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Paracoccus denitrificans Cytochrome c_1 Gene Replacement Mutants

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We describe the construction and characterization of gene replacement mutants for the respiratory chain component cytochrome c_1 in the bacterium *Paracoccus denitrificans*. Its structural gene (*fbcC*) was inactivated by insertion of the kanamycin resistance gene, introduced into a suicide vector, and conjugated into *Paracoccus*; chromosomal mutants obtained by homologous recombination were selected by antibiotic resistance screening and further characterized biochemically. They showed the complete spectral, enzymatic, and immunological loss of the *fbcC* gene product together with a serious defect in the assembly of the two other gene products of the *fbc* operon, cytochrome *b* and the FeS protein. A possible role of the cytochrome c_1 in the assembly process for the enzyme complex is discussed. A functional restoration to wild-type phenotype was achieved by complementing in *trans* with a newly constructed broad-host-range vector carrying the *fbcC* gene cassette. When the complete *fbc* operon was present on this vector, overexpression of complex III subunits was observed. Apart from their physiological significance, such mutants are a prerequisite for probing structure-function relationships by site-directed mutagenesis in order to understand molecular details of electron transport and energy transduction processes of this respiratory enzyme in bacteria and in mitochondria.

Essentially three features have made the gram-negative soil bacterium *Paracoccus denitrificans* an interesting organism for bioenergetic research and, in particular, a model organism for the mitochondrial electron transport. (i) Its close relationship to present-day mitochondria, indicated by a large number of mostly physiological and bioenergetic arguments, prompted John and Whatley (23, 24) to assign this bacterium a hypothetical precursor role in endosymbiotic organelle development; this early assumption of a close evolutionary homology has recently been confirmed by more direct evidence on phylogenetic grounds (59). (ii) At least two respiratory complexes, cytochrome *c* reductase (see below) and cytochrome *c* oxidase (17, 30, 32) isolated from this bacterium in a functional state, have basic enzymatic properties almost indistinguishable from those of their mitochondrial counterparts, but are far less complex in their structure. (iii) Due to the availability of the genes for their subunits, these enzymes are amenable to site-specific mutagenesis experiments to study stucture-function relationships and thus increase our knowledge on basic electron transport and energy transduction mechanisms both in bacteria and in mitochondria.

The cytochrome bc_1 complex (complex III; ubiquinol: cytochrome *c* oxidoreductase [EC 1.10.2.2]) is an obligate component in the electron transport chain of mitochondria and, in an analogous form, of photosynthetic organisms (19, 20, 43, 58). Moreover, several bacterial species have recently been shown to contain a functionally homologous complex, notably *Rhodobacter* species (8, 13, 14) and *Paracoccus denitrificans* (4, 33, 60). Three different subunits carrying the redox centers of this integral membrane enzyme, cytochromes *b* and c_1 and the so-called Rieske protein with its iron-sulfur center (FeS), are common to all bc_1 complexes. Whereas the well-studied enzyme complexes from mitochondria typically contain a large number of additional polypeptides of uncertain function, the bacterial enzymes mentioned above are composed of only the three essential redox center subunits. However, at least for *Paracoccus* spp., full enzymatic competence of this structurally simple complex has been reported in a reconstituted system, for both its electron transport and energy transduction capacity (60, 61). Molecular details of these two coupled processes have not been elucidated in mitochondrial bc_1 complexes, but hypotheses for structure-function relationships as well as tentative functional assignments for certain residues have been made (10, 22, 38, 45, 52, 57). Such assumptions can be tested in bacteria by performing site-directed mutagenesis on the isolated genes, followed by insertion into a suitable host to obtain expression of the redox complex. In *Paracoccus* spp., the genes for the bc_1 subunits have been cloned and sequenced (27), and, as in *Rhodobacter* spp. (8, 13), have been shown to be organized in an operon structure, subsequently referred to as the *fbc* operon. (Following general nomenclature, we here refer to the gene locus as the *fbc* operon, with the order of genes *fbcF*, *fbcB*, *fbcC*, coding for the FeS subunit, cytochrome *b*, and cytochrome c_1, respectively.)

As a first step towards such an approach, we here describe the construction and biochemical characterization of *Paracoccus* mutants deprived of their functional genomic copy of the *fbcC* gene, coding for the cytochrome c_1 polypeptide. Functional expression can be restored by complementing the inactivated gene in *trans* with an intact copy located on a newly constructed broad-host-range plasmid. Such mutants are not only a prerequisite for site-directed mutagenesis experiments, but are also of physiological interest per se.

MATERIALS AND METHODS

Wild-type *Paracoccus denitrificans* ATCC 13 543, parent strain PD1222 used for generation of mutants, and resulting mutant strains (Table 1) were grown aerobically at 32°C in succinate medium as previously described (30, 31), or in methanol medium (54). If not stated otherwise, cells were harvested at mid-log phase and membranes were obtained by mechanical rupture at 4°C by three passages through a Manton-Gaulin homogenizer (APV Schröder, Lübeck) operating at 500 kg/cm^2, followed by differential centrifugation. Spectral and enzymatic assays were run on a Uvikon 810

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
JM101	Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*ZΔM15*)	36
CU9276	*hsdR17 mcrAB recA1* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*ZΔM15*)	53
GM2929	F$^-$ *dam-13 dcm-6*	M. G. Marinus, Worcester, Mass.
P. denitrificans		
Wild type		ATCC 13 543
PD1222	Spc^r Rif^r, enhanced mating frequency, *fbc*$^+$	9
G299	PD1222 derivative, *fbcC*::*neo*	This work
G300	PD1222 derivative, Δ*fbcC*::*neo*	This work
G305	PD1222 derivative, Sm^r *neo* (single crossover; see Results)	This work
Plasmids		
Clone 100	*fbc* operon	27
Clone 250	*fbcC*	27
pSUP202	*mob* Cm^r Ap^r Tc^r	47
pHP45Ω	Ap^r Sm^r/Spc^r	40
pHP45	Ap^r	40
RP4-7	Ap^r Km^s Tc^r::Tn*7*	R. Simon, Bielefeld
pUC13	Multicopy plasmid, *lacZ′* Ap^r	37
pUC1813	Multicopy plasmid, *lacZ′*, symmetric mcs, Ap^r	25
pUL62	Vector carrying Tn*5* *Hin*dIII-*Sal*I *neo* fragment	44
pBluescript II SK$^-$	Multicopy plasmid, *lacZ′*, T3 promoter, T7 promoter, f1 *ori*, Ap^r	Stratagene, Heidelberg
pEG06	pUL62 *Hin*dIII-*Sal*I *neo* fragment in pUC13	This work
pEG012	pEG06 *Sma*I *neo* fragment in pUC1813	This work
pEG148	*Hin*dIII-*Sma*I fragment of clone 100 in pHP45	This work
pEG149	pEG148 with *Rsa*I-*Sal*I fragment of clone 250	This work
pEG150	1.4-kb *Xho*I-*Cla*I fragment of clone 250 in pBluescript II SK$^-$	This work
pEG151	pUC13 with 0.4-kb *Bam*HI-*Hin*dIII fragment of pEG150	This work
pEG152	*Eco*RV-*Bam*HI fragment of pEG149 in pEG151; corresponds to *fbcC* cassette	This work
pEG153	3.5-kb *Eco*RV-*Bam*HI of clone 100 in pEG150; corresponds to *fbc* cassette	This work
pEG268	Clone 100 with *Sal*I *neo* fragment of pEG012	This work
pEG270	*Cla*I fragment of pEG268 cloned into pSUP202	This work
pEG284	pEG270 with Sm^r/Spc^r	This work
pEG285	Clone 250 with *mob* fragment of pSUP202	This work
pEG286	pEG285 with *neo* fragment of pEG06 between *Xho*I and *Bam*HI sites	This work
pEG287	pEG286 with Sm^r/Spc^r	This work
pEG400	IncP broad-host-range vector, Sm^r/Spc^r pUC13 mcs, *lacZ′*	To be published elsewhere
pEG435	pEG400 with *fbcC* cassette of pEG152	This work
pEG436	pEG400 with *fbc* cassette of pEG153	This work

double-beam spectrophotometer/recorder unit (Kontron, Munich) at 25°C.

Restriction enzyme and related DNA-modifying enzyme incubations, plasmid DNA isolations, agarose DNA gel electrophoresis, and Southern blotting were performed essentially as described before (34). Probes for hybridization were labeled by random priming (12) with biotin-14-dATP; hybrid bands were detected by using the streptavidin-alkaline phosphatase conjugate assay (29). Triparental mating experiments were performed (48).

Antibiotic concentrations in agar plates and in liquid media were as follows: rifampin, 60 μg/ml; ampicillin, 50 μg/ml; streptomycin sulfate, 25 μg/ml; kanamycin sulfate, 25 μg/ml; spectinomycin hydrochloride, 100 μg/ml.

Sodium dodecyl sulfate (SDS) gel electrophoresis, electrophoretic transfer to nitrocellulose membranes, immunological detection of reactive bands by using the protein A-alkaline phosphatase conjugate, preparation of specific polyclonal antibodies, and heme staining were performed as published previously (30, 31, 33, 49).

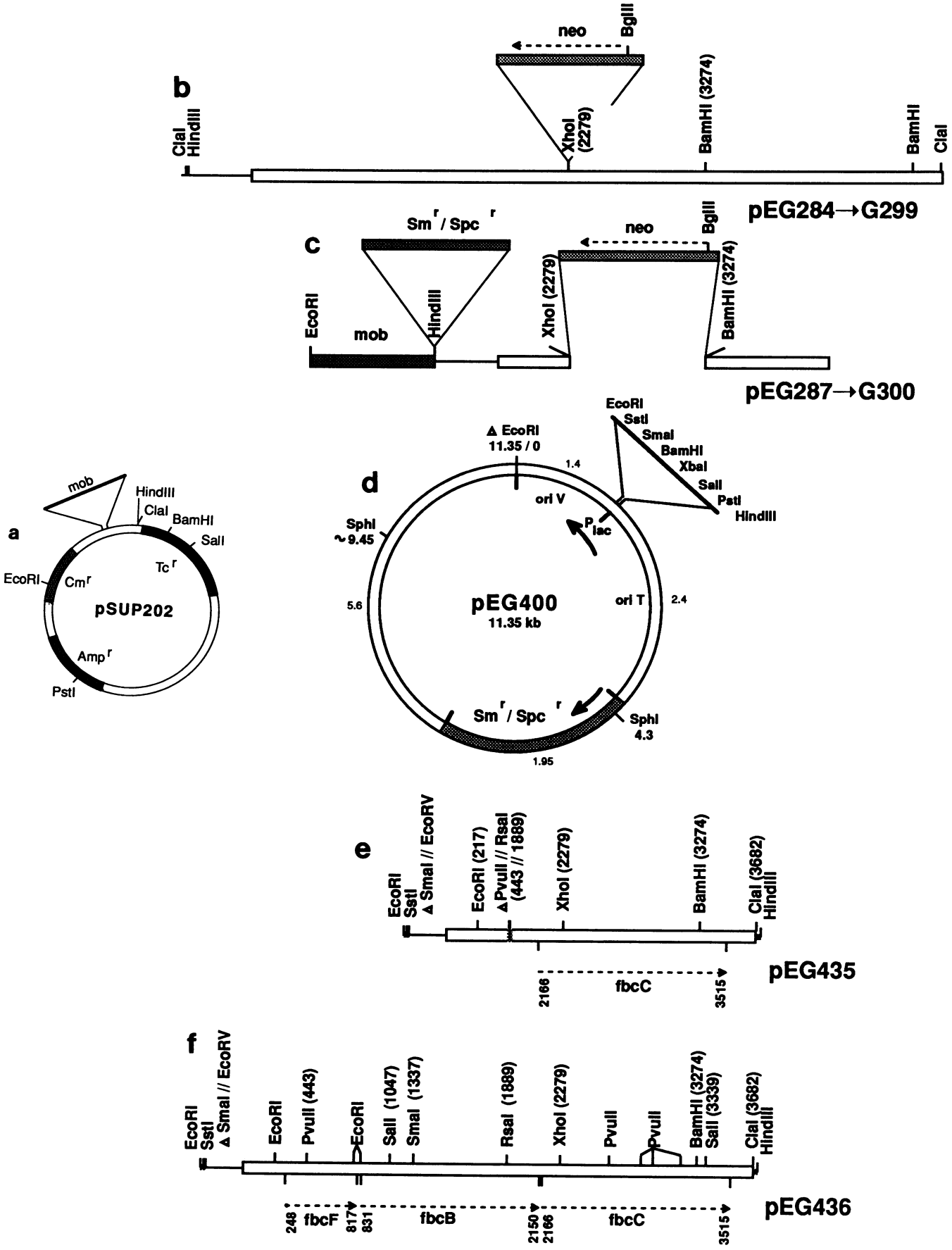
Restriction and other DNA-modifying enzymes, biotin-14-dATP, and random primers were obtained from Boehringer (Mannheim), Bethesda Research Laboratories (BRL) (Eggenstein), and Pharmacia (Freiburg), nitrocellulose membrane BA85 from Schleicher and Schüll (Dassel), and Immobilon-P membrane from Millipore (Eschborn); chemicals were obtained from Biomol (Hamburg) and Merck (Darmstadt) at the highest purity available.

For handling DNA sequence data, two computer programs were used: DNA Inspector IIe (Textco, West Lebanon, N.H.) and DNA Strider (35).

RESULTS

Construction of *Paracoccus* cytochrome c_1 mutants. By homologous recombination, the intact chromosomal copy of the *fbcC* gene (coding for cytochrome c_1) of *P. denitrificans* is replaced by a copy of the gene which was inactivated by the Tn*5* kanamycin resistance gene *neo* (2) in two different ways, either by insertion or by replacement of the native *fbcC*.

P. denitrificans PD1222 (Table 1) carrying the intact chromosomal copy of the *fbc* operon received by triparental mating, plasmid pEG284 or pEG287 (Fig. 1b and c). Due to their type of replicon (Col E1), such vectors, derivatives of the suicide vector pSUP202 (48) (see also Fig. 1a), can be


b
neo
BglII
ClaI
HindIII
XhoI (2279)
BamHI (3274)
BamHI
ClaI
pEG284→G299
c
Sm r / Spc r
neo
BglII
EcoRI
mob
HindIII
XhoI (2279)
BamHI (3274)
pEG287→G300
a
mob
HindIII
ClaI
BamHI
SalI
Tc r
EcoRI
Cm r
pSUP202
Amp r
PstI
d
EcoRI
SstI
SmaI
BamHI
XbaI
SalI
PstI
HindIII
Δ EcoRI
11.35 / 0
1.4
ori V
P lac
SphI
~9.45
5.6
pEG400
11.35 kb
ori T
2.4
Sm r / Spc r
SphI
4.3
1.95
e
EcoRI
SstI
Δ SmaI // EcoRV
EcoRI (217)
ΔPvuII // RsaI
(443 // 1889)
XhoI (2279)
BamHI (3274)
ClaI (3682)
HindIII
2166
fbcC
3515
pEG435
f
EcoRI
SstI
Δ SmaI // EcoRV
EcoRI
PvuII (443)
EcoRI
SalI (1047)
SmaI (1337)
RsaI (1889)
XhoI (2279)
PvuII
PvuII
BamHI (3274)
SalI (3339)
ClaI (3682)
HindIII
248
fbcF
817
831
fbcB
2150
2166
fbcC
3515
pEG436

conjugated into but cannot replicate in *Paracoccus* spp. Therefore, any kanamycin-resistant clone should be the result of a chromosomal recombination. In addition, both constructs contain the streptomycin resistance gene (Sm^r/Spc^r) within the vector sequence to differentiate between single and double crossover events.

Details of the construction of suicide plasmids. For convenient cloning, two cassettes were constructed containing the Tn*5* kanamycin resistance gene *neo* (2) flanked by suitable restriction sites.

The *neo* gene is isolated as a *Hin*dIII-*Sal*I fragment (1.3 kilobases [kb]) from the vector pUL62 (44), the *Hin*dIII site is end-filled, and the fragment is cloned into the multiple cloning site of pUC13 (55), which has been *Xba*I digested, end-filled, and further *Sal*I digested. This step leads to plasmid pEG06.

A *Sma*I digest of this plasmid cuts out the 1.1-kb *neo* gene, which is then subcloned into the symmetrical multiple cloning site (mcs) of pUC1813 (25) linearized by *Sma*I, to yield pEG012. This plasmid is *Sal*I digested, and the resulting kanamycin resistance gene cassette is used for insertional inactivation (see below).

pEG284. The plasmid, clone 100 (27), comprising the complete *fbc* operon, contains a unique *Xho*I site (nucleotide [nt] 2279, corresponding to amino acid residue 38 of the cytochrome c_1; see Fig. 1f). Disruption of the *fbcC* gene is achieved by inserting the *Sal*I *neo* cassette into this *Xho*I site. A *Cla*I cut in this pEG268 plasmid liberates the modified *fbcFBC* fragment, which is introduced into the single *Cla*I site of pSUP202 (Fig. 1a). In the resulting plasmid pEG270, the Tc^r gene is inactivated by this insertion. The *Hin*dIII streptomycin resistance gene cassette (Sm^r/Spc^r), removed from the pHP45Ω (40), is introduced into a partially *Hin*dIII-digested pEG270. A plasmid, termed pEG284, was isolated carrying the cassette in the *Hin*dIII site on the former pSUP202 part of pEG270 (Fig. 1b).

pEG287. The plasmid, clone 250 (27), containing part of *fbcB* and the complete *fbcC*, is used in the construction of a suicide plasmid with replacement of most of *fbcC*. The *Eco*RI-*Hin*dIII fragment containing the *mob* site of pSUP202 (Fig. 1a) is cloned into the *Eco*RI-*Hin*dIII-digested clone 250. This construct, pEG285, is *Bam*HI-*Xho*I digested; its internal fragment (1 kb) is replaced by the kanamycin resistance gene (1.3-kb *Bam*HI-*Sal*I fragment, see above) from pEG06, resulting in pEG286. To obtain pEG287 (Fig. 1c), the streptomycin cassette (see above) was introduced into the unique *Hin*dIII site of pEG286.

Recombination. Each of the two suicide plasmids, pEG284 and pEG287, was introduced into PD1222 by triparental mating (see Materials and Methods). Clones originating from recombination events between the intact genomic and the inactivated plasmid-borne copy of the gene and/or its flanking regions were selected for their kanamycin resistance. To select for double crossover events, clones with a Km^r Sm^s phenotype, which have lost the vector sequence, were selected. Two representative strains, termed G299 and G300 (Fig. 1 and Table 1), were chosen for further analysis. For occasional reference, results are included for strain G305 (Table 1); it showed both streptomycin and kanamycin resistance, carried both the intact genomic and the mutilated plasmid copy of the *fbcC* gene, and most likely resulted from a single crossover event. For pEG284, single crossover events were observed at a frequency of 4×10^{-5} (chromosomal recombinants per recipients), and double crossovers occurred at 2×10^{-6}. Corresponding figures for pEG287 are 3×10^{-7} and 6×10^{-9}, respectively, due to shorter flanking regions.

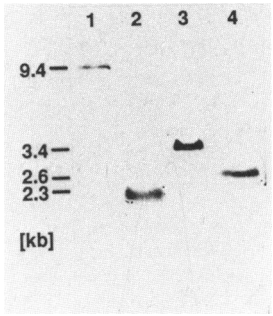


FIG. 2. Southern blot analysis confirming chromosomal location of mutation in *fbcC*. Plasmid DNA of clone 100 was *Hin*dIII digested (5 ng; lane 1), and 1 μg of genomic DNA from strain PD1222 (lane 2), the insertion mutant strain G299 (lane 3), and the deletion-insertion strain G300 (lane 4) was *Sal*I digested and, after agarose gel electrophoresis and transfer to Immobilon-P membrane, probed with the internal 2.3-kb *Sal*I fragment, representing major parts of the *fbcB* and *fbcC* genes (see Fig. 1f). For further details, see Materials and Methods and Results sections and Table 1.

Analysis of cytochrome c_1 mutants. The specificity of the recombination event, i.e, the replacement of the native chromosomal *fbcC* gene by the *neo*-inactivated copy offered on the suicide plasmid, was verified by Southern blot analysis of genomic DNA. Both mutants (Fig. 2) were characterized by probing with the internal 2.3-kb *Sal*I fragment of the *fbc* operon (Fig. 1f).

The expected loss of cytochrome c_1 expression should cause spectral changes in the mutants. Cytoplasmic membranes were isolated and solubilized, and optical difference spectra (reduced minus oxidized) were recorded. Figure 3a shows a clear shift of absorption maxima in the α region, most evident at around 553 nm typical for cytochrome c_1, and a slight decrease in the heme *b* region, when mutant membrane spectra were compared with those of the parent strain.

When membranes were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and assayed for peroxidase activity indicative of polypeptides with covalently bound heme, the prominent band of the cytochrome c_1 of about 62 kilodaltons (kDa) (27) of strain PD1222 was completely absent from the two mutant membrane lanes (Fig. 4). Like-

FIG. 1. Construction of plasmids used in generating and complementing specific mutations in the *Paracoccus fbcC* gene. All positional numbers for selected restriction enzyme sites in panels b, c, e, and f refer to clone 100, the original isolate of the *fbc* operon (27); open bars, drawn to scale and aligned vertically in the four maps, represent *Paracoccus* DNA; for a survey of the location of the three genes, see dotted arrows in panel f. Details of the constructions are given in the Results section and Table 1. (a) Partial physical map of the suicide vector pSUP202 (47). (b and c) Partial physical maps of DNA fragments with inactivated *fbcC* gene regions as contained in suicide plasmids pEG284 and pEG287, which gave rise to strains G299 and G300 after mating into PD1222. (d) Map of broad-host-range vector pEG400 used in complementation experiments. Apart from *Sph*I, all restriction sites listed are unique. (e and f) *fbcC* and *fbc* operon cassettes for cloning into pEG400 in the complementation assay of mutants. Outermost 5′- and 3′-end restriction sites are those of the mcs of pEG400.

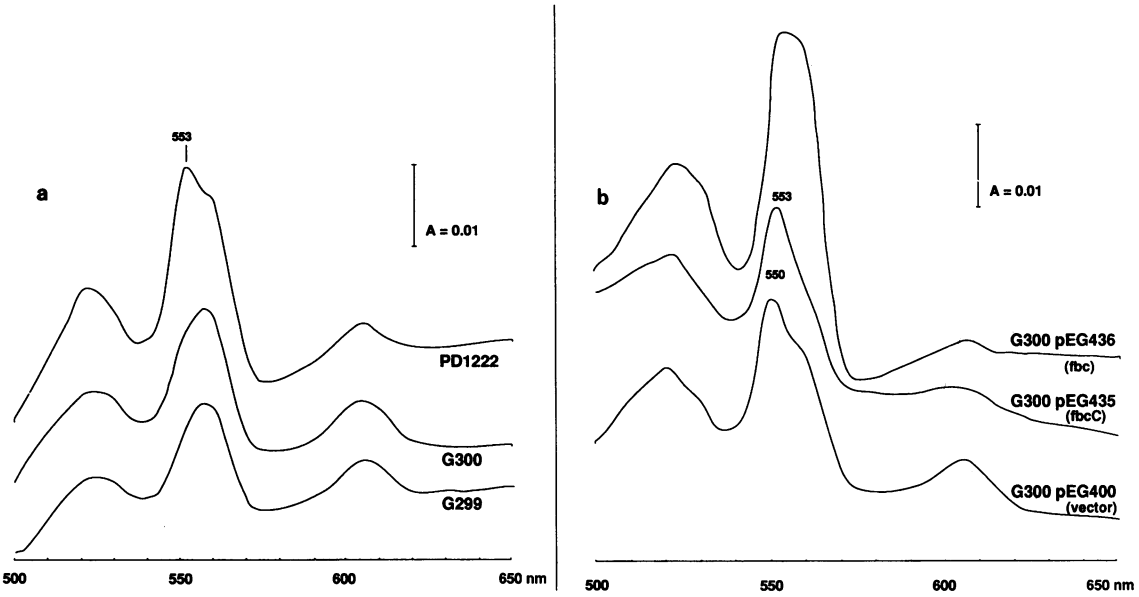


FIG. 3. Optical difference spectra of solubilized membranes isolated from parent, mutant, and reconstituted mutant strains. Membranes were isolated as described in Materials and Methods and solubilized with 2% Triton X-100, and their spectra were recorded from 650 to 500 nm, with the sample reduced by a grain of sodium dithionite, the reference left as isolated. For strain and plasmid designations, see Table 1 and Fig. 1. (a) Parent strain PD1222 and mutant strain G299 and G300 membranes at 6 mg/ml. (b) Mutant strain G300 carrying different plasmids for complementations; 5 mg of each membrane protein per ml.

wise, in addition to the missing heme cofactor, immunoblotting after SDS-PAGE with antibodies specific for the *Paracoccus* cytochrome c_1 revealed a complete loss of the polypeptide from mutant membranes (Fig. 5a) Immunological evidence shows that neither cytochrome c_1 nor fragment peptides could be observed in soluble cellular fractions (data not shown).

Cytochrome c_1 is an obligate component for electron transport through complex III, receiving electrons from the FeS center and donating them to a cytochrome *c* acceptor molecule, presumably the membrane-bound c_{552} (4). When isolated intact membranes of the mutants were assayed for NADH oxidase activity (Table 2), a drop in specific activity to about 4% compared with the parent strain membranes was observed. The residual activity was not further decreased by myxothiazol, a specific inhibitor of complex III (56). Table 2 also clearly shows that the NADH:cytochrome *c* limb of the chain was blocked by the mutation, whereas the cytochrome *c*:oxygen pathway (cytochrome *c* oxidase, complex IV) was more or less unaffected and functional; both parent strain and mutants oxidized reduced cytochrome *c* at about equal rates.

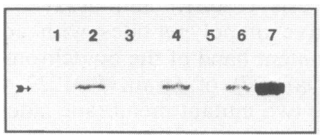


FIG. 4. Heme stain after SDS-PAGE of solubilized membranes of parent, mutant, and reconstituted mutant strains. SDS-solubilized membranes (100 μg each) after PAGE (12% acrylamide) were stained for covalently bound heme by the peroxidase assay (see Materials and Methods). Only the high-molecular-weight region of the gel is shown, and the position of cytochrome c_1 with an apparent molecular weight of 62,000 (27, 33) is marked by the arrow. Lanes 1 to 7: strains G299, G305 (resulting from single crossover event, see Results), G300, PD1222, G300(pEG400), G300(pEG435), and G300(pEG436), respectively (see also Fig. 3 for strain designations).

By using antibodies specific for the two other protein subunits of complex III, cytochrome *b* and the FeS protein, immunoblotting revealed that, under the conditions used here, these two components could not be detected in mutant membranes either (Fig. 5b), while traces were seen in corresponding supernatant fractions (not shown). This finding suggests that transcription/translation occurred at least to some extent for both genes, but both gene products were not inserted into or did not remain inserted in membranes with the cytochrome c_1 polypeptide missing (see also below).

Complementation of the mutation in *trans*. To be able to complement the mutation in *fbcC* in *trans*, a broad-host-range vector with an mcs was constructed to allow convenient insertion of various gene cassettes (pEG400, Fig. 1d; details of construction to be published elsewhere). Due to its type of incompatibility group (IncP [11]), this vector can be stably maintained and propagated in the *Paracoccus* host. This fact has been confirmed by Southern blotting and by transformation of *Escherichia coli* with *Paracoccus*-derived plasmid preparations (data not shown). As a marker, pEG400 carried the spectinomycin/streptomycin resistance gene (Sm^r/Spc^r). Since the *Paracoccus* host already has chromosomal spectinomycin resistance, we used streptomycin for plasmid selection. Two different cassettes were inserted into this vector (Fig. 1e and f): the *fbcC* structural gene along with a stretch of DNA representing the suspected promoter region upstream of *fbcF* (Fig. 1e and f), as well as the complete *fbc* operon (Fig. 1f).

Details of construction of *fbcC* gene cassette pEG435. To allow transcription of only *fbcC*, essential regions of *fbcF* and *fbcB* were deleted, leaving intact the assumed promoter upstream of *fbcF* (Fig. 1e).

The *Hin*dIII-*Sma*I fragment of clone 100 (27), *Hin*dIII site

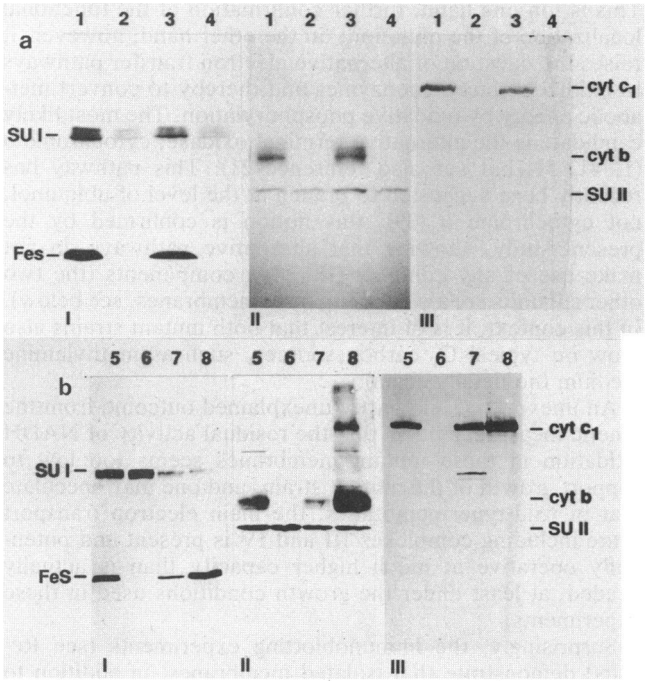


FIG. 5. Detection by immunoblotting of bc_1 complex subunits in parent, mutant, and complemented mutant strains. Specific polyclonal antibodies were used to detect the presence of complex III and IV subunits in membranes of the parent, mutant, and reconstituted mutant strains after SDS-PAGE and transfer to nitrocellulose membranes. Antibody binding was visualized by the protein A-alkaline phosphatase conjugate assay (see Materials and Methods). (I) A 1:1 mixture of antibodies (immunoglobulin fraction) specific for subunit I (SU I) of cytochrome *c* oxidase (apparent molecular weight, 45,000 [30, 42]) and for the FeS (Fes) subunit of complex III (20,000 [27, 60]) were used. (II) Antibody reaction, as above, against cytochrome *b* (cyt b) (39,000 [27, 60]) and subunit II (SU II) (28,000 [30, 42, 49]) of oxidase. (III) Antibodies directed against cytochrome c_1 (cyt c_1). (a) Lanes 1 to 4: membranes of strains PD1222, G299, G305 (resulting from single crossover event, see Results), and G300, respectively. (b) Lanes 5 to 8: PD1222, G300(pEG400), G300(pEG435), or G300(pEG436), respectively. For further details, see text.

on pBR322 to *Sma*I (nt 1337; see Fig. 1f), containing the proposed promotor region was subcloned into the *Hin*dIII-*Sma*I-digested pHP45 vector (40). In the resulting plasmid pEG148, the internal *Pvu*II-*Sal*I fragment was replaced by the 1.45-kb *Rsa*I-*Sal*I fragment (containing the *fbcC* gene lacking part of its 3′ end) from clone 250 (27), resulting in pEG149. By this, major parts of the *fbcF* and the *fbcB* genes are removed.

To subclone the missing 3′ end of the *fbc* operon for restoration of a functional *fbcC*, clone 250 DNA was isolated from GM2929 (*dam*; see Table 1) to allow restriction at the otherwise methylation-protected *Cla*I site (nt 3682). The 1.4-kb *Xho*I-*Cla*I fragment was subcloned into the mcs of pBluescript II SK$^-$. This plasmid, pEG150, supplies a 0.4-kb *Bam*HI-*Hin*dIII fragment, which represents the missing 3′ end of *fbcC*. It was subcloned into pUC13 with compatible ends. After *Sma*I-*Bam*HI digestion, the resulting plasmid pEG151 received the *Eco*RV-*Bam*HI fragment of pEG149 (see above), yielding pEG152. In this plasmid, the restored *fbcC* is now located downstream of the assumed promoter region.

The *Sst*I-*Hin*dIII fragment of plasmid pEG152 was removed as the final *fbcC* cassette for cloning into the suitably digested broad-host-range vector pEG400 (Fig. 1d, e, and f). The procedure described above also causes deletion of the tetracycline resistance gene promoter (located on the original pBR322 of plasmid clone 250) so that initiation of transcription should only be possible from the supposed *fbc* promoter region.

TABLE 2. Electron transfer rates in isolated membranes[a]

Strain	Sp act (mU/mg of protein)						
	NADH →→→ O_2			NADH →→ cyt *c*		Cyt *c* → O_2	
	None	Myx	KCN	None	Myx	None	KCN
PD1222 (parent)	645	50	4	617	24	758	6
G299 (insertion)	27	26	3	3	2	856	2
G300 (deletion)	29	23	3	4	3	730	4
PD1222	351	14	16	439		1,543	
G300(pEG435) (*fbcC*)	368	37	19	282		1,263	
G300(pEG436) (*fbc*)	130	32	11	454		743	

[a] Various sections of the respiratory chain were assayed spectroscopically at 25°C (number of arrows indicates number of respiratory complexes involved in parent strain), following the decrease in NADH (0.2 mM starting concentration) at 340 nm with a $\Delta\varepsilon$ of 6,300 M^{-1} cm^{-1}, recording reduction by NADH (0.2 mM) of 10 μM horse heart cytochrome *c* (cyt *c*) ($\Delta\varepsilon$ of 19,000 M^{-1} cm^{-1} at 550 nm) plus 1 mM KCN to inhibit terminal oxidases, and measuring oxidation of previously reduced cytochrome *c* (dithionite reduction followed by gel filtration on Sephadex G-25) at 25 μM and 550 nm, $\Delta\varepsilon$ as above. General assay conditions: potassium phosphate buffer, 20 mM, pH 7.5, containing 1 μM carbonylcyanide-*m*-chlorophenylhydrazone for uncoupling. Inhibitors used were myxothiazol (myx, 1 μM) and KCN (1 mM). Values are means of three determinations and given as specific activity. Due to different sets of cell and membrane preparations, the results in the upper half of the table are not strictly comparable to those in the lower half.

***fbc* operon cassette pEG436.** We started from the 3.5-kb *Eco*RV-*Bam*HI fragment of clone 100 (27), which comprises the complete *fbcF* and *fbcB* but only a fragment of *fbcC*. It was subcloned into *Sma*I-*Bam*HI-digested pEG150; the *Sst*I-*Hin*dIII fragment of this plasmid, pEG153, represents the *fbc* operon cassette for insertion into pEG400 (see Fig. 1e).

Complementation. When each construct was conjugated into each of the two mutants (G299 and G300), restoration of cytochrome c_1 expression was obtained. This was exemplified with the replacement mutant G300 harboring three different plasmids: the vector pEG400, the plasmid with the *fbcC* cassette (pEG435), and the plasmid with the complete *fbc* operon cassette (pEG436).

Figure 3b shows the reappearance of the cytochrome c_1 peak in the difference spectrum of membranes, Fig. 4b shows the positive heme stain, and Table 2 shows the restored enzymatic activity for the mutant complemented with the *fbcC* gene (pEG435). By immunoblotting (Fig. 5b), two features were evident: the relative amount of the cytochrome c_1 polypeptide roughly matched the wild-type level, and both cytochrome *b* and the FeS subunit could again be detected in the membrane fraction.

The same was essentially true for strain G300 carrying the complete operon structure in *trans* (pEG436); restoration of the mutation, but now accompanied by an elevated level of expression of all three subunits of complex III. Kinetic data (Table 2) showed restoration of enzymatic activity to about the wild-type level in the parent strain with respect to electron transport through complexes I and III. When the whole chain between NADH and oxygen (including complex IV) is considered, specific rates only reached about one-third of the comparable value; a similar reduction was also observed for electron transport by complex IV (cytochrome

aa_3) alone, which was only about 50% of that of the PD1222 level (see also Discussion).

DISCUSSION

Deletion of gene products of interest by insertional inactivation of their structural genes has been developed to become almost a standard procedure to obtain specific deletion mutants in several organisms, such as *E. coli*, *Saccharomyces cerevisiae*, and *Rhodobacter* (for some examples concerning respiratory enzyme subunits, see references 3, 5, 6, 7, 16, 18, 26, 28, and 46). For these organisms, efficient techniques for genetic transfer have been established to allow recombination processes to occur between plasmid-coded and genomically located gene copies. Availability of such mutants is a necessary prerequisite for in vitro mutagenesis experiments to avoid formation of hybrid protein complexes in the host organism.

Several reasons prompted us to attempt to establish *Paracoccus* as a homologous host for future expression and assembly studies of point-mutagenized gene products. (i) *E. coli*, although basically suitable because of lack of any bc_1 complex, is a questionable candidate for sustaining cofactor availability and insertion, in particular *c*-type heme (15). (ii) Since expression of the *fbc* operon may be regulated, a different genetic background could be disadvantageous. (iii) The GC content of *Paracoccus* DNA and promoter sequences differs widely from that of *E. coli* (see also below). On the other hand, as established recently (9), the *Paracoccus* wild-type strain, ATCC 13 543, used in previous studies, harbors a restriction-modification system(s), causing very low frequencies of exconjugates. For the present studies we therefore used strain PD1222, lacking the major restriction system (9).

To apply the above strategy for the generation of specific mutants in *P. denitrificans*, we constructed a plasmid derived from pSUP202 (Table 1) carrying a disrupted *fbcC* gene; inactivation was achieved by inserting the kanamycin resistance gene into the intact or previously truncated gene (see Fig. 1 and Results section). Upon conjugation into the *Paracoccus* host strain PD1222, homologous recombination can take place. Since this vector cannot replicate in *Paracoccus* spp., all kanamycin-resistant colonies should be products of recombination events between vector insert and genomic gene copy. Only those, however, which have lost their streptomycin resistance (conferred by the vector itself) simultaneously should represent double crossover events with a concomitant loss of all vector sequences. Specificity of this exchange (and exclusion of possible spontaneous kanamycin resistance) was verified by checking genomic DNA of the resulting mutant strains for loss of the streptomycin marker, presence of the kanamycin marker (not shown), and changes in the specific restriction enzyme pattern around the expected insertion locus by using hybridization with an *fbcC* gene probe (Fig. 2).

Both types of mutants, the insertion (G299) and the deletion-insertion (G300), exhibited the same phenotype, an expected complete loss of the *fbcC* gene product. This was shown unequivocally and independently by a number of techniques: loss of spectral properties and specific heme-staining ability, deficiency of the polypeptide as detected by antibodies, and loss of enzymatic properties (see Results section). This last criterion is of special interest; the mutation(s) caused a phenotype identical to that of an almost complete functional block in the electron transport between complexes I and IV as exerted by myxothiazol inhibition. This is, on one hand, further confirmation of the functional localization of the mutation; on the other hand, however, it raises the question of alternative electron transfer pathways to oxidize reduced coenzymes and thereby to convert metabolic energy by oxidative phosphorylation. The most likely candidate is the alternative terminal oxidase, cytochrome *o* (1, 41, 51; but see also reference 21). This pathway has recently been suggested to branch at the level of ubiquinol, not cytochrome *b* (39); this notion is confirmed by the present study, showing that alternative pathways do not make use of any complex III redox components (the two other subunits are also missing from membranes, see below). In this context, it is of interest that both mutant strains also grow on typical C_1 carbon sources, such as methylamine medium (no details given).

An unexpected and partly unexplained outcome from the kinetic measurements is that the residual activity of NADH oxidation in these mutant membranes seems too low to support growth of the mutant strain, and one may speculate that in wild-type membranes, the main electron transport route including complexes III and IV is present and potentially operative at much higher capacity than is actually needed, at least under the growth conditions used in these experiments.

Surprisingly, the immunoblotting experiments (see Results) demonstrate that isolated membranes, in addition to the missing cytochrome c_1, are also devoid of both of the two other protein components of complex III, cytochrome *b* and the FeS subunit, traces of which are recognized by specific antibodies in the supernatant fraction. This suggests that expression is basically still operative in these mutants, but, with the cytochrome c_1 polypeptide missing, membrane insertion and partial assembly to a membrane protein complex is defective, leaving these two subunits prone to proteolytic degradation. A similar assembly defect has recently been observed for the *Paracoccus* cytochrome *c* oxidase complex (16a; P. Steinrücke et al., unpublished).

The possible additional role of the cytochrome c_1 as a scaffold for assembly and/or as a protection against proteolytic attack of the other two subunits in the assembly process is in agreement with the results of the complementation experiment. The chromosomal genetic setup (in strain G300 with the *fbcC* gene cassette in *trans*) is that of the mutant, so that transcription of the *fbcF* and *fbcB* genes is physically separated from and independent of that of the *fbcC* gene on the plasmid. Due to the elevated copy number of the plasmid-coded *fbcC* gene, the most likely explanation is that the *fbcC* gene product is expressed at higher levels than the two other subunits coded on the genome. This could lead to two effects: on the one hand, cytochrome *b* and the FeS subunit assemble together with stoichiometric amounts of the cytochrome c_1 into a stable complex III in the membrane, whereas on the other hand any cytochrome c_1 in excess might be degraded proteolytically. Evidence for this is seen in Fig. 5b, lane 7, where the antibody recognized not only the authentic cytochrome c_1, but also a lower-molecular-weight band.

In the cytochrome c_1 mutant membranes, the additional loss of the two other components of the complex therefore cannot be explained by a potential interference in expression due to different directions of transcription of the kanamycin gene and of the first two genes of the *fbc* operon.

When complementation is performed with the complete *fbc* operon present on the vector, a severalfold overexpression of all three bc_1 components is observed. This may most easily be explained by the gene dose effect due to higher

plasmid copy numbers. Whether or not these overexpressed subunits, which by spectral evidence are fully associated with their heme cofactors, are indeed completely assembled into a functional bc_1 complex cannot be decided unequivocally on the basis of the kinetic data presented in Table 2 and may require more detailed kinetic and structural studies. Rates of NADH:cytochrome *c* oxidation show restoration to about parent-strain activity. If complex I were the rate-limiting electron transport component, any further increase in enzymatic activity due to overexpressed and correctly assembled complex III subunits above wild-type level would not show up. Considering all three complexes (I, III, and IV) in the overall reaction from NADH to oxygen, these membranes did not even reach parent strain activities. A likely explanation for this situation seems to be a limiting activity of complex IV (cytochrome *c* oxidase) (Table 2), matching the spectroscopic (Fig. 3b) and immunological (Fig. 5b) observation of reduced levels of aa_3 subunits under these complementation conditions. We explain this by a relative deficiency in heme biosynthesis and therefore a competition for heme precursor compounds in these cells.

The successful complementation experiments also illustrate that the DNA sequence insert in plasmid pEG435 (Fig. 1e) carries the signals essential to initiate transcription. *Paracoccus* promoter sequences are not well characterized at present (50), but appear to be different from typical *E. coli* sequences. For testing promoter activity of the short sequence upstream of the start of the *fbcF* gene (Fig. 1f), previous preliminary experiments had shown independently that a plasmid-borne *fbcF*::*lacZ'* gene fusion is expressed in *P. denitrificans* but not in *E. coli* (details not given).

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