Expression of the Gene Encoding Cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough) in *Escherichia coli*: Export and Processing of the Apoprotein

By W. BRENT R. POLLOCK,¹ PHILLIP J. CHERMERIK,¹
MARY E. FORREST,² J. THOMAS BEATTY² AND
GERRIT VOORDOUW^{1*}

¹Division of Biochemistry, Department of Biological Sciences, The University of Calgary,
Calgary, Alberta, Canada T2N 1N4

²Department of Microbiology, University of British Columbia, Vancouver, British Columbia,
Canada V6T 1W5

(Received 4 January 1989; revised 24 April 1989; accepted 16 May 1989)

The expression of cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough) was examined in *Escherichia coli* transformed with either of two plasmids, pJ8 and pJ81. The former has an 840 bp insert of *D. vulgaris* DNA, containing the structural gene for cytochrome c_3 (387 bp) and its promoter region. Plasmid pJ81 was generated from pJ8 by deoxyoligonucleotide-directed mutagenesis to direct the synthesis of a protein with an altered signal peptidase cleavage site [Ala(–1)→Asp(–1)]. Synthesis of the 14 kDa precursor, which was partly processed to the 12 kDa mature protein, was observed in cells of *E. coli* TG2(pJ8) by SDS gel electrophoresis and Western blotting. Analysis of spheroplasts revealed that the processed polypeptide was present in the periplasm while the precursor was found only in the membrane/cytoplasmic fraction. No processing was observed in *E. coli* TG2(pJ81) cells, due to the mutation of the signal peptide cleavage site. No insertion of haem into the *E. coli* product could be detected in *E. coli* TG2(pJ8) cells by post-electrophoretic protohaem fluorescence analysis. The sensitivity of the cytochrome c_3 synthesized in *E. coli* TG2(pJ8) to digestion by chymotrypsin also indicated that the apoprotein was formed. The results indicate that *E. coli* is capable of synthesizing and exporting the cytochrome c_3 polypeptide, but fails to insert the haems.

INTRODUCTION

Cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough) contains four c -type haems, attached to a polypeptide chain of 107 amino acid residues (Trousil & Campbell, 1974) via four pairs of definitive thioether bonds (Dickerson & Timkovich, 1975). First discovered by Postgate (1954) and Ishimoto *et al.* (1954), this tetrahaem cytochrome is now thought to occur exclusively in the genus *Desulfovibrio*. The amino acid sequences of cytochrome c_3 isolated from six different *Desulfovibrio* species have been determined (reviewed by Odom & Peck, 1984) and this has revealed a limited degree of sequence homology. Nevertheless, the three-dimensional structures of cytochrome c_3 from *D. vulgaris* Miyazaki (Higuchi *et al.*, 1984) and *D. desulfuricans* Norway (Pierrot *et al.*, 1982), which share only 30% amino acid sequence homology, appear to be very similar. In particular the relative positions of the four covalently bound haems, thought to function in the transport of electrons from hydrogen, via hydrogenase, into the sulphate-reduction chain, have been conserved in the two structures.

The gene encoding cytochrome c_3 from *D. vulgaris* (Hildenborough) has been cloned (Voordouw & Brenner, 1986) and its nucleotide sequence indicates that the mature protein of 107 residues is preceded by a signal sequence of 21 amino acid residues, which strongly resembles signal sequences of proteins exported by *E. coli* to the periplasmic space. In addition a

promoter sequence (TTGACA/TACCAT), resembling the *E. coli* consensus promoter (TTGACA/TATAAT) is present 60 base pairs (bp) upstream from the translational start of the structural gene. One may therefore expect *E. coli* to be capable of expressing the cytochrome c_3 gene from *D. vulgaris*, and this is analysed in the present paper at the level of translation. A preliminary report of some of this work has been published (Voordouw, 1987).

METHODS

Biochemical reagents. All enzymes used for cloning and sequencing work were obtained from Pharmacia. Reagents for colour development in Western blotting, alkaline phosphatase conjugated goat anti-rabbit IgG, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates were purchased from Promega Biotec.

Bacterial strains and plasmids. *Desulfovibrio vulgaris* subsp. *vulgaris* strain Hildenborough (NCIB 8303; Postgate, 1984), abbreviated as *D. vulgaris* (Hildenborough), served as the source for the cytochrome c_3 (*cyc*) gene, which was cloned on a 7.5 kb *HindIII*–*EcoRI* fragment of *D. vulgaris* DNA (Voordouw & Brenner, 1986) in pUC9 (Vieira & Messing, 1982). *Escherichia coli* TG2 [*F'*(*traD36 proAB⁺ lacZΔM15^P*) Δ (*lac-pro*) *supE thi hsdM hsdR recA*] was obtained from Toby Gibson (Laboratory of Molecular Biology, MRC Centre, Cambridge, UK). Several new plasmids carrying the *cyc* gene were constructed from the parent plasmid pCYC3, which carries the 7.5 kb *HindIII*–*EcoRI* insert (Fig. 1*a*). Plasmid pCYC3 was digested with restriction endonuclease *AccI* and the 840 bp fragment carrying the *cyc* gene was isolated by gel electrophoresis through low-melting-temperature agarose in 0.04 M-Tris/acetate, 0.002 M-EDTA, pH 8. The isolated fragment was end-repaired by Klenow polymerase in the presence of deoxynucleotide triphosphates. The resulting blunt-ended fragment was ligated into the *SmaI* site of pUC9 to give plasmid pJ2 (Fig. 1*b*). The insert of plasmid pJ2 was turned around by digestion with *EcoRI* and *HindIII* and ligation to the replicative form (RF) of M13mp8, which had been digested with these same two enzymes. The RF of the resulting recombinant phage, M13mpJ8, was isolated, digested with *EcoRI* and *HindIII* and ligated to pUC8 digested with these same two enzymes to give plasmid pJ8 (Fig. 1*c*), which features a tandem arrangement of the *lac* and *cyc* promoters. Approximately 200 bp of DNA upstream from the *cyc* promoter was deleted by digestion of pJ8 with *HinfI*, followed by end-repair with Klenow polymerase in the presence of deoxynucleotide triphosphates, digestion with *HindIII*, ligation to the RF of M13mp8 digested with *SmaI* and *HindIII*, isolation of the RF of the resulting recombinant phage M13mpJ800 and, finally, transfer of the insert of this RF to pUC8 as described for pJ8, to give plasmid pJ800 (Fig. 1*d*).

Site-directed mutagenesis. A deoxypolynucleotide, P25, designed to change the GCC codon for Ala(–1) of the signal peptidase cleavage site into a GAC (Asp) codon 5'T GGG AGC GTC CAC AAC C (the mutant nucleotide is shown in bold) was purchased from the Regional DNA Synthesis Laboratory of the University of Calgary. The double primer method (Zoller & Smith, 1984) in which both kinased P25 and the universal sequencing primer are hybridized to single-stranded M13mpJ8 DNA, followed by *in vitro* extension with Klenow polymerase and ligation with T4 DNA ligase, was used. Following screening, a mutant M13 clone (M13mpJ81) was obtained. The presence of the desired mutation was checked by dideoxy-sequencing (Sanger *et al.*, 1977; Bankier & Barrell, 1983). Plasmid pJ81 was obtained by transfer of the insert from the RF of M13mpJ81 to pUC8 following digestion by *EcoRI* and *HindIII* as described above.

Growth of bacteria. Transformed *E. coli* TG2 was grown in TY medium (Maniatis *et al.*, 1982) containing 100 µg ampicillin ml^{–1}. Aerobic growth was in 5 ml cultures shaken at 250 r.p.m. in a New Brunswick G25 shaker at 37 °C. Exponential-phase aerobic cultures had an OD₆₀₀ of 0.5–0.8, while stationary-phase (overnight) cultures had an OD₆₀₀ of > 2. Anaerobic growth was in 20 ml standing cultures at 37 °C in culture tubes closed by rubber stoppers. Sodium nitrate (20 mM) was added to some of these cultures, which were always grown to the stationary phase.

Slab SDS-PAGE. Bacterial cultures were chilled on ice and then harvested by centrifugation at 1750 *g* for 10 min. For whole-cell samples the bacterial pellet was resuspended in 1 ml cold H₂O, transferred to a microfuge tube and centrifuged at 16000 *g* for 1 min. The cells were then resuspended in a small volume of cold H₂O (50 µl for aerobically grown exponential-phase cells, 100 µl for stationary-phase cells), an equal volume of SDS incubation buffer [60 mM-Tris/HCl, pH 6.8, 10% (w/v) SDS, 10% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.4% (w/v) bromophenol blue] was added and the samples placed in a boiling water bath for 2 min.

For spheroplast and periplasmic samples the pellet of exponential-phase cells was resuspended in 50 µl 30 mM-Tris/HCl, pH 8, 20% (w/v) sucrose. Lysozyme (5 µl, 10 mg ml^{–1} in 100 mM-EDTA, pH 7.4) was then added. Following incubation for 2 min at 4 °C the cells were pelleted with a microfuge at 16000 *g* for 2 min. The supernatant, containing the periplasmic fraction, was removed. The pellet, containing the cytoplasmic and membrane fraction, was resuspended in 50 µl Tris/sucrose. SDS incubation buffer (50 µl) was then added to both samples and they were placed in a boiling water bath for 2 min. Specified periplasmic fractions were incubated with chymotrypsin (6 µg ml^{–1}) for 30 min at 37 °C. Proteolysis was terminated by adding incubation buffer and boiling.

Fig. 1. Representation of plasmids carrying the *cyc* gene from *D. vulgaris* (Hildenborough). The coding region for cytochrome c_3 (hatched boxes), the position of and direction of transcription from the *lac* (P_{lac}) and *cyc* (P_{cyc}) promoters, and sites for restriction endonucleases *Eco*RI (E), *Acc*I (A), *Hind*III (H) and *Hinf*I (Hf) are indicated. Their construction is described in Methods. (a) The parent plasmid pCYC3, (b) pJ2, (c) pJ8, (d) pJ800. The scale bar is 1 kb for (a) and 100 bp for (b–d).

Gel electrophoresis, essentially according to Laemmli (1970), was either on a homogeneous, 15% (w/v) acrylamide/5% (w/v) bisacrylamide gel, or on a gradient (15%/5% to 7%/3%) gel in 40 mM-Tris/HCl, pH 8.8, 0.1% (w/v) SDS. A 4.6%/3% stacking gel in 80 mM-Tris/HCl, pH 6.8, 0.06% (w/v) SDS and a running buffer containing 25 mM-Tris/HCl and 192 mM-glycine at pH 8.3 and 0.1% (w/v) SDS were used.

Western blotting. Following SDS-PAGE, polypeptides were transferred to nitrocellulose electrophoretically overnight at 20 V in a Bio-Rad transfer tank, containing 25 mM-Tris, 192 mM-glycine and 20% (v/v) methanol, according to Towbin *et al.* (1979). The nitrocellulose was then blocked by shaking in 0.7% (w/v) gelatin, 10 mM-Tris/HCl, pH 7.4, and 0.9% (w/v) NaCl for 1 h. After a wash with buffer A, containing 0.1% (v/v) Nonidet P-40, 10 mM-Tris/HCl, pH 7.4, and 0.9% (w/v) NaCl, the blot was incubated with a 1:2500 dilution of a pretreated polyclonal antiserum, raised against holocytochrome c_3 purified from *D. vulgaris* (Hildenborough), in buffer A for at least 2 h. The blot was then thoroughly washed with buffer A and incubated with 2 μ l of an alkaline phosphatase conjugated goat anti-rabbit IgG in 50 ml buffer A for 2 h. Following washes, first with buffer A and then with alkaline phosphatase buffer (APB: 100 mM-NaCl, 10 mM-Tris/HCl, pH 9.5, 5 mM-MgCl₂) the nitrocellulose was incubated with nitroblue tetrazolium (67 μ g ml⁻¹) and 5-bromo-4-chloro-3-indolyl phosphate (33 μ g ml⁻¹) in 30 ml APB for 15 min, shielded from light. The blot was finally washed with water and dried.

For pretreatment, 20 μ l serum was incubated with 5 ml serum pretreatment solution (SPS) at 10 °C overnight. The mixture was then centrifuged and the supernatant diluted with 45 ml buffer A to which 0.03% (w/v) NaN₃ was added. SPS was prepared from *E. coli* TG2(pUC8) cells. These were grown in 100 ml TY-ampicillin, harvested by centrifugation and resuspended in 7 ml cold H₂O. Following the addition of 3 ml 10% (w/v) SDS the lysed cells were boiled for 2 min and then dialysed against 500 ml 150 mM-NaCl, 10 mM-Tris/HCl, pH 7.4, and two changes of 500 ml buffer A. The solution was diluted to 50 ml with buffer A and stored as SPS at –20 °C.

Fluorophotography. Slab gels were placed on a short-wavelength-UV light box and photographed through a red filter using Polaroid type 52 film (ISO 400), f/4.5, and an exposure of 6 min (Katan, 1976).

RESULTS AND DISCUSSION

In exponential-phase *E. coli* TG2 cells, transformed with plasmid pCYC3, pJ2 or pJ8, the level of expression of cytochrome c_3 polypeptides was found to be dependent on the plasmid used for transformation (Fig. 2, lanes 2–4). The reduction of the insert size from 7.5 kb in pCYC3 to 0.84 kb in both pJ2 and pJ8 led generally to an increase in the expression of cytochrome c_3 polypeptides. Little difference was seen in the level of expression of cells transformed with pJ2, in which transcription from the *cyc* and *lac* promoters is in opposite directions, or pJ8, in which these two promoters are arranged in tandem. Addition of isopropyl β -D-thiogalactopyranoside was not found to increase expression (not shown). These results confirm that the *cyc* promoter,

Fig. 2. Expression of the *cyc* gene from *D. vulgaris* (Hildenborough) in *E. coli* TG2, transformed with the plasmids described in Fig. 1. Detection was by SDS-PAGE and Western blotting using a cytochrome c_3 specific polyclonal antiserum. Lane 1, 200 ng of holocytochrome c_3 purified from *D. vulgaris* (Hildenborough); lanes 2–4, exponential-phase *E. coli* TG2 cells transformed with pCYC3, pJ2 or pJ8, respectively; lane 5, stationary-phase *E. coli* TG2 cells transformed with pJ8. No expression of cytochrome c_3 polypeptides was observed in the *E. coli* TG2(pUC8) control (not shown; see Fig. 4). The 14 and 12 kDa polypeptides correspond to the precursor and processed forms, respectively, of apocytochrome c_3 .

which strongly resembles the *E. coli* consensus promoter sequence (see Introduction) functions well in *E. coli*. The observation that the level of expression of *E. coli* TG2 transformed with pJ800, in which 200 bp of DNA separating these two promoters has been deleted (Fig. 1), is similar to that in cells transformed with pJ8 (not shown) also indicates that the *lac* promoter contributes little to the expression observed in Fig. 2.

Two immunoreactive bands are seen in Fig. 2, lanes 3–5. As will be shown below there is good evidence to suggest that the upper band corresponds to the cytochrome c_3 precursor protein (128 amino acids, molecular mass 14.0 kDa, based on the sequence of the gene and the absence of bound haem), while the lower band corresponds to the processed apocytochrome c_3 (107 amino acids, molecular mass 11.6 kDa). For simplicity these two bands will be referred to as the 14 kDa and 12 kDa forms of cytochrome c_3 expressed in *E. coli*. The apparent molecular mass of these forms derived from a comparison with standards on an SDS gel (not shown) is higher: 17.5 and 15.5 kDa, respectively. The discrepancy may be due to their high net positive charge, which can be estimated as +10 and +8 at pH 8.3 for the precursor and processed forms of the apoprotein, respectively. Assuming a ratio of 1.4 g SDS per g of polypeptide (Reynolds & Tanford, 1970) one can estimate a negative charge contribution of –68 and –56 in the precursor and processed polypeptides due to bound SDS. Thus the net negative charge is substantially reduced and may cause an electrophoretic retardation of these polypeptides. The molecular mass of holocytochrome c_3 derived from SDS gels was 15.5–16.0 kDa, different from the 13.9 kDa value predicted by the structural gene sequence and the presence of four covalently bound haems. It is generally found to migrate as a somewhat broader band with a mobility similar to that of the 12 kDa form (Fig. 2, lanes 1 and 2). Good expression was observed in aerobic stationary phase cultures (Fig. 2, lane 5). A smaller polypeptide (11 kDa, relative to the migration of the 14 kDa and 12 kDa polypeptides) was also observed under these conditions, as indicated. The 11 kDa polypeptide is thought to be a proteolytic degradation product of the 12 kDa polypeptide, as discussed below. When periplasmic and membrane/cytoplasmic fractions were prepared by lysozyme treatment of *E. coli* TG2(pJ8) cells and blotted separately the results shown in Fig. 3(a) were obtained. The

Fig. 3. Localization of the precursor (14 kDa) and processed (12 kDa) forms of apocytochrome c_3 from *D. vulgaris* (Hildenborough) synthesized in *E. coli*. (a) Samples were run on a 15% acrylamide gel. Lane 1, periplasmic fraction of exponential-phase *E. coli* TG2(pJ8) cells; lane 2, membrane/cytoplasmic fraction of exponential-phase *E. coli* TG2(pJ8) cells. (b) Samples were run on a 7–15% acrylamide gradient gel. Lane 1, 250 ng of holocytochrome c_3 purified from *D. vulgaris* (Hildenborough); lane 2, stationary-phase *E. coli* TG2(pJ81) cells; lane 3, stationary-phase *E. coli* TG2(pJ8) cells.

processed 12 kDa polypeptide was found predominantly in the periplasm (Fig. 3a, lane 1) while the precursor 14 kDa polypeptide was found only in the membrane/cytoplasmic fraction (Fig. 3a, lane 2). The presence of the 12 kDa polypeptide in the non-periplasmic fraction could have been a result of incomplete release of the polypeptide from the periplasm, partial cytoplasmic localization (unlikely in the light of the signal peptide), or association of the positively charged apoprotein with the phospholipid bilayer as has been observed with apocytochrome c (Rietveld *et al.*, 1986; Li-Xin *et al.*, 1988).

The processing and export of the membrane-bound or cytoplasmic 14 kDa form to the periplasmic 12 kDa polypeptide involves cleavage at a typical Ala(−1)↓Ala(+1) signal peptidase cleavage site as deduced from the sequence of the *D. vulgaris* (Hildenborough) gene (Voordouw & Brenner, 1986). A mutation was introduced at this cleavage site by deoxyoligonucleotide-directed mutagenesis of the gene in order to confirm that the processing and export in *E. coli* involves cleavage at this same site. When cells of *E. coli* TG2(pJ81), in which the (−1) position

Fig. 4. (a) Post-SDS-PAGE protohaem fluorescence analysis of aerobically grown stationary-phase *E. coli* cells expressing the *cyc* gene. Lanes 1 and 7, 10 μ g of holocytochrome c_3 purified from *D. vulgaris* (Hildenborough); lane 2, *E. coli* TG2(pJ8) with 2.5 μ g of added holocytochrome c_3 ; lane 3, *E. coli* TG2(pJ8), no addition; lane 4, *E. coli* TG2(pUC8), no addition; lane 5, *E. coli* TG2(pUC8) with 2.5 μ g of added holocytochrome c_3 ; lane 6, 2.5 μ g holocytochrome c_3 . (b) Western blot of the same gel as in (a). The position of the 14 kDa and 12 kDa forms of apocytochrome c_3 synthesized by *E. coli* TG2 transformed with pJ8 (lanes 2 and 3) is indicated.

of the cleavage site has been mutated to Asp, were blotted only a minor amount of the 14 kDa form, but no 12 kDa polypeptide, was observed (Fig. 3*b*, lane 2). This result was surprising since an accumulation of the 14 kDa precursor was anticipated. A possible explanation is that the introduction of a negative charge in the signal peptide of the 14 kDa polypeptide synthesized in cells transformed with pJ81 prevents its association with the membrane, causing a rapid degradation by cytoplasmic proteases. These experiments clearly establish that *E. coli* can synthesize, export and process apocytochrome c_3 from *D. vulgaris* (Hildenborough).

It is unusual to find an accumulation of the precursor form of a periplasmic protein to the extent shown in Fig. 2, lanes 3–5, and this indicates that the export and processing of this polypeptide is slow. The ratio of 14 kDa to 12 kDa protein expressed in *E. coli* was variable. The increased expression in cells transformed with either pJ2 or pJ8 led to a further accumulation of the 14 kDa protein, compared with cells transformed with the parent plasmid pCYC3 (Fig. 2: lanes 3 and 4 vs lane 2). A significant accumulation of the 14 kDa form was also seen in stationary-phase cells transformed with pJ8 (Fig. 2, lane 5). It is unlikely that this accumulation is artefactual e.g. the result of the formation of inclusion bodies of the 14 kDa form in the cytoplasm, since no such accumulation is observed in *E. coli* TG2(pJ81), which produces a polypeptide that differs by only a single amino acid. The slow processing and export of the 14 kDa polypeptide in *E. coli* indicates a limited capacity of the *E. coli* system to translocate and process the apopolypeptide.

The biosynthesis of holocytochrome c_3 requires the covalent insertion of four haems into the apoprotein. The results shown in Figs 4 and 5 indicate that *E. coli* is not capable of inserting the haems, by two criteria: (a) direct visualization of haem fluorescence, and (b) sensitivity of the *E. coli* product towards proteolysis by chymotrypsin. It is shown in Fig. 4 that holocytochrome c_3 can be readily visualized following SDS-PAGE, by direct photography of haem fluorescence. This method allows the detection of 1 μ g of holocytochrome c_3 (not shown) either as the pure protein or in a cell extract. For instance, when samples prepared from *D. vulgaris* (Hildenborough) cells are run on an SDS gel both cytochrome c_3 and the mono-haem cytochrome c_{553} can be readily visualized (not shown). No haem fluorescence could be detected

in aerobically grown *E. coli* TG2(pJ8) cells (Fig. 4a, lane 3), while blotting of the gel (Fig. 4b, lane 3) showed the presence of a substantial amount of cytochrome c_3 polypeptide (estimated at 2–6 μ g). Of course failure of *E. coli* to insert haem under aerobic growth conditions does not mean that holocytochrome c_3 cannot be synthesized during anaerobic growth, or growth with a terminal electron acceptor other than oxygen. In particular, anaerobic growth in the presence of nitrate has been shown to induce the synthesis of a hexahaem cytochrome c_{552} , which has recently been extensively characterized. This c -type cytochrome functions as a nitrite reductase, is present in the periplasm of *E. coli* and was shown to be similar to a hexahaem cytochrome isolated from *D. desulfuricans* (Kajie & Anraku, 1986). *D. vulgaris* probably lacks this particular hexahaem cytochrome, since it is not capable of using nitrate as a respiratory substrate. Expression of apocytochrome c_3 in *E. coli* TG2(pJ8) can be readily demonstrated under anaerobic conditions both in the absence and presence of nitrate (Fig. 5a, lanes 2 and 3, respectively). The 14 kDa precursor form that is seen in aerobic cells (Fig. 5, lane 1) does not accumulate under these slower growth conditions. Instead the 11 kDa polypeptide is prominent in Fig. 5(a), lanes 2 and 3. This polypeptide is absent in pUC9-transformed control cells (not shown) and therefore presumably results from proteolysis of the 12 kDa polypeptide, which is not stable in *E. coli* possibly due to the absence of bound haem. This idea was tested further in the experiment shown in Fig. 5(b): addition of chymotrypsin leads to complete digestion of the *cyc* gene product in nitrate-grown cells, whereas holocytochrome c_3 isolated from *D. vulgaris* (Hildenborough) is resistant to proteolysis. No resistance to proteolysis of the cytochrome c_3 polypeptide produced in *E. coli* was observed under any of the growth conditions indicated above, suggesting that the apoprotein was formed under all of these conditions and that a specific system for haem insertion into cytochrome c_3 that exists in *D. vulgaris* is absent in *E. coli*.

Presently the mechanism of c -type haem insertion in prokaryotes is unknown, but the results obtained using the mitochondrial cytochrome c systems in yeast (Rothstein & Sherman, 1980; Taniuchi *et al.*, 1983; Dumont *et al.*, 1987) and *Neurospora crassa* (Hennig & Neupert, 1981; Nicholson *et al.*, 1987; Nargang *et al.*, 1988) indicate a requirement in eukaryotes for at least one protein, referred to as haem lyase, to covalently attach the haem to the apoprotein. Evidence for the haem lyase is both genetic (Rothstein & Sherman, 1980; Dumont *et al.*, 1987) and biochemical (Hennig & Neupert, 1981; Taniuchi *et al.*, 1983; Nicholson *et al.*, 1987; Nargang *et al.*, 1988), the latter indicating that the haem lyase is situated in the intermembrane space of *N. crassa* mitochondria.

Holocytochromes c and c_3 are similar in that (i) both contain the characteristic c -type haem attachment site Cys-(Xaa) $_n$ -Cys-His (Dickerson & Timkovich, 1975; $n=2$ for cytochrome c and 2 or 4 for c_3), (ii) their respective apoforms are both synthesized in the cytoplasm and subsequently translocated across a membrane and, as a result, (iii) they display homologous cellular localization, cytochrome c being located in the intermembrane space of mitochondria while cytochrome c_3 is periplasmic (Postgate, 1984). An important difference between the two is in the mechanism by which the apoforms are translocated across their respective membranes. Whereas cytochrome c_3 possesses an N-terminal signal peptide sequence (Voordouw & Brenner, 1986), cytochrome c apparently requires the presence of an apocytochrome c binding protein (Hennig *et al.*, 1983; Nicholson *et al.*, 1987) in addition to the haem lyase (Nargang *et al.*, 1988) for effective translocation. There is also evidence to suggest that the translocation of apocytochrome c across the outer mitochondrial membrane is directed by an N-terminal sequence which interacts electrostatically with negatively-charged phospholipids (Li-Xin *et al.*, 1988). However, unlike the prokaryotic signal peptide, this sequence is not removed by proteolysis. Cytochrome c haem lyase is able to use apocytochrome c from a number of different species as a substrate for haem attachment (Visco *et al.*, 1985; Scarpulla & Nye, 1986). However, no attempt has been made to assess its activity with c -type apocytochromes other than cytochrome c .

Use of *Rhodobacter capsulatus*, which has high levels of a monohaem cytochrome c_2 in its periplasm as well as several other c -type cytochromes (Bartsch, 1978), as the host organism for expression of the *D. vulgaris cyc* gene from an expression vector described elsewhere (Johnson *et al.*, 1986) also did not result in the expression of holocytochrome c_3 (results not shown). Given

Fig. 5. Expression of the *cyc* gene in stationary-phase *E. coli* cells under anaerobic conditions. (a) Comparison of expression under aerobic and anaerobic conditions. The positions of the 14 kDa precursor and the 12 kDa processed forms of apocytochrome c_3 expressed in *E. coli* are indicated, as well as that of a prominent 11 kDa proteolytic degradation product. Lane 1, aerobically grown *E. coli* TG2(pJ8); lane 2, anaerobically grown *E. coli* TG2(pJ8); lane 3, *E. coli* TG2(pJ8) grown anaerobically in the presence of 20 mM- NaNO_3 . (b) Chymotrypsin sensitivity of the *cyc* gene product synthesized in *E. coli*. Cells of *E. coli* TG2(pJ8) were grown anaerobically in the presence of 20 mM- NaNO_3 to stationary phase. Lane 1, periplasmic fraction isolated as described in Methods; lane 2, same as lane 1, but digested with chymotrypsin ($6 \mu\text{g ml}^{-1}$) for 30 min at 37°C ; lane 3, same as lane 2, but with *D. vulgaris* (Hildenborough) holocytochrome c_3 (200 ng) added. The same results were obtained using cells grown aerobically or anaerobically without nitrate (not shown).

the specificity of eukaryotic cytochrome *c* haem lyase (Visco *et al.*, 1985) and the ability of *E. coli* to express holocytochrome b_5 which contains a non-covalently bound *b*-type haem, under aerobic conditions (von Bodman *et al.*, 1986), it is likely that *E. coli* and *R. capsulatus* are unable to express holocytochrome c_3 due to the absence of a specific haem insertion system in these host organisms.

W.B.R.P. acknowledges the support of Graduate Teaching and Graduate Research Assistantships from the University of Calgary. This work was supported by an establishment grant from the Alberta Heritage Foundation for Medical Research (AHFMR) and an operating grant to G.V. from the National Science and Engineering Research Council of Canada (NSERC). P.J.C. acknowledges receipt of an AHFMR summer studentship award. The authors wish to thank Gijs van Rooijen for the construction of plasmid pJ800.

REFERENCES

- BANKIER, A. T. & BARRELL, B. G. (1983). Shotgun DNA sequencing. In *Techniques in the Life Sciences*, pp. 1–34, B5 Nucleic Acid Biochemistry, B508, Elsevier Scientific Publishers Ireland Ltd.
- BARTSCH, R. G. (1978). Cytochromes. In *The Photosynthetic Bacteria*, pp. 249–279. Edited by R. K. Clayton & W. R. Sistrom. New York: Plenum Press.
- VON BODMAN, S. B., SCHULER, M. A., JOLLIE, D. R. & SLIGAR, S. G. (1986). Synthesis, bacterial expression, and mutagenesis of the gene coding for mammalian cytochrome b_5 . *Proceedings of the National Academy of Sciences of the United States of America* **83**, 9443–9447.
- DICKERSON, R. E. & TIMKOVICH, R. (1975). Cytochromes c . In *The Enzymes*, vol. 11, 3rd edn, pp. 397–547. Edited by P. D. Boyer. New York: Academic Press.
- DUMONT, M. E., ERNST, J. F., HAMPSEY, D. M. & SHERMAN, F. (1987). Identification and sequence of the gene encoding cytochrome c heme lyase in the yeast *Saccharomyces cerevisiae*. *EMBO Journal* **6**, 235–241.
- HENNIG, B. & NEUPERT, W. (1981). Assembly of cytochrome c . Apocytochrome c is bound to specific sites on mitochondria before its conversion to holocytochrome c . *European Journal of Biochemistry* **121**, 203–212.
- HENNIG, B., KOEHLER, H. & NEUPERT, W. (1983). Receptor sites involved in posttranslational transport of apocytochrome c into mitochondria: specificity, affinity, and number of sites. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 4963–4967.
- HIGUCHI, Y., KUSUNOKI, M., MATSUURA, Y., YASOKA, N. & KAKUDO, M. (1984). Refined structure of cytochrome c_3 at 1.8 Å resolution. *Journal of Molecular Biology* **172**, 109–139.
- ISHIMOTO, M., KOYAMA, J. & NAGAI, Y. (1954). Role of a cytochrome in thiosulphate reduction by a sulphate reducing bacterium. *Seikagaku Zasshi* **26**, 303.
- JOHNSON, J. A., WONG, W. K. R. & BEATTY, J. T. (1986). Expression of cellulase genes in *Rhodobacter capsulatus* by use of plasmid expression vectors. *Journal of Bacteriology* **167**, 604–610.
- KAJIE, S. & ANRAKU, Y. (1986). Purification of a hexaheme cytochrome c_{552} from *Escherichia coli* K12 and its properties as a nitrite reductase. *European Journal of Biochemistry* **154**, 457–463.
- KATAN, M. B. (1976). Detection of cytochromes on sodium dodecylsulphate-polyacrylamide gels by their intrinsic fluorescence. *Analytical Biochemistry* **74**, 132–137.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LI-XIN, Z., JORDI, W. & DE KRUIJFF, B. (1988). Influence of heme and importance of the N-terminal part of the protein and physical state of model membranes for the apocytochrome c -lipid interaction. *Biochimica et biophysica acta* **942**, 115–124.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NARGANG, F. E., DRYGAS, M. E., KWONG, P. L., NICHOLSON, D. W. & NEUPERT, W. (1988). A mutant of *Neurospora crassa* deficient in cytochrome c heme lyase activity cannot import cytochrome c into mitochondria. *Journal of Biological Chemistry* **263**, 9388–9394.
- NICHOLSON, D. W., KÖHLER, H. & NEUPERT, W. (1987). Import of cytochrome c into mitochondria: cytochrome c heme lyase. *European Journal of Biochemistry* **164**, 147–157.
- ODOM, J. M. & PECK, H. D., JR (1984). Hydrogenase, electron transfer proteins and energy coupling in the sulfate-reducing bacteria *Desulfovibrio*. *Annual Review of Microbiology* **38**, 551–592.
- PEIRROT, M., HASER, R., FREY, M., PAYAN, F. & ASTIER, J. P. (1982). Crystal structure and electron transfer properties of cytochrome c_3 . *Journal of Biological Chemistry* **257**, 14341–14348.
- POSTGATE, J. R. (1954). Presence of cytochrome in an obligate anaerobe. *Biochemical Journal* **56**, xi.
- POSTGATE, J. R. (1984). *The Sulphate-reducing Bacteria*, 2nd edn. Cambridge: Cambridge University Press.
- REYNOLDS, J. A. & TANFORD, C. (1970). Binding of dodecyl sulfate to proteins at high binding ratios. Possible implications for the state of proteins in biological membranes. *Proceedings of the National Academy of Sciences of the United States of America* **66**, 1002–1007.
- RIETVELD, A., BERKHOUT, T. A., ROENHORST, A., MARSH, D. & DE KRUIJFF, B. (1986). Preferential association of apocytochrome c with negatively charged phospholipids in mixed model membranes. *Biochimica et biophysica acta* **858**, 38–46.
- ROTHSTEIN, R. J. & SHERMAN, F. (1980). Genes affecting the expression of cytochrome c in yeast: genetic mapping and genetic interactions. *Genetics* **94**, 871–889.
- SANGER, F., NICKLEN, S. & COULSEN, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCARPULLA, R. C. & NYE, S. H. (1986). Functional expression of rat cytochrome c in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 6352–6356.
- TANIUCHI, H., BASILE, G., TANIUCHI, M. & VELOSO, D. (1983). Evidence for formation of two thioether bonds to link heme to apocytochrome c by partially purified cytochrome c synthetase. *Journal of Biological Chemistry* **258**, 10963–10966.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- TROUSIL, E. B. & CAMPBELL, L. L. (1974). The amino acid sequence of cytochrome c_3 from *Desulfovibrio vulgaris*. *Journal of Biological Chemistry* **249**, 386–393.
- VIEIRA, J. & MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.

- VISCO, C., TANIUCHI, H. & BERLETT, B. S. (1985). On the specificity of cytochrome *c* synthetase in recognition of the amino acid sequence of apocytochrome *c*. *Journal of Biological Chemistry* **260**, 6133–6138.
- VOORDOUW, G. (1987). Molecular biology of redox proteins in sulphate reduction. In *The Nitrogen and Sulphur Cycles*, pp. 147–160. Edited by J. A. Cole & S. J. Ferguson. Cambridge: Cambridge University Press.
- VOORDOUW, G. & BRENNER, S. (1986). Cloning and sequencing of the gene encoding cytochrome *c*₃ from *Desulfovibrio vulgaris* (Hildenborough). *European Journal of Biochemistry* **159**, 347–351.
- ZOLLER, M. J. & SMITH, M. (1984). Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single stranded DNA template. *DNA* **3**, 479–488.