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Expression of plastocyanin and cytochrome *f* of the cyanobacterium *Phormidium laminosum* in *Escherichia coli* and *Paracoccus denitrificans* and the role of leader peptides

Beatrix G. Schlarb [a], Michael J. Wagner [a], Erik Vijgenboom [b], Marcellus Ubbink [b], Derek S. Bendall [a], Christopher J. Howe [a,*]

[a] *Department of Biochemistry and Cambridge Centre for Molecular Recognition, University of Cambridge, Cambridge CB2 1QW, UK*
[b] *Leiden Institute of Chemistry, Leiden University, Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands*

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Abstract

The gene for plastocyanin from the cyanobacterium *Phormidium laminosum* was successfully expressed in *Escherichia coli*. Expression of the gene for cytochrome *f* resulted in the production of holocytochrome *f* in the periplasmic space of *E. coli*, but the yield was low. Expression in *Paracoccus denitrificans* yielded no holoprotein. When the region encoding the cytochrome *f* leader sequence was replaced with more typical bacterial leader sequences (those from the *P. laminosum* plastocyanin gene and the *Paracoccus versutus* cytochrome *c*-550 gene), much higher yields were consistently obtained in both species. Overexpressed proteins were compared to those isolated from *P. laminosum* and found to be identical in mass, isoelectric point, redox midpoint potential and (for plastocyanin) ^{1}H-NMR spectrum.

Keywords: *petA*; *petE*; Photosynthetic electron transfer; Protein targeting

1. Introduction

Plastocyanin (Pc) and cytochrome *f* (Cyt f) are two proteins involved in the electron transfer chain of oxygenic photosynthesis. They represent a good model system for the study of protein–protein electron transfer. Each protein has only one redox centre and their reaction is fast ($2 \times 10^8\ M^{-1}\ s^{-1}$ in solution at 25°C, 100 mM ionic strength, pH 6.0; Kannt et al., 1996).

Pc is a soluble, type I copper protein of about 11 kDa which is situated in the thylakoid lumen and functions as an electron shuttle between Cyt f of the cytochrome *bf* (Cyt bf) complex and photosystem I (Ref. 13 in Wagner et al., 1996). Its three-dimensional structure is highly conserved among cyanobacteria, algae and higher plants (Refs. 5–11 in Ubbink et al., 1998). The distribution of charges on the protein surface varies considerably; whereas in higher plants an accumulation of negative charges can be found at the remote face around Tyr-83 (according to the higher plant numbering), basic and acidic residues are more evenly distributed in the cyanobacterial case.

Cyt f, a *c*-type cytochrome, is the largest component of the Cyt bf complex (Cramer et al., 1996). It is synthesised with a cleavable leader sequence and is anchored in the thylakoid membrane by a single C-terminal transmembrane helix. Its approx. 28 kDa N-terminal main domain, which is fully redox active, protrudes into the lumen. It receives electrons from the Rieske FeS–protein and passes them on to Pc. All known primary structures of Cyt f are highly conserved (Ref. 12 in Wagner et al., 1996). However, while Cyt f possesses a basic ridge in higher plants, it is predominantly acidic in cyanobacteria. So far, only one three-

Abbreviations: A_x, absorbance at *x* nm; Ap, ampicillin; ave, average; Cm, chloramphenicol; Cyt bf, cytochrome *bf*; Cyt f, cytochrome *f*; ESI-MS, electrospray ionisation mass spectrometry; NMR, nuclear magnetic resonance; *ori*, origin(s) of DNA replication; Pc, plastocyanin; ^{R} resistance/resistant; RBS, ribosome-binding site; Rif, rifampicin; rpm, revolutions per minute; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; Sm, streptomycin; Sp, spectinomycin; ϵ, absorption coefficient; $\varnothing$, diameter; ′ (prime), denotes a truncated gene at the indicated side; [] denotes plasmid-carrier state.

* Corresponding author. Tel.: +44-1223-333688; fax: +44-1223-333345.
E-mail address: c.j.howe@bioc.cam.ac.uk (C. Howe)

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dimensional structure of the truncated, soluble lumenal domain has been published, from turnip (Ref. 10 in Wagner et al., 1996).

In order to study the mechanism of electron transfer in vitro it is desirable to use homologous systems with Cyt f and Pc from the same source. This is especially so for the cyanobacterial proteins, which are less highly conserved than those of higher plants. However, to our knowledge, no systems exist so far for the overexpression of Pc and Cyt f from the same source. Expression of functional Cyt f is likely to be particularly difficult, given the need for incorporation of a haem group. We describe here expression systems for Pc and the redox active soluble domain of Cyt f from *Phormidium laminosum*. We found that the level of expression of Cyt f is affected by the leader sequence used to direct export to the periplasm. We compare the proteins produced with those isolated from *P. laminosum*.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli BL21(DE3)pLysS and W3110: see Studier and Moffatt (1986) and Studier et al. (1990), respectively. *Paracoccus denitrificans* Pd2131: see van Spanning et al. (1991).

References and characteristics of plasmids used in this work are given in Table 1. Plasmids pMU8, pTZ18R, pTZ18U, pTZ19U, pEG400, pMAU40 and pEG.Tv1 were a gift from Professor Dr G.W. Canters, Gorlaeus Laboratories, Leiden University, Netherlands. Plasmid pEC86 was a gift from Dr L. Thoeny-Meyer, Microbiology Institute, ETH Zurich, Switzerland. Plasmid pET11d was purchased from Stratagene.

2.2. Molecular biology

Molecular biological methods were essentially as described by Sambrook et al. (1989) and Ausubel et al. (1993). Constructs generated using PCR were sequenced to check that no mutations had been introduced. Enzymes were purchased from Boehringer (restriction enzymes), Pharmacia (restriction enzymes, T4 DNA ligase), Stratagene (Pfu-DNA-polymerase), Qiagen (*Taq* DNA polymerase) and New England Biolabs (Vent DNA polymerase). Dehydrated BHI- and LB-medium were bought from Difco Laboratories. Concentrations of antibiotics used were: chloramphenicol: 25 μg/ml; ampicillin: 100 μg/ml; rifampicin: 40 μg/ml; streptomycin: 50 μg/ml; spectinomycin: 100 μg/ml.

2.3. Protein expression and purification

Periplasmic extracts of the harvested cells were obtained by cold osmotic shock (Ref. 17 in Varley et al., 1995). For all protein work Milli-Q water was used. Protein solutions were oxidised with potassium ferricyanide or reduced with buffered sodium ascorbate and concentrated by ultrafiltration. Columns used for purification (at 4°C) were: *gel filtration* — Pharmacia Superdex G75 pre-poured FPLC-column, ∅2.5 cm × 1 m, flow rate 2 ml/min, equilibrated with 100 mM NaCl, 50 mM Tris–HCl (pH 7.5); *ion exchange* — Whatman DE52, self-poured, ∅ 5 cm × 8 cm, flow rate 4.5 ml/min; Pharmacia DEAE Sepharose® FastFlow self-poured FPLC-column, ∅2.5 cm × 12 cm, flow rate

Table 1
Description of plasmids

Plasmid	Relevant characteristics	Reference
pUC19PC	Ap^R; *petE* with endogenous leader cloned into *Eco*RI–*Bam*HI-site of pUC19	This work
pUC19CF	Ap^R; *petA* with endogenous leader and part of the expression platform of pET11d cloned into the *EcoR*I–*Xba*I-site of pUC19	This work
pUC19li5	Ap^R; *petA* with *P. laminosum* pc leader and part of the expression platform of pET11d cloned into the *EcoR*I–*Xba*I-site of pUC19	This work
pET11PC	Ap^R; *petE* cloned into the *Nco*I–*BamH*I-site of pET11d	This work
pET11CF	Ap^R; *petA* with its endogenous leader sequence cloned into the *Nco*I–*Bam*HI-site of pET11d	This work
pMU8	Ap^R (*cycA* cloned into *Sph*I-site of pUC18)	Ubbink et al. (1992)
pTZ18R, pTZ18U, pTZ19R	Ap^R, *lacZ′*, pBR322 *ori*, f1-*ori*	Mead et al. (1986)
pEG400	*IncP*, Sm^R, Sp^R, pUC12/13mcs, *lacZ′*	de Gier et al. (1994), Gerhus et al. (1990)
pMAU40	Sm^R, Sp^R (*pcycA* and *mauG* cloned into *Sac*I–*Sal*I-site of pEG400-derivative pEG401)	van der Palen et al. (1995)
pEG.Tv1	Sm^R, Sp^R (*cycA* cloned into *Bam*HI–*Hin*dIII-site of pEG400)	Ubbink et al. (1996)
pGC202	Sm^R, Sp^R (*petA* cloned into *Nde*I–*Bam*HI-site of pMAU40)	This work
pEG402	Sm^R, Sp^R (*petA* with *pcycA*, RBS and *cycA*-leader from pMU8 cloned into *Eco*RI–*Bam*HI-site of pEG400)	This work
pEC86	Cm^R,*ccm*ABCDEFGH (cassette of cytochrome *c* maturation genes of *E. coli*)	Arslan et al. (1998)

4 ml/min; Pharmacia HiTrap® Q-Sepharose pre-poured FPLC-column, flow rate 2 ml/min. Pc was eluted with 5–50 mM Tris–HCl (pH 7.5) and quantified by the absorbance at 598 nm of the oxidised form ($\epsilon_{598\ nm} = 4.3\ mM^{-1}\ cm^{-1}$; Varley et al., 1995), Cyt f with 0–200 mM NaCl, 50 mM Tris–HCl (pH 7.5) and quantified by the absorbance at 556 nm of the reduced form [$\epsilon_{556\ nm} = 31.5\ mM^{-1}\ cm^{-1}$, assuming ϵ of the maximum of the α-band to be identical to that of Cyt f from turnip (Ubbink et al., 1998)]. SDS–polyacrylamide gels (Ref. 20 in Varley et al., 1995) were stained for haem-containing bands according to Goodhew et al., 1986.

2.3.1. Wild-type plastocyanin

Pc from *P. laminosum* was prepared as described by Varley et al., 1995.

2.3.2. Recombinant plastocyanin

A single colony of *E. coli* BL21(DE3)-pLysS[pET11PC] (see Section 3.1) was suspended in 150 ml LB-medium supplemented with ampicillin and chloramphenicol. The starter culture was incubated overnight at 37°C shaking at 250 rpm. Ten conical flasks (2 l) containing 1 l LB-medium with ampicillin and chloramphenicol were inoculated with 10 ml starter culture each, grown into mid-log phase for 2.5 h in an orbital shaker (250 rpm) at 37°C and induced with IPTG and $CuSO_4$ to final concentrations of 100 mg/l and 0.2 mM, respectively. The temperature was reduced to 30°C and the culture harvested 16 h after induction. The protein was purified from periplasmic extracts using ion exchange chromatography and gel filtration [order of columns used (oxidation state of the protein): Whatman DE52 (reduced); Pharmacia Superdex® G75 (oxidised); Pharmacia HiTrap® Q-Sepharose (reduced); Pharmacia DEAE Sepharose FastFlow, (oxidised)]. A ratio A_{278}/A_{598} of 2.5 of the oxidised protein indicated sufficient purity for characterisation by NMR and further applications.

2.3.3. Wild-type cytochrome f

Cyt f from *P. laminosum* was prepared as described by Wagner et al. (1996).

2.3.4. Recombinant cytochrome f

2.3.4.1. Expression in E. coli strain W3110[pUC19CF] or [pUC19li5] Different expression conditions were used for expressing Cyt f in *E. coli* W3110, depending on the construct. Those for pUC19li5 are given in the text; those for pUC19CF are in (*italics*). A single colony from a plate was suspended in 40 ml 2 × YT medium containing ampicillin and grown for 9–16 h at 37°C. LB medium (1.7 l) containing ampicillin, 1 mM KNO_3, 3 μM haematin (stock solution prepared as described by McConville and Charles, 1979) and 4 mM sodium fumarate in 2 l conical flasks was inoculated with 12 ml (*1 ml*) of the starter culture per flask. The cultures were grown for 20 h (*38 h*) at 30°C in an orbital shaker at 180 rpm.

2.3.4.2. Expression in Pa. denitrificans strain Pd2131[pGC202] or [pEG402] A single colony from a plate was suspended in BHI medium containing rifampicin, streptomycin and spectinomycin and grown for 20–26 h at 30°C and 300 rpm. The starter culture was diluted 1:100 into BHI medium containing streptomycin and spectinomycin and grown for 20–24 h at 30°C and 300 rpm. Culture volumes up to 20 ml, 100 ml and 500 ml were grown in 100 ml, 250 ml and 2 l conical flasks, respectively.

2.3.4.2. Purification of periplasmic extracts containing Cyt f The purification procedure used for Cyt f expressed from pUC19CF is to be described elsewhere (C.J. Carrell, B.G. Schlarb, D.S. Bendall, C.J. Howe, W.A. Cramer, J.L. Smith, in preparation). Cyt f expressed from *E. coli* W3110[pUC19li5] and *Pa. denitrificans* Pd2131[pEG402] was purified from periplasmic extracts by a combination of ion exchange chromatography and gel filtration [order of columns used (oxidation state of the protein): Whatman DE52, (reduced); Pharmacia Superdex G75, (reduced); Pharmacia DEAE Sepharose FastFlow (reduced), Pharmacia HighTrap® Q-Sepharose (oxidised)]. A ratio A_{280}/A_{556} of 1.0 of the reduced protein indicated sufficient purity for further applications.

2.4. IEF gel electrophoresis

IEF gel electrophoresis was carried out using precast PhastGel® IEF pH 4–6.5 and 3–9 gels and a Pharmacia PhastSystem Separation-Control Unit according to the manufacturer's instructions. The proteins were reduced with sodium ascorbate before electrophoresis. The gel contained ampholytes in the pH range from 4 to 6.5. Sigma IEF-Mix (product No. I-3018) was used as markers (marker mix from pH 3.5–9.3). Staining was done by hand with fixing, staining and destaining solutions prepared as recommended in the PhastSystem manual.

2.5. Determination of midpoint potentials

Determination of oxidation–reduction potential by potentiometric titration was carried out as described by Wagner et al. (1996). For the *P. laminosum* Cyt bf complex, 10 μM hydroquinone was included in the mixture as mediator.

2.6. NMR spectroscopy

^{1}H-NMR spectra were taken as described by Kannt et al. (1996).

3. Results

3.1. Expression of P. laminosum Pc using its endogenous leader peptide: pET11PC in E. coli BL21(DE3)pLysS

For overexpression of *P. laminosum* Pc in *E. coli*, the *petE*-coding region (EMBL ID: PLPETE), including the region encoding the endogenous leader sequence, was amplified by PCR out of plasmid pPLL6b (Varley et al., 1995) using primers including an *Eco*RI site followed by an *Rca*I site (forward) and a *Bam*HI site (reverse). The PCR product was cloned into *Eco*RI/*Bam*HI cut pUC19, generating pUC19PC. The *petE* gene fragment of an *Rca*I/*Bam*HI-double digest of pUC19PC was ligated into *Nco*I/*Bam*HI cut pET11d to generate pET11PC and used to transform *E. coli* BL21(DE3)pLysS. A transformant was used for expression as described in Section 2.3.2. The yield of Pc after purification (see Section 2.3.2) was 5 mg/l culture. In some instances, filamentation of *E. coli* BL21-(DE3)pLysS[pET11PC] was observed, but not reproducibly and without effect on the level of expression of Pc.

The Pc expressed in *E. coli* BL21(DE3)pLysS-[pET11PC] was compared with Pc isolated from *P. laminosum* with regard to its molecular mass, redox potential, isoelectric point, optical and ^{1}H-NMR-spectra (Figs. 1 and 2). The molecular mass of the wild-type and recombinant proteins (11 421 $\pm$ 2 and 11 420 $\pm$ 1 Da, respectively), measured by ESI-MS, which causes loss of copper, were in agreement with the expected molecular mass (11 419.8 Da, predicted from the translated DNA sequence). The midpoint potential of the wild-type Pc was 378 $\pm$ 5 mV and that of the recombinant protein was 376 $\pm$ 5 mV (data not shown). The isoelectric point of the reduced form of both proteins was 4.95 (Fig. 1). The spectra of both wild-type and recombinant Pc showed a broad absorbance peak between 510 nm and 670 nm with a maximum at 598 nm and no shoulders (data not shown). The ^{1}H-NMR-spectra of wild-type and recombinant Pc were essentially indistinguishable (Fig. 2).

It was concluded that the recombinant protein is identical to the wild type isolated from *P. laminosum*, both in primary and 3D-structure, and it has the same redox properties.

3.2. Expression of P. laminosum Cyt f using its endogenous leader peptide: pUC19CF in E. coli W3110 and pGC202 in Paracoccus denitrificans Pd2131

Preliminary trials to express Cyt f (*petA* gene; EMBL ID: PLPETAGCG) truncated after Arg-250, and therefore lacking the C-terminal transmembrane anchor, in *Anacystis nidulans* R2 Pim9[pTRC99S], *E. coli* JCB387[pKK223-3] and BL21(DE3)pLysS[pET11d] gave no or very low yield.

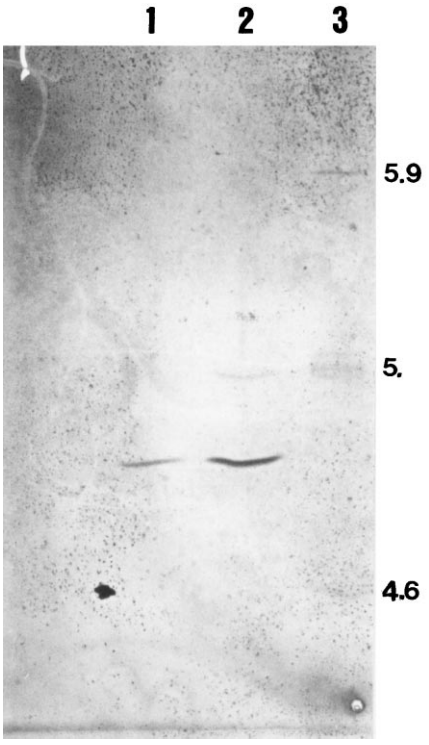

Fig. 1. Isoelectric point of Pc. IEF-gel of recombinant and wild-type plastocyanin. The gels were run as described in Section 2.4 and stained with Coomassie Brilliant Blue R. Lane 1, reduced wild-type plastocyanin; lane 2, reduced recombinant plastocyanin; lane 3, IEF-Marker mix (pH).

Ubbink et al. (1992) obtained 2 mg/l *Pa. versutus* cytochrome *c*-550 with a pUC19-derivative in *E. coli* W3110. W3110[pUC19CF] (see Tables 1 and 2 a), however, yielded an average of 0.2 mg/l Cyt f with low reproducibility.

Paracoccus denitrificans Pd2131 (which is depleted of endogenous cytochrome *c*-550) has been used by Ubbink et al. (1996) to express *Pa. versutus* cytochrome *c*-550 with yields of up to 10 mg/l culture. Expression in *Pa. denitrificans* Pd2131[pGC202] (see Tables 1 and 2) gave no detectable holo-Cyt f under aerobic, semi-anaerobic and anaerobic conditions in both rich (Brain Heart Infusion Broth) and minimal (succinate) medium.

An explanation for the low levels of holo-Cyt f might be that the *P. laminosum* Cyt f leader peptide is not adequately recognised by the protein translocation machinery of *E. coli* or *Pa. denitrificans*. It was therefore decided to determine the effects on expression of exchanging the Cyt f leader sequence for leaders that were known to function effectively in the respective host systems. These were the *P. laminosum* Pc leader for *E. coli* and the cytochrome *c*-550 (*cycA* gene) leader from *Pa. versutus* for *Pa. denitrificans*.

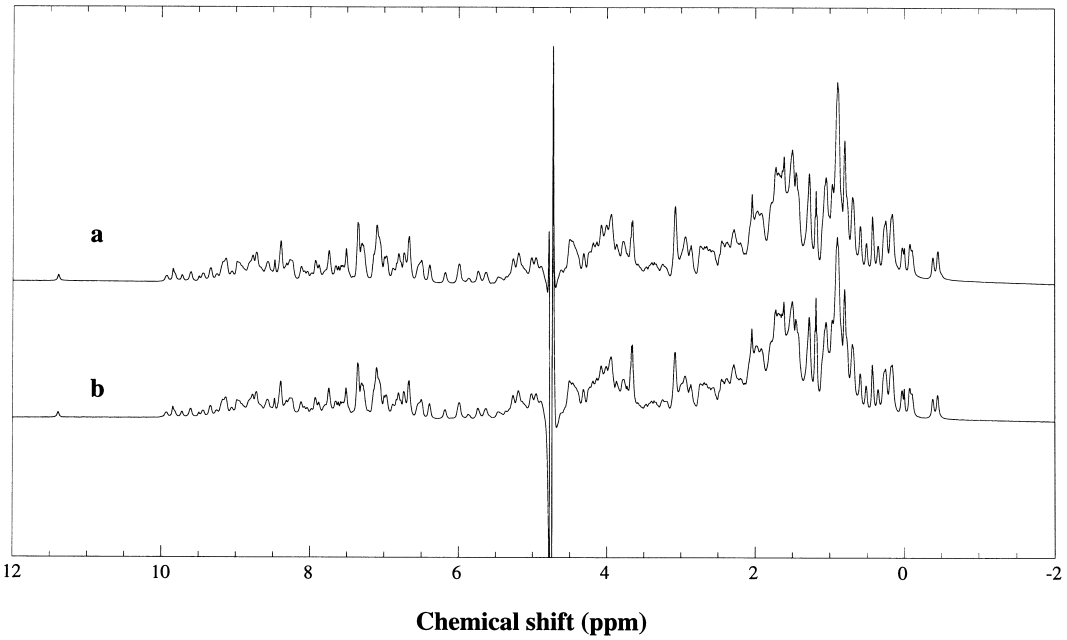

Fig. 2. ^{1}H-NMR-spectra (at pH 6, 300 K, in potassium phosphate buffer) of (a) wild-type and (b) recombinant Pc.

3.3. Expression of P. laminosum Cyt f using bacterial leader peptides

3.3.1. Use of the P. laminosum Pc leader peptide in E. coli W3110 (plasmid pUC19li5)

For exchanging the leader peptide with that from *P. laminosum* Pc, two PCRs were carried out in parallel. The *P. laminosum* Pc leader was amplified from linearised pET11PC, using a forward primer that introduced an *Eco*RI–*Rca*I-site upstream of the 5′ end of the leader and a reverse primer complementary to the last 15 bases of the leader and the first six nucleotides of the mature protein that introduced a *Pst*I-site into the cleavage site. The sequence encoding the mature, truncated *P. laminosum* Cyt f was amplified from linearised pET11CF (see Table 1) using a forward primer complementary to the last 15 nucleotides of the leader-coding region and the first six nucleotides of the mature protein-coding region, which introduced a *Pst*I-site into the cleavage-site, and a reverse primer introducing a *Bam*HI-site downstream of the stop-codon.

The products from both PCRs were gel-purified, digested with *Eco*RI/*Pst*I or *Pst*I/*Bam*HI as appropriate and cloned into pUC18 cut with *Eco*RI and *Bam*HI to generate pUC18li. The *Rca*I–*Bam*HI-fragment of pUC18li was ligated into *Nco*I/*Bam*HI cut pET11d, resulting in pET11li. The *Xba*I–*Eco*RI fragment of pET11li was ligated into pUC19, placing the *petA* gene in the same orientation as the *lacZ* promoter for a transcriptional, but not a translational fusion. The resulting construct, pUC19li5, was introduced into *E. coli* W3110. The new strain had a shorter doubling-time than W3110[pUC19CF] (30 min and 44 min, respectively). Optimal yield of Cyt f was obtained after 19–20 h growth (compared to 38 h with the endogenous leader peptide), using 12 ml inoculant for 1.7 l medium rather than 1 ml. The maximum yield obtained was 2 mg/l, the average 1.1 mg/l, with improved reproducibility.

Aerobic overnight cultures of W3110[pUC19CF] or [pUC19li5] were compared under the light microscope. The former were found to be partially filamentous and contained cell clumps, the latter appeared normal. Expression in these aerobic cultures was not investigated.

A cloned cytochrome *c* maturation cassette (plasmid pEC86; Arslan et al., 1998) has increased reproducibility

Table 2
Yields of the different expression systems

	Expression system	Plasmid	Leader peptide	Yield (mg/l), after 1st column
P. laminosum Pc (*petE*)	*E. coli* BL21(DE3)pLysS	pET11PC	Endogenous	5
P. laminosum Cyt f (*petA*)	*E. coli* W3110	pUC19CF	Endogenous	Ave 0.2 (max. 0.6, min 0)
	Pa. denitrificans Pd2131	pGC202	Endogenous	0
	E. coli W3110	pUC19li5	*P. laminosum* Pc (*petE*)	Ave. 1.1 (max 2, min 0)
	Pa. denitrificans Pd2131	pEG402	*Pa. versutus* cyt *c-550* (*cycA*)	ave. 2.7 (max. 4.5, min 0)

of expression of *Chlamydomonas reinhardtii* Cyt f in *E. coli* W3110 and MV1190 when a pUC19-derivative analogous to pUC19li5 was used as an expression vector (Ponamarev and Cramer, 1998). Co-transformation of pEC86 with pUC19li5 in *E. coli* strains W3110 and MV1190 did not increase the yield of *P. laminosum* Cyt f.

3.3.2. Use of the Pa. versutus cytochrome c-550 leader in Pa. denitrificans Pd2131 (plasmid pEG402)

For the exchange of the leader peptide in the *Pa. denitrificans*-system, a two-step PCR reaction was carried out. The promoter, RBS and leader peptide of *Pa. versutus* cytochrome *c*-550 (template: linearised pMU8) were amplified using a forward primer which introduced an *Eco*RI-site 25 bp upstream of the promoter region, and a reverse primer complementary to the last 21 nucleotides of the leader with a 24 nucleotide extension at the 3′ end complementary to the mature *petA* sequence. The product was used as a forward primer in a second PCR with linearised pET11CF as template and the reverse primer used for *petA*-amplification (see Section 3.3.1.). The 1 kb product was cloned into *Eco*RI/*Bam*HI cut pEG400, generating pEG402, which was then introduced into Pd2131 by triple mating (Gerhus et al., 1990). The average yield of a small number of expression trials was 2.7 mg/l, the maximum obtained was 4.5 mg/l.

The doubling times for Pd2131[pGC202] or [pEG402] were virtually identical. The endogenous *P. laminosum* Cyt f leader peptide did not lead to filamentation of *Pa. denitrificans*.

The properties of truncated Cyt f expressed and purified from *E. coli* and *Pa. denitrificans* were compared. The isoelectric points were found to be identical at 4.2 on an IEF-gel (Fig. 3). The redox midpoint potentials were determined to be 300 ± 2 mV for the protein expressed in *E. coli* and 299 ± 1 mV for that expressed in *Pa. denitrificans* (data not shown). The value obtained for Cyt f in the Cyt bf complex isolated from thylakoids of *P. laminosum* is slightly higher (323 ± 3 mV). This small difference is unlikely to indicate an alteration in the structure of the soluble domain and could either be due to residual detergent necessary to keep the complex in solution or to the protein environment of Cyt f in the Cyt bf complex.

The molecular mass expected for the protein truncated after Arg-250 (predicted from the DNA sequence), including the covalently bound haem, was 27 212.4 Da. The molecular mass as detected by ESI-MS was $27\,213 \pm 5$ Da for the protein expressed in *E. coli* and $27\,222 \pm 10$ Da for the protein expressed in *Pa. denitrificans*. The expected mass lies within the error of the measurement for both protein preparations. The visible spectrum of Cyt f expressed in *E. coli* is shown in Fig. 4. Spectra of Cyt f preparations from thylakoids of *P. laminosum* or from *Pa. denitrificans* Pd2131[pEG402] were identical to Fig. 4 (data not shown). Reduced *P. laminosum* cytochrome *f* has a Soret band maximum at 421 nm, a β-band at 524 nm and an α-band at 556 nm. The asymmetry of the α-band is more obvious in the enlarged section of the spectrum (shown as inset). The β-band is split and shows a second maximum at 532 nm wavelength.

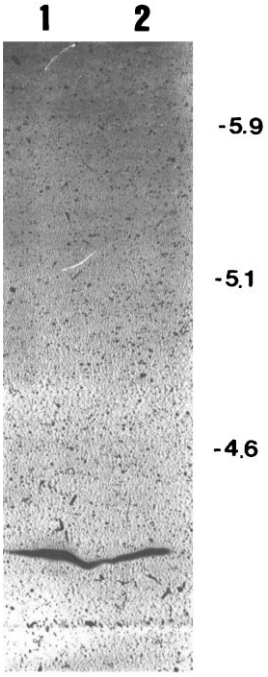

Fig. 3. IEF-gel of Cyt f expressed in *E. coli* W3110 [pUC19li5] and *Pa. denitrificans* Pd2131 [pEG402]. Lane 1, Cyt f isolated from *E. coli*; lane 2, Cyt f isolated from *Pa. Denitrificans*. The gels were run as described in Section 2.4 and stained with Coomassie Brilliant Blue R.

The proteins expressed in *E. coli* or *Pa. denitrificans* were essentially indistinguishable from each other and from the protein isolated from thylakoids of *P. laminosum*.

4. Discussion

The yields obtained are summarised in Table 2. The unmodified *P. laminosum* Pc gene was expressed efficiently. However, the Cyt f holoprotein was expressed only at very low levels, or not at all from the unmodified *P. laminosum* gene. Difficulties with expression of *c*-type cytochromes in *E. coli* are not unusual (Pollock et al., 1989; Sambongi and Ferguson, 1994). The absence of detectable levels of Cyt f in *Pa. denitrificans* P2131 was surprising given the yields of 10 mg/l *Pa. versutus* cytochrome *c*-550 obtained by Ubbink et al. (1996). When the region encoding the *P. laminosum* Cyt f leader sequence was replaced with a region encoding the leader sequence from Pc or from *Pa. versutus* cytochrome *c*-550, the observed levels of expression were greatly

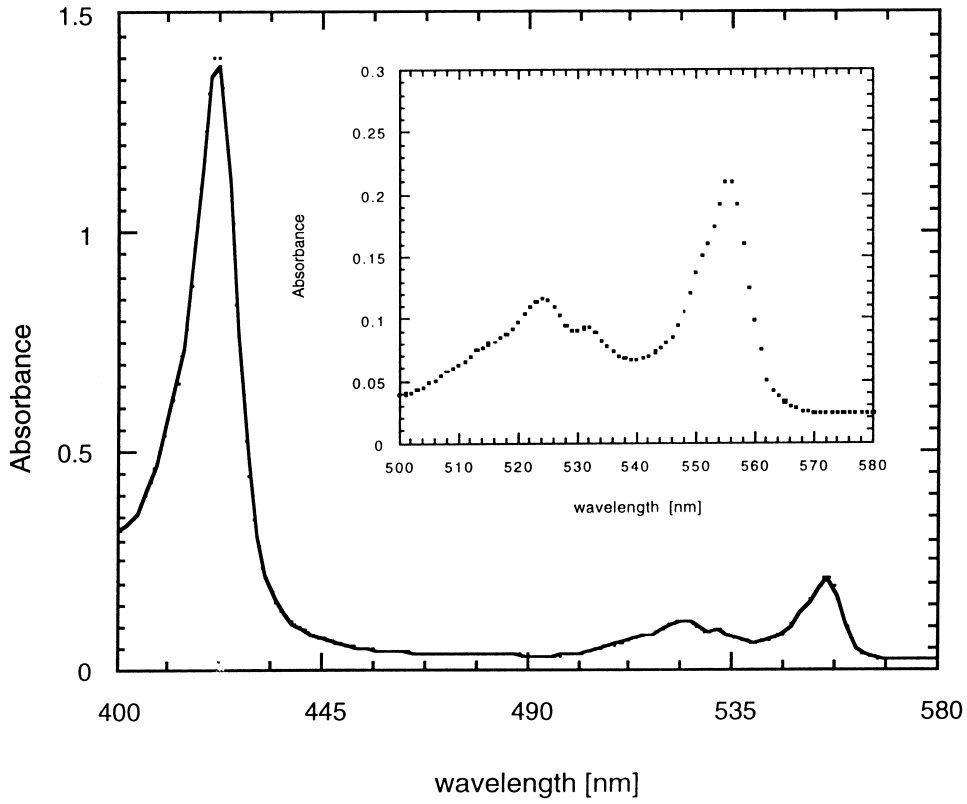

Fig. 4. Optical spectra of the reduced lumenal fragment of Cyt f isolated from *E. coli* W3110 [pUC19li5], recorded on a Cary 219 spectrophotometer and digitised by hand. The inset shows an enlargement of the α-band. The protein was reduced with sodium ascorbate prior to recording the spectrum.

increased in *E. coli* and *Pa. denitrificans*, respectively. For expression in *E. coli* the promoters and RBS remained unchanged, whereas for expression in *Pa. denitrificans* both promoter and RBS were changed with the leader peptide. This is unlikely to be the reason for improved expression, however, as the promoter and RBS used originally stemmed from a gene (*cycA*) from the host *Pa. denitrificans* itself, whereas the replacement stemmed from *Pa. versutus* cytochrome *c*-550.

The leader sequences are shown in Table 3. The *P. laminosum* Cyt f leader sequence differs from typical bacterial leader sequences in its length and the presence of an acidic residue within the hydrophobic domain. It is not clear which of these characteristics is responsible for the low level of expression of holoprotein. The presence of an acidic residue in this position has been shown to interfere with the export process (von Heijne, 1985). Other cyanobacterial proteins that have been expressed successfully in *E. coli* with transport into the periplasm include WoxA from *Anabaena* sp. PCC 7120 (Borthakur and Haselkorn, 1989), WoxA from *Anacystis nidulans* R2 (Kuwabara et al., 1989), Pc and

Table 3
Leader sequences

	N-terminal pos. domain	hydrophobic stretch (charged residues in *italics*)	cleavage site
***Pa. versutus* cyt *c*-550**	+ MK	ISIYATLAALSLAL	PAVA
P. laminosum* cyt *f	+ ++ + MNFKVCSFPSRRQSIAAFVR	– VLMVILLTLGALVSS***D***VLL	PQPAAA
***P. laminosum* pc**	+ + MKLIAQISR	SLSLALFALVLMVGSFVAVM	SPAAA
E. coli* cyt *c	+ MR	FLLGVLMLMIS	GSALA
***E. coli* OmpA**	++ MKK	TAIAIAVALAGFA	TVAQA

cytochrome c_6 from *Anabaena* sp. PCC 7119, (Molina-Heredia et al., 1998), Pc (Romero et al., 1998; Hervas et al., 1993) and cytochrome c_6 (Diaz et al., 1994) from *Synechocystis* sp. PCC 6803. These all have leader sequences more similar in length to those of *E. coli* and do not have an acidic residue within the hydrophobic span.

The results indicate the importance of the leader sequence in determining successful export of overexpressed heterologous proteins into the periplasm. They also demonstrate that there may be subtle differences between some cyanobacterial leader sequences and those from *E. coli*.

The failure of the cytochrome *c* maturation cassette (Arslan et al., 1998) to increase yields indicates that none of its products is rate-limiting for production of holo-Cyt f. The maximal yield reported here of 2 mg/l is equal to the highest reported so far for the expression of *c*-type cytochromes in *E. coli*, that of 2 mg/l for *Pa. versutus* cytochrome *c*-550 (Ubbink et al., 1992). This indicates that the substrate specificity of the *E. coli* haem lyase is rather low, although Pollock et al. (1989) reported that haem was not attached to the apo-cytochrome c_3 from *Desulfovibrio vulgaris* when expressed in *E. coli* TG2.

It is not clear why filamentation of cells was observed under certain circumstances. However, filamentation of *E. coli* cells depleted of Ffh, the homologue of the 54 kDa subunit of the signal recognition particle, has been reported by Phillips and Silhavy (1992). Since plant Cyt f has been shown to interact with the chloroplast SRP (High et al., 1997) it may be that the overexpressed Cyt f precursor with its cognate leader sequence is binding to Ffh, leading to the Ffh-phenotype. Although there are two adjacent arginines in the N-terminal domain of Cyt f (see Table 3), other features of the Sec-independent pathway of bacteria or chloroplasts are not found, suggesting this is not a genuine 'twin-arginine' motif (Wexler et al., 1998). However, this does not explain why filamentation in some instances was observed in BL21(DE3)pLysS[pET11PC], since Pc is believed to be exported by the Sec-pathway (Varley et al., 1995).

To our knowledge, this is the first example of the successful overexpression of Pc and Cyt f from the same organism. The results indicate the importance of the leader sequence in determining successful export of overexpressed heterologous proteins into the periplasm and show that haem can be successfully inserted into proteins expressed in *E. coli*. They also demonstrate that there may be subtle differences between some cyanobacterial leader sequences and those from *E. coli*. The over-expressed proteins were functionally identical to those isolated from *P. laminosum* cells, and will therefore be suitable for studies on electron transfer in vitro.

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