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Mutagenesis of the Gene Encoding Cytochrome c_{550} of *Paracoccus denitrificans* and Analysis of the Resultant Physiological Effects

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By using synthetic oligonucleotides, the gene encoding soluble cytochrome c_{550} was isolated from a genomic bank of *Paracoccus denitrificans*. The nucleotide sequence of the gene was determined, and the deduced amino acid sequence of the mature protein was found to be similar to the primary structure of purified cytochrome c_{550} except for the presence of seven additional amino acid residues at the C terminus. At the N terminus of the primary structure was found an additional stretch of 19 amino acid residues that had the typical features of the signal sequence of the cytochrome. Comparison of the nucleotide sequences of the upstream regions of the *P. denitrificans* cytochrome c_{550} gene and bc_1 operon revealed three regions with a distinct organization that showed strong similarity. Downstream of the c_{550} gene was found part of another gene, the deduced amino acid sequence of which showed strong homology with subunit 1 of the cytochrome aa_3 oxidase. For gene replacement experiments, the suicide vector pGRPd1 was constructed. The cytochrome c_{550} gene was inactivated by insertion of a kanamycin resistance gene, and the mutated gene was cloned into this vector. Recombination with the wild-type gene resulted in a mutant strain with an inactivated cytochrome gene. Isolated mutant strains were unable to synthesize the soluble cytochrome, as judged by spectrum analysis and analysis of periplasmic proteins by gel electrophoresis and heme staining. The mutation resulted in a 14% decrease in the growth yield during aerobic heterotrophic growth and in a 40% decrease in the maximum specific growth rate during growth on methylamine. Furthermore, a longer lag phase was observed under both growth conditions. The mutation had no effect on growth yield, maximum specific growth rate, and duration of the lag phase during anaerobic growth in the presence of nitrate. In addition, there was no accumulation of nitrite and nitrous oxide.

Paracoccus denitrificans is a gram-negative bacterium capable of growing under various growth conditions. The bacterium can grow heterotrophically with a great variety of multicarbon compounds at aerobic conditions but also under anaerobic conditions with nitrate, nitrite, or nitrous oxide as a terminal electron acceptor (23, 53). In addition, the bacterium can grow autotrophically on hydrogen and carbon dioxide as well as methylotrophically with methanol or methylamine (15). The composition of the electron transport chain varies with the growth condition. During aerobic heterotrophic growth, the electron transport chain contains many mitochondrionlike components, such as NADH dehydrogenase, the bc_1 complex, and the aa_3 oxidase (1, 27, 52). Periplasmic dehydrogenases, such as methanol dehydrogenase and methylamine dehydrogenase, are induced during methylotrophic growth and enable the bacterium to grow on methanol and methylamine (4, 26). *P. denitrificans* can also synthesize various terminal oxidoreductases (55). During anaerobic growth, the expression of nitrate, nitrite, and nitrous oxide reductases is induced. During aerobic growth, the most abundant oxidases are the proton-translocating cytochrome aa_3 and an *o*- and/or *co*-type oxidase. The first is synthesized mainly at high oxygen tension, whereas the latter one(s) is synthesized at low oxygen tensions (11, 16; G. Bosma, Ph.D. thesis, Vrije Universiteit, Amsterdam, The Netherlands, 1989).

The distribution of the electron flow from the dehydrogenases to the terminal oxidoreductases is not well understood. Specific cytochromes *c* are implicated in this process, especially at the terminal part of the various electron transport branches. In addition to cytochrome c_1, at least eight membrane-bound and periplasmic cytochromes *c* are reported in *P. denitrificans* (11, 12). Most of them are synthesized under specific growth conditions, indicating their possible function in the involved branches. Two cytochromes *c* with respective relative molecular masses of 30 and 45 kilodaltons (kDa) are found in the cytoplasmic membrane, especially after growth at oxygen limitation, which suggests that they function in the electron transport to the *o*- or *co*-type oxidase (Bosma, Ph.D. thesis, 1989). Two other membrane-bound cytochromes *c* of 14 and 22 kDa are synthesized in high amounts during anaerobiosis and aerobiosis, respectively. Two soluble cytochromes *c*, cytochrome c_{552} and cytochrome c_{553}, of 22 and 30 kDa, respectively, are induced predominantly during methylotrophic growth. Another periplasmic cytochrome of 45 kDa, containing both a *b* and a *c* heme, is found during oxygen limitation and anaerobiosis. The fourth cytochrome found in the periplasm is cytochrome c_{550}, which has a relative molecular mass of 14 kDa. This cytochrome is the only one that is present under all growth conditions and therefore might have a crucial or nonspecific function in the different electron transport branches.

With respect to primary (2, 49) and secondary (48) structure, cytochrome c_{550} shows strong homology with cytochrome *c* from mitochondria and with the so-called cytochrome c_2 found in the purple nonsulfur bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (49), organisms that are phylogenetically closely related to *P. denitrificans*. In the electron transport chains of these purple nonsulfur bacteria and of mitochondria, this cytochrome functions in the periplasm and cytosol, respectively, and

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facilitates electron flow between the bc_1 complex and the aa_3 oxidase (5, 6, 22). It has also been shown that the aa_3 oxidase of *P. denitrificans* can oxidize at very high rates both the bacterial and eucaryotic cytochromes *c* (42). Despite these observations, it is not known whether cytochrome c_{550} in *P. denitrificans* functions as an electron carrier between the bc_1 complex and the aa_3 oxidase. In some reports, it was suggested that the membrane-bound cytochrome *c* of 22 kDa, mentioned above, is the direct electron carrier between the bc_1 complex and aa_3 oxidase (7, 11, 12, 28). Cytochrome c_{550} might also function in the electron flow from methylamine dehydrogenase to cytochrome aa_3, analogous to the small cytochrome c_h, which has been found in other methylotrophic bacteria (3, 13). Recently, proof for this suggestion was obtained from in vitro experiments involving purified soluble cytochromes from cells of *P. denitrificans* grown on methylamine (18). A cytochrome *c* of 14 kDa is expressed at high levels in the cytoplasmic membrane during anaerobiosis, which suggests that this cytochrome is involved in electron transport to the anaerobic electron acceptors. It is not known whether this membrane-bound cytochrome *c* is the same as its periplasmic counterpart (11).

Thus, although biochemically well characterized, little is known about the exact physiological functions of cytochrome c_{550} in *P. denitrificans*. To study these functions, a mutation was introduced in the structural gene for this electron carrier and the physiological effects of this mutation during growth under various conditions were studied. The advantage of this strategy was that these effects could be studied in an in vivo situation.

In this report, the isolation and analysis of the structural gene for cytochrome c_{550}, the construction of mutants in this gene, and a brief characterization of these mutants are described. Furthermore, the possible function of this cytochrome in several electron transport branches is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. *P. denitrificans* was grown either in batch cultures or in continuous cultures at 30°C. For aerobic batch cultures, brain heart infusion (BHI) broth or mineral salts medium with 50 mM methylamine, 25 mM succinate, or 10 mM choline chloride as the carbon and energy source was used. The mineral salts medium was as described by Chang and Morris (14), supplemented with 1.0 ml of trace element solution (containing 100 mM $CaCl_2$, 90 mM $FeCl_3$, 50 mM $MnCl_2$, 25 mM $ZnCl_2$, 10 mM $CoCl_2$, 5 mM $CuCl_2$, 5 mM H_3BO_3, 10 mM Na_2MoO_4, and 5 ml of concentrated H_2SO_4 per liter plus 3 M HCl) per liter and 0.01% yeast extract. During anaerobic growth, the medium was supplemented with 50 mM KNO_3; these cultures were incubated in 30-ml flasks completely filled with medium.

Chemostat cultures were run at a dilution rate of 0.07 and at a controlled pH of 7.0, with 100 mM methylamine as the carbon and energy source in a medium with 9.6 mM K_2HPO_4, 6.3 mM KH_2PO_4, 30 mM NH_4Cl, 0.66 mM Na_2MoO_4, 0.26 mM Titriplex I, 0.8 mM $MgSO_4$, 0.01% yeast extract, and 1.0 ml of the trace element solution per liter. The carbon sources were added from filter-sterilized stock solutions. When necessary, antibiotics were added to final concentrations of 40 μg of rifampin per ml, 25 μg of kanamycin per ml, 25 μg of streptomycin per ml, and 50 μg of ampicillin per ml.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacteria		
E. coli		
S17-1	Sm^r *pro* r$^-$ m$^+$, RP4-2 integrated (Tc::Mu)(Km::Tn*7*)	41
JM103	Δ(*lac pro*)*thi endA sbrB hsdR4 rpsL supE* F′ *traD36 proAB lacI^{q}ZΔM15*	59
NF1:K-12	ΔHI Δ*trp* λ Δ*lacZ* Nam7 Nam53 *c*I857 ΔHI	44
P. denitrificans		
Pd1235	Rif^r, enhanced conjugation frequencies, m$^-$	20
Pdcytcm1	Pd1235, cyt c_{550}::Km^r	This study
Pdcytcwtm1	Pd1235, pRcTd1 integrated	This study
Plasmids		
pBR322	*oriV* (ColE1) Amp^r	8
pUC4K	Km^r	54
pRTP1	*oriT*	46
pGRPd1	*oriV* (ColE1) Amp^r *oriT* Sm^r (Tn*1831*)	This study
pRcTd1	*oriV* (ColE1) Amp^r *oriT* Sm^r cyt c_{550}::Km^r	This study
pRS59	cyt c_{550}, pEX2 derivative	This study
pRS59K	cyt c_{550}::Km^r, pRS59 derivative	This study

[a] Sm, streptomycin; Km, kanamycin; Rif, rifampin; cyt, cytochrome.

Plasmid pRTP1 was kindly provided by F. R. Mooi, Rijks Instituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands.

Screening of a *P. denitrificans* genomic bank. The *P. denitrificans* genomic bank was constructed in the pEX2 vector (24). The bank was screened for the cytochrome c_{550} gene with two 17-mer mixed oligonucleotides, which were synthesized on a 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.). The sequences were deduced from the amino acid sequence of the purified protein (49). The bank was spread on six round-cut GeneScreen Plus hybridization filters (Dupont, NEN Research Products, Boston, Mass.) at a dilution of 10,000 colonies per filter, and the filters were incubated on BHI agar plates at 30°C. Colonies were transferred by replica plating. Original filters were stored at 4°C, and sets of replica filters were incubated on BHI agar plates at 30°C until suitable colonies appeared. Lysis of bacteria and binding of the liberated DNA to the nitrocellulose filters were carried out as described by Maniatis et al. (32). These blots were then used for hybridization experiments. The hybridization probes were labeled at the 5′ end with [γ-^{32}P]ATP (>3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in the presence of T4 polynucleotide kinase (New England BioLabs, Inc., Beverly, Mass.). Free label was separated from the labeled DNA probe by column chromatography on Sephadex G-25. The six blots were pretreated with 50 ml of 1% (wt/vol) sodium dodecyl sulfate (SDS), 1 M sodium chloride, and 10% (wt/vol) dextran sulfate during 6 h at 45°C. This is 10°C below the theoretical melting temperatures of the oligonucleotides, which were calculated by assuming hybridization temperatures of 2°C for each A · T base pair and 4°C for each G · C base pair, according to the Wallace rule (57). Hybridization was started with the addition of 50 pmol of either of the labeled probes in parallel experiments. After overnight incubation, blots were washed twice with 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate) during 5 min and twice with a solution of 2×

SSC supplemented with 0.1% (wt/vol) SDS during 30 min. All washing steps were carried out at 45°C and were sufficient to remove the background radioactivity. Clones reacting positively with both probes were picked from the original filters and rescreened at a colony density of 200 per filter. After this selection procedure, recombinant plasmids were isolated and used for the transformation of *Escherichia coli* K-12 strain NF-1.

DNA manipulations. General cloning techniques were carried out essentially as described by Maniatis et al. (32). Plasmid DNA was isolated from *E. coli* by the cleared-lysate method (51) and purified by using CsCl-ethidium bromide density gradients. For rapid screening, plasmid DNA was isolated by the alkaline lysis method (32). Chromosomal DNA from *P. denitrificans* was isolated from a stationary-phase culture. Cells from a 10-ml culture were harvested by centrifugation, washed with 5 ml of washing buffer (50 mM Tris hydrochloride, 50 mM EDTA [pH 8.0]), and then resuspended in 3 ml of this buffer. After addition of SDS to a final concentration of 0.15%, 5 U of pronase E, and 5 U of Prolase (Sigma Chemical Co., St. Louis, Mo.), the cell suspension was incubated for 60 min at 37°C. Subsequently, DNA was purified by phenol extraction and ethanol precipitation. DNA fragments were purified from agarose gels by using GeneClean (Bio 101, Inc., San Diego, Calif.).

Chromosomal DNA (10 μg per lane) was loaded on 0.7% agarose gels, denatured, and transferred to GeneScreen Plus filters according to the method of Southern (43). Southern analysis of chromosomal restriction fragments was done by random-primed DNA labeling of cloned sequences with digoxigenin and subsequent detection of hybrids by an enzyme immunoassay according to the protocol of the manufacturer (Boehringer GmbH, Mannheim, Federal Republic of Germany).

For sequencing, DNA fragments were inserted in derivatives of bacteriophage M13 (35, 37) and amplified in *E. coli* JM103, after which the single-stranded copies were used as templates in a Klenow polymerase reaction in the presence of [^{35}S]ATP, deoxynucleotides, and dideoxynucleotides as described by Sanger et al. (38). Because of the high G+C content of *P. denitrificans*, deaza-dGTP (Boehringer GmbH) was used instead of dGTP. The sequence reaction started from the M13 linker region with the universal primer or was primed by internally annealing 17-mer oligonucleotides synthesized on a 381A DNA synthesizer.

Conjugations. Cells of donor and recipient strains were cultured to the exponential phase of growth, harvested, and suspended in BHI broth to an optical density of 5.0 cm^{-1} at 660 nm. A mixture of 50 μl of the donor and 100 μl of the recipient cell suspensions was spread onto a BHI agar plate. After incubation for 48 h at 30°C, cells were collected and appropriate dilutions were plated on selective plates. Conjugation frequencies and combined conjugation-recombination frequencies were expressed as the number of exconjugants and exconjugants-recombinants per recipient cell.

Isolation of periplasmic proteins. Cells were suspended to an optical density of 200.0 cm^{-1} at 660 nm, and the periplasmic fraction of cells was isolated by preparing spheroplasts essentially as described by Witholt et al. (58). The spheroplasts were removed by centrifugation for 30 min at 40,000 × *g* and 4°C. The supernatant fraction was then saturated with ammonium sulfate, stirred for 16 h on ice, and centrifuged for 1 h at 100,000 × *g*. The precipitate was suspended in a minimal volume of 10 mM Tris hydrochloride (pH 8.0) and dialzyed for 16 h against the same buffer. Periplasmic proteins were routinely stored in equal samples at −80°C at a concentration of 25 mg of protein per ml. The concentration of protein was determined by the method of Lowry et al. (31), using bovine serum albumin as a standard.

Gel electrophoresis and heme staining. SDS-polyacrylamide gel electrophoresis was carried out on 13% slab gels. Periplasmic proteins were incubated for 15 min at 20°C in 0.0625 mM Tris hydrochloride (pH 6.8)–2% (wt/vol) SDS–10% (vol/vol) glycerol–0.001% (wt/vol) bromophenol blue–5% (vol/vol) β-mercaptoethanol according to the method of Laemmli (31) except that the samples were not boiled before electrophoresis. Gels were stained for covalently bound heme-containing proteins with 3,3′,5,5′-tetramethylbenzidine according to the method of Thomas et al. (47).

Spectrophotometry. Cytochrome studies were carried out by using an Aminco DW-2 UV/V is spectrophotometer. Dithionite-reduced spectra of cells, grown in the chemostat under methylamine limitation, were recorded at 77 K in the dual-wavelength mode, with the reference wavelength set at 578 nm.

Analysis of products. Anaerobic cultures were incubated in 30-ml flasks almost completely filled with minimal medium and sealed with a rubber septum. Samples of 25 μl of gas were withdrawn through the septum and analyzed for N_2O in a gas chromatograph (model 5370 A; Hewlett Packard Co., Palo Alto, Calif.) with two Porapak Q columns (6 ft [ca. 1.8 m]; outer diameter, 0.125 inch [ca. 3.2 mm]; 80/100 mesh; Chrompak Nederland BV, Middelburg, The Netherlands) as described by Boogerd et al. (9). The elution behavior of N_2O was determined by a calibrated gas mixture containing 2% N_2O in helium (Aga Gas BV, Amsterdam, The Netherlands). Analysis of nitrite in these cultures was carried out with test strips from Nitur-Test (Boehringer GmbH).

RESULTS

Cloning and analysis of the gene for cytochrome c_{550}. On the basis of the amino acid sequence of cytochrome c_{550} of *P. denitrificans*, two mixed oligonucleotides were synthesized. The first was deduced from amino acid residues 89 through 95, and the second was deduced from residues 100 through 106. These probes were then used to isolate clones containing the gene encoding cytochrome c_{550} from a genomic library constructed in the pEX2 vector. Two positive clones, designated pRS30 and pRS59, were selected from 60,000 colonies, using both probes in parallel experiments. A more precise location of the gene encoding cytochrome c_{550} and physical maps of the plasmids were determined by restriction enzyme analysis and by Southern hybridization using the same probes. Positively reacting DNA fragments were then purified by electrophoresis and subcloned into M13 vectors to determine the nucleotide sequence. A map of the approximately 4.5-kilobase-pair (kb) *P. denitrificans Sau*3A restriction fragment of pRS59, containing the complete structural gene for cytochrome c_{550}, is shown in Fig. 1 along with the sequencing strategy. The resulting nucleotide and deduced amino acid sequences are presented in Fig. 2. The amino acid sequence is in close agreement with that of purified cytochrome c_{550} as published by Ambler et al. (2). The nucleotide sequence, however, revealed the presence of seven additional amino acid residues at the C terminus of the mature protein. This raises the total number of amino acid residues to 135. This number as well as the amino acid composition are in agreement with the findings of Scholes et al. (39).

The sequence also revealed the presence of a typical ribosome-binding site 8 base pairs (bp) upstream of the start

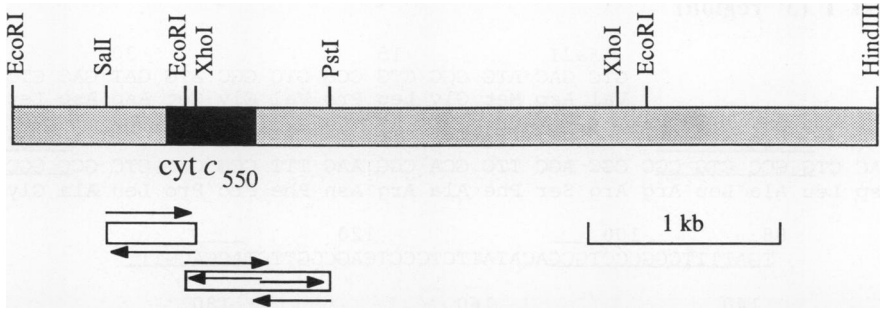

FIG. 1. Physical map and sequencing strategy of a *P. denitrificans* DNA fragment containing the cytochrome (cyt) c_{550}-coding region. Restriction sites for *Eco*RI and *Hin*dIII at both sides of the fragment are located on the pEX2 vector; the relevant restriction sites of the *P. denitrificans* DNA are shown in between. The direction and extent of the nucleotide sequence are indicated by the direction and length of the arrows. Restriction fragments (▭) were ligated in the linker region of M13mp18 or -mp19. In these clones, the polymerase reaction was primed from M13 by using the universal primer (arrows outside the rectangles) or from internally annealing 17-mer synthetic primers (arrows inside the rectangles).

codon at the 5′ end of the sequenced gene, the Shine-Dalgarno sequence GAGGA (41; underlined in Fig. 2). The deduced amino acid sequence showed 19 additional amino acid residues compared with the sequence of the purified protein. These additional amino acid residues form a typical signal peptide for periplasmic proteins in gram-negative bacteria (56). A positively charged residue (Lys) is followed by a central hydrophobic core, required for initiation of transport of the native cytochrome across the inner membrane. A helix-breaking residue (Pro) is found four residues before the peptidase cleavage site, which is between the Ala residue at the carboxyl terminus and the Gln residue at the N terminus of the mature protein. A comparable organization of amino acid residues has been found for all signal peptides of *P. denitrificans* examined so far (24, 29, 36, 45). The relative molecular mass of the deduced precursor protein is 16,379 Da. As a consequence of processing of this precursor protein, involving the attachment of the heme and the cleavage of the signal peptide, a relative molecular mass of 15,023 Da can be calculated for the mature cytochrome c_{550}.

The high G+C content of the gene (63.5%) is similar to the G+C content of other *P. denitrificans* genes (24, 29, 36) as well as that of the total *P. denitrificans* genome (34). A strong preference (90%) for a G or a C at the third position in the codons was observed. This phenomenon of avoiding an A or T ending in triplets is found in all *P. denitrificans* genes analyzed thus far and seems to be the consequence of the high G+C content (genes in the bc_1 operon [29], genes for the aa_3 oxidase [36, 45], and genes involved in methanol oxidation [24; manuscript in preparation]).

In addition to the apparent similarity between the amino acid sequences of cytochrome c_{550} of *P. denitrificans* and cytochrome c_2 of *R. capsulatus* (53%) (49), both the signal sequences and the nucleotide sequences of the genes in these related bacteria are strikingly similar (homologies of, respectively, 70 and 64%) (17). There is no similarity between the nucleotide sequences directly up- and downstream of the genes. More upstream, however, *P. denitrificans* contains a DNA region from bp 1 through 90, which is largely similar to a DNA region of *R. capsulatus* (bp 72 through 164) (17). This similarity ends directly after the stop codon TGA in both sequences, and more careful examination of these regions revealed no other stop codons. These findings suggested that these sequences are part of open reading frames, especially since the codon usage in these putative coding regions shows a strong bias for G or C at the third position of the triplets (90%), a typical feature of genes in these organisms (see also orf [open reading frame] 1 in Fig. 2). The 3′ part of the open reading frame of *P. denitrificans* showed no similarity to the 3′-terminal regions of other *P. denitrificans* genes characterized thus far (genes in the bc_1 operon [29], genes for the aa_3 oxidase [36, 45], and genes involved in methanol oxidation [24; manuscript in preparation]). Additional sequencing must be done to elucidate the nucleotide sequence of the remaining 5′ terminus of this gene.

Downstream of the stop codon at the 3′ end of the c_{550} gene, a long inverted repeat (nucleotides 734 through 766) followed by a uracyl-rich area was found. After transcription, this sequence might form a stem-loop structure in the mRNA with a stem of 13 bp, for which a free energy value of −35.6 kcal (ca. −149 J) was calculated and which could act as a typical rho-independent terminator. Further downstream, starting at position 874, part of a third structural gene was detected. This gene has been found to be largely similar to the gene coding for subunit 1 of cytochrome aa_3 oxidase, the COI gene (36; M. Raitio and M. Saraste, personal communication). The deduced amino acid sequence of the part of this gene, tentatively called iso-COI, showed an overall homology of 66% with the amino acid sequence, deduced from the corresponding part of the COI gene. When a particular nonhomologous region involving the first 15 amino acid residues encoded by the iso-COI gene and the first 13 amino acid residues encoded by the COI gene were not taken into account, the similarity of the remaining N-terminal regions increased to 88%.

It is generally assumed that *P. denitrificans* promoter structures do not resemble the consensus promoter of *E. coli* (25). It is often suggested that promoters in *P. denitrificans* might consist of G+C-rich elements (24, 29, 45), which are also found in the promoter regions of genes isolated from closely related bacteria, such as *R. sphaeroides* (21) and *Rhodopseudomonas blastica* (50). Therefore, it seemed reasonable to carry out similarity studies between the upstream region of the c_{550} gene and the genes in the bc_1 operon of *P. denitrificans*, since the soluble cytochrome as well as the bc_1 complex might be formed constitutively, and therefore these regions might be organized similarly. The transcriptional start of the bc_1 operon is at position 173 of the published sequence (29). Starting at position 103, which is 70 nucleotides upstream of this transcriptional start and 145 nucleotides separated from the gene, a DNA region of 20 nucleotides was found which showed striking similarity to a DNA region upstream of the c_{550} gene, at about the same distance of the gene (Fig. 3). About 22 nucleotides further down-

Orf 1 (3' region)

```
                    SalI                15                  30
                GTC GAC ATG GGC CTG CCG GTC GGC ATG GAT GAC CTG
                Val Asp Met Gly Leu Pro Val Gly Met Asp Asp Leu

      45                        60                  75          87
GAC CTG GCC CTG CGC CGC AGC TTC GCA CGG AAC TTT CCG CCG CTC GCC GGC
Asp Leu Ala Leu Arg Arg Ser Phe Ala Arg Asn Phe Pro Pro Leu Ala Gly

     88        100                 120
     TGATTTTCGGCCCTGCGACATATTCTCCCTCAGCCGTTCTACGACGTTT

     140               160                 180
     TTCCTGTATTCTGCCCGCTTGGCACATGATAGCCCTGTCAATCGGGGAA

                200                 220          234
     GCGGAGAGGTCCGTTGCCCGGACATGAGCGATACCCAAGAGGAAACGCG
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Cytochrome *c* 550

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    240                 255                 270                 285
ATG AAG ATC AGC ATC TAT GCC ACT CTC GCC GCC ATC ACC CTC GCC CTG CCC
Met Lys Ile Ser Ile Tyr Ala Thr Leu Ala Ala Ile Thr Leu Ala Leu Pro

                300                 315                 330
GCT GCG GCC CAG GAT GGC GAC GCC GCC AAA GGC GAG AAA GAA TTC AAC AAG
Ala Ala Ala*Gln Asp Gly Asp Ala Ala Lys Gly Glu Lys Glu Phe Asn Lys

        345                 360                 375
TGC AAG GCT TGC CAC ATG ATC CAG GCG CCG GAC GGC ACC GAC ATC ATC AAG
Cys Lys Ala Cys His Met Ile Gln Ala Pro Asp Gly Thr Asp Ile Ile Lys

390                 405                 420                 435
GGC GGC AAG ACC GGG CCC AAC CTT TAC GGC GTC GTC GGC CGC AAG ATC GCC
Ser Glu Glu Gly Phe Lys Tyr Gly Glu Gly Ile Leu Gly Gly Lys Thr Gly

            450                 465          XhoI   480
TCG GAG GAG GGC TTC AAA TAC GGC GAA GGC ATC CTC GAG GTC GCC GAA AAG
Pro Asn Leu Tyr Gly Val Val Gly Arg Lys Ile Ala Glu Val Ala Glu Lys

    495                 510                 525                 540
AAC CCC GAC CTG ACC TGG ACC GAG GCC GAC CTG ATC GAA TAC GTC ACC GAC
Asn Pro Asp Leu Thr Trp Thr Glu Ala Asp Leu Ile Glu Tyr Val Thr Asp

                555                 570                 585
CCC AAG CCC TGG CTG GTC AAG ATG ACC GAC GAC AAG GGC GCC AAG ACC AAG
Pro Lys Pro Trp Leu Val Lys Met Thr Asp Asp Lys Gly Ala Lys Thr Lys

        600                 615                 630
ATG ACC TTC AAG ATG GGC AAG AAC CAG GCC GAC GTG GTG GCC TTC CTG GCC
Met Thr Phe Lys Met Gly Lys Asn Gln Ala Asp Val Val Ala Phe Leu Ala
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FIG. 2. Nucleotide sequence of the 3' region of orf 1, the cytochrome c_{550} gene, and the 5' region of the iso-COI gene (GenBank accession no. M27304). The 1,047 nucleotides from pRS59 (Fig. 1) between the *SalI* and *PstI* restriction sites are shown. Predicted amino acid sequences are given for the C-terminal part of the putative gene product of orf 1 (29 residues), cytochrome c_{550} and its signal sequence (135 and 19 residues, respectively), and the N-terminal part of iso-COI gene product (58 residues). The signal sequence of cytochrome c_{550} is in italics, the putative signal sequence cleavage site is indicated by an asterisk, putative Shine-Dalgarno sequences are underlined, and the inverted repeat is indicated by double underlining. Sequences similar to those found in the promoter region of the bc_1 operon (Fig. 3) are between lines.

stream of these homologous sequences and 18 nucleotides from the transcriptional start, a second region of homology was found in both sequences. Furthermore, the area around the transcriptional start of the bc_1 operon showed similarity to a sequence in the upstream region of the c_{550} gene at the same position. Part of the first region of similarity is repeated in the promoter region of the cytochrome *c* gene at position 42. The latter sequence, GGCCCTGCG, might act as a region involved in regulation of gene expression. A comparable organization of homologous sequences was found in the upstream region of the iso-COI gene except that in this case the first box of homology was restricted to the sequence GCCCCTGCG (Fig. 3). No comparable sets of these homologous sequences were found in the upstream regions of other *P. denitrificans* genes such as the gene encoding methanol dehydrogenase (24) and the genes encoding the subunits of aa_3 (36, 45).

Construction of the suicide vector pGRPd1. The strategy for introduction of a specific mutation in the chromosomal gene encoding cytochrome c_{550} of *P. denitrificans* is based on the exchange of DNA via homologous recombination in vivo of the wild-type gene and a gene that has been inactivated by an insertion mutation in vitro. To introduce such an in vitro-altered sequence into *P. denitrificans* as well as to select proper recombinants, a new vector was constructed. A scheme of the construction of this plasmid is presented in Fig. 4. Starting material was the cloning vector pBR322, containing the origin of replication derived from pColE1.

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645                 660                 675                 690
CAG AAC TCG CCC GAT GCG GGC GGC GAC GGC GAG GCT GCG GCC GAG GGC GAA
Gln Asn Ser Pro Asp Ala Gly Gly Asp Gly Glu Ala Ala Ala Glu Gly Glu

   699
TCG AAC
Ser Asn

 700                 720                 740
  TGATCCCGACGCCTCCGAGATGTCTCGACGGCCCCGCCCCTGCGTGCGACAAAAGCGC

  760                 780                 800
  GCAGGGGCGCGACTTTTAGTCCTTTCTTCGCATTGCGAGGGGTAGGATTGTGGTCTAG

    820                 840                 860          873
  AAACAGGCGAGTCCGTCGGCCTTTGCGCCGGCCAGCCGCATGTCTAGGGAGTCCACGC
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iso-COI (5' region)

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            885                 900                 915
ATG GCA GAC GCA GCC GTT CAC GGC CAC GGT GAC CAT CAT GAC ACC CGC GGG
Met Ala Asp Ala Ala Val His Gly His Gly Asp His His Asp Thr Arg Gly

   930                 945                 960                 975
TTC TTC ACC CGC TGG TTC ATG TCA ACA AAC CAC AAG GAT ATC GGT ATC CTT
Phe Phe Thr Arg Trp Phe Met Ser Thr Asn His Lys Asp Ile Gly Ile Leu

                990                1005                1020
TAC CTG TTC ACG GCC GGC ATC GTC GGC CTG ATC TCG GTA TGC TTC ACC GTC
Tyr Leu Phe Thr Ala Gly Ile Val Gly Leu Ile Ser Val Cys Phe Thr Val

      1035          PstI
TAT ATG CGG ATG GAA CTG CAG
Tyr Met Arg Met Glu Leu Gln
```

FIG. 2—*Continued*

The plasmid was restricted with *Bam*HI, *Sal*I, and a 0.7-kb *Bam*HI-*Sal*I fragment of pRTP1, which contained the origin for conjugative transfer (*oriT*) from the broad-host-range plasmid RK2. This *oriT* is necessary for the transfer of the pBR322 derivatives via conjugation from *E. coli* S17-1 into the recipient *P. denitrificans*. Since the plasmid-encoded ampicillin resistance will not be expressed in *P. denitrificans* (19), another selection marker had to be introduced into the constructed vector. For this purpose, the vector was restricted with *Bam*HI and *Hin*dIII, and a gene coding for streptomycin resistance, part of a 2.3-kb *Bam*HI-*Hin*dIII fragment of transposon Tn*1831*, was introduced into the plasmid. The resulting vector pGRPd1 had unique *Eco*RI, *Cla*I, *Hin*dIII, *Sph*I, *Bam*HI, *Sma*I, *Sal*I, and *Bal*I restriction sites. Derivatives of pGRPd1, containing in vitro-mutated DNA sequences of *P. denitrificans*, cannot be maintained in this bacterium in an episomal form because of the lack of a suitable origin for replication in *P. denitrificans*. Hence, when exconjugants are grown in the presence of streptomycin, only those bacteria will be selected in which the plasmid has been integrated into the chromosome via homologous recombination.

In vitro insertional mutagenesis of the gene coding for cytochrome c_{550}. Plasmid pRS59 contained the complete gene of cytochrome c_{550} and adjacent DNA fragments large enough to allow recombination events at both sides of the gene. The c_{550} gene was mutated in vitro by partial digestion of the sequence at the *Xho*I site in the middle of the gene and subsequent ligation of a 1.5-kb *Sal*I fragment of pUC4K, containing the kanamycin resistance gene from transposon Tn*5*. This kanamycin marker was also used for selection of the inactivated gene during the successive steps of conjugation and recombination. The resulting vector pRS59K was partially digested with *Eco*RI, and the 8.2-kb *Eco*RI fragment, containing the interrupted gene, was isolated and ligated into the *Eco*RI site of pGRPd1. This construct, pRcTd1, was then used for transformation of the mobilizing strain, *E. coli* S17-1.

Gene	Position		D		D		D	Start
		-55		-20		+1		
		|		|		|		
bc_1 operon[a]	bp103	CGGC GATTTGCGGCGCTGCC	22N	ACGGGGATTT	11N	CCGCGCGGC	73N	GTG
		**** ***** **** ****		*** * ***		**** ***		
c_{550} gene[b]	bp 84	CGGCTGATTTTCGGCCCTGCG	23N	ACGACGTTTT	13N	CCGCTTGGC	75N	ATG
		** *******		**** ***		*** * **		
iso-COI gene[b]	bp734	CGCCCCTGCG	22N	GCGACTTTTA	9N	TCGCATTGC	80N	ATG

FIG. 3. Similarity between the nucleotide sequences in the promoter region of the bc_1 operon and the upstream regions of the genes encoding cytochrome c_{550} and iso-COI. Identical nucleotides (*) are indicated. Base pair numbers, indicating the first nucleotide of each set of homologous sequences, correspond with the position numbers in the published sequences ([a]Kurowski and Ludwig [29]; [b]this study). Distances (D) between regions of similarity are given in number of nucleotides (N). GTG and ATG codons are the translational starts of the genes. The transcriptional start of the bc_1 operon is indicated by nucleotide +1. The locations of the regions of similarity with respect to this transcriptional start are indicated by −55 and −20.

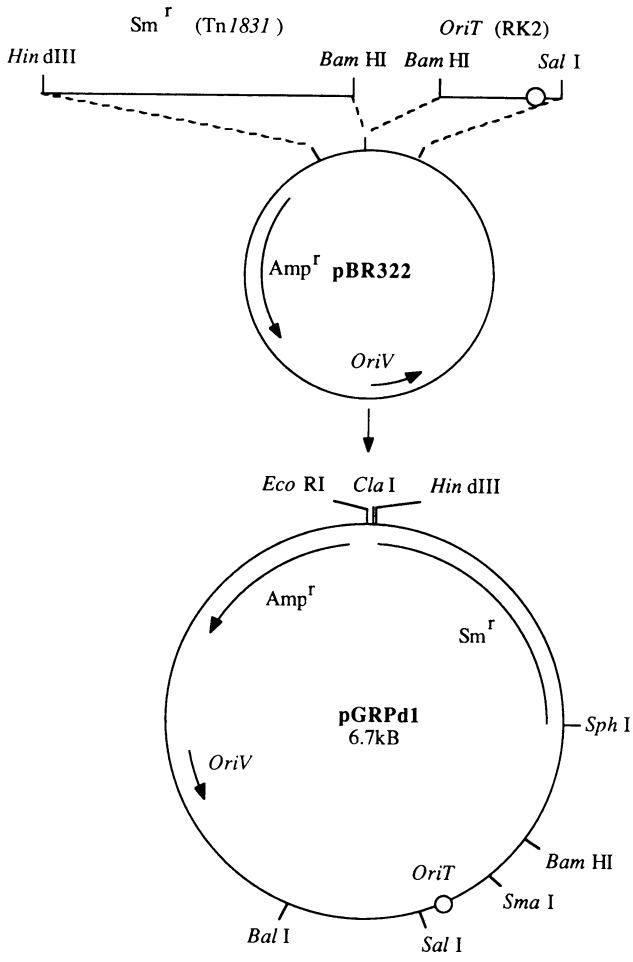

FIG. 4. Construction of the suicide vector pGRPd1. Starting material was the cloning vector pBR322, which was supplemented with the origin for conjugative transfer of RK2 (part of a 0.7-kb *Bam*HI-*Sal*I fragment obtained from plasmid pRTP1) and with a gene encoding streptomycin resistance (part of a 2.3-kb *Hin*dIII-*Bam*HI fragment of transposon Tn*1831*). Only unique restriction sites are indicated.

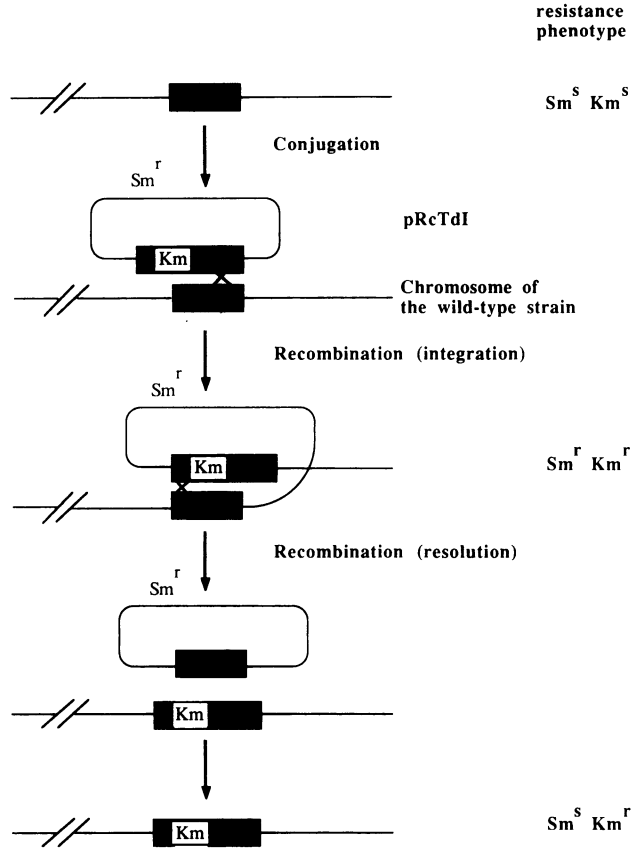

FIG. 5. Scheme of the expected mechanism for replacement of a chromosomal segment by an in vitro-mutated DNA segment. Relevant resistance phenotypes of the selected strains are shown at the right.

In vivo replacement of the gene coding for cytochrome c_{550}. *E. coli* S17-1 harboring pRcTd1 was used as the donor strain in the biparental mating with *P. denitrificans*. A scheme of the possible recombination events after the transfer, resulting in the exchange of the wild-type cytochrome c_{550} gene for the insertional inactivated gene, is shown in Fig. 5. The overall process may be divided into two main stages. First, is integration of the whole plasmid due to a single crossover event in the region of flanking homology at one side of the kanamycin resistance gene. Isolation of these so-called integrants was carried out by selection of both kanamycin and streptomycin resistance in addition to the rifampin resistance already present in the wild type. A combined conjugation-recombination frequency of 10^{-4} was found, and this value was compared with known conjugation frequencies of some broad-host-range vectors under the same conjugation conditions (19). From these results, a frequency of 10^{-1} to 10^{-2} could be deduced for the recombination event alone. The integration of pRcTd1 resulted in the presence of two copies of the cytochrome c_{550} gene in the chromosome, a mutated one and a wild type one. This situation still enables the bacterium to synthesize an active gene product. To achieve the complete process of gene replacement, a second recombination is necessary, now at the other side of the kanamycin resistance gene, resolving the plasmid with the wild-type gene and leaving the mutated gene in the chromosome. This procedure results in a kanamycin-resistant, streptomycin-sensitive phenotype. To isolate these mutants, integrants were plated on kanamycin plates to allow for the desired recombination. The resulting colonies were plated on kanamycin plates again to separate cells of different phenotypes. About 500 of the resulting colonies were tested for streptomycin sensitivity, but none of them possessed the desired phenotype. This seemed rather peculiar, since both genes are in a tandem configuration in the chromosome of the integrant, and therefore a frequency of more than 10^{-1} to 10^{-2} was expected for this second crossover. Therefore, another strategy was used to avoid the intermediate stage of the isolation of integrants. Now exconjugants were selected in the presence of kanamycin alone, allowing both single and double crossovers directly after the transfer of pRcTd1. The resulting colonies were then tested for resistance to streptomycin. Forty percent of the tested colonies had the desired kanamycin-resistant, streptomycin-sensitive phenotype, which should be the result of a complete gene replacement. Apparently, when the homologous sequences are situated in the proper orientation directly after transfer of the construct, both the single and the double crossovers occur at about equal frequencies.

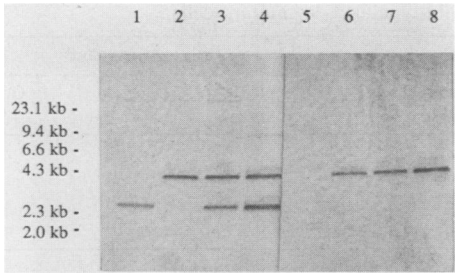

FIG. 6. Blot of *Eco*RI-digested chromosomal DNA of the *P. denitrificans* wild-type strain (lanes 1 and 5), the cytochrome c_{550} insertion mutant strain (lanes 2 and 6), and the pRcTD1 integrant strain (lanes 3 and 7) and of the *Eco*RI-digested vectors pRS59 (lower band) and pRS59K (upper band, lanes 4 and 8). DNA fragments were visualized with either the labeled 2.3-kb *Eco*RI fragment of pRS59 (lanes 1 to 4) or a labeled 1.5-kb fragment containing the gene encoding kanamycin resistance (lanes 5 to 8). Fragment sizes are indicated at the left.

Southern analysis of the c_{550} locus. To confirm the presence of the in vitro-mutated cytochrome c_{550} gene at the proper location in the genome of *P. denitrificans*, chromosomal DNA of the parental, integrant, and mutant strains was isolated and restricted with *Eco*RI for Southern analysis. This Southern blot was used for hybridization with the internal 2.4-kb *Eco*RI fragment of clone pRS59, containing the gene for the cytochrome (Fig. 6). The results showed that the *Eco*RI probe hybridized with a 2.4-kb DNA fragment of the chromosomal DNA from the wild-type strain and, as expected, with a 3.9-kb DNA fragment of the mutant strain. In the integrant strain, both the wild-type and the inactivated gene were detected. To demonstrate the presence of the gene encoding kanamycin resistance, the 1.5-kb *Sal*I fragment of pUC4K was used as a probe, and hybridization was observed with a 3.9-kb DNA fragment of chromosomal DNA from both the integrant and mutant strains but not from the wild-type strain. These results clearly indicated that the wild-type cytochrome c_{550} gene was replaced by the in vitro-mutated gene. In addition, it could be concluded that the wild-type *P. denitrificans* strain has only one copy of this gene.

Cytochrome analysis of the c_{550} mutant. To investigate whether the insertional inactivation of the gene had resulted in the absence of soluble cytochrome c_{550}, periplasmic proteins were isolated from methylamine-limited cultures of both the wild-type and the mutant strain and analyzed by SDS-polyacrylamide gel electrophoresis and subsequent heme staining (Fig. 7). *P. denitrificans* wild-type cells contained soluble cytochromes *c* with relative molecular masses of 14, 22, and 30 kDa. The mutant strain had the same set of cytochromes except one with a relative molecular mass of 14 kDa.

Freshly prepared suspensions of methylamine-grown cells from both the wild-type and the c_{550} mutant strain were used for spectral analysis at low temperature. The spectrum of the wild type is shown in Fig. 8A; the spectrum of the mutant, fitted with that of the wild-type strain by using a vector method, is shown in Fig. 8B (K. Krab, personal communication). The cytochrome spectrum was changed as a consequence of the mutation. The shape of the residual spectrum (Fig. 8C) clearly indicated the difference in cytochrome composition between the two strains. Cytochrome c_{550}, having its absorption maximum at 546.5 nm at a temperature of 77 K, was absent, and the amount of cytochrome aa_3 oxidase was less in the mutant strain than in the wild-type

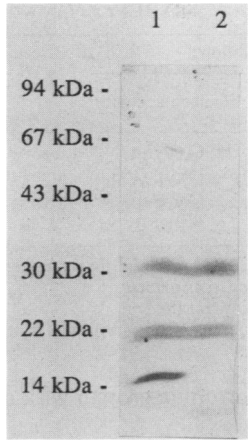

FIG. 7. SDS-polyacrylamide gel electrophoresis of periplasmic proteins of the *P. denitrificans* wild-type strain (lane 1) and the c_{550} mutant strain (lane 2). Cells were grown in methylamine-limited chemostat cultures. Covalently bound heme was stained with tetramethylbenzidine. The positions of the marker proteins are indicated by their relative molecular masses.

strain. Furthermore, in the spectrum of the c_{550} mutant strain, the A_{552} and A_{558} were raised relatively, indicating an increased amount of one or more cytochromes *b* and *c* in the mutant strain.

Growth characteristics of the c_{550} mutant. Growth characteristics of *P. denitrificans* wild-type and c_{550} mutant strains were determined in batch cultures. Cells of both strains were cultured in BHI broth to the late exponential phase of growth and then diluted with mineral salts medium with either succinate, choline, or methylamine as the carbon and energy source to an optical density of 0.1 at 660 nm and further incubated (Table 2). During aerobic growth with all tested carbon sources, a fourfold increased lag phase was observed for the mutant strain as compared with the parental strain. In addition, the growth yield of the mutant was about 14% lower after growth at these conditions. The maximum specific growth rate, however, was not changed during growth in the presence of succinate or choline but decreased about

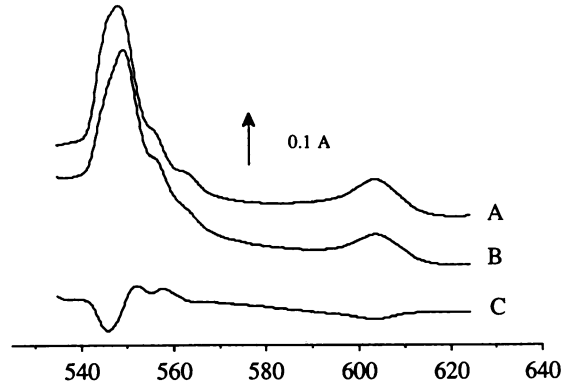

FIG. 8. Dithionite-reduced absorption spectra at 77 K of *P. denitrificans* wild-type cells (A) and c_{550} mutant cells (B). Cells were grown in methylamine-limited chemostat cultures. The spectrum of the mutant strain was fitted with that of the wild-type strain. The residual spectrum (C) is as shown.

TABLE 2. Growth characteristics of *P. denitrificans* wild-type and c_{550} mutant cells

Growth condition[a]		Wild type			c_{550} mutant[b]		
Carbon source	Electron acceptor	Lag phase (min)	μ_{max}[c] (h^{-1})	Growth yield[d] (A_{660} units)	Lag phase (min)	μ_{max} (h^{-1})	Growth yield (A_{660} units)
Succinate	Oxygen	15	0.350	1.44	50	0.350	1.24
Methylamine	Oxygen	90	0.115	1.25	390	0.066	1.05
Choline	Oxygen	15	0.170	1.51	60	0.170	1.29
Succinate	Nitrate	60	0.210	1.29	60	0.210	1.29

[a] Cells were grown in BHI broth and then transferred in mineral media with the carbon sources and electron acceptors listed.
[b] Mean values of three experiments.
[c] Maximum specific growth rate.
[d] Units represent optical density per centimeter at 660 nm.

40% during methylotrophic growth with methylamine as the carbon source.

During anaerobic growth in the presence of succinate and nitrate, no differences between the two strains were observed with respect to length of the lag phase, maximum specific growth rate, and growth yield. In addition, there was no accumulation of nitrite or nitrous oxide in either of the cultures, indicating that the absence of cytochrome c_{550} does not affect the electron pathways to the nitrite and nitrous oxide reductases.

DISCUSSION

The results presented above clearly demonstrate that the structural gene for cytochrome c_{550} of *P. denitrificans* was isolated. On the basis of the deduced amino acid sequence, it was concluded that the amino acid sequences presented in previous papers (2, 49) contained some mistakes. The deduced amino acid composition, however, agreed with the one published by Scholes et al. (39). The nucleotide sequence also revealed the presence of a typical signal sequence (56). About 170 nucleotides downstream of the gene, the beginning of a second open reading frame, which showed large similarity to the COI gene of *P. denitrificans* aa_3 oxidase, was found. This open reading frame was tentatively called the iso-COI gene (36; Raitio and Saraste, personal communication).

No sequences resembling the *E. coli* consensus promoter were found in the upstream regions of the c_{550} gene, the iso-COI gene, or any other *P. denitrificans* gene (24, 29, 36, 45). Upstream of the c_{550} gene as well as the iso-COI gene, DNA regions were found with a distinct organization of homologous sequences, similar to the promoter region of the bc_1 operon of *P. denitrificans*. These regions consisted of a conserved sequence with a high G+C content, a conserved sequence with a rather high A+T content, and a putative initiation site. The three boxes are separated by 22 and 11 nucleotides, respectively. Surprisingly, all three initiation sites are at about the same distance from the beginning of the genes. The high G+C content of the first box might be a reflection of the overall high G+C content of the total *P. denitrificans* genome. Similar G+C-rich areas have been found in the promoter regions of genes of the phylogenetically related species *R. sphaeroides* and *R. capsulatus* (results not shown). These sites might be involved in the recognition or binding of RNA polymerases specific for this group of procaryotic organisms. The second box contains a stretch of A+T residues. This relatively weakly bound duplex sight might easily be involved in the formation of an open complex during the initiation of transcription.

Whether the gene product of this iso-COI gene forms part of the cytochrome aa_3 complex has not yet been proven. However, one could argue that the location of this gene close to the cytochrome c_{550} gene and the described upstream similarities indicate that these genes are expressed coordinately and suggest that their gene products are synthesized at the same specific growth conditions. To obtain more information on the regulation of transcription of the gene encoding cytochrome c_{550}, the putative promoter will be further characterized by performing RNA and transcriptional fusion experiments.

The growth yield of the c_{550} mutant strain during aerobic heterotrophic growth is about 14% lower than that of the wild-type strain, indicating a less efficient respiration-driven energy conservation during electron transport to the terminal oxidases. This difference in growth yield could be the result of the difference in charge separation during electron transport to oxygen. Bearing this in mind, one could predict a maximum difference in growth yield for growth on succinate, as discussed by Boogerd et al. (10). For the *P. denitrificans* wild-type strain at growth yields of between 50 and 30 g/mol of succinate, one could expect a maximum decrease of this yield for the mutant strain of 11 to 17%, assuming that in the wild-type strain the net charge separation is 9 during electron transport from NADH to oxygen exclusively via the aa_3 oxidase and that in the mutant strain it is 7, exclusively via an alternative oxidase. Thus, the observed decrease of the growth yield of about 14% under aerobic heterotrophic growth conditions strongly indicated that the absence of cytochrome c_{550} causes a shift of the electron flow to a less proton-pumping route, probably via an alternative oxidase. On the other hand, in the wild-type strain, a substantial part of the electron flow apparently takes the cytochrome aa_3 route. This is consistent with the situation in mitochondria and in *R. capsulatus* and *R. sphaeroides*. However, despite the altered electron flow, the mutant strain had a maximum specific growth rate during aerobic heterotrophic growth comparable with that of the wild-type strain, which suggested that the electron transport chain has been adapted in a proper way to avoid a rate limitation in the electron transport. Therefore, it can be concluded that the alternative electron route in this respiratory chain is mainly expressed when an increased electron flow is required to obtain an optimum internal energy charge. The adaptation of the respiratory chain to a more intensive use of the alternative oxidase would explain the observation that the mutant strain had a lag phase about four times longer than that of the wild-type strain. Probably, this lag phase is required to synthesize all of the necessary components for the alternative route in proper amounts.

In addition to the latter observation, these results explain why the desired mutant strain could not be selected by starting with cells having the plasmid with the mutated gene

integrated in the chromosome. When a second recombination event is allowed, the resultant colonies consist of cells still having the wild-type gene and of cells in which the wild-type gene is completely replaced. As a consequence of the long adaptation time, however, the latter cells are just a minor fraction of the whole population of cells, despite the relatively high recombination frequency. Therefore, direct selection appeared to be the most successful procedure for the isolation of double recombinants, avoiding the possible disadvantageous influence of altered growth characteristics that exists when the integrant is used as the starting material for selection.

During growth in the presence of methylamine, the maximum specific growth rate of the c_{550} mutant strain is about 40% lower than that of the wild-type strain. The absence of this cytochrome apparently causes a rate limitation in the oxidation of methylamine. Since a normal maximum specific growth rate was observed during growth in the presence of choline, a substrate which gives rise to the production of formaldehyde during oxidation in the periplasm, this observed rate limitation cannot be a consequence of a decreased capacity to oxidize formaldehyde. Apparently, the absence of cytochrome c_{550} introduces a rate limitation in the electron transport chain from the methylamine dehydrogenase to the oxidases. A similar conclusion was recently reported by Davidson and Kumar (18). On the basis of in vitro experiments, it was suggested that electron transport during methylamine oxidation involves the blue copper protein amicyanin, cytochrome c_{551}, and cytochrome c_{550} successively. However, although a major electron transport route for the oxidation of methylamine in *P. denitrificans* includes cytochrome c_{550}, alternative pathways have to exist. The c_{550} mutant strain was still capable of growing in the presence of methylamine, albeit with a 40% decreased maximum specific growth rate.

In conclusion, electron transport during methylamine oxidation in *P. denitrificans* is rather flexible, involving periplasmic electron carriers, some of which are able to interact with each other and with the terminal oxidase(s). As a consequence, they can take over each other's functions, but not completely. This emphasizes that electron transport from methylamine to oxygen is well balanced and most efficient in the presence of soluble cytochrome c_{550}.

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