JOURNAL OF BACTERIOLOGY, Jan. 2001, p. 257–263 Vol. 183, No. 1
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.1.257–263.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Identification of *ccdA* in *Paracoccus pantotrophus* GB17: Disruption of *ccdA* Causes Complete Deficiency in *c*-Type Cytochromes

FRANK BARDISCHEWSKY AND CORNELIUS G. FRIEDRICH*

Lehrstuhl für Technische Mikrobiologie, Fachbereich Chemietechnik, Universität Dortmund, D-44221 Dortmund, Germany

Received 13 June 2000 /Accepted 5 October 2000

A transposon Tn*5-mob* insertional mutant of *Paracoccus pantotrophus* GB17, strain TP43, was unable to oxidize thiosulfate aerobically or to reduce nitrite anaerobically, and the cellular yields were generally decreased by 11 to 20%. Strain TP43 was unable to form functional *c*-type cytochromes, as determined by difference spectroscopy and heme staining. However, formation of apocytochromes and their transport to the periplasm were not affected, as seen with SoxD, a *c*-type cytochrome associated with the periplasmic sulfite dehydrogenase homologue. The Tn*5-mob*-containing DNA region of strain TP43 was cloned into pSUP205 to produce pE18TP43. With the aid of pE18TP43 the corresponding wild-type gene region of 15 kb was isolated from a heterogenote recombinant to produce pEF15. Sequence analysis of 2.8 kb of the relevant region uncovered three open reading frames, designated ORFA, *ccdA*, and ORFB, with the latter being oriented divergently. ORFA and *ccdA* were constitutively cotranscribed as determined by primer extension analysis. In strain TP43 Tn*5-mob* was inserted into *ccdA*. The deduced ORFA product showed no similarity to any protein in databases. However, the *ccdA* gene product exhibited similarities to proteins assigned to different functions in bacteria, such as cytochrome *c* biogenesis. For these proteins at least six transmembrane helices are predicted with the potential to form a channel with two conserved cysteines. This structural identity suggests that these proteins transfer reducing equivalents from the cytoplasm to the periplasm and that the cysteines bring about this transfer to enable the various specific functions via specific redox mediators such as thioredoxins. CcdA of *P. pantotrophus* is 42% identical to a protein predicted by ORF2, and its location within the *sox* gene cluster coding for lithotrophic sulfur oxidation suggested a different function.

The neutrophilic facultatively lithoautotrophic gram-negative bacterium *Paracoccus pantotrophus* grows aerobically with a large variety of carbon sources and with molecular hydrogen or thiosulfate as energy source, and nitrate serves as electron acceptor under anaerobic conditions (41). *P. pantotrophus* (formerly *Paracoccus denitrificans* and *Thiosphaera pantotropha* [31, 38, 41]) has a branched electron transfer network. The electron flux is regulated by the reduction of the Q pool (33). While the transfer of electrons by the terminal cytochrome oxidases aa_3 and cbb_3 results in a translocation of six protons ($6H^+/2e^-$) (36, 54), the reduction of a terminal electron acceptor by the quinol oxidase *o* results in a translocation of only four protons ($4H^+/2e^-$) (39).

To identify the components involved in energy transformation by oxidation of reduced inorganic sulfur compounds (Sox) of *P. pantotrophus* GB17, genetic studies were initiated by the isolation of mutants. Transpositional mutagenesis with Tn*5-mob* coding for kanamycin resistance yielded mutants defective in Sox. One mutant, TP43, is unable to grow with thiosulfate (Sox^-) or reduce nitrite under anaerobic conditions ($Nitd^-$), while reduction of nitrate to nitrite is not affected (11).

Cytochromes of the *c* type are distinguished from cytochromes of other classes by the covalent attachment of their prosthetic heme group to the conserved CXXCH motif of the apoprotein. The biogenesis of *c*-type cytochromes consists of several steps performed in the cytoplasm and in the periplasm (reviewed in reference 53). Two different systems with respect to the late steps of cytochrome *c* biogenesis have been described for bacteria (reviewed in reference 28). Most gram-positive bacteria possess the class II system, consisting of at least 4 proteins (ResA, CcsA, CcsI, and CcdA), while most gram-negative bacteria harbor the class I system, consisting of at least 11 proteins (DsbABD and CcmABCDEFGH). In gram-negative bacteria the addition of the heme group to the apoprotein takes place in the oxidative environment of the periplasm, where disulfide bonds between pairs of protein cysteine thiols are generated by the DsbA/B system (4). Thus, cytochrome *c* apoproteins have to be rereduced prior to heme binding. Recently three proteins, CcmH, CcmG, and DsbD, were proposed to be responsible for this step in cytochrome *c* maturation in *Escherichia coli* (15). According to this proposal, the oxidized cytochrome *c* apoprotein is reduced by CcmH and CcmG and the latter is rereduced by DsbD. In *Rhodobacter capsulatus*, CcdA has been described as homologous to DsbD of *E. coli*, having similar predicted structural characteristics and essential cysteine residues (14). In *P. pantotrophus* within the *sox* gene cluster coding for lithotrophic sulfur oxidation, ORF2 has been identified, with a predicted product that is about 35% identical to CcdA of *Bacillus subtilis* and about 45% identical to CcdA of *R. capsulatus*. Eight genes required for cytochrome *c* biogenesis including *ccmG* have been identified

* Corresponding author. Mailing address: Lehrstuhl für Technische Mikrobiologie, Fachbereich Chemietechnik, Universität Dortmund, D-44221 Dortmund, Germany. Phone: (49)-231–755 5115. Fax: (49)-231–755 5118. E-mail: friedric@ct.uni-dortmund.de.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant geno- or phenotype[a]	Reference or source
E. coli		
S17-1	*recA pro thi hsdS*, RP4 *tra* functions, *supE44*	48
XL1-Blue	*hsdR17 recA1 endA1 gyrA46 thi relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet^r)]	9, Stratagene
P. pantotrophus		
GB17	Sox$^+$ Hox $^+$	31, 38, 41; L. A. Robertson
TP43	Sox$^-$ Nitd$^-$ cyt *c*$^-$	11; this study
TPX18	Sox$^+$ Nitd$^+$ cyt *c*$^+$ Km^r Tc^r, heterogenote of GB17	This study
TP43(pVK1)	Sox$^+$ Nitd$^+$ cyt *c*$^+$ Tc^r	This study
Plasmids		
pBluescript SK$^-$	Ap^r *lacZ*, f1 ori, T7 Phil 10 promoter	Stratagene
pSUP205	Cm^r Tc^r Tra$^-$ Mob$^+$	48
pVK101	Km^r Tc^r Tra$^-$ Mob$^+$	27
pE18TP43	18-kb *Eco*RI fragment containing Tn*5-mob* from TP43 in pSUP205	This study
pEF15	15.2-kb *Eco*RI wild-type fragment extending the corresponding insert of pE18TP43 by 1.5 kb	This study
pBSK$^-$XP6.8	6.8-kb *Xho*I-*Pst*I fragment from pEF15 in pBSK$^-$	This study
pBSK$^-$B3.8	3.8-kb *Bam*HI fragment from pEF15 in pBSK$^-$	
pBSK$^-$*ccdA*	744-bp PCR amplification product homologous to *ccdA* in pBSK$^-$	This study
pVK1	744-bp PCR amplification product homologous to *ccdA* in pVK101	This study

[a] Sox, lithotrophic growth with thiosulfate; Hox, lithotrophic growth with molecular hydrogen; Nitd, dissimilatory nitrite reduction; Tra, transfer of mobilizable plasmids; Mob, mobilizability; cyt, cytochrome.

so far in *P. denitrificans*. CcmG of *P. denitrificans* was proposed to reduce disulfide bonds in vivo (34) and may have the same function in cytochrome *c* biogenesis as that proposed for CcmG of *E. coli*.

To determine the nature of the mutation in the Sox$^-$ mutant TP43, we have reexamined its pleiotropic character and have identified the complete absence of *c*-type cytochromes in this strain. We have cloned the Tn*5-mob*-containing DNA region of strain TP43 (11) and have isolated and sequenced the corresponding wild-type gene region. Below we describe a new gene, *ccdA*, of *P. pantotrophus* GB17 which is essential for cytochrome *c* biogenesis and which is suggested to be involved in the transfer of reducing equivalents from the cytoplasm to the periplasm.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used and constructed in this study are listed in Table 1.

Media and growth conditions. Mineral media were identical for heterotrophic and lithotrophic growth of *P. pantotrophus* and were described previously (11). *P. pantotrophus* was cultivated at 30°C. For mixotrophic growth with thiosulfate, mineral media contained 20 mM sodium succinate and 20 mM sodium thiosulfate at an initial pH of 8. For anaerobic growth, bacteria were cultivated in Luria-Bertani broth containing 0.1% (wt/vol) sodium nitrite or 0.1% (wt/vol) potassium nitrate. The following antibiotics were used when appropriate for *P. pantotrophus* GB17: kanamycin (KM) at 300 μg/ml, tetracycline (TC) at 5 μg/ml, and chloramphenicol (CM) at 5 μg/ml. For *E. coli* the antibiotics were KM at 50 μg/ml, ampicillin (AP) at 50 μg/ml, TC at 12.5 μg/ml, and CM at 30 μg/ml. Antimycin A was used at a concentration of 50 μg/ml. Cellular yields of *P. pantotrophus* were determined as described for *Ralstonia eutropha* (19).

Enzyme assays. Whole cells were used for enzyme assays. Thiosulfate oxidizing activity was determined with an oxygen electrode as described elsewhere (56). *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity was measured in an oxygen electrode in the presence of 1 mM TMPD. One unit of enzyme activity was defined as 1 μmol of substrate converted per min at 30°C.

Immunoblot analysis. The level of Sox-specific *c*-type cytochrome SoxD and the purity of periplasmic extracts were determined by immunoblot analysis as described elsewhere (56). Antibodies against SoxD antigens were obtained against the oligopeptide FYPDDRDQTEYPLF, deduced from the *soxD* nucleotide sequence. Antibodies were raised in rabbits at the facilities of Eurogentec (Seraing, Belgium). Antibodies against ribulose-bisphosphate carboxylase of *R. eutropha* were obtained from B. Bowien, Goettingen, Germany (7).

Cytochrome analysis. Cytochromes of *P. pantotrophus* GB17 and its derivative strain TP43 were analyzed by using cell extracts. Cells were cultivated as specified below, washed twice at 0°C, and resuspended in 10 mM HEPES buffer, pH 7.4. Cells were disrupted by French press, and cell extracts were obtained from the 30,000 × *g* supernatant (16). For analysis of *c*-type cytochromes from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were prepared without boiling. Cytochromes were stained as described by Francis and Becker (18). For identification of the cytochromes, cell extracts (10 mg of protein/ml) were subjected to redox difference spectroscopy. Cell extracts reduced by addition of solid sodium dithionite were measured at 20°C against air-oxidized extracts with a Shimadzu UV 160 A spectrophotometer. Cytochromes were quantified from the 30,000 × *g* supernatant by the pyridine extraction procedure (5).

Periplasmic proteins were selectively extracted from whole cells by the chloroform procedure described in reference 3. Periplasmic extracts were examined for contamination by cytoplasmic proteins by immunoblot analysis of ribulose-bisphosphate carboxylase antigens.

Analytical procedures. Formate, thiosulfate, and nitrite were quantified by colorimetric methods. Formate was measured by the procedure described by Lang and Lang (30), thiosulfate was measured by the procedure described by Sørbø (49), and nitrite was measured by the sulfanilamide method (23). Protein from cell extracts was quantified by the procedure described by Bradford (8).

DNA and RNA techniques. Standard DNA techniques (43) were used. Plasmid DNA was isolated by the procedure described by Birnboim and Doly (6) or by using the high pure-plasmid isolation kit (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. Total RNA was isolated as described elsewhere (24) or by using the high pure-RNA isolation kit of Boehringer. DNA sequencing was performed by primer walking with the thermostable DNA polymerase of *Thermus aquaticus* and 7-deaza-dGTP (Amersham-Buchler, Braunschweig, Germany) by the dideoxy-chain termination method (44) with an automated DNA sequencing system (Li-Cor; MWG-Biotech, Munich, Germany). The plasmids pBSK$^-$XP6.8 and pBSK$^-$B3.8, used for sequencing, are described in Table 1.

For primer extension experiments *P. pantotrophus* strains were grown heterotrophically with glucose in mineral media as described above. RNA was isolated from cells in the late exponential growth phase and quantified at 260 nm. Primers were designated PE1 (5′-CGGTCATATAGGCCAGATAGGGG-3′), complementary to bases 1204 to 1227; PE2 (5′-GTGTCCAGGAAGGGGATGCGGAT-3′), complementary to bases 1436 to 1452; PE3 (5′-CCTCGGCGGGTTCCTCGTCATCG-3′), complementary to bases 261 to 284; and PE4 (5′-CGGCGCGGTCTTCCTCCCCGGCG-3′), complementary to bases 300 to 322. The

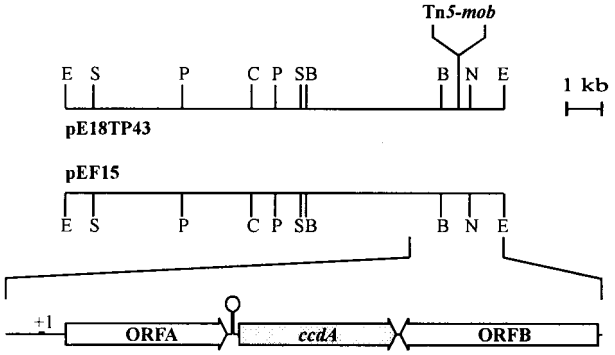

FIG. 1. Physical map of the insert DNAs of the plasmids pE18TP43 and pEF15 and ORFs within the 2.8-kb *ccdA* gene region. E, *Eco*RI; S, *Sma*I; P, *Pst*I; C, *Cla*I; B, *Bam*HI; N, *Not*I.

oligonucleotides were labeled at the 5′ end with the fluorescent dye IRD-800 (MWG Biotech). One picomole of primer was annealed to 1 μg of total RNA and extended for 1 h at 42°C by using reverse transcriptase of a variant of Moloney murine leukemia virus (Boehringer) according to the manufacturer's protocol. The primer extension products were analyzed with a sequencing gel as described above.

Sequence analysis was done with the DNASIS (Hitachi Software Engineering, San Bruno, Calif.) and PC/GENE (IntelliGenetics Inc., Mountain View, Calif.) software packages. Nucleotide and amino acid homology searches were done with the BLAST algorithm (2). Higher-order structure analysis of amino acid sequences was done with the PROSIS (Hitachi Software Engineering) and TMpred (25) software packages.

Construction of pEF15. The Tn*5-mob*-containing *Eco*RI fragment of strain TP43 was cloned in pSUP205 at the facilities of Biodelta (Bad Oeynhausen, Germany). Total DNA of strain TP43 was digested with *Eco*RI. Fragments of 18 to 6 kb were separated by agarose gel electrophoresis and purified with the extraction kit Jetsorb (Genomed, Bad Oeynhausen, Germany). Fragments were ligated with pSUP205, and the resulting plasmids were transformed into *E. coli* XL1-Blue. Transformants exhibiting the Tc^r, Km^r, Ap^r, and Cm^S phenotypes were examined for hybrid plasmids. One clone contained the 18-kb hybrid plasmid pE18TP43. The insertion of Tn*5-mob* was verified by Southern hybridization, and its location was determined by physical mapping as well as by DNA sequencing (Fig. 1).

For isolation of the respective wild-type gene region, plasmid pE18TP43 was transformed into *E. coli* S17–1 and conjugated therefrom into *P. pantotrophus* GB17. Heterogenote recombinants such as strain TPX18 exhibiting the Km^r Tc^r phenotype were isolated. Total DNA of strain TPX18 was partially digested with *Eco*RI, religated, and transformed into *E. coli* S17–1. Transformants exhibiting the Km^S Tc^r phenotype were examined for hybrid plasmids by colony hybridization using the 1,560-bp *Cla*I-*Bam*HI fragment of pE18TP43. Positive clones were screened for insert sizes, and plasmid pEF15 was selected (Table 1).

Construction of pVK1 to complement *ccdA*::Tn*5* in TP43. Primer C1 (5′-ATGTTGGGAATCGAGCTTGCA-3′) and primer C2 (5′-CTAACCCAGCGTGGCCAGCCA-3′) were used to amplify the *ccdA* gene region by PCR (32). The resulting PCR product was cloned into the *Eco*RV site of pBSK$^-$ to produce pBSK−*ccdA*. The insert DNA was then isolated by *Eco*RI and *Hin*dIII and cloned into the plasmid pVK101. The resulting plasmid pVK1 was transformed into *E. coli* S17–1 and conjugated therefrom into the mutant strain TP43 to produce *P. pantotrophus* TP43(pVK1).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the EMBL and GenBank databases under accession number AF308446.

RESULTS

Physiological characterization of strain TP43. Strain TP43 is unable to grow lithotrophically with thiosulfate and unable to reduce nitrite anaerobically (11). The pleiotropic character may have resulted from a mutation in a regulatory mechanism or may have been related to the energy metabolism. To distinguish between these possibilities, the cellular yields were determined. When cultivated with glucose, glyoxylate, or formate, respectively, strain TP43 exhibited generally lower cellular yields of 11.3 to 20.7% compared to the wild type (Table 2). The reduced yield was evidence that the mutation (i) was not restricted to the phenotypes described initially (11) but was a general characteristic of strain TP43 and (ii) was linked to the energy metabolism. Therefore, the effect of antimycin A, an inhibitor of the electron transfer to the bc_1 complex of the respiratory chain in various bacteria (reviewed in reference 40), was examined under anaerobic growth conditions with nitrate as electron acceptor. These conditions allowed the determination of whether *c*-type or other types of cytochromes were affected. Nitrate reduction does not require *c*-type cytochromes in *P. denitrificans*, whereas nitrite reduction involves different *c*-type cytochromes linked to the bc_1 complex, nitrite reductase, nitric oxide, and nitrous oxide reductase (10, 52). Under anaerobic conditions in the presence of antimycin A the yield of the wild type was reduced by 55% and the specific growth rate was reduced from 0.38 to 0.25 per h. The extent and the rate of growth of strain TP43 without antimycin A were identical to those of the wild type cultivated with antimycin A, and addition of the drug did not affect the growth characteristics of TP43 (data not shown). Under anaerobic growth conditions addition of antimycin A to the wild type allowed quantitative reduction of nitrate; however, nitrite was not metabolized further (data not shown). These results pointed to the inability of strain TP43 to form a component essential for the cytochrome bc_1 complex or the electron flow via this complex. This conclusion was supported by the complete absence of TMPD oxidase activity in strain TP43 (Table 2). For oxidation of TMPD, *c*-type cytochromes are essential (26).

Addition of hemin (45 μM) to glucose mineral medium led to an increase in the concentration of *b*-type cytochromes but did not enable strain TP43 to form *c*-type cytochromes or to grow anaerobically with nitrite (data not shown). This suggested that hemin was taken up by the cell and that heme deficiency was not responsible for the phenotype of TP43.

Cytochrome *c* analysis. To analyze components involved in electron transport, cytochromes were analyzed for *P. pantotrophus* GB17 and TP43. Redox difference spectroscopy of extracts from glucose-grown cells of *P. pantotrophus* GB17 showed an absorption maximum at 551 nm, diagnostic of *c*-type cytochromes, while extracts from glucose-grown cells of TP43 showed an absorption maximum at 560 nm, typical of *b*-type cytochromes (Fig. 2). Quantitative analysis of pyridine hemochromes (5) from the wild type and strain TP43 revealed a maximum concentration of 1.83 μmol of cyto-

TABLE 2. Growth yields of *P. pantotrophus* GB17 and strain TP43 with different carbon sources[a] and specific TMPD oxidase activity

Strain	Y_S[b]			TMPD oxidase[c]
	Glucose	Glyoxylate	Formate	
GB17	0.627	0.118	0.080	1.0
TP43	0.546	0.105	0.064	0
TP43(PVK1)	0.620	0.120	0.078	0.95

[a] Media and growth conditions are given in the text.
[b] In grams (dry weight) per gram of substrate.
[c] O_2 consumption (in micromoles per minute per milligram of protein). Cells were grown with glucose as carbon source.

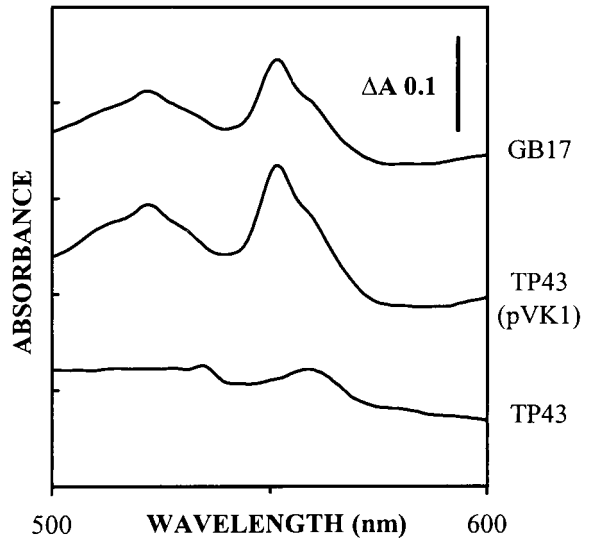

FIG. 2. Visible absorption spectra of total soluble cell extracts from *P. pantotrophus* GB17, strain TP43, and strain TP43(pVK1). The cuvettes contained 10 mg of protein per ml. The samples were reduced by addition of solid sodium dithionite, and spectra were measured against air-oxidized references.

chrome *c* per g of protein for the wild type, while only traces (0.09 μmol of cytochrome *c* per g of protein) were detected from strain TP43. Using this method, the concentrations of *b*-type cytochromes (5) from the wild type and strain TP43 were identical (0.3 μmol per g of protein; data not shown). The *c*-type cytochromes observed for strain TP43 were less than 5% of those for the wild type. This amount was attributed to the method of detection and its interference with the spectrometric determination of *b*- and *a*-type cytochromes as previously discussed (5).

Using heme staining (18), major *c*-type cytochromes were detected for crude extracts of the wild-type GB17 after separation of proteins by SDS-PAGE while no *c*-type cytochromes were detected for extracts of strain TP43 (Fig. 3). Therefore, *c*-type cytochromes were absent in strain TP43.

Cloning and physical map of the *ccdA* gene region. The wild-type gene region of 15 kb was cloned as described in Materials and Methods and was physically mapped. The map was compared with the Tn*5-mob*-containing insert cloned in pE18TP43. Identical restriction sites were obtained within the 12.5-kb *Eco*RI fragments of the two plasmids. The position of Tn*5-mob* mapped between the *Eco*RI and *Bam*HI sites of pE18TP43. The precise position of the Tn*5-mob* inverted-repeat chromosome junction was determined by sequencing to be between nucleotides 1697 and 1698 (Fig. 1).

Sequence analysis. The nucleotide sequence of 2.8 kb was determined for both strands starting from the *Eco*RI restriction site. The sequence was consistent with the restriction enzyme cleavage map of pEF15. Analysis of this sequence revealed three open reading frames (ORFs) designated ORFA, *ccdA*, and ORFB. These ORFs revealed coding characteristics according to codon preference analysis (50). ORFA was separated from *ccdA* by 48 nucleotides. Within this stretch was located an inverted repeat of 23 nucleotides with the potential to form a hairpin structure with a free energy of formation of −100.4 kJ/mol. ORFB was separated from *ccdA* by 9 bp and transcribed in the opposite direction.

ORFA predicted a protein of 251 amino acids (27,995 Da). A well-conserved ribosome binding site was located eight nucleotides before the start codon. The putative ORFA gene product was hydrophilic, with a hydropathy index of −1.55, and exhibited a very alkaline pI of 12.95 (29). The predicted protein had a high content of 54 proline residues distributed over the whole sequence and accumulating at the C terminus (data not shown). Amino acid sequence analysis suggested only a weak possibility for a signal peptide of 20 amino acids, consistent with the −3/−1 rule (55). Since the stretch of hydrophobic amino acids was missing, it was questionable whether the ORFA gene product was located in the periplasm. Amino acid sequence comparison of the deduced ORFA gene product revealed no significant similarity to other proteins.

The *ccdA* gene predicted a protein of 247 amino acids (25,838 Da). A putative ribosome binding site was located six nucleotides 5′ of the start codon. The *ccdA* gene product was highly hydrophobic, with an overall hydropathy index of +1.05 (29). The pI was 9.25. The deduced CcdA exhibited an identity of 55% to proteins involved in cytochrome *c* biogenesis of *R. capsulatus* (14) or 32% to a protein involved in sporulation of *B. subtilis* (46) or in heavy metal resistance of different gram-negative bacteria (22, 47), while an identity of 42% was observed to the ORF2 gene product of the *sox* gene cluster of *P. pantotrophus* (data not shown). Therefore, the CcdA protein was analyzed in detail. Amino acid sequence analysis suggested six transmembrane helices of 19 to 26 amino acids each. These helices were separated by segments of 7 to 25 amino acids. According to the algorithm used (25) the amino- and carboxy-terminal ends faced the periplasm. Helix 1 and helix 4 each contained one highly conserved cysteine residue flanked by conserved proline residues previously described for a putative protein disulfide reductase of *Pseudomonas aeruginosa* (35). The essential function of these cysteines of CcdA of *R. capsu-*

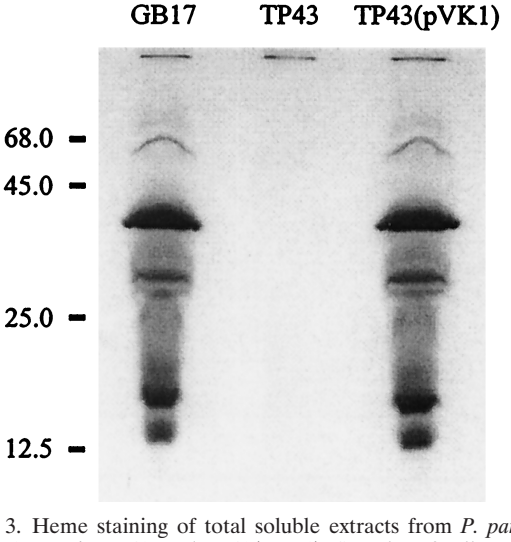

FIG. 3. Heme staining of total soluble extracts from *P. pantotrophus* GB17, strain TP43, and TP43(pVK1). Samples of cell extract of each strain (0.3 mg of protein) were subjected to SDS-PAGE without boiling.

latus related to DipZ of *P. aeruginosa* with respect to transport of reductant from the cytoplasm to the periplasm has recently been demonstrated (14).

ORFB predicted a protein of 273 amino acids (29,015 Da) with a well-conserved putative ribosome binding site six nucleotides 5′ of the start codon. The predicted protein was slightly hydrophobic, with a hydropathy index of +0.06 and a pI of 4.87 (29). Amino acid sequence comparison of the deduced ORFB gene product revealed an identity of 30% to a putative enoyl coenzyme A hydratase of *E. coli* (1).

Complementation of the mutation in strain TP43. To analyze if the insertion of the transposon Tn*5-mob* in *ccdA* caused any polar effects which could have been responsible for the pleiotropy of TP43, plasmid pVK1 was constructed. The *ccdA* gene region was cloned into the vector pVK101 and used for complementation. Strain TP43 harboring plasmid pVK1 in *trans* was able to grow aerobically with thiosulfate and anaerobically with nitrite (data not shown). When cultivated heterotrophically with glucose, glyoxylate, or formate, the cellular yields of TP43(pVK1) were similar to those of the wild type (Table 2). Spectrophotometric analysis demonstrated that the ability to form holo- *c*-type cytochromes was fully restored (Fig. 2), as was also evident from heme staining (Fig. 3). These data were convincing evidence that the pleiotropy of strain TP43 resulted from the inactivation of *ccdA*.

Transcription of ORFA and *ccdA*. Transcription of ORFA started with an adenine 114 nucleotides before the translation start codon as determined by primer extension analysis using the oligonucleotides PE3 and PE4 (data not shown). Using the oligonucleotides PE1 and PE2, complementary to the beginning of the subsequent *ccdA* gene, the signal appeared at sizes of about 1,000 and 1,200 nucleotides, respectively (data not shown). This result was evidence that ORFA and *ccdA* were cotranscribed. Constitutive expression of ORFA and *ccdA* was deduced from primer extension analysis using oligonucleotide PE3. Signals were equally intense in cells grown aerobically with glucose or succinate and cells grown anaerobically with nitrate as electron acceptor (data not shown).

Biochemical analysis of the *ccdA* function. To determine the function of *ccdA* expression of SoxD, a *c*-type cytochrome was monitored. The *c*-type cytochrome SoxD is associated with a sulfite dehydrogenase homologue, located in the periplasm and beneficial for thiosulfate oxidation (37, 56). SoxD antigens were formed by strain TP43 when cultivated in the presence of thiosulfate, demonstrating that expression of the *sox* genes was not affected by the mutation. Moreover, SoxD antigens were detected in the periplasm using the chloroform procedure which specifically extracts proteins therefrom (Fig. 4). This result demonstrated that the *ccdA* gene was not involved in the transport of the apoprotein SoxD to the periplasm but was involved in a late step of cytochrome *c* maturation.

A mutation in the *dipZ* gene of *E. coli* inactivates DipZ, and this inactivation has been overcome by adding compounds containing thiol groups (42). In *P. pantotrophus* addition of cysteine, cystine, or other thiols did not compensate the mutation described for strain TP43.

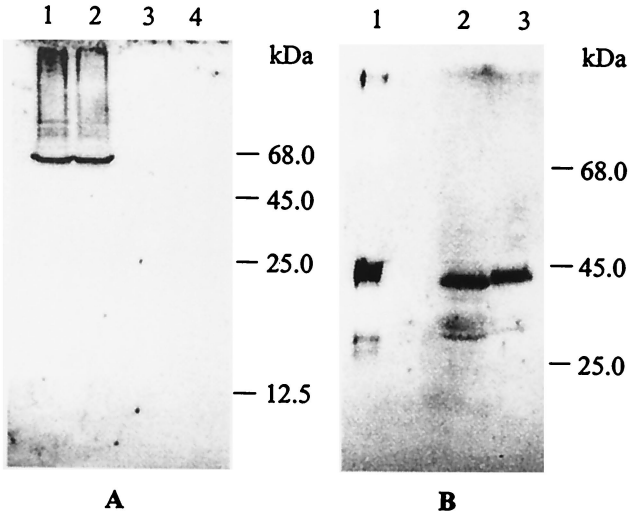

FIG. 4. Western blot analysis after SDS-PAGE of cell extracts of *P. pantotrophus* GB17 and strain TP43 for ribulose-bisphosphate carboxylase (A) and cytochrome SoxD antigens (B). (A) Proteins (15 μg) of the cytoplasmic and periplasmic fractions of strain GB17 (lanes 1 and 3, respectively) and strain TP43 (lanes 2 and 4, respectively). (B) homogenous SoxD (2 μg; lane 1), periplasmic fraction of strain GB17 (10 μg of protein; lane 2), and periplasmic fraction of strain TP43 (20 μg of protein; lane 3).

DISCUSSION

Three new ORFs, ORFA, *ccdA*, and ORFB, were identified for the genome of *P. pantotrophus* GB17. ORFA and *ccdA* were constitutively expressed and cotranscribed. Disruption of *ccdA* by transposon Tn*5-mob* caused inability to form functional *c*-type cytochromes. Convincing evidence for the requirement of *ccdA* in cytochrome *c* biogenesis was obtained from (i) physiological studies, (ii) complementation analysis, (iii) biochemical and immunochemical analysis, and (iv) sequence analysis of the deduced *ccdA* gene product.

Strain TP43 not only was impaired in aerobic thiosulfate oxidation or dissimilatory nitrite reduction but also exhibited significantly reduced cellular yields compared to the wild type. The reduction in cellular yields was evidence of a general defect in energy metabolism. This evidence was supported by the inability of strain TP43 to oxidize TMPD and the absence of any effect of antimycin A, an inhibitor of electron transfer to the bc_1 complex, which caused reduction in the cellular yield of the wild type but not in that of strain TP43. Without antimycin A the yield of strain TP43 was identical to that of the wild type grown in the presence of antimycin A. This was evidence that electron transfer was abolished from ubiquinone to the bc_1 complex and further to cytochrome c_{551} (40).

Analysis of cytochromes revealed the complete absence of holocytochromes *c*, whereas apoproteins of *c*-type cytochromes were still formed in strain TP43, as demonstrated by the immunochemical detection of SoxD, the periplasmic *c*-type cytochrome which is part of the sulfur-oxidizing enzyme system in *P. pantotrophus* GB17 (37, 56). Moreover, the SoxD apoprotein was transported to the periplasm as demonstrated by its specific extraction and by immunochemical analysis. Therefore, disruption of *ccdA* eliminated an essential late step in

cytochrome *c* biogenesis. This in turn affected energy conservation and caused reduced cellular yields.

To exclude a polar effect of the Tn*5-mob* which could have been responsible for the pleiotropy of strain TP43, the *ccdA* gene region was cloned into pVK101. TP43 harboring pVK1 was fully restored in its ability to form *c*-type cytochromes. This demonstrated that the inactivation of only *ccdA* caused the inability to form holo- *c*-type cytochromes, and thiol compounds could not compensate this defect as described for *E. coli* (42).

CcdA of *P. pantotrophus* is homologous to proteins of other bacteria involved in copper tolerance (17, 22), mercury resistance (47), spore synthesis (46), and cytochrome *c* biogenesis (13, 35, 45). CcdA was not cotranscribed with a thioredoxin in *P. pantotrophus*, and it missed the thioredoxin stretch at the C terminus as described for DipZ homologous proteins found in *E. coli* (13) or *P. aeruginosa* (35). CcdA homologues have been proposed to participate in class II cytochrome *c* biogenesis systems, whereas the class I cytochrome *c* biogenesis systems present in most gram-negative bacteria are proposed to involve a DipZ homologue (28). The participation of a CcdA homologue in the biogenesis of a class I cytochrome *c* has been described only for *R. capsulatus* (14) and CcdA of *P. pantotrophus*, closely related to *R. capsulatus* (31).

In analogy to previous findings we suggest that CcdA of *P. pantotrophus* transports electrons from the cytoplasm to the periplasm via the two cysteine residues, although its electron donor is still unknown. Such involvement has been suggested for CcdA of *R. capsulatus* (14) and for DsbD of *E. coli* (12, 21, 51).

The disruption of *ccmG* of *P. denitrificans* caused a partial deficiency in holo- *c*-type cytochromes, and it was proposed that CcmG participates in cytochrome *c* biogenesis by reducing disulfide bonds in the periplasm (34). However, it is still unknown in which way CcmG derives the reducing equivalents. On the basis of the data presented we suggest that CcdA may act in reducing CcmG directly or via an unknown mediator in *P. pantotrophus* GB17 which seems to play an important role in the rereduction of cytochrome *c* apoproteins in the periplasm prior to heme binding.

The primary structure of CcdA is 42% identical and 80% similar to that of the predicted ORF2 gene product of *P. pantotrophus* located within the *sox* gene cluster (20). ORF2, however, cannot complement the disrupted *ccdA*, as shown with strain TP43. Therefore, the possible ORF2 function is proposed to be different from that of *ccdA*; whether it is specific for or linked to lithotrophic sulfur oxidation remains to be analyzed.

REFERENCES

1. **Aiba, H., T. Baba, K. Hayashi, T. Inada, K. Isono, T. Itoh, H. Kasai, K. Kishimoto, S. Kimura, M. Kitakawa, K. Makino, T. Miki, K. Mizobuchi, H. Mori, T. Mori, K. Motumura, S. Nakade, Y. Nakamura, H. Nashimoto, Y. Nishio, T. Oshima, N. Saito, G. Sampei, T. Horiuchi, et al.** 1996. A 570-kb DNA sequence of the *Escherichia coli* K-12 genome corresponding to the 28.0 - 40.1 min region on the linkage map. DNA Res. **3:**363–377.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Ames, G. F.-L., C. Prody, and S. Kustu.** 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. **160:**1181–1183.
4. **Bardwell, J. C., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulfide bond formation *in vivo*. Cell **67:**581–589.
5. **Berry, E. A., and B. L. Trumpower.** 1987. Simultaneous determination of hemes *a*, *b*, and *c* from pyridine hemochrome spectra. Anal. Biochem. **161:** 1–15.
6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
7. **Bowien, B., F. Mayer, G. A. Codd, and H. G. Schlegel.** 1976. Purification, some properties, and quaternary structure of the D-ribulose 1,5-diphosphate carboxylase of *Alcaligenes eutrophus*. Arch. Microbiol. **110:**157–167.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
9. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strains with beta-galactosidase selection. BioTechniques **5:**376–378.
10. **Carr, G. J., and S. J. Ferguson.** 1990. The nitric oxide reductase of *Paracoccus denitrificans*. Biochem. J. **269:**423–429.
11. **Chandra, T. S., and C. G. Friedrich.** 1986. Tn*5*-induced mutations affecting sulfur-oxidizing ability (Sox) of *Thiosphaera pantotropha*. J. Bacteriol. **166:** 446–452.
12. **Chung, J., T. Chen, and D. Missiakas.** 2000. Transfer of electrons across the cytoplasmic membrane by DsbD, a membrane protein involved in thiol-disulphide exchange and protein folding in the bacterial periplasm. Mol. Microbiol. **35:**1099–1109.
13. **Crooke, H., and J. Cole.** 1995. The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain. Mol. Microbiol. **15:**1139–1150.
14. **Deshmukh, M., G. Brasseur, and F. Daldal.** 2000. Novel *Rhodobacter capsulatus* genes required for the biogenesis of various *c*-type cytochromes. Mol. Microbiol. **35:**123–138.
15. **Fabianek, R. A., T. Hofer, and L. Thöny-Meyer.** 1999. Characterization of the *Escherichia coli* CcmH protein reveals new insights into the redox pathway required for cytochrome *c* maturation. Arch. Microbiol. **171:**92–100.
16. **Fischer, J., A. Quentmeier, S. Kostka, R. Kraft, and C. G. Friedrich.** 1996. Purification and characterization of the hydrogenase from *Thiobacillus ferrooxidans*. Arch. Microbiol. **165:**289–296.
17. **Fong, S.-T., J. Camakaris, and B. T. O. Lee.** 1995. Molecular genetics of a chromosomal locus involved in copper tolerance in *Escherichia coli* K-12. Mol. Microbiol. **15:**1127–1137.
18. **Francis, R. T., and R. R. Becker.** 1984. Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. Anal. Biochem. **136:** 509–514.
19. **Friedrich, C. G., B. Bowien, and B. Friedrich.** 1979. Formate and oxalate metabolism in *Alcaligenes eutrophus*. J. Gen. Microbiol. **115:**185–192.
20. **Friedrich, C. G., A. Quentmeier, F. Bardischewsky, D. Rother, R. Kraft, S. Kostka, and H. Prinz.** 2000. Novel genes coding for lithotrophic sulfur oxidation in *Paracoccus pantotrophus* GB17. J. Bacteriol. **182:**4677–4687.
21. **Gordon, E. H. J., M. D. Page, A. C. Willis, and S. J. Ferguson.** 2000. *Escherichia coli* DipZ: anatomy of a transmembrane protein disulphide reductase in which three pairs of cysteine residues, one in each of three domains, contribute differentially to function. Mol. Microbiol. **35:**1360–1374.
22. **Gupta, S. D., H. C. Wu, and P. D. Rick.** 1997. A *Salmonella typhimurium* genetic locus which confers copper tolerance on copper-sensitive mutants of *Escherichia coli*. J. Bacteriol. **179:**4977–4984.
23. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 355. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general microbiology. American Society for Microbiology, Washington, D.C.
24. **Heilmann, H.-J.** 1991. Isolierung von RNA aus *E. coli*, p. 199–200. *In* S. Bertram and H. G. Gassen (ed.), Gentechnische Methoden. Gustav Fischer, Stuttgart, Germany.
25. **Hoffmann, K., and W. Stoffel.** 1993. TMBASE—a database of membrane spanning protein segments. Biol. Chem. Hoppe-Seyler **374:**166.
26. **Keilin, D. (ed.).** 1966. The history of cell respiration and cytochrome. Cambridge University Press, Cambridge, United Kingdom.
27. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmic clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
28. **Kranz, R., R. Lill, B. Goldman, G. Bonnard, and S. Merchant.** 1998. Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems. Mol. Microbiol. **29:**383–396.
29. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
30. **Lang, E., and H. Lang.** 1972. Spezifische Farbreaktion zum direkten Nachweis von Ameisensäure. Z. Anal. Chem. **260:**8–10.
31. **Ludwig, W., G. Mittenhuber, and C. G. Friedrich.** 1993. Transfer of *Thiosphaera pantotropha* to *Paracoccus denitrificans*. Int. J. Syst. Bacteriol. **43:** 363–367.
32. **Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich.** 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. **51:**263–273.
33. **Otten, F. M., W. N. M. Reijnders, J. J. M. Bedaux, H. V. Westerhoff, K. Krab, and R. J. M. Van Spanning.** 1999. The reduction state of the Q-pool regulates the electron flux through the branched respiratory network of *Paracoccus denitrificans*. Eur. J. Biochem. **261:**767–774.

34. **Page, D. M., and S. J. Ferguson.** 1997. *Paracoccus denitrificans* CcmG is a periplasmic protein-disulphide oxidoreductase required for *c*- and aa_3-type cytochrome biogenesis; evidence for a reductase role *in vivo*. Mol. Microbiol. **24:**977–990.
35. **Page, D. M., N. F. Saunders, and S. J. Ferguson.** 1997. Disruption of the *Pseudomonas aeruginosa dipZ* gene, encoding a putative protein-disulfide reductase, leads to partial pleiotropic deficiency in *c*-type cytochrome biogenesis. Microbiology **143:**3111–3122.
36. **Puustinen, A., M. Finel, M. Virkki, and M. Wikström.** 1989. Cytochrome *o* (*bo*) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli*. FEBS Lett. **249:**163–167.
37. **Quentmeier, A., R. Kraft, S. Kostka, R. Klockenkämper, and C. G. Friedrich.** 2000. Characterization of a new type of sulfite dehydrogenase from *Paracoccus pantotrophus* GB17. Arch. Microbiol. **173:**117–125.
38. **Rainey, F. A., D. P. Kelly, E. Stackebrandt, J. Burhardt, A. Hiraishi, Y. Katayama, and A. P. Wood.** 1999. A re-evaluation of the taxonomy of *Paracoccus denitrificans* and a proposal for the combination *Paracoccus pantotrophus* comb. nov. Int. J. Syst. Bacteriol. **49:**645–651.
39. **Raitio, M., and M. Wikström.** 1994. An alternative cytochrome oxidase of *Paracoccus denitrificans* functions as a proton pump. Biochim. Biophys. Acta **1186:**100–106.
40. **Rieske, J. S.** 1967. Antimycin A, p. 542–580. *In* D. Gottlieb (ed.), Antibiotics I. Mechanism of action. Springer, Berlin, Germany.
41. **Robertson, L. A., and J. G. Kuenen.** 1983. *Thiosphaera pantotropha* gen. nov. sp. nov., a facultatively anaerobic, facultative autotrophic sulphur bacterium. J. Gen. Microbiol. **129:**2847–2855.
42. **Sambongi, Y., and S. J. Ferguson.** 1994. Specific thiol compounds complement deficiency in *c*-type cytochrome biogenesis in *Escherichia coli* carrying a mutation in a membrane bound disulphide isomerase-like protein. FEBS Lett. **353:**235–238.
43. **Sambrook, J., T. Maniatis, and E. F. Fritsch.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Schiött, T., C. von Wachenfeldt, and L. Hederstedt.** 1997. Identification and characterization of the *ccdA* gene, required for cytochrome *c* synthesis in *Bacillus subtilis*. J. Bacteriol. **179:**1962–1973.
46. **Schiött, T., and L. Hederstedt.** 2000. Efficient spore synthesis in *Bacillus subtilis* depends on the CcdA protein. J. Bacteriol. **182:**2845–2854.
47. **Sedlmeier, R., and J. Altenbuchner.** 1992. Cloning and DNA sequence analysis of the mercury resistance genes of *Streptomyces lividans*. Mol. Gen. Genet. **236:**76–85.
48. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–790.
49. **Sørbø, B.** 1957. A colorimetric method for the determination of thiosulfate. Biochim. Biophys. Acta **23:**412–416.
50. **Steinrücke, P., and B. Ludwig.** 1993. Genetics of *Paracoccus denitrificans*. FEMS Microbiol. Rev. **104:**83–118.
51. **Stewart, E. J., F. Katzen, and J. Beckwith.** 1999. Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of *Escherichia coli*. EMBO J. **18:**5963–5971.
52. **Stouthamer, A. H.** 1991. Metabolic regulation including anaerobic metabolism in *Paracoccus denitrificans*. J. Bioenerg. Biomembr. **23:**163–185.
53. **Thöny-Meyer, L.** 1997. Biogenesis of respiratory cytochromes in bacteria. Microbiol. Mol. Biol. Rev. **61:**337–376.
54. **Trumpower, B. L.** 1991. The three subunit cytochrome bc_1 complex of *Paracoccus denitrificans*. It's physiological function, structure, and mechanism of electron transfer and energy transduction. J. Bioenerg. Biomembr. **23:**241–255.
55. **von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184:**99–105.
56. **Wodara, C., F. Bardischewsky, and C. G. Friedrich.** 1997. Cloning and characterization of sulfite dehydrogenase, two *c*-type cytochromes, and a flavoprotein of *Paracoccus denitrificans* GB17: essential role of sulfite dehydrogenase in lithotrophic sulfur oxidation. J. Bacteriol. **179:**5014–5023.