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Cytochrome c_{550} from *Thiobacillus versutus*: Cloning, Expression in *Escherichia coli*, and Purification of the Heterologous Holoprotein

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The gene coding for cytochrome c_{550} from *Thiobacillus versutus*, *cycA*, has been cloned and sequenced. It codes for a protein of 134 amino acids plus a 19-amino-acid-long signal peptide. Both coding and noncoding DNA sequences of the clone are homologous to the *Paracoccus denitrificans* DNA sequence. An expression vector was constructed by cloning the *cycA* gene directly behind the *lac* promoter of pUC. The *cycA* gene was expressed in *Escherichia coli* under semianaerobic conditions, and mature holo-cytochrome c_{550} was isolated with the periplasmic soluble protein fraction. Under both aerobic and anaerobic conditions, significantly less cytochrome c_{550} was produced. The heterologously expressed cytochrome c_{550} was isolated and purified to better than 95% purity and was compared with cytochrome c_{550} isolated and purified from *T. versutus*. No structural differences could be detected by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis UV-visible light spectroscopy, and ^{1}H nuclear magnetic resonance spectroscopy, indicating that *E. coli* produces the cytochrome and attaches the heme correctly.

When *Thiobacillus versutus* is grown on methylamine as its sole carbon and nitrogen source, it produces a set of proteins which enable the bacterium to grow on this substrate. Methylamine is oxidized to formaldehyde by methylamine dehydrogenase (MADH; EC 1.4.99.3), and the electrons which are produced in this reaction are transported via the blue copper protein amicyanin and, presumably, cytochrome c_{550} to cytochrome *c* oxidase (EC 1.9.3.1). The latter enzyme is able to reduce oxygen and to establish a proton gradient across the cell membrane (12, 39, 41, 42).

This redox chain represents a good model for the study of electron transport between proteins, since it is short and the proteins are relatively easy to manipulate and rather well characterized (18–22, 38, 42); structure determinations of MADH (43) and amicyanin (13) are under way. Furthermore, the genes for both subunits of MADH and the gene for amicyanin have been cloned and sequenced (12a, 37), which allows the use of site-directed mutagenesis.

The protein reported upon in this paper is cytochrome c_{550} of *T. versutus*. This cytochrome was first described by Lu and Kelly (23) and has been extensively characterized by Lommen et al. (21). From protein sequencing studies, it has emerged that this cytochrome is homologous to that of *Paracoccus denitrificans* (38a).

For site-directed mutagenesis, it is necessary to clone the gene for cytochrome c_{550} and express it in a heterologous expression system. Several systems for the expression of different cytochromes *c* have been described (2–4, 14, 28, 30, 45). However, heterologous expression of cytochromes *c* in *Escherichia coli* in quantities which enable isolation and purification of the holoprotein generally appears to be difficult (10, 25, 29, 33, 35, 44).

In this report, we describe the cloning and sequencing of the gene, *cycA*, coding for cytochrome c_{550} from *T. versutus*. It is demonstrated that under semianaerobic conditions, heterologous expression of the *cycA* gene can be achieved in *E. coli* so that the holo-cytochrome *c* is present in the periplasmic space at levels that permit isolation of the protein. We have purified the heterologously expressed protein and compared it with the cytochrome isolated from *T. versutus*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *T. versutus* ATCC 25364^T (11) cells grown on methylamine (19) were obtained from J. Frank, Delft University of Technology. *E. coli* JM105 and JM101 were used as hosts for cloning purposes. *E. coli* W3110 was obtained from R. J. M. van Spanning, Free University, Amsterdam, The Netherlands, and used for expression studies. Plasmids pUC18 and pUC19 were used as cloning vectors. Plasmid pCYA2 consists of pUC19 and a 1.0-kbp *Sal*I-*Pst*I restriction fragment containing the gene for cytochrome c_{550} of *P. denitrificans* (40); it was a kind gift from R. J. M. van Spanning and was used in hybridization studies. M13mp18 and M13mp19 phages were used for DNA sequencing. pMU8, pUC19EE, and pMU19 are described below.

Hybridization of genomic DNA and cloning. Chromosomal DNA of *T. versutus* was isolated from 3 g of bacterial paste as described by Chater et al. (5). The DNA was digested with several restriction enzymes, and the fragments were separated on a 1% agarose gel and blotted via capillary transfer to a Nytran (NY13N) membrane (Schleicher & Schuell, Dassel, Germany) (32). Plasmid pCYA2 was digested with restriction enzymes *Bam*HI and *Hin*dIII, and the 1.0-kbp fragment carrying the cytochrome c_{550}-encoding gene from *P. denitrificans* was isolated and labelled uniformly with [α-^{32}P]dCTP, using random priming with hexadeoxyribonucleotides (7, 8, 32). The labelled probe was hybridized at 65°C for 16 h (32) to the chromosomal DNA immobilized on the Nytran membrane. An autoradiogram showed that various restriction fragments gave positive hybridization signals, among them a *Sph*I-*Sph*I fragment of 2.6 kbp (see Fig. 2, lane 5). A band of this size was isolated from a similar gel and cloned into pUC18 (32). Of 320 screened colonies, one hybridized strongly with the probe mentioned above.

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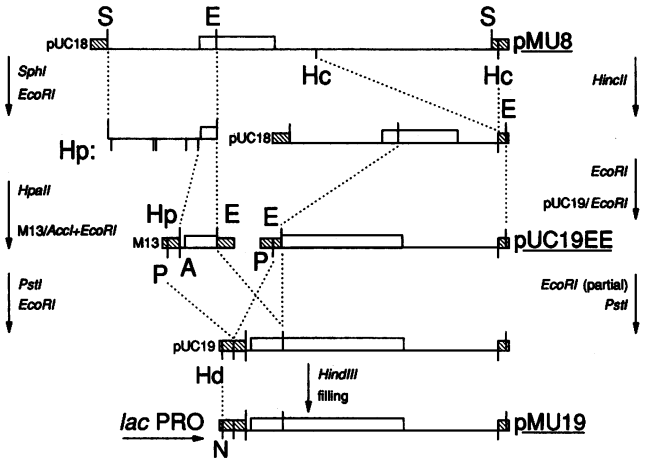

FIG. 1. Construction of expression vector pMU19. Open boxes represent parts of the *cycA* gene; hatched boxes represent multiple cloning sites of vectors; plasmid names are underlined; vectors (pUC18, M13, and pUC19) are indicated at the left of the constructs. Restriction sites are as follows: A, *Acc*I; E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; Hp, *Hpa*II; N, *Nhe*I; P, *Pst*I; and S, *Sph*I. *lac* PRO and horizontal arrow indicate the *lac* promoter and its direction. The 1.3-kbp *Hin*cII-*Hin*cII fragment was removed from pMU8, and the *Eco*RI-*Eco*RI fragment (0.6 kbp) was isolated from the resulting plasmid and cloned into pUC19 with the *lac* promoter and the *cycA* gene in the same orientation (pUC19EE). The 0.7-kbp *Sph*I-*Eco*RI restriction fragment of pMU8 was isolated and digested with *Hpa*II. Fragments were cloned into M13 phages; the *Hpa*II-*Eco*RI fragment, containing the 5′ part of the *cycA* gene, was isolated as a *Pst*I-*Eco*RI fragment from M13 replicative-form DNA and inserted between the *lac* promoter and the 3′ part of the *cycA* gene in pUC19EE. Finally, the *Hin*dIII site of this pUC19-derived expression vector was filled to introduce a stop codon in frame in the coding sequence of the *lacZ′* gene, thereby creating a unique *Nhe*I site.

Characterization of pMU8. Cells of the positive colony were cultured, and plasmid DNA (pMU8) was isolated (1). To determine the location of the *cycA* gene on the cloned 2.6-kbp *Sph*I fragment, restriction fragments of pMU8 were separated by electrophoresis and blotted. These blots were incubated with three synthetic deoxyribonucleotides (5′ labelled with ^{32}P) under same conditions described above but at 35°C. The sequence of these probes was designed on the basis of the amino acid sequence of cytochrome c_{550} (38a). DNA sequencing was performed according to Sanger et al. (34) by using subclones of pMU8 in M13 phages. 7-DeazadGTP was used to relieve band compression in the gels (26).

Construction of expression vector pMU19. The expression plasmid pMU19 was constructed as shown in Fig. 1; the region upstream of the *cycA* gene has been deleted, and the *cycA* gene itself is situated directly behind the *lac* promoter. The complete insert (nucleotides 582 to 1313 in Fig. 4) in pUC19 was isolated as a *Pst*I-*Eco*RI fragment, cloned in M13, and sequenced to ascertain that no mutations had taken place. The newly introduced stop codon was created to prevent translational read-through of the *lacZ′* gene into the *cycA* gene. The *coxI* gene fragment was fused to the remaining of the *lacZ′* gene.

Culture conditions. *E. coli* cells were grown either in 2-liter flasks or in a 40-liter fermentor (NBS MPP40; New Brunswick Scientific, New Brunswick, N.J.) at 37°C for 18 h under semianaerobic conditions: 2-liter flasks were filled with 1.7 liters of culture medium and were sealed with a nonairtight seal. The flasks were shaken vigorously. The 40-liter fermentor was filled with 30 liters of culture medium, and the air flow was adjusted to ca 0.7 liters/min. During growth, the relative dissolved oxygen concentration dropped to 2 to 4%. When the air flow was shut off, almost no holocytochrome could be isolated. The culture was stirred at 80 to 250 rpm.

The culture medium consisted of Luria-Bertani (LB) medium (32) to which Tris-HCl and KNO_3 had been added to final concentrations of 20 and 1 mM, respectively. The pH was ca. 7.4 but exhibited a tendency to drop during semianaerobic growth. Therefore, the pH was kept constant in fermentor cultures by addition of small amounts of 4 N NaOH. Also, 1 ml of antifoam (Sigma, St. Louis, Mo.) was added per 30 liters of fermentor culture medium.

To prevent loss of the expression vector, *E. coli* was freshly transformed with pMU19, and one colony of transformants was suspended in 30 ml of LB medium and grown for 6 to 7 h under aerobic conditions. This culture was then diluted 1:1,000 into the culture medium. Ampicillin was added to media and plates at 0.2 g/liter.

Isolation and purification of cytochrome c_{550}. To isolate cytochrome c_{550} from *T. versutus*, 200 g of cell paste was resuspended in 300 ml of 10 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride and incubated for 1 h with 200 mg of lysozyme. DNase was added to lower the viscosity. Next, the suspension was diluted four times with demineralized water and stirred for 90 min. Cell debris was removed by centrifugation, and the solution was applied to a DEAE-Sepharose (fast-flow) column (2.5 by 50 cm). The column was eluted at a flow rate of ca. 4 ml/min with a gradient of 0 to 500 mM NaCl in sodium phosphate buffer (10 mM, pH 7.0), and the cytochrome c_{550} came off the column at ca. 100 mM NaCl. The fractions containing cytochrome c_{550} were pooled, and the pH was lowered to 4.5 with 1 M acetic acid; precipitates were removed by centrifugation.

The final steps of the purification were the same as for cytochrome c_{550} isolated from *E. coli* (see below). The yield was 60 mg of cytochrome c_{550} (4 μmol) from 200 g (wet weight) of cell paste.

The *E. coli* cell culture was centrifuged, and the pellet was resuspended in 1/75 volume of sucrose buffer (20% [wt/vol] sucrose, 30 mM Tris-HCl [pH 8], 1 mM EDTA) and vigorously stirred for 15 min. Cells were collected by centrifugation, resuspended in 2/75 volume of ice-cold water, stirred for 15 min, and centrifuged again. The respective supernatants represent the sucrose and water fractions of the osmotic shock treatment (27). Usually, ca. 5% of the cytochrome c_{550} was found in the sucrose fraction and 95% was found in the water fraction. The water fraction was applied to a DEAE-Sepharose (fast-flow) column as described above but with a gradient of 0 to 200 mM NaCl.

Cytochrome c_{550} isolated from *T. versutus* or *E. coli* was oxidized with $K_3[Fe(III)(CN)_6]$ and further purified by using Hiload 16/10 S- and Q-Sepharose (fast-flow) columns by fast protein liquid chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). The protein was first applied to the S-Sepharose column and eluted with a 0 to 200 mM NaCl gradient (in 10 mM sodium phosphate buffer [pH 6.0]). It was then loaded onto the Q-Sepharose column and eluted with the same gradient (in 10 mM sodium acetate [pH 5.0]). The A_{525}/A_{280} of the oxidized protein was used as an index of purity (0.41) (21).

Nucleotide sequence accession number. The sequence presented in Fig. 4 has been entered into GenBank under accession number X62808.

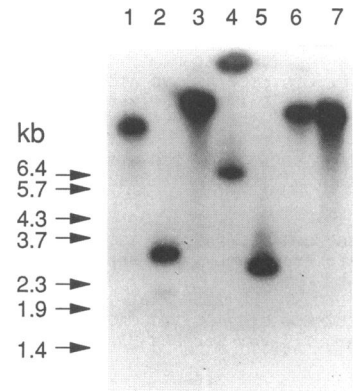

FIG. 2. Autoradiogram of Southern blot of chromosomal DNA of *T. versutus*. Chromosomal DNA digested with several restriction enzymes was separated on a 1% agarose gel, blotted to a nylon membrane, and hybridized with a labelled restriction fragment containing the gene for the cytochrome c_{550} from *P. denitrificans*. Lanes: 1, *Bam*HI; 2, *Hinc*II; 3, *Kpn*I; 4, *Sal*I; 5, *Sph*I; 6, *Xba*I; 7, *Xma*I. Restriction fragment size is indicated on the left.

RESULTS

Cloning of the gene for cytochrome c_{550}. A 2.6-kbp *Sph*I-*Sph*I restriction fragment (Fig. 2) was cloned and isolated as described in Materials and Methods, and a limited restriction map was constructed (Fig. 3A). The *Sph*I-*Hinc*II fragment containing the gene was sequenced in both directions (Fig. 3B).

The nucleotide sequence of this fragment is presented in Fig. 4. One complete open reading frame (ORF) is present. The amino acid sequence derived from this ORF can be aligned with the protein sequence of cytochrome c_{550}, starting with the glutamine coded by nucleotides 673 to 675 and ending at the asparagine at nucleotides 1072 to 1074. The two sequences are identical, although in protein sequence analysis, a minor molecular species that is shorter by a few residues at the C-terminal end is detected (38a). The ORF also encodes a leader peptide of 19 amino acids (not counting the Met starting codon). It is concluded that this ORF codes for cytochrome c_{550}. The calculated molecular weights are

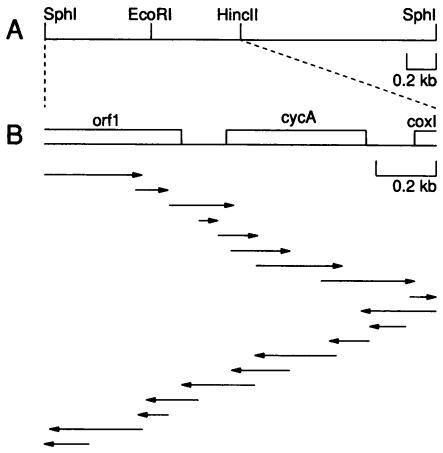

FIG. 3. Restriction map and sequencing strategy. (A) Restriction map of the cloned 2.6-kbp *Sph*I-*Sph*I restriction fragment from *T. versutus* chromosomal DNA; (B) sequencing strategy for sequencing of the *cycA* gene.

14,756 for the mature holo-cytochrome c_{550} and 16,138 for the preapocytochrome.

Downstream of the *cycA* gene is another ORF which is tentatively identified as the gene coding for subunit I of cytochrome *c* oxidase by homology to one of the *coxI* (*ctaDII*) genes of *P. denitrificans* (31, 40). It is therefore called *coxI*. Shine-Dalgarno sequences (36) are present in front of both the *cycA* and *coxI* genes (singly underlined in Fig. 4). Upstream of the *cycA* gene is the 3′ end of another ORF (orf 1) which shows no obvious homology with sequences present in GenBank.

Expression of cytochrome c_{550} in *E. coli*. The vector pMU19 was transformed into *E. coli* W3110, and transformants were cultured, harvested, and submitted to an osmotic shock to obtain the protein fraction from the periplasmic space. To test for expression of the *cycA* gene, UV-visible light (Vis) difference spectra of fractions obtained from cells transformed with pMU19 and pUC19 as a control were taken. Absorption peaks at 414 and 550 nm were used as indicators for expression of holo-cytochrome c_{550}. When the transformants were grown aerobically, very little expression of holo-cytochrome c_{550} was observed (ca. 0.1 mg/liter of culture). Approximately the same amount was found when cells were grown anaerobically in airtight flasks which were completely filled with LB medium supplemented with KNO_3 and Tris buffer. However, when cells were grown in the same medium under semianaerobic conditions, 1 to 2 mg of protein per liter of culture was present in the periplasmic protein fraction. For 2-liter flasks, conditions were optimal when 1.7 liters of medium and a nonairtight seal were used; for a 40-liter fermentor, a low air flow (of ca. 0.7 liters/min) had to be used (see Materials and Methods for details).

Figure 5A shows the results from sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of water fractions of the osmotic shock. Lane 3 shows the water fraction of cells containing pMU19, and lane 2 shows the result for cells containing pUC19 as a control. A faint but significant band that comigrates with cytochrome c_{550} can be detected in the pMU19 sample. This band is not present in the pUC19 sample. Upon heme staining, peroxidase activity of this band is observed (Fig. 5B, lane 3). The presence of this activity proves that the heme present in this protein is not removed by treatment with SDS and boiling and thus is bound covalently (9). The strong band which is present in both samples is thought to be due to β-lactamase.

Lanes 4 to 6 of Fig. 5A show the analysis of protein contents of the spheroplasts remaining after osmotic shock and two wash steps. The samples represent the same amount of cells as do the samples of the water fractions. It is clear that no large quantities of either cytochrome c_{550} or its precursor are present in the remaining spheroplasts. No proteins with peroxidase activity are present in spheroplasts (Fig. 5B, lanes 4 to 6). (Note that the strong, extra band in lanes 6 in Fig. 5A and B is produced by cytochrome c_{550} that was added to the sample of lane 5 as an internal marker to distinguish between possibly unprocessed and processed cytochrome.)

Purification and characterization of heterologously expressed cytochrome c_{550}. Cytochrome c_{550} expressed in *E. coli* could be isolated as a soluble protein from the periplasmic space by using osmotic shock. The protein was purified in three steps of ion-exchange chromatography at different pHs to better than 95% purity, as judged by the A_{525}/A_{280} ratio (21) and SDS-PAGE (Fig. 5A, lane 1).

To investigate whether the heterologously expressed cytochrome c_{550} shows any structural differences from the

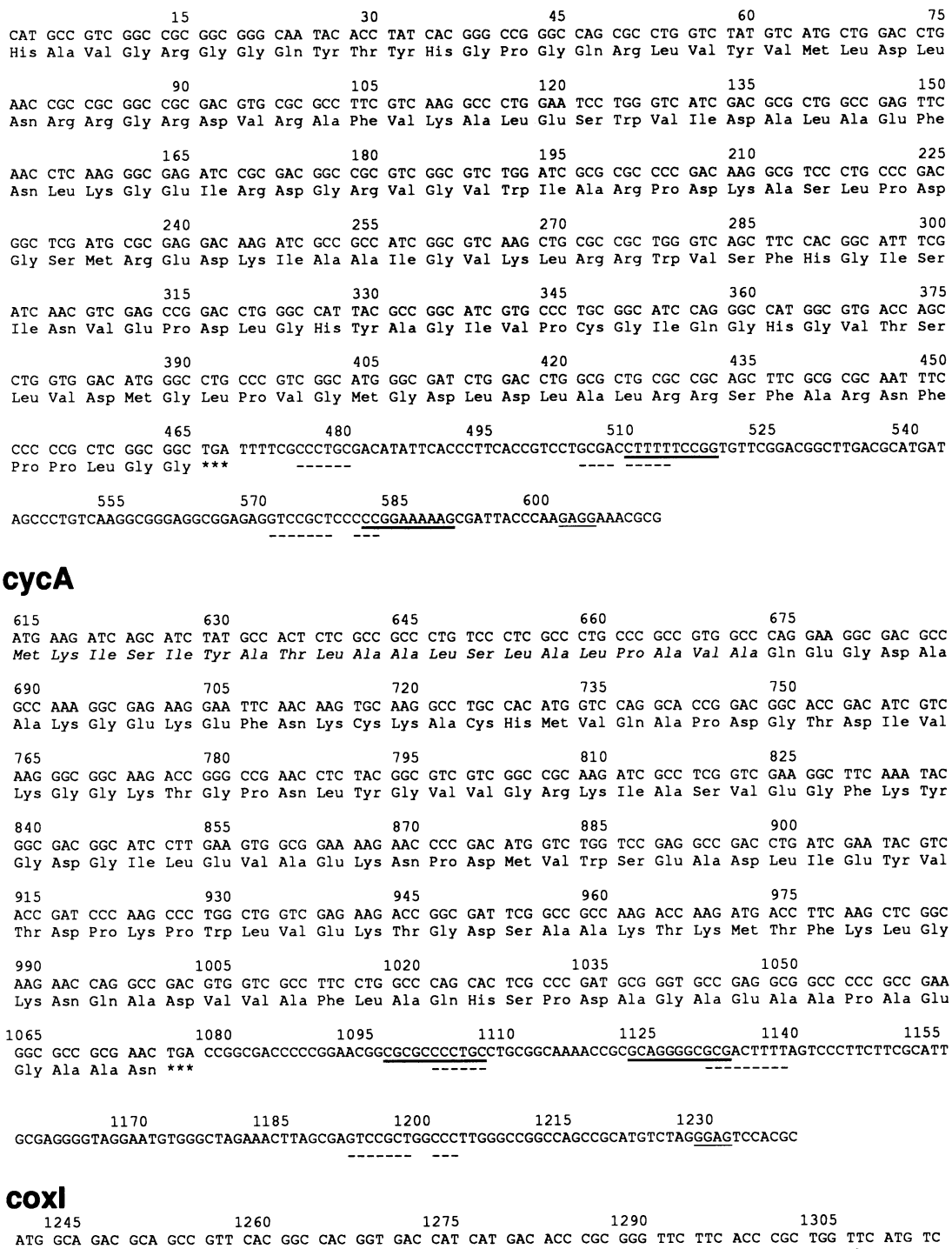

FIG. 4. Nucleotide sequence of the *cycA* gene. ORFs are translated; signal peptide is in italics; Shine-Dalgarno (36) sequences are underlined; dyad symmetries are underlined in bold; stretches of homology upstream of the *cycA* and *coxI* genes are underlined with a broken line.

protein isolated from *T. versutus*, several characterizations were performed. Both proteins behave identically in an SDS-polyacrylamide gel (Fig. 5, lanes 7 and 8). Therefore, the signal peptide of cytochrome c_{550} produced in *E. coli* must have been removed. Lanes 7 and 8 of Fig. 5B show that the heterologously produced protein stains well upon heme-specific staining. Also, a red band was directly visible in the running gel when the protein was overloaded, another strong indication that the heme group is bound covalently.

Figure 6 shows the UV-Vis spectra of the two proteins in

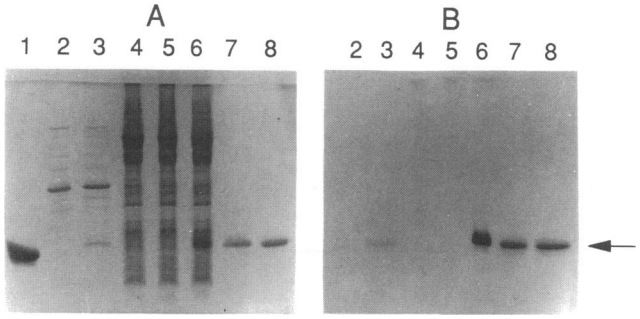

FIG. 5. SDS-PAGE analysis of heterologously expressed cytochrome c_{550}. Spheroplast pellets, obtained after osmotic shock treatment, were washed twice and resuspended in 10 mM sodium phosphate buffer (pH 7.0). To these suspensions and to protein samples, 2% (wt/wt) SDS was added; samples were heated at 100°C for 5 min. SDS–20% polyacrylamide homogeneous gels were run on a Phastsystem (Pharmacia) (16). For heme staining (9), the gel was submerged for 45 min in a fresh 0.1 mM solution of 3,3′,5,5′-tetramethylbenzidine in 30% (vol/vol) methanol and 70% 0.25 M sodium acetate buffer (pH 5.0). Then 30 μl of 30% H_2O_2 was added, and the gel was incubated for 10 to 15 min. To stop the reaction, the gel was washed with water and submerged in 30% (vol/vol) isopropanol and 70% of the same buffer as described above. (A) Coomassie brilliant blue-stained gel; (B) gel stained with heme-specific stain. Lanes: 1, purified *T. versutus* cytochrome c_{550} isolated from *E. coli*, overloaded; 2, periplasmic protein fraction from *E. coli*/pUC19; 3, periplasmic protein fraction from *E. coli*/pMU19; 4, spheroplast protein fraction from *E. coli*/pUC19; 5, spheroplast protein fraction from *E. coli*/pMU19; 6, as lane 5 plus purified cytochrome c_{550} isolated from *E. coli*; 7, purified cytochrome c_{550} isolated from *E. coli*; 8, purified cytochrome c_{550} isolated from *T. versutus*. The arrow indicates. . .

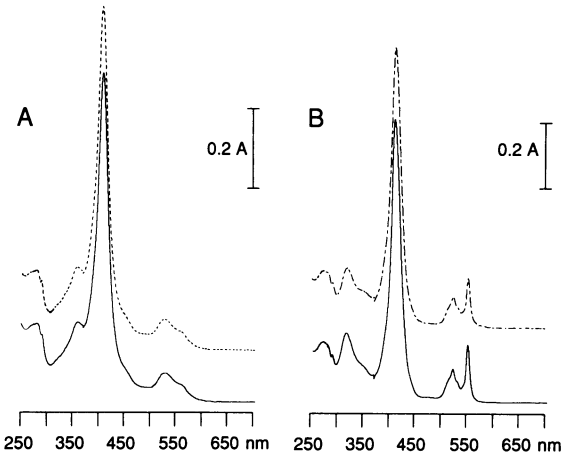

FIG. 6. UV-Vis spectra of purified cytochrome c_{550}. Spectra of oxidized (5.4 μM; A) and reduced (5.0 μM; B) cytochrome c_{550} in 10 mM sodium phosphate buffer (pH 7.0), isolated from *T. versutus* (solid line) and from *E. coli* after heterologous expression (broken line). Spectra were take at room temperature; path length was 1 cm.

both redox states. The spectra of cytochrome c_{550} produced by *E. coli* and *T. versutus* show no differences. The ratios of the extinction coefficients agree with those published previously (21). At high concentrations of the cytochromes in the oxidized form, the absorption peak at 695 nm, indicative for Met ligation of the heme iron, was also detected in the both samples (results not shown).

^{1}H nuclear magnetic resonance (NMR) spectra of cytochrome c_{550} isolated from *E. coli* and *T. versutus* are shown in Fig. 7. Peaks which are shifted downfield and upfield outside the bulk of the resonances occur at identical positions in the spectra of the reduced proteins. A number of these peaks have been assigned to the mesoprotons of the heme and to protons of the axial Met ligand of the heme (21). Also, all of the paramagnetic shifts of peaks in the oxidized form of the proteins are identical. The peaks at 29.5, 28.7, 16.9, and 12.8 ppm of the oxidized proteins have been assigned to the heme methyl groups (21).

Considering the presence of heme proton resonances and the fact that shifts of paramagnetically shifted protons are highly sensitive to structural changes, and also taking into account the information from the experiments presented above, it is concluded that the heme group is present in heterologously expressed cytochrome c_{550} and that its orientation inside the protein matrix is the same as in cytochrome c_{550} from *T. versutus*.

DISCUSSION

The gene for cytochrome c_{550} from *T. versutus* has been cloned. It codes for a protein of 134 amino acids with a single consensus sequence for binding of heme (CXXCH) (nucleotides 715 to 729 in Fig. 4). The ORF also encodes a leader peptide of 19 amino acids (not counting the Met starting codon) which probably represents a signal peptide for protein translocation across the cytoplasmic membrane, since cytochrome c_{550} is a periplasmic protein (41) and the leader peptide has the characteristics of a signal peptide of gram-negative bacteria (6).

Figure 8 shows the sequences of the cytochromes of *T. versutus* and *P. denitrificans* as derived from their DNA sequences. Of the total of 155 residues (including those of the signal peptides), 130 are identical. The homology between the restriction fragments containing the two cytochrome genes in fact extends throughout the two fragments: 88% of nucleotides 379 to 1313 (this work) are identical with nucleotides 1 to 947 of *P. denitrificans* (40), supporting the finding (17) that the organisms are closely related.

In the noncoding regions in Fig. 4, small stretches of homologous sequence appear at roughly the same distances (around 136, 104, and 38 nucleotides) upstream of the *cycA* and *coxI* genes (at nucleotides 477, 509, and 575 for the *cycA* gene and at nucleotides 1104, 1136, and 1202 for the *coxI* gene; these stretches are indicated in Fig. 4 by broken lines). Also, the *P. denitrificans cycA* and *coxI* genes and the first gene of the bc_1 operon (15) are preceded by such conserved sequences (located around 136, 104, and 80 nucleotides upstream of the respective start codons [40]). Apparently, the sequences around 136 and 104 nucleotides in front of the start codons for these genes are conserved in both organisms. Their function is still unclear.

Dyad symmetries are present in the sequences in front of both the *cycA* and *coxI* genes. Only the latter is conserved in the *P. denitrificans* sequence. Whether these sequences have any function remains to be established. It was observed that *E. coli* containing pUC19 in which nucleotides 1 to 1313 instead of 582 to 1313 (Fig. 4) had been cloned showed significantly reduced expression of cytochrome c_{550} (results not shown). This finding might be attributable to terminator activity of the dyad symmetry sequence in front of the *cycA* gene. When pMU19 was transformed into *E. coli* JM101, the transformant colonies were blue, but only very weakly. This finding suggests that the 5′ part of the *coxI* gene is indeed

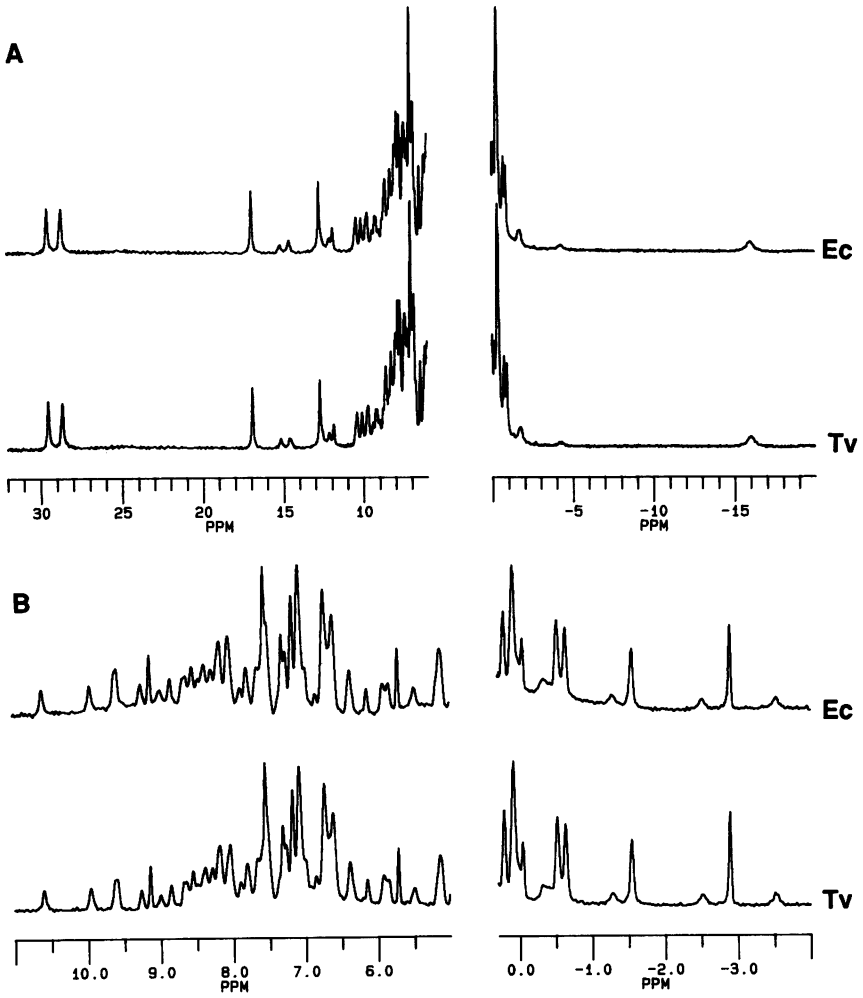

FIG. 7. ^{1}H NMR spectra of cytochrome c_{550}. Spectra of oxidized (A) and reduced (B) cytochrome c_{550} isolated from *T. versutus* (Tv) and from *E. coli* after heterologous expression (Ec). Samples of 1 mM protein were prepared in D_2O with 20 mM sodium phosphate (pH 7.0) as described previously (21). Reduction with a 0.1 M solution of sodium dithionite and pH adjustments were performed as described previously (21). Spectra were recorded at 300 MHz on a Bruker WM300 spectrometer at a temperature of 300 K. Free induction decays of the reduced protein were recorded in 8K memory with the spectral width set to 6,000 Hz; for oxidized samples, these values were 16K and 18,000 Hz, respectively.

fused to the *lacZ'* gene and that the fusion product is produced only in low amounts. This finding could be explained by assuming partial terminator activity of the dyad symmetry sequence in front of the *coxI* gene.

This study has shown that it is possible to express the *cycA* gene of *T. versutus* in *E. coli*. The cytochrome is isolated with the periplasmic protein fraction, and it contains a heme group that is covalently bound to the protein. Previous attempts to use *E. coli* as a host system to obtain suitable expression of cytochrome *c* genes have met with only limited success. In some cases, heme incorporation appeared to be absent (10, 29); in other cases, expression of holo-cytochromes *c* in *E. coli* was obtained under both aerobic and anaerobic conditions but not in amounts that permitted isolation and purification of the protein (25, 35, 44). Sanbongi et al. (33) recently reported on the expression of cytochrome c_{552} from *Hydrogenobacter thermophilus* in the cytoplasm of *E. coli* grown under completely anaerobic conditions (and also under aerobic conditions). The level of production is same as we have found for *T. versutus* cytochrome c_{550}. However, the protein contained an extra, N-terminal methionine residue that had been introduced on the DNA level as a start codon.

Other host systems have also been used. The first expression system for cytochromes *c* was established in *Saccharomyces cerevisiae* and was used to express a mutant iso-1-cytochrome *c*, yielding 3 mg of protein per liter of culture (28). Other eukaryotic cytochromes *c* have also been expressed in *S. cerevisiae* (14). This expression system has the disadvantage that it is suitable only for the expression of cytochrome *c* (mutants) that is physiologically competent in electron transfer (24). Heterologous expression of cytochrome c_3 from *Desulfovibrio vulgaris* has been achieved in *Rhodobacter sphaeroides* (2 mg/100 g of cells) (4); this protein was also overexpressed (20 mg/100 g of cells) in an expression system in *D. desulfuricans* G200 (30, 45). Cytochrome c_2 has been expressed in *Rhodobacter capsulatus* (2, 3).

The remarkable finding of the present study is that compared with aerobic or anaerobic growth, cytochrome c_{550}

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Tv   1 MKISIYATLAALSLALPAVA/QEGDAAKGEKEFNKCKACHMVQAPDGTDIV 50
       ***********  ***** * * ****************** ********
Pd   1 MKISIYATLAAITLALPAAA/QDGDAAKGEKEFNKCKACHMIQAPDGTDII 50

Tv  51 KGGKTGPNLYGVVGRKIASVEGFKYGDGILEVAEKNPDMVWSEADLIEYV 100
       ******************** ****** ***********  * ********
Pd  51 KGGKTGPNLYGVVGRKIASEEGFKYGEGILEVAEKNPDLTWTEADLIEYV 100

Tv 101 TDPKPWLVEKTGDSAAKTKMTFKLGKNQADVVAFLAQHSPDAGAEA-APA 149
       ********  * *  ******** ************* *****    * *
Pd 101 TDPKPWLVKMTDDKGAKTKMTFKMGKNQADVVAFLAQNSPDAGGDGEAAA 150

Tv 150 EGAAN 154
       **  *
Pd 151 EGESN 155
```

FIG. 8. Comparison of DNA-derived protein sequences of pre-cytochromes c_{550} from *T. versutus* (Tv) and *P. denitrificans* (Pd) (40). Identical residues are indicated by asterisks.

production can be increased at least by an order of magnitude by using semianaerobic growth conditions, yielding 1 to 2 mg of holoprotein per liter of culture medium. This finding suggests that increased production of the holoprotein can perhaps be achieved under semianaerobic conditions for other cytochromes *c* as well.

Another notable finding of this study is that pre-cytochrome c_{550} is processed by a signal peptidase when expressed in *E. coli*. Protein sequence analysis (38a) indicates that the mature cytochrome c_{550} starts with the same amino acid sequence as does the cytochrome produced in *T. versutus*; the *E. coli* peptidase thus recognizes the same cleavage site as the one in *T. versutus*. Only one small difference was found between the two cytochromes: while the N terminus of the cytochrome from *T. versutus* is pyroglutamated, this is not the case for the cytochrome produced in *E. coli*. However, since no other differences could be detected between the cytochrome c_{550} produced by *T. versutus* and the same protein heterologously expressed in *E. coli*, using SDS-PAGE, UV-Vis spectroscopy, and ^{1}H NMR spectroscopy, the expression system described here is considered appropriate for the production of site-specific mutants of cytochrome c_{550}; the occurrence of the holoprotein in the periplasmic space of *E. coli* permits the use of standard mutagenesis techniques and ensures easy isolation and purification of the mutant proteins.

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