JOURNAL OF BACTERIOLOGY, Nov. 1991, p. 6962–6970
0021-9193/91/216962-09$02.00/0

Vol. 173, No. 21

A Method for Introduction of Unmarked Mutations in the Genome of *Paracoccus denitrificans*: Construction of Strains with Multiple Mutations in the Genes Encoding Periplasmic Cytochromes c_{550}, c_{551i}, and c_{553i}

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Received 18 June 1991/Accepted 5 September 1991

A new suicide vector, pRVS1, was constructed to facilitate the site-directed introduction of unmarked mutations in the chromosome of *Paracoccus denitrificans*. The vector was derived from suicide vector pGRPd1, which was equipped with the *lacZ* gene encoding β-galactosidase. The reporter gene was found to be a successful screening marker for the discrimination between plasmid integrant strains and mutant strains which had lost the plasmid after homologous recombination. Suicide vectors pGRPd1 and pRVS1 were used in gene replacement techniques for the construction of mutant strains with multiple mutations in the *cycA*, *moxG*, and *cycB* genes encoding the periplasmic cytochromes c_{550}, c_{551i}, and c_{553i}, respectively. Southern analyses of the DNA and protein analyses of the resultant single, double, and triple mutant strains confirmed the correctness of the mutations. The wild type and mutant strains were all able to grow on succinate and choline chloride. In addition, all strains grew on methylamine and displayed wild-type levels of methylamine dehydrogenase activities. *cycA* mutant strains, however, showed a decreased maximum specific growth rate on the methylamine substrate. The wild-type strain, *cycA* and *cycB* mutant strains, and the *cycA cycB* double mutant strain were able to grow on methanol and showed wild-type levels of methanol dehydrogenase activities. *moxG* mutant strains failed to grow on methanol and had low levels of methanol dehydrogenase activities. The maximum specific growth rate of the *cycA* mutant strain on methanol was comparable with that of the wild-type strain. The data indicate the involvement of the soluble cytochromes *c* in clearly defined electron transport routes.

Paracoccus denitrificans is a gram-negative soil bacterium, which is well adapted for aerobic and anaerobic growth on a variety of carbon sources. During aerobic heterotrophic growth, electrons can be passed from reducing compounds to oxygen via at least three different routes. One of these routes has typical mitochondrionlike characteristics, involving NADH dehydrogenase, the bc_1 complex, and the aa_3-type oxidase (1, 5, 6, 12, 19, 43, 44, 46). Electron transport from the bc_1 complex to the aa_3-type oxidase is mediated by the constitutively formed cytochrome c_{550}, which operates in the periplasm (32, 41). An alternative route between the latter two proton-translocating complexes is suggested to involve membrane-bound cytochrome c_{552} (4, 21). Besides the two routes to the aa_3-type oxidase, *P. denitrificans* has the disposal of a third aerobic electron transport route in which oxygen reduction is mediated by a quinol oxidase (10, 28).

For growth on methanol or methylamine, additional periplasmically located dehydrogenases and electron carriers are induced to enable the bacterium to use the C_1 compounds concerned as carbon and energy sources (2, 6, 15–18). Methylamine is oxidized by methylamine dehydrogenase, and electrons are transferred to their natural electron acceptor, amicyanin (16, 18, 42). This blue copper enzyme seems to be the branching point of two electron transport routes to the aa_3-type oxidase (41, 42), one of which is suggested to involve periplasmic cytochrome c_{550} (8, 41). The alternative route is currently unknown. During this growth condition, cytochromes c_{551i} and c_{553i} are also found in the periplasm (6). Not only electron transport routes during methylamine oxidation but also the nature and interactions of the electron carriers involved in methanol oxidation have not been completely elucidated. Methanol is oxidized by methanol dehydrogenase (MDH) (2, 14), and electrons are passed to their natural electron acceptor, cytochrome c_{551i} (23, 40). However, it is not known whether additional components are involved in the electron transport route(s) to the aa_3-type oxidase. In addition to cytochrome c_{551i}, cytochromes c_{550} and c_{553i} are found in the periplasm during growth on methanol; thus, the cytochrome composition is similar to that found during methylamine oxidation (6).

By using different approaches, elucidation of the nature of the different electron transport routes in *P. denitrificans* is in progress. One important strategy is the mutagenesis by marker insertion of the genes encoding components of the respiratory chain, followed by bioenergetical characterization of the resultant mutant strains (10, 13, 29, 30, 41, 42). However, elucidation of simultaneously operating electron transport routes is difficult. Simultaneous mutagenesis of the involved key components, resulting in a complete loss of the ability to grow under that specific growth condition, may offer important evidence in those cases. Up to now, the method for the construction of strains of *P. denitrificans* with multiple mutations was hampered by the limitation of usable resistance markers, of which only kanamycin and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
S17-1	Sm^r *pro* r$^-$ m$^+$ RP4-2 integrated (Tc::Mu)(Km::Tn*7*)	31
JM101	Δ(*lac pro*)*thi supE* F′ *traD36 proAB lacI*^{q}*Z* Δ*M15*	26
P. denitrificans		
Pd1235	Rif^r, enhanced conjugation frequencies, m$^-$	9
Pd2121	Pd1235, *cycA*::Km^r	41
Pd2131	Pd1235, Δ*cycA*	This study
Pd0321	Pd1235, *moxG*::Km^r	40
Pd0331	Pd1235, Δ*moxG*	40
Pd6121	Pd1235, *cycB*::Km^r	30
Pd9201	Pd2131, Δ*cycA moxG*::Km^r	This study
Pd9203	Pd9201, Δ*cycA* Δ*moxG*	This study
Pd9204	Pd2131, Δ*cycA cycB*::Km^r	This study
Pd9205	Pd0331, Δ*moxG cycB*::Km^r	This study
Pd9303	Pd9203, Δ*cycA* Δ*moxG cycB*::Km^r	This study
Plasmids		
pRTd2121	*cycA*::Km^r, pGRPd1 derivative	41
pRTd2131	Δ*cycA*, pRVS1 derivative	This study
pRTd0321	*moxG*::Km^r, pGRPd1 derivative	40
pRTd0331	Δ*moxG*, pRVS1 derivative	40
pRTd6121	*cycB*::Km^r, pGRPd1 derivative	30
pRS59	*cycA*, pEX2 derivative	41
pRS59B	*cycA*, pEX2 (*Sma*I::*Bam*HI), pRS59 derivative	This study
pCYA1	*cycA*, pUC19 derivative	This study
pCYA1D	Δ*cycA*, pUC19 derivative	This study
pNH33	*moxJ moxG moxI moxR*, pEX2 derivative	40
pWR20	*cycB*, pUC13 derivative	30
pMP190	mcsp, *lacZ*	34
pUC4K	Km^r (Tn*903*)	45
pUC4KIXX	Tn*5p*	3
pRSPL1	Tn*5p* mcsp, *lacZ*	This study
pRSPL2	pRSPL1 derivative, Δmcsp	This study
pGRPd1	*oriV* (ColE1) Amp^r *oriT* Sm^r (Tn*1831*)	41
pRVS1	*oriV* (ColE1) Amp^r *oriT* Sm^r (Tn*1831*) Tn*5p lacZ*	This study

[a] Sm, streptomycin; Km, kanamycin; Rif, rifampin; Tn*5p*, Km^r promoter of Tn*5*; mcsp, multiple-cloning-site polylinker.

streptomycin resistance genes were shown to be expressed properly (27, 35).

In this study, the construction of the new suicide vector pRVS1 is described to accommodate the problems mentioned above. The vector was used for the replacement in the chromosome of *P. denitrificans* of insertionally inactivated genes with genes bearing unmarked mutations. The main advantage of the method is demonstrated for the introduction of multiple mutations in the chromosomal genes encoding periplasmic cytochromes c_{550}, c_{551i}, and c_{553i} of *P. denitrificans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. *Escherichia coli* and *P. denitrificans* strains were grown in batches with brain heart infusion broth at 37 and 30°C, respectively. For determination of growth characteristics, *P. denitrificans* strains were grown at 30°C on plates with mineral salts medium with 100 mM methanol, 100 mM methylamine, 25 mM succinate, or 20 mM choline chloride as the carbon and energy source, supplemented with 0.02% yeast extract. For determination of maximum specific growth rates on methylamine, *P. denitrificans* strains were grown in mineral salts medium at 30°C in batches with 100 mM methylamine as the carbon and energy source, supplemented with 0.02% yeast extract. The mineral salts medium for growth on plates and for batch cultures was prepared by the method of Chang and Morris (7), with some minor modifications (41). The maximum specific growth rate of the c_{550} mutant on methanol was determined in the chemostat. Chemostat cultures were run at a controlled pH of 7.0, with 100 mM methylamine or 100 mM methanol as the carbon and energy source in a mineral medium essentially as described earlier (41). The strain was first grown at a dilution rate of 0.12 with methylamine as the carbon and energy source, after which the incoming medium of the culture was switched to a medium containing methanol as the only carbon and energy source. After incubation during six generation times, the dilution rate was increased to 0.20, after which the optical density at 660 nm was monitored. The maximum specific growth rate was deduced from the discrepancy between the established rate of washout and the adjusted dilution rate. 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.

DNA manipulations. General cloning techniques were carried out essentially as described by Maniatis et al. (25).

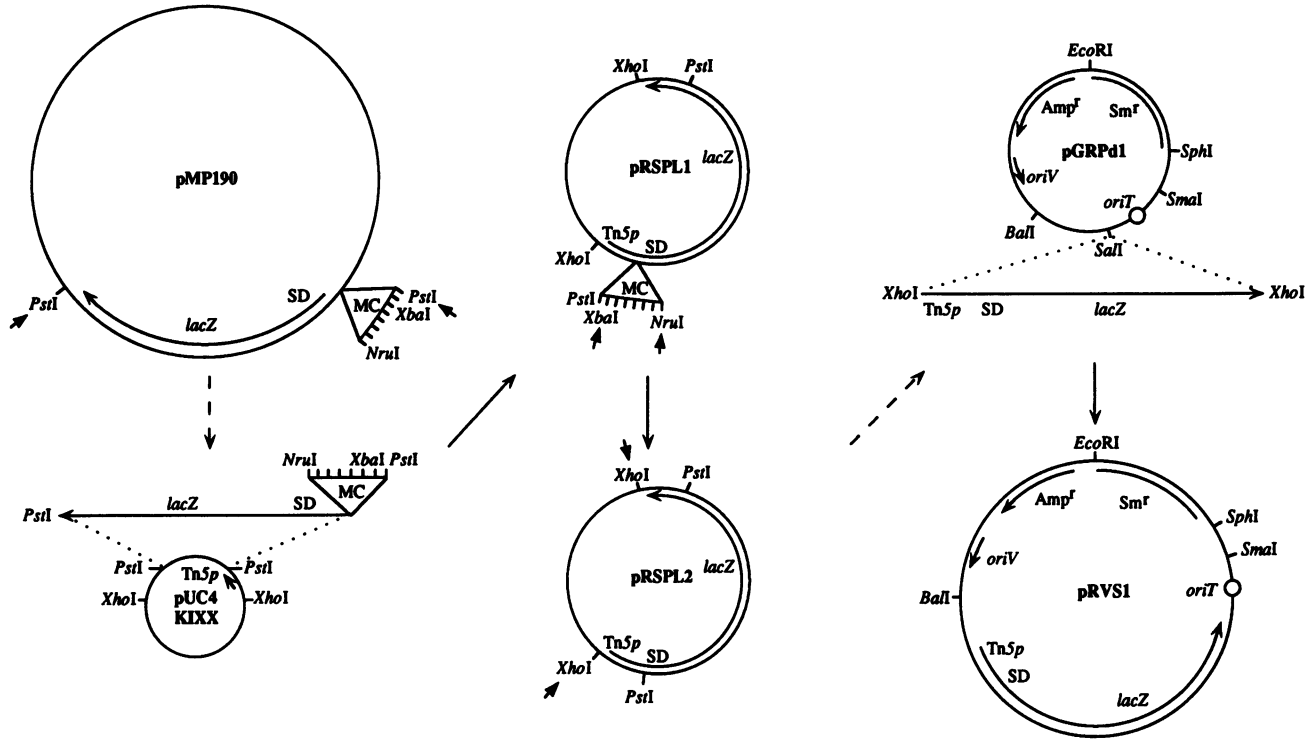

FIG. 1. Scheme of the construction of suicide vector pRVS1. Details of the construction are mentioned in Results. Relevant functional elements of participating plasmids and relevant restriction sites are indicated. Plasmid pMP190 was from Spaink et al. (34), pUC4KIXX was from Pharmacia, and plasmid pGRPd1 was from Van Spanning et al. (41). MC, multiple-cloning-site polylinker; SD, Shine-Dalgarno sequence; Tn*5p*, promoter of Tn*5* resistance genes.

Plasmid DNA was isolated from *E. coli* by the cleared-lysate method (38) and purified by using Qiagen. For rapid screening, plasmid DNA was isolated by the alkaline lysis method (25). Chromosomal DNA of *P. denitrificans* was isolated as described earlier (41). DNA restriction fragments were purified from agarose gels by using GeneClean (Bio 101, Inc., San Diego, Calif.). Chromosomal DNA (10 μg per lane) was loaded on 1% agarose gels, denatured, and transferred to GeneScreen Plus filters according to the method of Southern (33). Southern analysis of chromosomal restriction fragments was done by random-primed DNA labeling of cloned sequences with digoxigenin, and subsequent detection of hybrids was done by an enzyme immunoassay according to the protocol of the manufacturer (Boehringer GmbH, Mannheim, Germany). In vitro inactivation of isolated genes by insertion of a kanamycin resistance cartridge and in vivo exchange of these genes with the wild-type genes were done essentially as described previously (40–42).

Cytochrome analysis. For the isolation of whole-cell extracts, cells were suspended in a 10 mM potassium phosphate buffer (pH 7.0) to an optical density of 50.0 cm^{-1} at 660 nm and then broken in a French pressure cell (American Instrument Company, Silver Spring, Md.). Membranes were removed by centrifugation for 60 min at 100,000 × *g* and 4°C. The concentration of protein was determined by the method of Lowry et al. (24), with bovine serum albumin as the standard. Periplasmic proteins were incubated in a solubilization mixture as described earlier (40). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out on 14% slab gels (22). Gels were stained for covalently bound heme-containing proteins with 3,3′,5,5′-tetramethylbenzidine according to the method of Thomas et al. (37).

MDH and methylamine dehydrogenase activities. Methylamine-grown cells were harvested, washed with 10 mM Tris hydrochloride (pH 7.0), and resuspended in the same buffer to an optical density of 100.0 cm^{-1} at 660 nm. The assay mixture of 3 ml contained 0.3 ml of this cell suspension, 100 mM Tris hydrochloride (pH 9.0), 15 mM NH_4Cl, 1 mM KCN, 0.1 mM 2,6-dichlorophenol-indol-phenol, and 0.1 mM phenazine methosulfate and was kept anaerobically with argon. The reaction was started with the addition of 10 μl of 2.5 M methanol or 10 μl of 2.5 M methylamine. The A_{610} changes were recorded by using an Aminco DW-2 UV/Vis spectrophotometer with the reference wavelength set at 750 nm.

RESULTS

Construction and properties of the suicide vector pRVS1. The strategy for the construction of the suicide vector pRVS1 is presented in Fig. 1. The starting material was the suicide vector pGRPd1, the construction and properties of which were presented in an earlier paper (41). Additional elements were obtained from plasmid pMP190 (the complete *lacZ* gene) (34) and plasmid pUC4KIXX (the promoter region of the Tn*5* resistance genes) (3). The latter promoter element is one of the few known promoters which is active in *P. denitrificans* but bears no homology with its DNA.

The *lacZ* gene of pMP190 was isolated as a 4-kb *Pst*I restriction fragment. This fragment was used to replace the 1-kb *Pst*I fragment of pUC4KIXX just downstream from the

Tn*5* promoter. Plasmids with the *lacZ* gene in the proper orientation with regard to the Tn*5* promoter were designated pRSPL1. Plasmid pRSPL1 was restricted with *Xba*I and *Nru*I to delete part of the multiple-cloning-site polylinker linked to the *lacZ* gene of plasmid pMP190. The sticky ends were then filled in, after which the vector was ligated to yield plasmid pRSPL2. The *lacZ* gene with the Tn*5* promoter in front could be isolated as a 4.9-kb *Xho*I fragment, and this fragment was incorporated in the unique *Sal*I site of suicide vector pGRPd1. The orientation of the *lacZ* gene was determined by restriction enzyme analysis and those with the transcription in the same direction as that of the ampicillin resistance gene were designated pRVS1. Suicide vector pRVS1 is 11.6 kb and has unique cloning sites for *Eco*RI, *Bal*I, *Sma*I, and *Sph*I. The plasmid can be transferred to *P. denitrificans* by means of conjugation but cannot be maintained in an episomal form. Derivatives of pRVS1 were shown to be preserved in *P. denitrificans* as a result of integration into its chromosome after homologous recombination. The resulting plasmid integrant strains are selected in the presence of kanamycin and streptomycin. They were shown to synthesize β-galactosidase, since they formed colonies with blue centers on plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). X-Gal was dissolved in dimethylformamide at a concentration of 20 mg/ml. The final concentration of X-Gal in the plates was 20 μg/ml. However, the growth of the integrant strains on these plates was diminished compared with the growth on control plates to which only the solvent dimethylformamide was added. This effect became clearer at higher X-Gal concentrations. At concentrations of 80 μg of X-Gal per ml, cells of the integrant strains appeared as very tiny blue colonies and they remained that small, despite longer incubation times. Integrant strains, growing in the absence of X-Gal, displayed normal wild-type growth. In addition, growth of the *P. denitrificans* wild-type strain was not affected by the presence of X-Gal. These data strongly suggest that the extent of growth of the integrant strains is related to the formation of the degradation product of X-Gal. The blue indolyl derivative probably becomes more growth inhibiting at increasing concentrations. At a certain stage, the concentration reaches a critical value, after which growth is fully repressed. This phenomenon is rather convenient, since it can be used to facilitate the isolation of mutant strains from the plasmid integrant strains.

Introduction of unmarked deletions by the use of suicide vector pRVS1. A scheme of the expected stages in the introduction of unmarked deletions into the chromosome of *P. denitrificans* is presented in Fig. 2. The starting material in this procedure is a *P. denitrificans* mutant strain, in which the target gene is inactivated by insertion of a gene encoding kanamycin resistance. A copy of the target gene is mutated in vitro by making a deletion linked to or overlapping the same site which was chosen for insertion of the kanamycin resistance gene. The mutated gene is inserted in pRVS1, and the resulting construct is transferred by conjugation from *E. coli* S17-1 to the *P. denitrificans* insertion mutant strain. Plasmid integrant strains which have integrated the construct after a single crossover are selected on plates with kanamycin and streptomycin, and the resulting colonies are blue in the presence of 20 μg of X-Gal per ml. Cells of these colonies are suspended in liquid medium, and the suspension is diluted to a concentration of 10^5 cells per ml. Samples (100 μl) are spread on plates without antibiotics to allow for recombinational loss of the plasmid. Arising colonies consist predominantly of cells with the vector still integrated into the chromosome but also consist of cells in which the vector is lost after recombination. The crossover could have been either at the former integration site or at the opposite side of the marker gene, yielding either the original insertion mutant strain or the desired deletion mutant strain, respectively. Cells from the colonies containing the different genotypes are collected in liquid medium, and the suspension is diluted at a concentration of 10^5 cells per ml. Samples (100 μl) are spread on plates, again in the absence of antibiotics but in the presence of 80 μg of X-Gal per ml. Under this condition, cells of the integrant strain form only tiny blue colonies; the relatively large white colonies of recombinant cells could be discriminated easily. Cells of these colonies are plated in duplicate both in the absence and in the presence of kanamycin. Mutant strains, which after a proper recombination event, had replaced the insertionally inactivated gene for the one with a deletion in it, show a kanamycin- and streptomycin-sensitive phenotype.

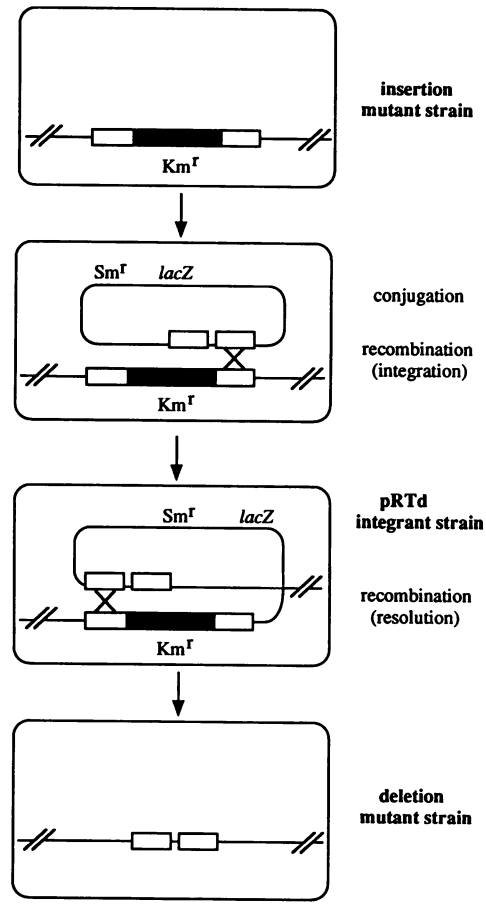

FIG. 2. Scheme of the expected mechanism for replacement by homologous recombination of an insertionally inactivated chromosomal gene for a gene with a deletion made in vitro. Relevant selection markers and resistance phenotypes of the selected strains are shown. The chromosome is represented by a line interrupted at both sides. The target gene and the kanamycin resistance gene are shown as white and black rectangles, respectively.

Introduction of single and multiple unmarked mutations in the genes encoding cytochromes c_{550}, c_{551i}, and c_{553i}. The construction of the c_{550} insertion mutant strain Pd2121 was described earlier (41). This strain was the starting point for the in vivo replacement of the kanamycin resistance gene in

the *cycA* gene for a gene with an unmarked mutation made in vitro. For the latter purpose, the 3-kb *Bam*HI-*Sph*I fragment of pRS59B, containing the *cycA* gene, was cloned in pUC19. The resulting plasmid, pCYA1, was partially restricted with *Eco*RI and *Xho*I to remove the approximately 0.15-kb *Eco*RI-*Xho*I fragment in the middle of the *cycA* gene, after which the sticky ends were filled in and ligated to yield plasmid pCYA1D. As a consequence, part of the gene, including the heme-binding site, was deleted. In addition, a frameshift was introduced in the coding region downstream from the mutation. Together with its flanking regions, the *cycA* gene mutated in this way was isolated as a 2.85-kb *Sma*I-*Sph*I fragment from pCYA1D and inserted into the *Sma*I and *Sph*I sites of suicide vector pRVS1 to yield pRTd2131. Integration of this plasmid in the genome of *P. denitrificans* occurred at a frequency of 10^{-5}. Subsequently, colonies of cells in which the plasmid was resolved from the chromosome were isolated at a frequency of 5×10^{-4}. Of these strains, 25% had a kanamycin-sensitive phenotype, one of which was designated Pd2131 and used for further analyses.

The construction and properties of *moxG* mutant strains were described in another report (40). In strain Pd0321, the *moxG* gene is interrupted by insertion of the kanamycin resistance gene. Strain Pd0331 is derived from Pd0321 and harbors a gene with an unmarked deletion instead of the kanamycin resistance gene. The mutant strains were constructed with the use of suicide vectors pRTd0321 and pRTd0331, respectively. By using plasmid pRTd6121, the *cycB* mutant strain Pd6121, the properties of which are described in a forthcoming paper (30), was isolated. The *cycA* mutant strain Pd2131 was the starting point for the construction of the *cycA moxG* and *cycA cycB* double mutant strains. In parallel experiments, the *moxG* and *cycB* genes of Pd2131 were inactivated by marker insertion by using suicide vectors pRTd0321 and pRTd6121, respectively. The resulting strains were designated Pd9201 and Pd9204, respectively. The *moxG cycB* double mutant strain was derived from the *moxG* mutant strain Pd0331 after a gene replacement procedure involving the suicide vector pRTd6121. In a final experiment, a mutant strain was constructed with mutations in *cycA*, *moxG* mutant strain Pd0331 after a gene replacement procedure involving the suicide vector pRTd6121. In a final experiment, a mutant strain was constructed with mutations in *cycA*, *moxG*, and *cycB*. The starting point was the *cycA moxG* double mutant strain Pd9201. By using suicide vector pRTd0331, the mutation by marker insertion in *moxG* was replaced by an unmarked deletion after homologous recombination. In the resulting *cycA moxG* double mutant strain, designated Pd9203, the *cycB* gene was subsequently mutated by using suicide vector pRTd6121. The resulting triple mutant strain was designated Pd9303.

Southern analysis of the *cycA*, *moxG*, and *cycB* loci. The correctness of the different types of mutations in all single, double, and triple mutant strains was confirmed by Southern analysis of chromosomal DNA. The results of the hybridization experiments with the wild-type strain and single mutant strains are presented in Fig. 3. The results with the double and triple mutant strains were consistent with those of the single mutant strains (results not shown). Chromosomal DNA of the parental and of the *cycA* mutant strains was restricted with *Eco*RI. *cycA*-containing fragments were visualized by using the 0.6-kb *Xho*I-*Pst*I fragment of plasmid pRS59 as the probe. Hybridization with a 2.4-kb fragment is observed in the lane with the wild-type strain and with a 3.8-kb fragment in the lane with the *cycA* mutant strain Pd2121. This result reflects the proper insertion of the kanamycin marker gene, the size of which is 1.4 kb. In the *cycA* deletion mutant strain Pd2131, the *Eco*RI site in the middle of the *cycA* gene was deleted as part of the mutagenesis strategy, and in this lane, hybridization with a large *Eco*RI fragment of unknown size was observed. Chromosomal DNA of the parental strain and of the *moxG* mutant strains was restricted with *Pvu*II, and hybridization was carried out with the labeled 1-kb *Pst*I-*Bam*HI fragment of plasmid pNH33. In the lane with the wild-type strain and in the lane with the *moxG* insertion mutant strain Pd0321, hybridization with a 2.0- and 3.4-kb fragment, respectively, was observed. The *moxG* deletion mutant strain Pd0331 lacks the internal 0.2-kb *Bam*HI fragment of *moxG*, and hybridization with an 1.8-kb fragment was observed, as expected. Chromosomal DNA of the wild type and the *cycB* insertion mutant strain Pd6121 was restricted with *Eco*RI, and *cycB*-containing fragments were analyzed by using the labeled 1.4-kb *Eco*RI fragment of plasmid pWR20 as the hybridization probe. In the lane with the wild-type strain, this fragment was recovered. In the lane with the *cycB* insertion mutant strain Pd6121, staining of a 2.8-kb fragment confirmed the correctness of the insertional mutagenesis of *cycB* in this strain.

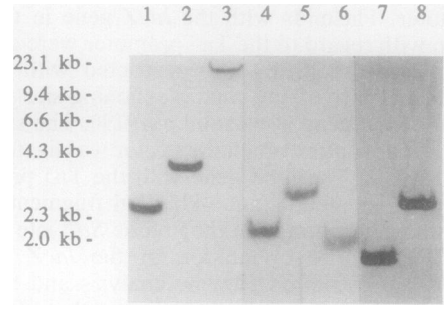

FIG. 3. Blots of digested chromosomal DNA of the *P. denitrificans* wild-type strain (lanes 1, 4, and 7), *cycA* insertion mutant strain Pd2121 (lane 2), *cycA* deletion mutant strain Pd2131 (lane 3), *moxG* insertion mutant strain Pd0321 (lane 5), *moxG* deletion mutant strain Pd0331 (lane 6), and *cycB* insertion mutant strain Pd6121 (lane 8). In lanes 1 through 3, chromosomal DNA was digested with *Eco*RI and DNA fragments were visualized with the 0.6-kb *Xho*I fragment of plasmid pRS59. In lanes 4 through 6, DNA was digested with *Pvu*II and DNA fragments were visualized with the 1.0-kb fragment of plasmid pNH33. In lanes 7 and 8, chromosomal DNA was digested with *Eco*RI and DNA fragments were visualized with the 1.4-kb fragment of plasmid WR20. Fragment sizes are indicated at the left.

Cytochrome *c* analyses of the *cycA*, *moxG*, and *cycB* mutant strains. *P. denitrificans* wild-type and mutant strains were grown in batches on mineral medium with methylamine as the carbon and energy source. Cells from these cultures were harvested at the late exponential phase of growth, after which cell extracts were prepared. Following SDS gel electrophoresis, cytochromes *c* were visualized by means of heme staining (Fig. 4). In the wild-type strain, cytochromes of apparently 50, 30, 22, and 15 kDa, corresponding with a heme *c*-containing peroxidase (11), cytochrome c_{553i} (30), cytochrome c_{551i} (40), and cytochrome c_{550} (41), respectively, are found. Cytochrome c_{550} is absent in all single, double, and triple mutant strains in which the *cycA* gene was mutated. This is also true for *moxG* and *cycB* mutant strains lacking the cytochromes c_{551i} and c_{553i}, respectively. The

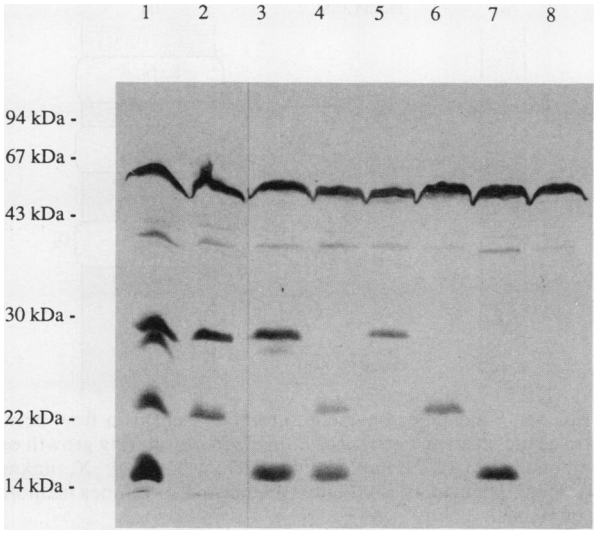

FIG. 4. SDS-polyacrylamide gel electrophoresis of soluble cytochromes c of the *P. denitrificans* wild-type strain (lane 1), Pd2131 (lane 2), Pd0331 (lane 3), Pd6121 (lane 4), Pd9203 (lane 5), Pd9204 (lane 6), Pd9205 (lane 7), and Pd9303 (lane 8). Proteins were run in 14% gels. Cells were grown in batch cultures with methylamine as the carbon and energy source. Covalently bound heme was stained with tetramethylbenzidine. The positions of the marker proteins are indicated by their relative molecular masses.

triple mutant strain Pd9303 is completely devoid of the cytochromes concerned and contains only the periplasmically located peroxidase. The data above clearly show that the single- and multiple-site-directed mutations in the chromosomal *cycA*, *moxG*, and *cycB* genes have resulted in the desired loss of one or more of the periplasmic cytochromes *c*. In the lane with the wild-type strain, an additional heme-stained protein of about 27 kDa was observed. The stock solution of proteins from the wild-type strain was stored in the freezer prior to electrophoresis. All other samples were freshly prepared solutions. After storage of these solutions in the freezer, the 27-kDa cytochrome was also observed in protein samples containing the 30-kDa cytochrome but not in samples of mutant strains lacking the 30-kDa cytochrome (results not shown). Therefore, the 27-kDa cytochrome is most probably a breakdown product of cytochrome c_{553i}.

Effects of multiple mutations in the *cycA*, *moxG*, and *cycB* genes. Properties of the *P. denitrificans* wild-type strain and mutant strains are presented in Table 2. All strains tested were able to grow heterotrophically with succinate or choline chloride as the sole carbon and energy source. In addition, the wild-type strain as well as the mutant strains was able to grow methylotrophically with methylamine as the substrate. When methanol was the substrate, growth on plates inoculated with the wild-type strain, the two *cycA* single mutant strains, the *cycB* single mutant strain, or the *cycA cycB* double mutant strain was observed. All of the mutant strains lacking cytochrome c_{551i} were unable to grow on methanol.

The effects of the mutations on the oxidation of the latter two C_1 substrates were studied in more detail. Maximum specific growth rates on methylamine and on methanol were determined in batch cultures and in continuous cultures, respectively. The wild-type strain, the two *moxG* mutant strains, the *cycB* mutant strain, and the *moxG cycB* double mutant strain exhibited comparable maximum specific growth rates on methylamine. The maximum specific growth rate of mutant strains lacking cytochrome c_{550} was about 35% lower compared with that of the wild-type strain. These data are in agreement with observations made earlier (41). With methanol as the carbon and energy source, similar growth rates were determined for the wild-type strain and the *cycA* mutant strain Pd2121. The *moxG* mutant strain Pd0321 failed to grow in the chemostat under methanol limitation. Finally, an approximately 20% decrease in the rate was determined for the *cycB* mutant strain Pd6121; these results will be described in another report (30).

MDH and methylamine dehydrogenase activities were both measured in suspensions of batch-cultured methylamine-grown cells. All of the strains tested displayed comparable methylamine dehydrogenase activities. *cycA* and *cycB* single mutant strains showed MDH activities similar to that of the wild-type strain. MDH activity was absent in the *moxG* insertion mutant strains Pd0321 and Pd9201. Partial activity was observed in the mutant strains Pd0331, Pd9203, Pd9205, and Pd9303, which bear an unmarked deletion in *moxG*. The extent of MDH activities in these *moxG* mutant strains was shown to be dependent on the extent of synthesis

TABLE 2. Properties of *P. denitrificans* wild-type and mutant strains

Strain	Relevant characteristic(s)	Growth[a] on:				μ_{max}[b] with:		Activity[c]	
		Succ	Chol	MA	MeOH	MA	MeOH	MADH	MDH
Wild type		+	+	+	+	0.15	0.16[d]	194	96
Pd2121	*cycA*::Km^r	+	+	+	+	0.10	0.15	180	95
Pd2131	Δ*cycA*	+	+	+	+	0.10	ND	173	106
Pd0321	*moxG*::Km^r	+	+	+	−	0.15		190	0
Pd0331	Δ*moxG*	+	+	+	−	0.15	ND	201	13
Pd6121	*cycB*::Km^r	+	+	+	+	0.15	0.12[d]	184	99
Pd9201	Δ*cycA moxG*::Km^r	+	+	+	−	0.10	ND	197	0
Pd9203	Δ*cycA* Δ*moxG*	+	+	+	−	0.10	ND	197	11
Pd9204	Δ*cycA cycB*::Km^r	+	+	+	+	0.10	ND	206	89
Pd9205	Δ*moxG cycB*::Km^r	+	+	+	−	0.15	ND	188	13
Pd9303	Δ*cycA* Δ*moxG cycB*::Km^r	+	+	+	−	0.10	ND	216	14

[a] Growth of bacteria on plates with mineral medium and the carbon sources listed; +, growth; −, no growth; ND, not determined; succ, succinate; Chol, choline chloride; MA, methylamine; MeOH, methanol.

[b] μ_{max}, maximum specific growth rate (h^{-1}) of bacteria with the carbon sources listed.

[c] Enzyme activity (nmol of DCPIP · min^{-1} · mg^{-1} of protein) in whole cells. MADH, methylamine dehydrogenase.

[d] Data from reference 30.

of the small subunit of MDH, its gene located directly downstream from the *moxG* gene (40).

DISCUSSION

By using suicide vectors pGRPd1 and pRVS1, insertion markers and unmarked mutations, respectively, can be introduced at specific sites in the chromosome of *P. denitrificans*. Different strains, obtained during the intermediate stages of the mutagenesis, could easily be discriminated from each other by the use of three marker genes. Two of these encode resistance to kanamycin and streptomycin as markers for insertional inactivation of the target gene and integration of the suicide vector in the chromosome, respectively. The third gene, *lacZ*, encodes β-galactosidase and marks the presence or excision of the suicide vector. Strains which have incorporated the suicide vector in the genome after homologous recombination also possess this reporter gene, and these integrant strains were shown to cleave X-Gal successfully. At high concentrations, the released blue indolyl derivative turned out to be toxic for growth of *P. denitrificans*. Therefore, on the basis of both the size and color of the colonies, integrant strains could easily be discriminated from the desired mutant strains, which had resolved the suicide vector pRVS1. Analogous mechanisms for the resolution of plasmids from the chromosome have been described for *E. coli* and *Bordetella pertussis*. *E. coli* mutant strains which had lost the λ*c*Its857 phage vector were selected, while the integrant strains were killed after thermal induction of the prophage (20). *B. pertussis* mutant strains which had lost the plasmid-encoded streptomycin-sensitive ribosomal protein S12 were selected, while the integrant strains were killed in the presence of streptomycin (36). The first method is specifically applicable to *E. coli*. Also, the latter system was proven to be unsuitable with *P. denitrificans*. Apparently, the S12 gene is not expressed efficiently or the S12 protein is not assembled in *P. denitrificans* ribosomes (39). Therefore, the screening method described in this report offers the first possibility to introduce successfully unmarked mutations of any extent into the genes of *P. denitrificans*. In addition, the method might easily be used with other bacterial species, too, especially cases in which known screening or selection systems are not applicable. It has to be emphasized that the *lacZ* gene function might be used as a positive (formation of blue colonies in the presence of X-Gal) or negative (diminished growth as a result of the toxic indolyl degradation product of X-Gal) screening principle.

The technique described in this report has important advantages over the insertion mutagenesis technique. First, mutant strains which have the same phenotype as the wild-type strain with respect to antibiotic sensitivity can be isolated. Therefore, single and multiple mutations can be introduced in *P. denitrificans* strains; each time, the procedure can be started by use of the kanamycin resistance gene as the insertion marker and then that gene can be exchanged for a gene with an unmarked mutation. As a consequence, this strategy is a very powerful tool for the mutagenesis of several genes in an organism in which only a few resistance markers are expressed properly. Second, the method permits site-directed alterations of codons in the genome, which is of great importance for future studies of the function of specific amino acids in *P. denitrificans* proteins of interest. The effects of the mutations can be interpreted properly, since the target genes are present in single copies on the chromosome. Third, the method provides the possibility to introduce unmarked in-frame deletions by which polar effects on downstream transcription and translation can be restricted to a minimum. As a consequence, mutations in operonlike organizations of genes should effect mainly the expression of the desired gene, unless its gene product involves the induction or stability of other proteins. Some preliminary data on the comparison between marker insertion mutations and of unmarked mutations in target genes with respect to the expression of genes located downstream are reported elsewhere (40). In this paper, the first point is emphasized. Mutant strains are constructed with single, double, and triple mutations in the genes encoding periplasmic cytochromes c_{550}, c_{551i}, and c_{553i}. Through physiological studies of these mutant strains, insight into the nature of the periplasmic electron transport routes during methylotrophic growth is extended. A schematic model of the routes concerned is presented in Fig. 5 and discussed below.

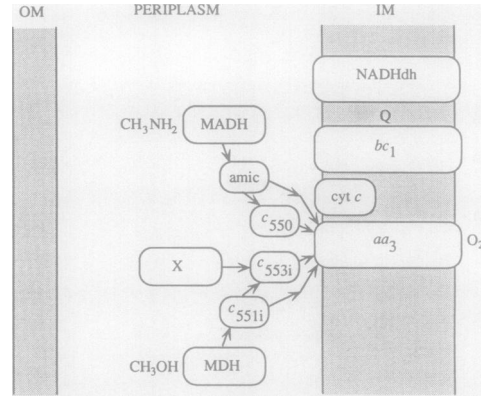

FIG. 5. Schematic representation of the electron flow between periplasmic electron carriers of *P. denitrificans* during growth on C_1 compounds. MADH, methylamine dehydrogenase; X, unknown (31); amic, amicyanin; OM, outer membrane; IM, inner membrane; Q, quinones.

Methylamine dehydrogenase catalyzes the oxidation of methylamine, and released electrons are passed to amicyanin, this blue copper protein being the irreplaceable electron acceptor of the dehydrogenase (16, 18, 42). Electron transport from amicyanin to the oxidase is suggested to be mediated by cytochrome c_{550} (41). Compared with the wild-type strain, single and multiple mutants with either an insertion marker or an unmarked deletion in *cycA* showed diminished growth rates on methylamine because of the absence of cytochrome c_{550}. Methylamine dehydrogenase itself is not affected by the mutation, since the mutant strains displayed wild-type levels of dehydrogenase activities. Although these results suggest the involvement of the cytochrome in methylamine oxidation, they also show that this involvement is not indispensable. Therefore, additional electron transfer between the blue copper protein and the oxidase may be either direct or mediated through an alternative electron carrier. Probable candidates for the latter function seem to be one of the periplasmically located cytochromes, c_{551i} or c_{553i}, since these cytochromes are also induced during growth on methylamine. However, from the studies above, it is shown that mutant strains lacking either one of these cytochromes still displayed wild-type growth rates on methylamine. These results are in agreement with earlier studies of the c_{553i} and c_{551i} single mutant strains (30, 40). Also, the cytochrome c_{551i} c_{553i} double mutant strain showed a maximum specific growth rate on methylamine similar to that of the wild-type strain. However, with respect

to electron transfer rates, the absence of a particular electron carrier could be masked by the presence of an alternative operating carrier. Therefore, double and triple mutant strains lacking cytochrome c_{550} as well as one or both of the cytochromes c_{551i} and c_{553i} were constructed. These mutant strains were all able to grow on methylamine with rates comparable to that of the c_{550} single mutant strain. These data demonstrate that when cytochrome c_{550} is involved in methylamine oxidation, periplasmic cytochromes c_{551i} and c_{553i} are not its substitutes. Since other periplasmic cytochromes c are not found during this growth condition, possible options for the alternative electron transport route are limited. Electrons from amicyanin may be transferred to the aa_3-type oxidase either directly or indirectly, mediated by a membrane-associated component.

Methanol is oxidized by MDH, and electrons are passed to cytochrome c_{551i}, the physiological electron acceptor of MDH (23, 40). Single, double, and triple mutant strains lacking cytochrome c_{551i} are all unable to grow on methanol, suggesting the relevance of this cytochrome in methanol oxidation. However, the way electrons are transported between cytochrome c_{551i} and the aa_3-type oxidase is not yet elucidated. In another report, it was shown that mutants lacking cytochrome c_{553i} were still able to grow on methanol, although with a somewhat decreased growth rate (30). From these data, it was suggested that in one way or another, this cytochrome might be involved in methanol oxidation. In this report, it was shown that the mutant strain lacking cytochrome c_{550} was still able to grow on methanol with rates comparable to that of the wild-type strain. However, the absence of cytochrome c_{550} could be masked by the presence of cytochrome c_{553i}. Therefore, a double mutant strain lacking both cytochromes c_{550} and c_{553i} was constructed. This strain was still able to grow on methanol, indicating that these two cytochromes are not each other's counterpart in possible parallel operating electron transport routes. In addition, wild-type levels of MDH activities were observed in the *cycA* and *cycB* mutant strains, indicating that neither of the mutations affected the synthesis of native MDH. In conclusion, the data above do not suggest that cytochrome c_{550} of *P. denitrificans* is an intermediate electron carrier between cytochrome c_{551i} and the aa_3-type oxidase during methanol oxidation. Electron transport between cytochrome c_{551i} and the aa_3-type oxidase might involve cytochrome c_{553i}. Work in our laboratory suggests that this cytochrome is part of a periplasmically located dehydrogenase system, resembling the *mox* system. The nature and properties of this system, however, are not yet elucidated (30). With respect to the alternative operating route during methanol oxidation, suggestions similar to those made for methylamine oxidation have to be taken into consideration. These options are currently under investigation.

ACKNOWLEDGMENTS

This research was supported by the Netherlands Foundation for Chemical Research, with financial aid from the Netherlands Organization for the Advancement of Science.

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