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Protein Expression and Purification **20,** 444–450 (2000)
doi:10.1006/prep.2000.1318, available online at http://www.idealibrary.com on IDEAL®

High Yield of *Methylophilus methylotrophus* Cytochrome *c*″ by Coexpression with Cytochrome *c* Maturation Gene Cluster from *Escherichia coli*

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Received June 2, 2000, and in revised form July 19, 2000

Heterologous expression of *c*-type cytochromes in the periplasm of *Escherichia coli* often results in low soluble product yield, apoprotein formation, or protein degradation. We have expressed cytochrome *c*″ from *Methylophilus methylotrophus* in *E. coli* by coexpression of the gene encoding the cytochrome (*cyc*A) with the host-specific cytochrome *c* maturation elements, within the *ccm*A-H gene cluster. Aerobic cultures produced up to 10 mg holoprotein per liter after induction with IPTG. In the absence of the maturation factors *E. coli* failed to produce a stable haem protein. Cytochrome *c*″ isolated from the natural host was compared with the recombinant protein. No structural differences were detected using SDS–PAGE, UV-Visible spectroscopy, differential scanning calorimetry, and ^{1}H-NMR spectroscopy. The success in expressing the mature cytochrome *c*″ in *E. coli* allows the engineering of the *cyc*A gene by site-directed mutagenesis thereby providing an ideal method for producing mutant protein for studying the structure/function relationship.

Cytochrome *c*″ is a unique monohaem protein isolated from the obligate aerobe *Methylophilus methylotrophus* that undergoes a major redox-linked spin-state transition from a low-spin state in the oxidised form to a high-spin state in the reduced form. The axial ligands are two histidinyl residues in the oxidised form, and a single histidinyl residue in the reduced form. Upon reduction a proton is taken up through protonation of the histidine that detaches, and this unique property makes cytochrome *c*″ an interesting example of a soluble protein capable of coupling electron and proton transfer *in vitro* (1–3).

Although there is considerable information on the haem electronic structure (4), ligand orientation (2), and characteristics of the haem pocket (5) of cytochrome *c*″, the physiological role of this unusual protein is undetermined as yet. The numerous attempts to obtain crystals of cytochrome *c*″ have failed, and solution structure determination by NMR[2] has been hampered by the need to produce a large quantity of isotopically labelled protein. Moreover, structure/function studies would be facilitated by having the means to produce site-directed mutants. Therefore, the continuing interest in this unusual cytochrome has directed our attention towards the development of a suitable heterologous expression system. Hence, we attempted to obtain strains of *E. coli* transformed for the overexpression of cytochrome *c*″. However, early attempts within our laboratory to express cytochrome *c*″ in *E. coli* met with little success.

Heterologous expression in *E. coli* of *c*-type cytochromes has not always been successful. Some cytochromes *c*, such as the tetrahaem cytochrome c_3 from *Desulfovibrio vulgaris* are exported to the periplasm but are unable to convert into their mature forms (6). Others, such as *c*-type cytochromes from *Rhodopseudomonas viridis*, only accumulate as precursors in the cytoplasmic membrane (7). Alteration in culture condi-

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[2] Abbreviations used: *ccm*A-H, *Escherichia coli* cytochrome *c* maturation gene cluster; *cyc*A, gene encoding cytochrome *c*″; IPTG, isopropyl β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

1046-5928/00 $35.00

tions can improve soluble cytochrome yield and decrease toxicity.

Cytochromes *c* from several organisms have been expressed in *E. coli* with varying degrees of success (8–17), indicating that expression of *c*-type cytochromes still tends to be optimised on a case by case basis. The recent isolation and characterisation of the *E. coli* cytochrome *c* maturation factors by Thöny-Meyer and coworkers (18) has led to the improved expression of cytochrome c_{552} from *Paracoccus denitrificans* (16) and cytochrome *c* subunits of the cbb_3 oxidase from *Bradyrhizobium japonicum* (17) in *E. coli*. We now report that the synthesis of cytochrome *c″* is achieved efficiently in *E. coli* by coexpression with the complete *ccm*A-H gene cluster.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli XL1-Blue (Stratagene) was used for subcloning, and BL21(DE3) (Stratagene) for expression of the *M. methylotrophus* cytochrome *c″* (see below); plasmids were maintained in the presence of chloramphenicol (25 μg/ml) or ampicillin (100 μg/ml) where appropriate. Expression was performed as described by Reincke and coworkers (16).

Recombinant DNA Techniques

DNA manipulations followed published procedures (19). Restriction sites needed for cloning of *cyc*A were introduced by PCR. A 372-bp *Nde*I–*Bam*HI fragment was generated using the primers CCDP1 [5′-CTC CAT ATG AAA ATC AAA ACA ATC ATT GCC G-3′] and CCDP2 [5′-CTC GGA TCC TTA TTT GGT AGG CTT GGT TTC TG-3′]) with pSAL1, containing *cyc*A, as template (20). After digestion with *Nde*I and *Bam*HI, the PCR product was cloned into the corresponding sites of pT7-7, resulting in pHS1. The plasmid was introduced into strain BL21(DE3) harboring chloramphenicol resistant plasmid pEC86 encoding the complete *ccm*A-H gene cluster from *E. coli* under the control of the *tet* promoter from pACYC184 (17). Plasmid pEC86 was a generous gift from L. Thöny-Meyer.

Cell Fractionation and Purification of the Recombinant Protein

E. coli cells were harvested by centrifugation and periplasmic fractions isolated by osmotic shock treatment as previously described (21) except that cell pellets were routinely frozen before osmotic shock. Cytochrome *c″* was purified by column chromatography in three steps (see Table 1). (i) Ion exchange by applying the soluble fraction to a DEAE-Fast Flow column equilibrated with 5 mM Tris–HCl (pH 7.6 at 25°C). Cytochrome *c″* does not adsorb to this column and elutes with the equilibrating buffer. (ii) Ion exchange by applying the red fractions of (i) to an S-Sepharose column equilibrated with the same buffer. This column was eluted with a linear NaCl gradient (0–1 M) prepared in 5 mM Tris–HCl, pH 7.6. The red fractions containing cytochrome *c″* were eluted at about 150 mM NaCl. (iii) These fractions were pooled and concentrated by ultrafiltration (Amicon YM3) before being applied on a Superdex-75 column equilibrated with 100 mM NaCl in 50 mM Tris–HCl, pH 7.6. Purity of the resulting protein fractions was determined by comparison with the absorption ratio A_{406}/A_{280} of 8.5 for pure cytochrome (1) and by SDS–PAGE. Protein concentration was determined in a Shimadzu UV-1601 spectrophotometer using an absorption coefficient of 97.5 mM^{-1} cm^{-1} at 406 nm for oxidized cytochrome *c″* (1).

NMR Spectroscopy

Cytochrome *c″* was purified from cell mass of the natural host, *M. methylotrophus,* as previously reported (3). Two samples were prepared for the NMR experiments, one of the native protein and one of the recombinant protein. Samples of the reduced protein were obtained by addition of sodium dithionite. The proteins were dissolved in 90% 10 mM phosphate buffer, pH 5.6/10% ^2H_2O to make final concentrations of ca 3 mM and 1.3 mM for the native and recombinant protein, respectively. ^{1}H-NMR spectra were acquired on a Bruker DRX 500 spectrometer at 294.4K. One-dimensional spectra were recorded with a spectral width of 60 ppm with 128 scans for the oxidized protein and 256 scans for the reduced protein. NOESY spectra were recorded with mixing times of 100 ms using the WATERGATE sequence for water suppression (22,23). TOCSY spectra were recorded using the clean TOCSY pulse sequence with mixing times of 50 ms and 75 ms (24,25). All the spectra were recorded with 4×1k data points and with 32 or 48 scans per increment. The spectra were processed using Bruker software.

Differential Scanning Calorimetry

Measurements were performed in a MicroCal VP DSC calorimeter. The sample buffer was changed to sodium phosphate buffer 50 mM, pH 5.5, by several cycles of concentration/dilution using a centricon tube. The reference cell was filled with the buffer obtained from the last concentration step. Protein scans were baseline corrected by subtracting the baselines collected for the buffer under identical experimental conditions. Samples were vacuum degassed for 5 min immediately before the assay, and an overpressure of about 29 psi was applied to the cells. Scans were performed from 20 to 90°C with a heating rate of 1°C per min. Samples with protein concentrations in the range 0.07 to 0.37 mg/ml were examined. Reversibility was

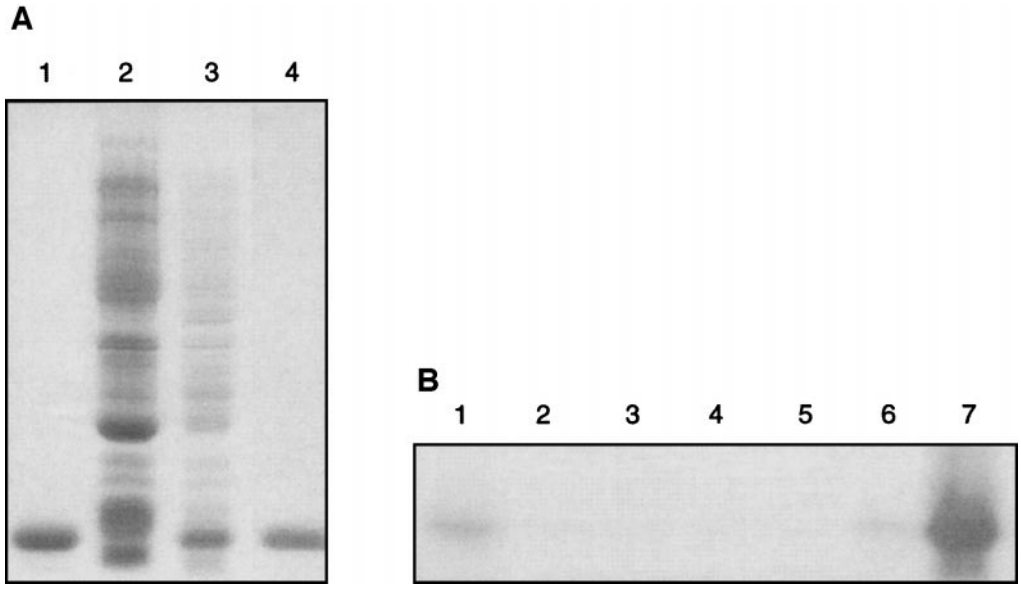

FIG. 1. Expression of the *cyc*A gene from *M. methylotrophus* in *E. coli* BL21[DE3], transformed with plasmids described under Materials and Methods. Detection was by SDS–PAGE (A) and haem stain (B): (A) Lane 1, holocytochrome *c*″ from *M. methylotrophus;* Lane 2, total protein extract from *E. coli* cells transformed with pEC86 and pHS1; Lane 3, periplasmic extract of total protein extract of lane 2; Lane 4, purified recombinant cytochrome *c*″; (B) Lane 1, holocytochrome *c*″ from *M. methylotrophus;* Lane 2, *E. coli;* Lane 3, *E. coli* transformed with pT7-7; Lane 4, *E. coli* transformed with pHS1; Lane 5, *E. coli* transformed with pEC86; Lane 6, *E. coli* transformed with pEC86/pHS1 (cytoplasmic extract after osmotic shock); Lane 7, *E. coli* transformed with pEC86/pHS1 (periplasmic extract after osmotic shock). Lanes 1–5 represent total protein extracts.

assessed by performing a second heating scan. Data were analyzed with the software provided by MicroCal.

RESULTS

Expression Studies of Cytochrome c″ in E. coli

We have investigated several strategies of overexpression of the gene encoding cytochrome *c*″ (*cyc*A). Small-scale expression studies routinely performed after cloning the *cyc*A gene into a variety of commercially available expression vectors and transforming into *E. coli* strains (including JM109, W3110, M15L, SG13009, and MC1061) failed to produce the holoprotein. Transformed strains were routinely grown to midexponential phase and then induced with IPTG. The conditions assayed included growth under variation in O_2 levels, pH, temperature, and by supplementing the media with iron citrate. To detect cytochrome *c*″ in the recombinant *E. coli* strain, cells were osmotically treated to obtain cytoplasmic and periplasmic fractions (21). SDS–PAGE and immunoblotting with polyclonal antiserum raised against purified cytochrome *c*″ detected a small amount of smeared protein from the expected molecular weight (15 kDa) present in the periplasmic fraction, suggesting proteolytic degradation. Furthermore, no haem was detected by haem stain or spectrophotom-

TABLE 1

Summary of Purification and Yield of the Heterologously Expressed *M. methylotrophus* Cytochrome *c*″ from Strain BL21 (DE3)

Purification step	Protein (mg)[a]	Cytochrome *c*″ (mg)[b]	A_{406}/A_{275}	Yield (%)	Purification (fold)
Periplasmic extract[c]	1260	26	0.2	(100)	—
First ion exchange	313	23	0.7	89	4
Second ion exchange	41	21	4.2	80	22
Gel filtration	19[b]	19	8.9	75	47

[a] Determined from A_{275} using the extinction coefficient of 0.7 $mg^{-1} \cdot ml \cdot cm^{-1}$ determined for albumin.
[b] Calculated from A_{406} using ϵ_{406} of 97.5 $mM^{-1} \cdot cm^{-1}$ (1).
[c] Corresponding to 3 liters of growth medium.

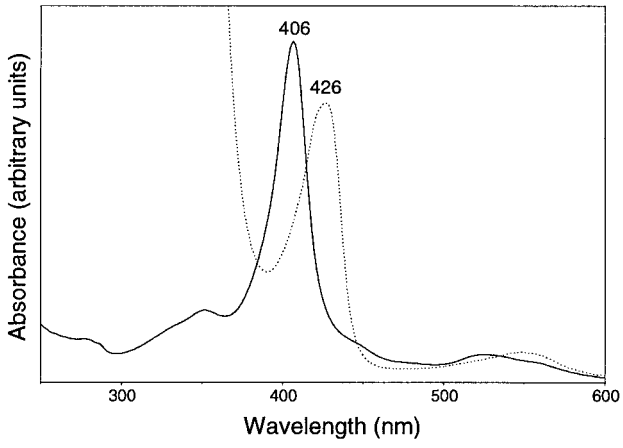

FIG. 2. Absorption spectra of *Methylophilus methylotrophus* cytochrome *c″* at pH 5.5. The solid line represents the oxidized form and the dotted line the reduced form obtained by addition of sodium dithionite. Extinction coefficients determined at pH 4.3: oxidized form, $\epsilon_{406} = 97.5\ mM^{-1} \cdot cm^{-1}$; reduced form, $\epsilon_{426} = 90.0\ mM^{-1} \cdot cm^{-1}$. Data from reference (1).

etry. This suggests *E. coli* is capable of synthesising and exporting the apoform of cytochrome *c″* but fails to incorporate the haem moiety. Moreover, all *cyc*A expressing clones grew slower than nontransformed cells (data not shown), indicating a detrimental effect of the gene product on *E. coli* metabolism.

Further attempts utilized the cytochrome *c* maturation factor from yeast, haem lyase (on plasmids pBPCYC1(wt/3) and pBTR1) cloned downstream from the *cyc*A gene, lacking the 5′ region encoding the signal peptide, into the expression vector pQE60 (Qiagen). Mauk and coworkers obtained high yields of yeast *iso*-1-cytochrome *c* in *E. coli* when the mitochondrial cytochrome was cloned adjacent to the haem lyase gene (14). Here, however, not even degradation products were observed by haem staining or immunoblotting with polyclonal antiserum in total cell extracts of transformants grown under a variety of conditions (data not shown). This suggests rapid proteolytic degradation of the apoprotein, indicating that the haem lyase is not effective in the addition of haem to cytochrome *c″*.

Effect of ccmA-H Gene Cluster on Cytochrome c″ Expression

Successful expression of cytochrome *c″* as a holoform was achieved by coexpression of *cyc*A with the *ccm* gene cluster of *E. coli.* The *cyc*A gene was cloned in pT7-7 under control of the *lac* promoter and transformed into *E. coli* strain BL21(DE3) harboring pEC86 (containing the *ccm* genes). Expression was induced by the addition of IPTG. The expressed protein was effectively transported to the periplasm of the bacterium using its own signal sequence, indicating correct targeting within the cell, signal peptide cleavage, and haem association, as detected by SDS–PAGE and immunoblotting (see Fig. 1). Several strategies of growth were tested on small-scale (100 ml) before attempting scale-up. Highest yields were obtained when the cell cultures were induced with IPTG at the late exponential phase. Routinely, small-scale studies produced up to 1 mg of cytochrome *c″* per 100 ml.

SDS–PAGE analysis (Fig. 1A) of extracts revealed high levels of the expected 15-kDa band. Meanwhile, no holocytochrome *c″* was detected in *E. coli* cells bearing the gene for cytochrome *c″* in the absence of the cytochrome *c* maturation gene cluster, regardless of the scale of the culture (see Fig. 1B). Likewise, an untransformed host strain showed virtually no *c*-type cytochromes expression under aerobic growth conditions, either in the presence or the absence of pEC86. When scaled-up to 3 liters, almost 30 mg of the cytochrome was obtained from periplasmic extracts as determined spectrophotometrically (Table 1). Higher levels of expression might be obtained by using an *E. coli* strain with diminished periplasmic proteolytic activity. Following purification (see Materials and Methods), a final yield of 19 mg of purified cytochrome *c″* was obtained. In order to verify that the expressed cytochrome *c″* had similar properties to the native protein, several physical measurements were performed.

UV-Visible Spectrophotometry

Purified recombinant cytochrome *c″* presented an A_{406}/A_{275} ratio typical of the native protein obtained from *M. methylotrophus* (≈ 8.5). The UV-Visible absorption spectra of oxidized and reduced recombinant

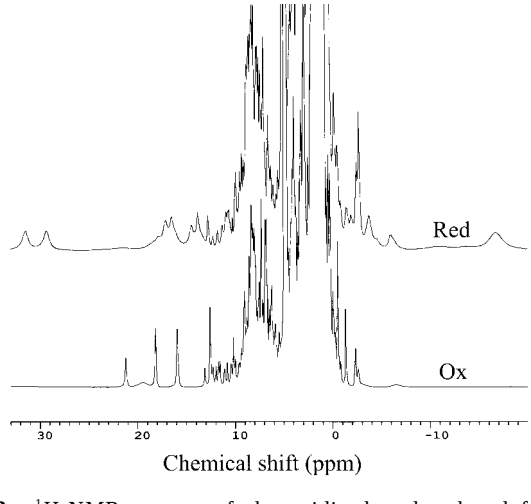

FIG. 3. ^{1}H-NMR spectra of the oxidized and reduced forms of recombinant cytochrome *c″*.

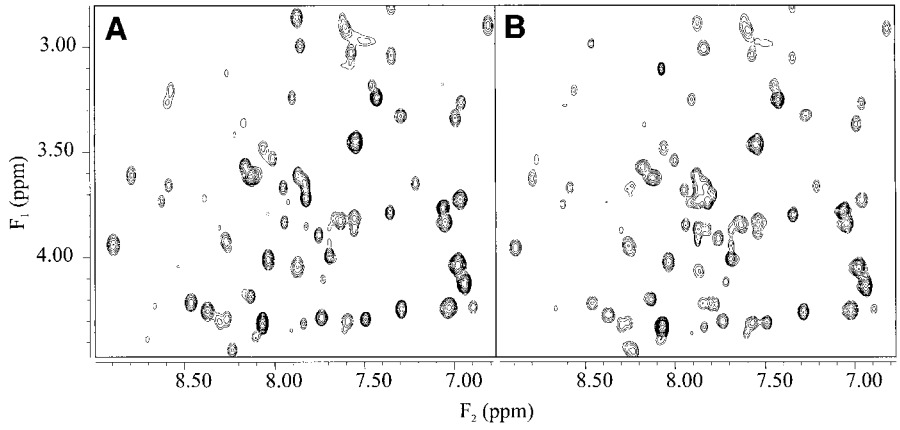

FIG. 4. A section from the fingerprint region of the two-dimensional TOCSY spectra of the native protein (A), and the recombinant protein (B). The similarity of the cross peak patterns and chemical shifts in both spectra is apparent. Mixing times: 50 and 75 ms for (A) and (B), respectively.

cytochrome *c″* (Fig. 2) were identical to those of the native protein (1).

^{1}H-NMR Spectroscopy

The one-dimensional spectra of the oxidized and reduced forms of the recombinant protein (Fig. 3) are indistinguishable from those previously reported for the protein isolated from the natural host (1). In particular, the similarity of the pattern of resonances, shifted outside the main envelope by paramagnetic effects, and the redox-dependent reversible change of spin state, provide strong evidence for the similarity of the haem structure. Two-dimensional spectra (TOCSY and NOESY) of the oxidized recombinant protein were acquired and compared to those of the native protein. The same pattern of chemical shifts and spin systems was observed (see Fig. 4, for illustration of TOCSY spectra), indicating that the folding of the two proteins is similar. This is further supported by the patterns of NOEs observed in the NOESY spectra (spectra not shown).

Differential Scanning Calorimetry

Differential scanning calorimetry measurements were performed to confirm the similarity of the folding of the native and recombinant proteins. Measurements performed with the oxidized form of the two proteins showed two transitions with midpoint temperatures of 57 ± 2°C and 68 ± 1°C. A second heating scan of the same sample led to loss of the lower temperature transition with about 75% of the intensity of the first run being recovered on the single observed signal (Fig. 5). The intensity ratio of the two transitions was markedly concentration dependent, the contribution of the lower temperature transition being decreased by 40% when the protein concentration was increased from 0.22 to 0.37 mg/ml. In every experiment, the behavior of the recombinant and native proteins was identical.

DISCUSSION

We have utilized an expression vector containing *cyc*A that gave rise to enhanced levels of cytochrome *c″*

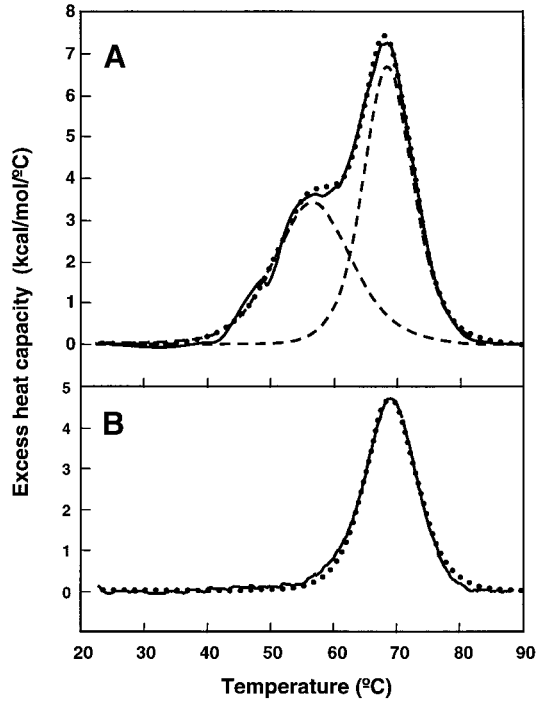

FIG. 5. Differential scanning calorimetric scans of recombinant *M. methylotrophus* cytochrome *c″* (0.22 mg/ml) in 50 mM phosphate buffer, pH 5.5, after baseline subtraction. (A) First scan. (B) Second scan of the same sample. Experimental data are shown as solid lines, and circles are from the theoretical fit to a two-transition process (A) or a single transition process (B). The deconvoluted endotherms of the two transitions are shown in dashed lines.

by coexpression with the cytochrome *c* maturation factors, recently isolated and characterized by the team of Thöny–Meyer (18). Following numerous failures to heterologously express cytochrome *c*″, the present work shows that it is possible to overexpress the *cyc*A gene of *M. methylotrophus* in *E. coli*, with high yield, only in the presence of haem maturation factors. Our earlier attempts did not produce mature cytochrome *c*″: haem could not be detected by spectrophotometry or by haem stain. However, some growth conditions led to expression of the *cyc*A gene, showing that *E. coli* was capable of synthesising and translocating the cytochrome *c*″ but failed to incorporate the haem moiety.

The recombinant cytochrome isolated with the periplasmic fraction contained a haem group and its structure was identical to that of the protein obtained from the natural host, as probed by UV-Visible and NMR spectroscopies, and differential scanning calorimetric measurements. This would suggest that the signal peptide of cytochrome *c*″ is correctly targeted to the periplasm and processed, thereby indicating recognition of the same cleavage sequence as in the natural host.

Heterologous expression has been shown to be successful for a variety of *c*-type cytochromes (9–13). The coexpression of cytochromes *c* with the *ccm*A-H gene cluster increases the level of expression by an order of magnitude compared to those previously reported. The yield of recombinant cytochrome *c*″ obtained from *E. coli* in the current work is similar to that obtained by coexpression of *ccm*A-H with cytochrome c_{552} (16) and the *c*-type cytochrome subunits of the cbb_3 oxidase (17).

In summary, our success in expressing cytochrome *c*″ reinforces the view that the haem maturation gene cluster could be used for expression of a wide range of haem-*c* proteins thereby making *E. coli* a more reliable and suitable host. This methodology can now be utilized to heterologously express site-directed mutants to study structure/function relationship in this unusual *bis*-histidinyl *c*-type cytochrome.

ACKNOWLEDGMENTS

We are indebted to Professor B. Ludwig for suggestion of the *ccm* elements and to Professor L. Thöny-Meyer for the generous gift of pEC86. We thank Professor A. G. Mauk for providing us with pBPCYC1(wt/3) and pBTR1, Dr. F. Molina-Heredia for providing the *E. coli* strain MC1061 and Isabel Pacheco for her help in purification of the protein. This work was supported by the E.C. Project FMRX-CT98-0218.

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