succinate, and in chemostat cultures, 100 mM methylamine or 100 mM methanol, was used as the carbon source. Continuous cultures on methanol were started on methylamine as the sole carbon source, a growth condition during which methanol dehydrogenase is also induced in *P. denitrificans* (16). When a steady state was obtained, the culture was switched to methanol as the sole carbon source. During anaerobic growth, the medium described above was supplemented with 40 mM potassium

nitrate. The cultures were incubated in 30-ml flasks completely filled with medium.

Isolation of periplasmic and membrane-bound proteins. Cells were harvested and suspended to an optical density of 200 cm^{−1} at 660 nm. Cells were broken by an osmotic shock after treatment with lysozyme as described by Witholt et al. (50). The resulting spheroplasts were removed by centrifugation for 30 min at 40,000 × g and 4°C. The supernatant fraction, containing the periplasmic proteins, was saturated with ammonium sulfate (0.6 g/ml), stirred for 1 h on ice, and centrifuged for 1 h at 100,000 × g. For further purification of cytochrome c_{553i}, the precipitate was dissolved in a minimal volume of 100 mM Tris hydrochloride (pH 8.0) containing 1.5 M ammonium sulfate. For gel electrophoresis of the periplasmic proteins, the protein precipitate was dissolved in a minimal volume of 100 mM Tris hydrochloride (pH 8.0). The resulting solution was dialyzed overnight at 4°C against 100 mM Tris hydrochloride (pH 8.0), using a dialyzing tube with a cutoff of 3,500. Periplasmic proteins were stored at −70°C.

Membrane-bound proteins were isolated from membrane vesicles, which were prepared by an osmotic shock of the spheroplasts (27). A centrifugation step of 10 min at 20,000 × g and 4°C was used to remove cell debris. Vesicles were collected by centrifugation for 45 min at 50,000 × g and 4°C. To solubilize the membrane proteins, the vesicles were suspended in 1% dodecyl-β-D-maltoside in 50 mM Tris hydrochloride (pH 7.5) and incubated for 1 h on ice, according to Berry and Trumpower (6). The membrane particles were removed by centrifugation for 1 h at 100,000 × g and 4°C. The extract containing the membrane proteins was stored at −70°C.

Purification of periplasmically located cytochrome c_{553i}. The concentrated periplasmic protein fraction was applied to a hydrophobic interaction high-pressure liquid chromatography (HPLC) column (TSK-Phenyl-5-PW [7.5 by 75 mm]; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 100 mM Tris hydrochloride (pH 8.0) containing 1.5 M ammonium sulfate. The proteins were eluted with a linear gradient of 1.5 to 0 M ammonium sulfate in 100 mM Tris hydrochloride (pH 8.0). The flow rate was 0.8 ml/min. Proteins and heme were monitored by the A_{280} and A_{417}, respectively. Cytochrome c_{553i}-containing fractions were concentrated by ammonium sulfate precipitation (0.6 g/ml). The proteins were collected by centrifuging for 1 h at 100,000 × g and 4°C and then dialyzing overnight at 4°C against 100 mM Tris hydrochloride (pH 8.0). Cytochrome c_{553i} was further purified by elution over an anion-exchange HPLC column (TSK-DEAE-5-PW [7.5 by 75 mm]; Pharmacia LKB, Uppsala, Sweden) equilibrated with 20 mM Tris hydrochloride (pH 8.0). The proteins were eluted with a linear gradient of 20 to 500 mM Tris hydrochloride (pH 8.0) and a flow rate of 0.8 ml/min. Proteins were monitored by the A_{280}, and heme was monitored by the A_{417}. Cytochrome c_{553i} was identified by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and subsequent heme staining. The fractions containing cytochrome c_{553i} were collected and loaded on a preparative SDS gel. After gel electrophoresis, a reddish heme-containing band was obtained and cut out from the gel. To remove the polyacrylamide, electroelution was performed using a dialyzing membrane with a cutoff of 3,500. Proteins were eluted for 45 min at 20 mA with an elution buffer containing 8.0 mM Tris hydrochloride (pH 7.8), 1.1 mM citric acid, and 2.5 mM EDTA.

N-terminal amino acid sequence determination. Trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ke-
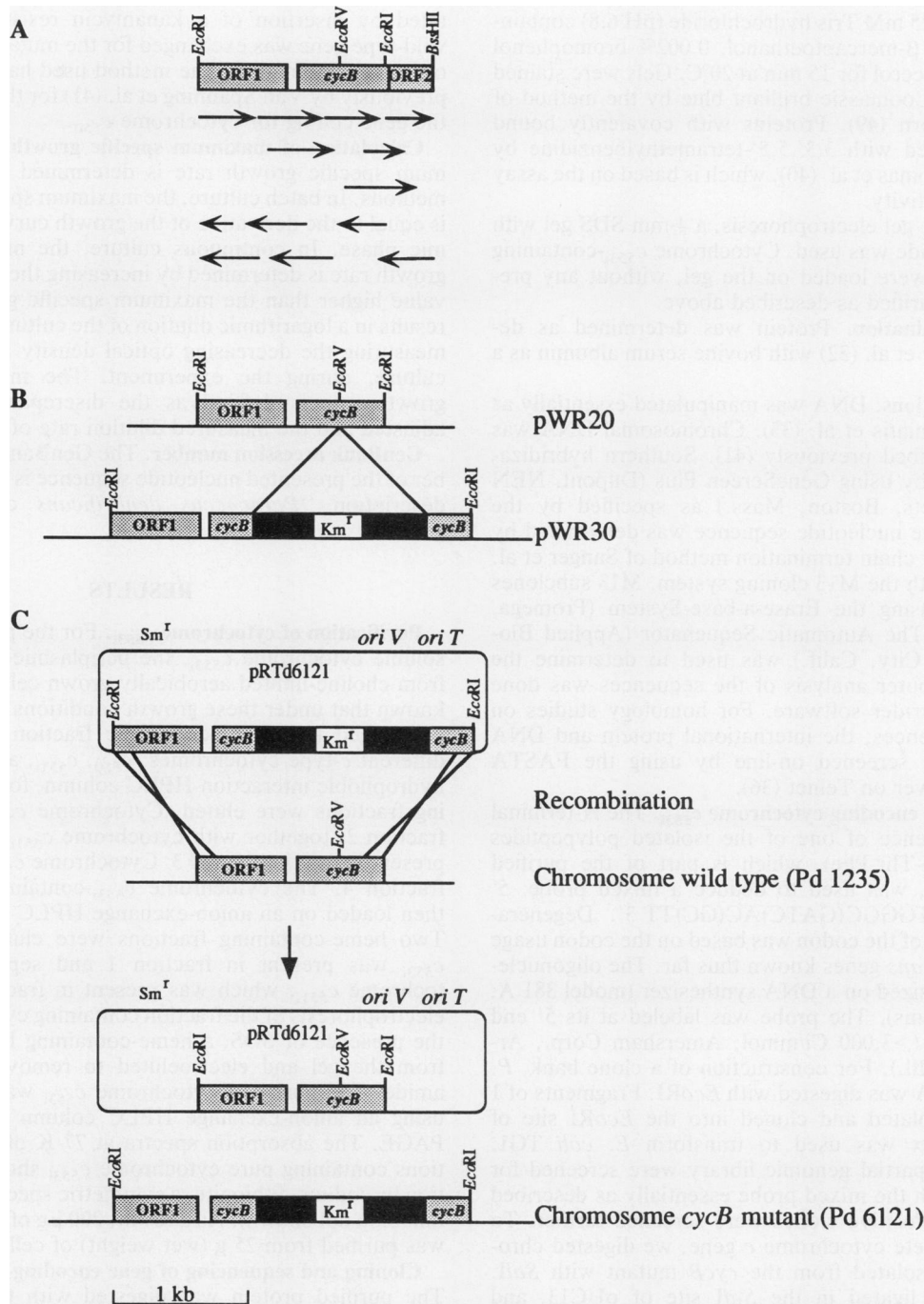
FIG. 1. Restriction map and cloning strategy for a *P. denitrificans* DNA fragment containing the gene encoding cytochrome c_{553i} (*cycB*) and part of the open reading frames ORF1 and ORF2 at both sides of the *cycB* gene (A). Scheme of the inactivation of the structural gene encoding cytochrome c_{553i} (*cycB*) by insertion of a kanamycin resistance gene (Km^r) (B), and the exchange of the wild-type gene with the mutated gene by homologous recombination (C). Sm^r, streptomycin resistance.

tone; Sigma Chemical Co., St. Louis, Mo.) was used to digest the purified protein into several polypeptides. The peptides were separated with a reversed-phase HPLC column (RP318 [4.6 by 250 mm]; Bio-Rad Laboratories) preequilibrated with 0.1% trifluoracetic acid. Peptides were eluted with 75% acetonitrile in 0.08% trifluoracetic acid (pH 2.0) with a flow rate of 1 ml/min. The effluent was monitored for peptides at A_{220}. The N-terminal regions of the purified

polypeptides were sequenced by Edman degradation with a gas-phase amino acid sequenator (19) at the Laboratory for Medical Biochemistry, Sylvius Laboratories, Leiden, The Netherlands.

Gel electrophoresis, and protein and heme staining. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 1.5-mm-thick slab gels with 11 to 13% polyacrylamide prepared by the method of Laemmli (29). Samples were

incubated in 0.0625 mM Tris hydrochloride (pH 6.8) containing 2% SDS, 5% β-mercaptoethanol, 0.002% bromophenol blue, and 10% glycerol for 15 min at 20°C. Gels were stained for protein with Coomassie brilliant blue by the method of Weber and Osborn (49). Proteins with covalently bound heme were stained with 3,3',5,5'-tetramethylbenzidine by the method of Thomas et al. (40), which is based on the assay for peroxidase activity.

For preparative gel electrophoresis, a 4-mm SDS gel with 11% polyacrylamide was used. Cytochrome c_{553i}-containing HPLC fractions were loaded on the gel, without any pretreatment, and purified as described above.

Protein determination. Protein was determined as described by Lowry et al. (32) with bovine serum albumin as a standard.

DNA manipulations. DNA was manipulated essentially as described by Maniatis et al. (33). Chromosomal DNA was isolated as described previously (41). Southern hybridizations were done by using GeneScreen Plus (Dupont, NEN Research Products, Boston, Mass.) as specified by the manufacturer. The nucleotide sequence was determined by using the dideoxy chain termination method of Sanger et al. (38) combined with the M13 cloning system. M13 subclones were made by using the Erase-a-base-System (Promega, Madison, Wis.). The Automatic Sequenator (Applied Biosystems, Foster City, Calif.) was used to determine the sequences. Computer analysis of the sequences was done with the DNA-Strider software. For homology studies on amino acid sequences, the international protein and DNA data banks were screened on-line by using the FASTA program mail server on Telnet (36).

Cloning of gene encoding cytochrome c_{553i}. The N-terminal amino acid sequence of one of the isolated polypeptides (Val-Asp-Trp-Ala-Thr-Phe), which is part of the purified cytochrome c_{553i}, was used to deduce a mixed probe: 5' GT(GC)GA(CT)TGGGC(GATC)AC(GC)TT 3'. Degeneration on position 3 of the codon was based on the codon usage of all *P. denitrificans* genes known thus far. The oligonucleotide was synthesized on a DNA synthesizer (model 381 A; Applied Biosystems). The probe was labeled at its 5' end with [γ-^{32}P]ATP (>3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). For construction of a clone bank, *P. denitrificans* DNA was digested with *Eco*RI. Fragments of 1 to 3 kb were isolated and cloned into the *Eco*RI site of pUC13. This mix was used to transform *E. coli* TG1. Colonies of this partial genomic library were screened for hybridization with the mixed probe essentially as described previously (41). Positive clones were screened further. To isolate the complete cytochrome *c* gene, we digested chromosomal DNA isolated from the *cycB* mutant with *Sal*I. Fragments were ligated in the *Sal*I site of pUC13, and colonies were selected that were resistant to kanamycin, the resistance being encoded by the marker present in the mutated gene. Positive clones were further analyzed.

Site-directed mutagenesis of the gene encoding cytochrome c_{553i}. The gene encoding cytochrome c_{553i} (*cycB*) was mutated by insertion of a kanamycin resistance gene. The wild-type gene was exchanged for the mutant one by homologous recombination. The method used has been described previously by Van Spanning et al. (41) for the mutagenesis of the gene coding for cytochrome c_{550}.

Calculation of maximum specific growth rate. The maximum specific growth rate is determined by two different methods. In batch culture, the maximum specific growth rate is equal to the derivative of the growth curve in the logarithmic phase. In continuous culture, the maximum specific growth rate is determined by increasing the dilution rate to a value higher than the maximum specific growth rate. This results in a logarithmic dilution of the culture, determined by measuring the decreasing optical density at 660 nm of the culture, during the experiment. The maximum specific growth rate is defined as the discrepancy between the adjusted and the measured dilution rate of the culture.

GenBank accession number. The GenBank accession number of the presented nucleotide sequence is M75583, with the description "*Paracoccus denitrificans* cytochrome c_{553i} gene."

RESULTS

Purification of cytochrome c_{553i}. For the purification of the soluble cytochrome c_{553i}, the periplasmic fraction isolated from choline-limited aerobically grown cells was used. It is known that under these growth conditions cytochrome c_{553i} is induced (7). The periplasmic fraction contained three different *c*-type cytochromes; c_{550}, c_{551i}, and c_{553i}. Using a hydrophobic interaction HPLC column, four heme-containing fractions were eluted. Cytochrome c_{553i} was found in fraction 2, together with cytochrome c_{551i}, which was also present in fractions 1 and 3. Cytochrome c_{550} was present in fraction 4. The cytochrome c_{553i}-containing fraction was then loaded on an anion-exchange HPLC column (DEAE). Two heme-containing fractions were eluted. Cytochrome c_{553i} was present in fraction 1 and separated from cytochrome c_{551i}, which was present in fraction 2. After gel electrophoresis of the fraction containing cytochrome c_{553i} in the presence of SDS, a heme-containing band was cut out from the gel and electroeluted to remove the polyacrylamide. The purity of cytochrome c_{553i} was determined by using an anion-exchange HPLC column as well as SDS-PAGE. The absorption spectra at 77 K of the HPLC fractions containing pure cytochrome c_{553i} showed after reduction by sodium dithionite, a symmetric spectral band at 553.5 nm (data not shown). As a result, 200 μg of cytochrome c_{553i} was purified from 25 g (wet weight) of cells.

Cloning and sequencing of gene encoding cytochrome c_{553i}. The purified protein was digested with trypsin to obtain several polypeptides. The N-terminal regions of these peptides were sequenced. On the basis of the amino acid sequence of one of these peptides, a mix of 17-mer oligonucleotides was synthesized. Clones from the *Eco*RI bank reacting positively on this probe were isolated. From one of

FIG. 2. Nucleotide sequences and deduced amino acid sequences of the 3' part of ORF1, the *cycB* gene, and the 5' part of ORF2 of *P. denitrificans*. Indicated are the putative Shine-Dalgarno sequences (underlined), signal sequences (in italics), signal peptide cleavage sites (*), and inverted repeat (double underline). The heme-binding site and the possible sixth iron ligand methionine of cytochrome c_{553i} are indicated in boldface. The amino acid sequences which are underlined with wavy lines represent the N-terminal sequences from the isolated polypeptides obtained after digestion of the purified cytochrome c_{553i} with trypsin. The nucleotide sequence used for construction of the mixed oligonucleotide is also indicated by wavy underlining. The slash (/) indicates the position of the last amino acid of cytochrome c_{553i} and the first amino acid of the ORF2 gene product.

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        N    S    E    T    Y    G    R    P    A    V    V    A    E    Y    S    T    A    Q    N    G    E    D    E    N    T
     GAAT TCG  GAA  ACC  TAT  GGC  CGT  CCG  GCC  GTG  GTG  GCC  GAA  TAC  TCG  ACC  GCC  CAG  AAC  GGC  GAG  GAC  GAG  AAC  ACC
     EcoRI           20                          40                          60


        T    G    V    C    P    A    A    L    G    T    K    D    Q    Q    P    A    A    F    S    P    K    T    N    L    F
     ACC  GGC  GTC  TGC  CCT  GCG  GCA  CTG  GGC  ACC  AAG  GAC  CAG  CAG  CCG  GCG  GCC  TTC  TCG  CCC  AAG  ACC  AAC  CTG  TTC
           80                         100                         120                         140


        Y    V    P    T    N    H    V    C    M    D    Y    E    P    F    R    V    A    Y    T    A    G    Q    P    Y    V
     TAT  GTG  CCG  ACG  AAC  CAC  GTC  TGC  ATG  GAT  TAC  GAG  CCG  TTC  CGG  GTG  GCC  TAT  ACC  GCC  GGC  CAG  CCC  TAT  GTC
          160                         180                         200                         220


        G    A    T    L    S    M    Y    P    A    P    N    S    H    G    G    M    G    N    F    I    A    W    H    N    T
     GGC  GCC  ACG  CTG  TCC  ATG  TAT  CCC  GCG  CCG  AAC  AGC  CAT  GGC  GGC  ATG  GGC  AAC  TTC  ATC  GCC  TGG  CAC  AAC  ACC
          240                         260                         280                         300


        T    G    E    I    K    W    S    V    P    E    Q    F    S    V    W    S    G    A    L    A    T    A    G    D    V
     ACG  GGC  GAG  ATC  AAG  TGG  TCC  GTT  CCC  GAG  CAG  TTC  TCG  GTC  TGG  TCG  GGC  GCT  TTG  GCT  ACG  GCG  GGC  GAC  GTG
          320                         340                         360


        V    F    Y    G    T    L    E    G    Y    L    K    P    V    D    A    Q    T    G    E    E    L    Y    K    F    K
     GTC  TTC  TAC  GGC  ACG  TTG  GAG  GGC  TAT  CTG  AAG  CCC  GTC  GAT  GCC  CAG  ACG  GGC  GAG  GAG  CTT  TAC  AAG  TTC  AAG
          380                         400                         420                         440


        T    P    S    G    I    I    G    N    V    M    T    Y    E    H    G    G    K    Q    Y    V    G    I    L    S    G
     ACC  CCC  TCG  GGC  ATC  ATC  GGC  AAC  GTG  ATG  ACC  TAC  GAA  CAT  GGC  GGC  AAG  CAG  TAT  GTC  GGC  ATC  CTG  TCG  GGT
          460                         480                         500                         520


        V    G    G    W    A    G    I    G    L    A    A    G    L    T    N    P    N    D    G    L    G    A    V    G    G
     GTC  GGC  GGC  TGG  GCC  GGG  ATC  GGC  CTT  GCC  GCG  GGC  CTG  ACC  AAT  CCC  AAC  GAT  GGG  CTT  GGC  GCG  GTG  GGC  GGC
          540                         560                         580                         600


        Y    A    S    L    S    Q    Y    T    E    L    G    G    Q    L    T    V    F    E    L    P    G
     TAT  GCC  TCG  CTG  TCG  CAA  TAT  ACC  GAG  CTG  GGC  GGC  CAG  CTG  ACC  GTG  TTC  GAA  CTG  CCG  GGC  TAATACTTTGGTGCC
          620                         640                         660

                                                                                                         M    T    S
     GGAAAGCAAACTGTGACTCGAAACTGGGCGCCCCAGGGGTAACCTTGGGGCGCGCCATCATCTTCGGGTGACAGCCAGGGAGGAAATTCATG ACC  AGC
     680              700              720              740              760


        K    T    T    A    S    L    L    A    I    C    V    A    C    A    A    S    A    I    A    *    G    T    A    L    C    A
     AAG  ACG  ACC  GCC  AGT  CTT  TTG  GCG  ATC  TGT  GTC  GCA  TGC  GCG  GCC  TCT  GCC  ATC  GCC  GGA  ACG  GCC  CTT  TGC  GCA
          780                         800                         820                         840


        D    R    R    N    A    P    A    Q    A    G    A    G    A    A    A    V    S    G    D    A    H    E    Q    P
     GAC  CGC  CGA  AAC  GCC  CCG  GCC  CAG  GCC  GGG  GCA  GGC  GCT  GCC  GCC  GCC  GTC  TCG  GGC  GAT  GCG  CAC  GAG  CAA  CCG
          860                         880                         900                         920


        A    A    E    A    P    A    E    E    E    E    T    P    A    V    A    A    T    D    G    K    L    V    L    P
     GCC  GCG  GAA  GCG  CCC  GCC  GAA  GAG  GAG  GAG  GAG  ACG  CCA  GCG  GTC  GCC  GCA  ACG  GAC  GGC  AAG  CTT  GTG  CTG  CCG
          940                         960                         980                        1000


        N    G    Q    D    I    T    P    D    H    M    E    N    G    R    W    Y    T    A    E    D    I    P    T    Y    K
     AAC  GGC  CAG  GAC  ATC  ACC  CCG  GAC  CAT  ATG  GAG  AAC  GGC  CGG  TGG  TAT  ACG  GCC  GAG  GAT  ATC  CCG  ACC  TAC  AAG
          1020                        1040                        EcoRV


        I    A    E    E    G    A    V    D    W    A    T    F    S    G    Y    R    R    Y    S    A    E    C    H    V    C
     ATC  GCC  GAG  GAA  GGC  GCG  GTG  GAT  TGG  GCG  ACC  TTC  TCG  GGC  TAT  CGC  CGC  TAT  TCC  GCC  GAA  TGC  CAT  GTC  TGC
          1080                        1100                        1120                        1140


        H    G    P    D    G    E    G    S    T    Y    A    P    L    R    K    S    V    L    T    M    G    Y    Y    D
     CAC  GGC  CCG  GAT  GGC  GAG  GGT  TCG  ACC  TAT  GCC  CCG  GCC  CTG  CGG  AAA  TCG  GTG  CTG  ACC  ATG  GGC  TAT  TAC  GAT
          1160                        1180                        1200                        1220


        F    L    E    I    A    A    S    G    K    Q    E    V    N    T    A    A    N    L    V    M    P    A    F    G    T
     TTC  CTG  GAG  ATC  GCC  GCC  TCG  GGC  AAG  CAA  GAG  GTC  AAC  ACC  GCC  GCC  AAT  CTG  GTC  ATG  CCG  GCC  TTT  GGC  ACC
          1240                        1260                        1280                        1300


        N    K    N    V    W    C    Y    I    D    D    I    Y    A    Y    L    L    A    R    G    T    G    D    L    P    R
     AAC  AAG  AAC  GTC  TGG  TGC  TAT  ATC  GAC  GAC  ATC  TAT  GCC  TAT  CTG  CTG  GCG  CGG  GGC  ACG  GGA  GAC  TTG  CCG  CGC
          1320                        1340                        1360


        G    R    P    A    K    R    E    D    K    S    D    E    F    V    A    Q    E    D    S    C    M    S    G/M  R
     GGC  CGC  CCG  GCG  AAG  AGA  GAG  GAC  AAG  TCC  GAT  GAA  TTC  GTT  GCG  CAG  GAA  GAT  TCC  TGC  ATG  AGC  GGATGAGA
          1380                        1400      EcoRI     1420                        1440


        R    A    L    T    L    A    V    L    A    T    C    A    V    L    P    A    L    A    *    Q    V    A    D    L    R    S
     CGC  GCC  CTT  ACC  CTT  GCC  GTG  CTG  GCG  ACC  TGC  GCC  GTG  CTG  CCC  GCC  TTG  GCG  CAG  GTT  GCC  GAC  CTG  CGC  TCC
          1460                        1480                        1500                        1520


        K    T    E    F    R    V    C    A    D    P    A    A    V    P    M    S    S    Q    D    G    K    G    F    E    N
     AAG  ACC  GAG  TTC  CGG  GTC  TGT  GCC  GAT  CCC  GCC  GCG  GTG  CCG  ATG  TCG  TCC  CAG  GAC  GGC  AAG  GGC  TTC  GAG  AAC
          1540                        1560                        1580                        1600


        R    I    A    Q    L    F    A    E    K    L    G    V    P    V    A    Y    T    W    F    P    Q    S    R    L    H
     CGC  ATC  GCC  CAG  CTT  TTC  GCC  GAA  AAG  CTG  GGC  GTG  CCG  GTC  GCC  TAT  ACC  TGG  TTC  CCG  CAG  TCC  CGG  CTT  CAT
          1620                        1640                        1660


        P    Q    E    P    A    R
     CCG  CAA  GAA  CCT  GCG  CGC
          1680          BssHII
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these, its plasmid (pWR20) was isolated for further characterization and sequence analysis. A restriction map of pWR20 and the sequencing strategy are shown in Fig. 1. A major part of the cycB gene was located on plasmid pWR20 containing a 1.4-kb chromosomal EcoRI fragment. An additional 278 bp were located on a 7-kb SalI fragment.

The gene encoding cytochrome c_{553i}, as presented in Fig. 2, starts at position 769. The sequences underlined with wavy lines represent the sequences from the polypeptides from which the N-terminal regions were sequenced. These sequences confirm the correctness of the chosen reading frame. A putative Shine-Dalgarno sequence (AGGGAGGA), indicating a ribosome-binding site, was present 6 bp upstream from the initiation codon. The deduced amino acid residues at the N-terminal region form a typical signal peptide. A positively charged residue (Lys) at the N terminus is followed by a central hydrophobic core, presumably responsible for initiating export of the native protein across the cytoplasmic membrane. This was to be expected, since cytochrome c_{553i} is a periplasmic protein. The signal peptide-processing site was determined by the method of Von Heijne (48) and is located between the alanine residue at position 832 and the glycine residue at position 835. The cycB gene encodes a precursor protein of 226 amino acids with a putative signal peptide of 22 amino acid residues. Taking into account the cleavage of the signal peptide (2.077 kDa) and assembly of the heme (0.640 kDa), the calculated molecular weight of the mature protein is 22,427. The codon usage is typical for P. denitrificans genes. The G+C content of the cycB gene is 65.0%. There is a 79.2% preference for a G or a C at position 3 in the codons. Based on the homology of several cytochromes c of different bacteria (1), the methionine residue in position 1285 is probably the sixth ligand of the heme iron. The deduced amino acid sequence of cytochrome c_{553i} at its N terminus in a 102-amino-acid overlap is 25.5% identical with cytochrome c_1 of P. denitrificans (28). The sequence shows at its C-terminal part in a 104-amino-acid overlap an identity of 35% with the determined amino acid sequence of cytochrome c_{555} of Methylococcus capsulatus (1). No significant homology was found between

TABLE 2. Homology of the deduced amino acid sequences of the 3' part of ORF1 and the 5' part of ORF2 of P. denitrificans with several quinoproteins

Gene product	ORF1		ORF2	
	Amino acid overlap	% Identical amino acids	Amino acid overlap	% Identical amino acids
MoxF[a]	204	47.1	—[h]	—
MoxF[b]	220	49.5	—	—
ADH[c]	216	28.7	—	—
GDH-A[d]	74	37.8	—	—
GDH[e]	74	36.5	—	—
MoxJ[f]	—	—	124	31.0
MoxJ[g]	—	—	126	35.0

[a] The moxF gene product of methanol dehydrogenase of P. denitrificans (22).
[b] The moxF gene product of methanol dehydrogenase of M. extorquens AM1 (2).
[c] Alcohol dehydrogenase of Acetobacter aceti (26).
[d] Glucose dehydrogenase of Acinetobacter calcoaceticus (12).
[e] Glucose dehydrogenase of E. coli (11).
[f] The moxJ gene product of methanol dehydrogenase of P. denitrificans (42).
[g] The moxJ gene product of methanol dehydrogenase of M. extorquens AM1 (2).
[h] —, no homology.

cytochrome c_{553i} and cytochrome c_{551i}, the cytochrome that is encoded by the moxG gene.

The gene encoding cytochrome c_{553i} was flanked by two interesting open reading frames. As shown in Table 2, the deduced amino acid sequence of the open reading frame upstream of the cycB gene (designated ORF1) shows homology with several quinoproteins, such as methanol dehydrogenase from P. denitrificans (22) and Methylobacterium extorquens AM1 (2), alcohol dehydrogenase from Acetobacter aceti (26), and glucose dehydrogenase from both Acinetobacter calcoaceticus (12) and E. coli (11). It was shown by Cleton-Jansen et al. (11) that these quinoproteins contain in a stretch of 50 amino acids several conserved amino acids. Figure 3 shows that these sequences could also be found in the gene product of ORF1. The deduced amino acid sequence of the open reading frame downstream of cycB (designated ORF2) shows homology with the moxJ gene products of both P. denitrificans (42) and M. extorquens AM1 (2).

The start codon of the deduced amino acid sequence of ORF1 is not present on the isolated EcoRI fragment. The termination codon TAA is located at position 665. Downstream from this stop codon, a 26-bp long inverted repeat was found, which might form a hairpin structure that could act as a transcription terminator (nucleotides 707 to 733 as shown in Fig. 2). The 3' part of the open reading frame sequenced so far has a G+C content of 64.6% and an 86.4% preference for a G or a C at position 3 of the codons.

Immediately downstream the cycB gene, ORF2 was found. Its possible start codon (at position 1446) shows overlap with the termination codon of the cycB gene. The positions of two possible ribosome-binding sites (AGGA at position 1424 and GAG at position 1440) are indicated. The N-terminal part of the sequence has characteristics of a signal sequence. Positively charged residues (Arg, Arg) are followed by a central hydrophobic core. A helix-breaking residue (Pro) could be found four residues before the cleavage site, which was determined by the method of Von Heijne (48). This peptidase cleavage site is located between the alanine residue at position 1503 and the glutamine residue at position 1506. Therefore, the protein contains a 20-residue signal peptide. The part of ORF2 sequenced so far has a G+C content of 67.1% and an 80.7% preference for a G or a C at position 3 in the codons.

Construction of cycB mutant. The plasmid WR20, harboring a major part of the gene encoding cytochrome c_{553i}, was digested with EcoRV (Fig. 1). In this unique EcoRV site, a 1.4-kb HincII fragment of pUC4K, containing the kanamycin resistance gene from transposon Tn903, was inserted. To exchange the interrupted gene for the wild-type gene in the chromosome of P. denitrificans, we cloned the 2.8-kb EcoRI fragment in the EcoRI site of the suicide vector pGRPd1 (41). The wild-type gene in the chromosome of P. denitrificans was exchanged with the mutated gene by homologous recombination. For a complete recombination, resulting in a mutant strain, a crossover event at both sides of the kanamycin resistance gene was necessary. Kanamycin-resistant colonies were obtained with a frequency of 10^{-5}, of which 36% were shown to have streptomycin resistance, caused by a single crossover and subsequent insertion of the vector which contains the streptomycin marker.

The exchange of the wild-type gene with the mutated gene was verified by Southern analysis of genomic DNA. Chromosomal DNA of the parent and mutant strains was isolated and restricted with EcoRI. The DNA was characterized by probing it with the 1.4-kb fragment of pWR20. From DNA of

ORF1 *P. d*	EGYLKPVDAQT**GEEL**YKFKT**P**SGIIGNV**MTY**EHGGKQ**Y**VGILSGVGGWAG
	524　　　　　　　　　　　　　　　　　　　　　　　573
MoxF *P. d*	DGFIK**A**RDSDT**G**D**LLW**KFKL**P**SGVIGH**PMTY**HKDGRQ**Y**VAIMYGVGGW**P**G
	519　　　　　　　　　　　　　　　　　　　　　　　568
MoxF *M. e* AM1	DGYLK**A**RDSDT**G**D**LLW**KFKI**P**SGAIGY**PMTY**THKGTQ**Y**VAIYYGVGGW**P**G
	537　　　　　　　　　　　　　　　　　　　　　　　584
ADH *A. a*	NGEF**H**A**Y**DATNGSD**L**YKFDAQSGIIA**PPMTY**SVNGKQ**Y**VAVEVGWGGI--
	736　　　　　　　　　　　　　　　　　　　　　　　785
GDH-A *A. c*	DNYLR**A**FNVTNGKK**LW**EARI**P**AGGQAT**PMTY**EINGKQ**Y**VVIMAGGHGSFG
	730　　　　　　　　　　　　　　　　　　　　　　　779
GDH *E. c*	DNYLR**A**YNMSNGEK**LW**QGRL**P**AGGQAT**PMTY**EVNGKQ**Y**VVISAGGHGSFG

FIG. 3. Comparison of homologous domains of the deduced amino acid sequence of ORF1 of *P. denitrificans* (ORF1 *P. d*), the gene product of *moxF* of methanol dehydrogenase of *P. denitrificans* (MoxF *P. d*) (22), the gene product of *moxF* of methanol dehydrogenase of *M. extorquens* AM1 (MoxF *M. e* AM1) (2), alcohol dehydrogenase of *Acetobacter aceti* (ADH *A. a*) (26), glucose dehydrogenase of *Acinetobacter calcoaceticus* (GDH-A *A. c*) (12), and glucose dehydrogenase of *E. coli* (GDH *E. c*) (11). Identical amino acid residues are indicated in boldface.

the parent strain, a 1.4-kb *Eco*RI fragment, corresponding in size to the wild-type DNA fragment, showed hybridization with the probe. From the *cycB* mutant, a 2.8-kb *Eco*RI fragment, corresponding in size to the mutated DNA fragment, showed hybridization with the probe. This clearly demonstrates that in the mutant strain the wild-type *cycB* was replaced by the mutated one (data not shown).

Biochemical and physiological analysis of *cycB* mutant. SDS-PAGE was used to investigate whether cytochrome c_{553i} was absent in the *cycB* mutant. Periplasmic and cytoplasmic membrane-bound proteins, isolated from parent and mutant strains, both grown in batch culture with methylamine as the sole carbon source, were analyzed. As shown in Fig. 4, it is clear that in the periplasmic fraction isolated from the mutant, no protein with covalently bound heme of the estimated 30 kDa was found. This is in contrast to the cytoplasmic membrane fraction of the mutant, in which a *c*-type cytochrome of about 30 kDa was present. The apparently increased levels of the 22-kDa cytochrome c_{551i} in the periplasmic fraction of the mutant strain are caused by decreased levels of this cytochrome in the periplasmic fraction of the parent strain resulting from degradation of this unstable cytochrome *c*. For the other *c*-type cytochromes, no differences were observed between the mutant and the parent strain.

The physiological consequences of the mutation were studied by growth experiments under different conditions (Table 3). The growth parameters for heterotrophic growth on succinate, under aerobic as well as under anaerobic conditions in the presence of nitrate, were determined in batch cultures. No differences could be detected for growth yield or maximum specific growth rate between the parent and mutant strain. Growth on methylamine was studied in both batch and continuous cultures. Also in this case, no differences between the parent and the mutant strain were observed in either growth yield or maximum specific growth rate. Chemostat cultures were used for studies of growth on methanol. No differences in specific growth yield between the parent strain and the mutant strain were observed. The maximum specific growth rate of the mutant strain growing on methanol, however, was decreased about 25%.

DISCUSSION

The experimental results pertaining to cytochrome c_{553i} described in this report should be discussed in connection with previously published results on the study of cytochromes *c* in *P. denitrificans* and in other methylotrophic bacteria as well. Upon previous analyses, it appeared that *P. denitrificans* can form a membrane-bound and a soluble *c*-type cytochrome, both of which have apparent molecular masses of around 30 kDa on SDS gels (7, 9). Whether or not these cytochromes are different entities was unknown at that time, but from the results presented here it can be concluded that they are encoded by different structural genes. Mutation in *cycB*, the gene encoding cytochrome c_{553i}, results in the
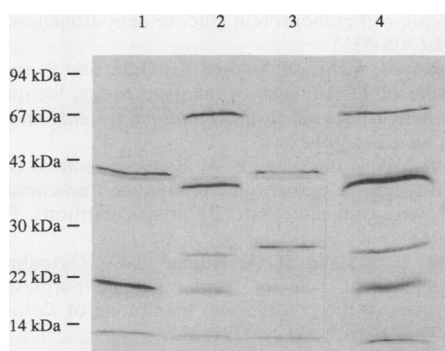

FIG. 4. SDS-PAGE of periplasmic proteins (lanes 1 and 3) and membrane-bound proteins (lanes 2 and 4) of the *P. denitrificans* parent strain (lanes 3 and 4) and the *cycB* mutant strain (lanes 1 and 2). Cells were grown in batches on methylamine. Covalently bound heme was stained with 3,3′,5,5′-tetramethylbenzidine. The relative molecular masses are indicated at the left.

TABLE 3. Growth characteristics of the parent strain (WT) and the *cycB* mutant strain (*cycB*-MT) under different growth conditions

Carbon source	Electron acceptor	Growth yield[a]		Growth rate[b]	
		WT	*cycB*-MT	WT	*cycB*-MT
Batch cultures					
Glucose	Oxygen	2.0	2.0	0.26	0.26
Succinate	Oxygen	2.1	2.1	0.42	0.40
Succinate	Nitrate	2.2	2.2	0.32	0.33
Methylamine	Oxygen	1.8	1.9	0.14	0.14
Continuous cultures					
Methylamine	Oxygen	11.0	11.7	0.14	0.14
Methanol	Oxygen	10.5	10.5	0.16	0.12

[a] For batch cultures, in maximum optical density at 660 nm (A_{660} units); for continuous cultures, in molecular growth yield (grams of biomass per mole of substrate).

[b] For both batch and continuous cultures, in maximum specific growth rate (hour^{-1}).

disappearance of the soluble cytochrome but leaves its membrane-bound equivalent unimpaired.

During methylotrophic growth, four soluble cytochromes c are located in the periplasm of P. *denitrificans*: c_{550}, c_{551i}, c_{553i}, and a peroxidase. Their functions were studied by in vitro experiments as well as by physiological experiments on mutant strains specifically lacking one of these cytochromes. The in vitro studies did not reveal corresponding results about the function of cytochrome c_{553i}. Previously, cytochrome c_{553i} was thought to be the electron acceptor of methanol dehydrogenase (14). Recently, however, it was found that during in vitro experiments with purified cytochrome c_{553i} and methanol dehydrogenase, no reduction of the cytochrome or production of formaldehyde occurs (31). Our physiological studies, using a mutant strain lacking cytochrome c_{553i} in the chemostat, exclude a function of cytochrome c_{553i} in the electron transfer from methylamine dehydrogenase to oxygen, since neither the molecular growth yield nor the maximum specific growth rate was affected by this mutation. Also, the function of c_{553i} in transport of electrons from methanol dehydrogenase to oxygen cannot be of decisive importance. The mutant still grows on methanol as the exclusive carbon and energy source, albeit with a lowered maximum specific growth rate. From the latter result, an involvement of the cytochrome in methanol oxidation might be deduced. It is unlikely that this involvement includes the transfer of electrons between cytochrome c_{551i} and cytochrome c_{550}. The latter was found to mediate transfer of electrons to the cytoplasmic membrane in vitro (14), since the depletion of cytochrome c_{550} does not affect the maximum specific growth rate on methanol (43). Decisive for the future elucidation of the more specific function of cytochrome c_{553i} might be the nature of the location of its structural gene, *cycB*, which is centered between two open reading frames. The deduced amino acid sequences of ORF1 and ORF2 show homology with the gene products of, respectively, *moxF* and *moxJ* of *P. denitrificans* (22, 42) and *M. extorquens* AM1 (2). The genetic organization ORF1-*cycB*-ORF2, however, is different from the sequence *moxF-moxJ-moxG-moxI*, where *moxF* and *moxI* encode the subunits of methanol dehydrogenase, *moxJ* probably encodes a chaparoninlike protein, and *moxG* encodes cytochrome c_{551i} (22, 42). Moreover, there is no homology in the amino acid sequences of the cytochromes c_{553i} and c_{551i}. Since it is also known that deletions in the *moxFJGI* cluster of *P. denitrificans* unambiguously result in the impossibility of growth on methanol (42), ORF1-*cycB*-ORF2 apparently is unlikely to be encoding part of an *iso*-methanol oxidation system.

ORF1 also exhibits homology with PQQ-dependent enzymes other than methanol dehydrogenase, like glucose dehydrogenases from *Acinetobacter calcoaceticus* (12) and *E. coli* (11) and alcohol dehydrogenase from *Acetobacter aceti* (26). In all cases, a stretch of 50 amino acids at the C terminus might be involved in PQQ binding (11). This might indicate that cytochrome c_{553i} is part of a periplasmically located, PQQ-dependent dehydrogenase system with a substrate specificity unknown at the moment.

Although the cytochrome is hardly detectable, *M. extorquens* AM1 also contains a periplasmic cytochrome c_{553} in addition to cytochromes c_L and c_H, which are equivalents of cytochromes c_{551i} and c_{550} in *P. denitrificans*. However, a *moxD* mutant of *M. extorquens* AM1, which contains no methanol dehydrogenase protein, forms large amounts of that c_{553}-type cytochrome (15). Its molecular mass of 23 kDa and its ability to bind CO, equal to that of cytochrome c_{553i}

in *P. denitrificans* (CO binding not shown here), suggest similarity between these c-type cytochromes in both organisms. The physiological function of cytochrome c_{553} in *M. extorquens* AM1 is unknown at the moment. In vitro experiments show that this cytochrome mediates electrons between the cytochromes c_L and c_H (15), but the physiological relevance of this phenomenon is unknown.

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