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Expression of the blue copper protein azurin from *Pseudomonas aeruginosa* in *Escherichia coli*

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The structural gene for the blue copper protein azurin from *Pseudomonas aeruginosa* has been subcloned in different expression plasmid vectors. The highest yield of expression was obtained when the gene with its native ribosome-binding site was placed downstream of the *lac* promoter in plasmid pUC18. The protein is exported to the periplasmic space in *Escherichia coli* and the amount corresponds to 27% of the total protein content in the periplasmic space. The preprotein is cleaved correctly according to N-terminal sequencing of the purified protein. Azurin has been purified in large amounts and is spectroscopically indistinguishable from the protein purified from *P. aeruginosa*.

Azurin; Cloning; Expression; Purification

1. INTRODUCTION

The type I 'blue' copper proteins are involved in electron-transfer reactions in both bacteria and plants. Structural information, and spectroscopic and electron-transfer kinetic data are available for a number of them [1]. The single-copper protein azurin from different *Pseudomonas* and *Alcaligenes* bacterial strains is a simple redox protein involved in denitrification metabolism. The three-dimensional structures of azurins from these two bacterial species have been determined by X-ray crystallography [2] and the structure around the copper site has been discussed [3].

Recently, we cloned and characterized the azurin structural gene [4] as a prerequisite for our protein engineering studies on azurin. Here, we show that azurin can be expressed in large amounts in *Escherichia coli* and that the purified protein is indistinguishable from that purified from its natural host *P. aeruginosa*.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli K12 strains TG1 [5] and KK2174 [6] were used in this study. *P. aeruginosa* strain ATCC 10145 was obtained from the Deutsche Sammlung von Mikroorganismen. Plasmids pUC18 and pUC19 [7] were obtained from Pharmacia (Uppsala), plasmid pCP3 [8] being a kind gift from Dr Per Olof Nyman (University of Lund, Sweden). The pUC vectors carrying the azurin structural gene (pUG1, pUG2) have been described earlier [4].

2.2. *Growth media*

The growth medium was either LB medium [9] or TB medium supplemented with the required selective agent (ampicillin 50 μg/ml and tetracycline 12 μg/ml) when needed. TB medium

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Abbreviations: Ap, ampicillin; BSA, bovine serum albumin; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEG, polyethylene glycol

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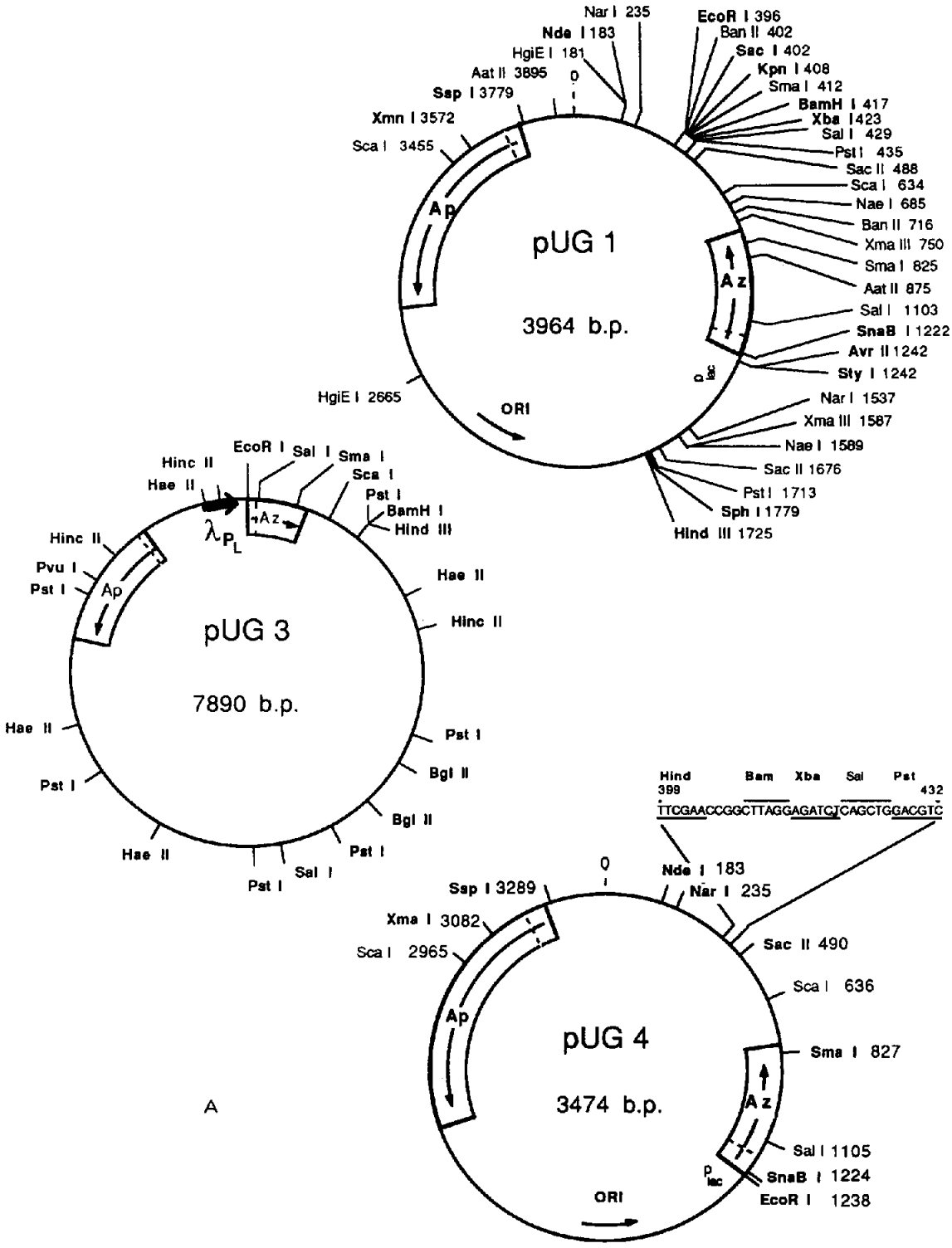
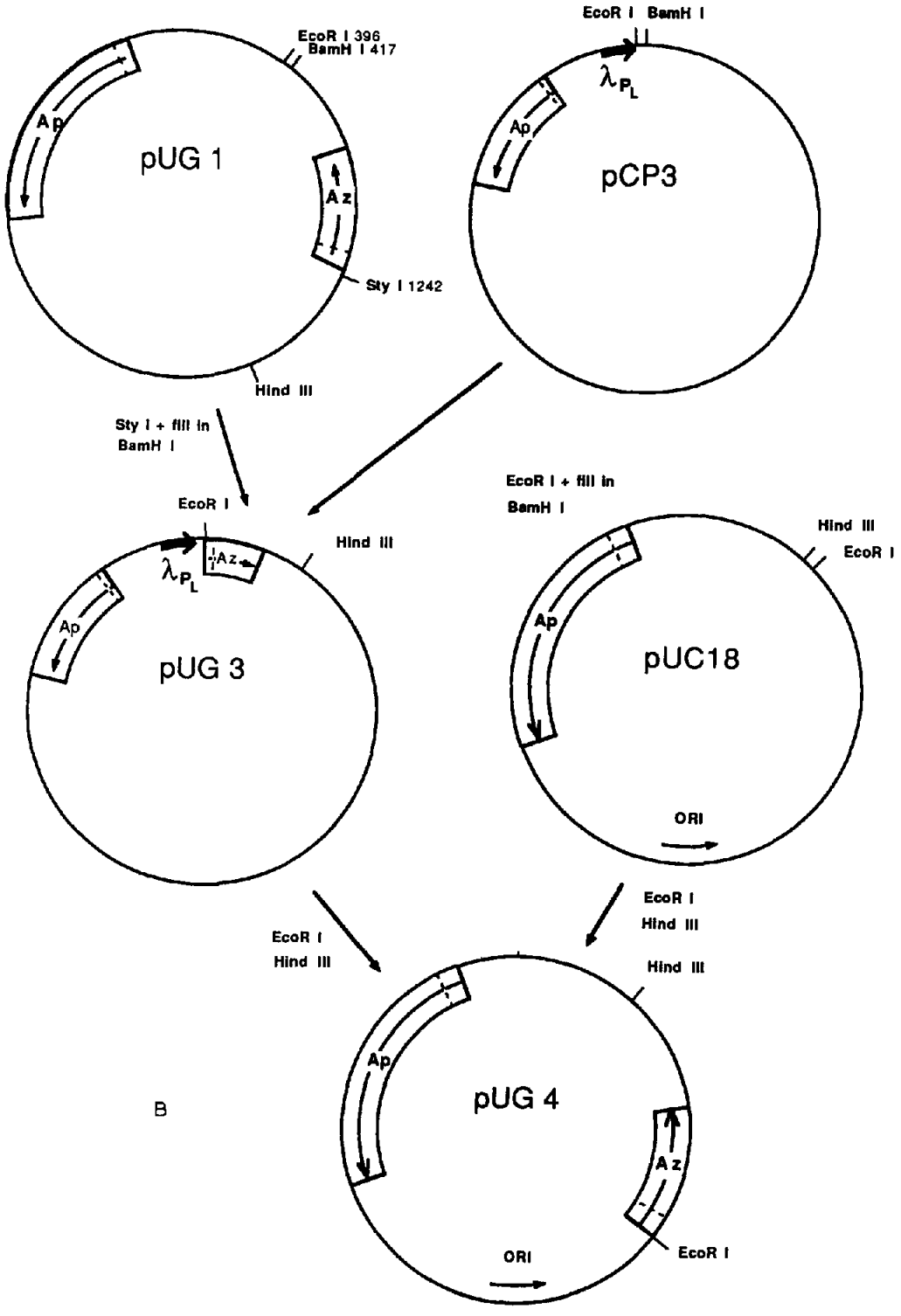


Fig.1. (A) Detailed restriction enzyme digestion maps of vectors pUG1, pUG3 and pUG4. (B) Subcloning of the azurin structural gene Steps for constructing the different expression vectors are described in the text.


EcoR I 396
BamH I 417
pUG 1
Ap
Az
Sty I 1242
Hind III
EcoR I BamH I
λ_{P_L}
pCP3
Sty I + fill in
BamH I
EcoR I
Hind III
pUG 3
EcoR I + fill in
BamH I
Hind III
EcoR I
pUC18
ORI
EcoR I
Hind III
Hind III
pUG 4
ORI
EcoR I

B

was prepared by adding 100 ml of a sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 to a sterile solution (900 ml) of 12 g tryptone, 24 g yeast extract, 4 ml glycerol and water. For *P. aeruginosa* medium A [4] was used. Growth temperature was 37°C, unless otherwise stated. Induction of *E. coli* cultures for expression of azurin was carried out as described below.

2.2.1. Temperature induction

Strain KK2174 carrying pUG3 was inoculated 1:100 from an overnight culture and grown at 28°C until $A_{550} = 1.0$. Runaway replication and induction of the λP_L promoter were carried out by adding an equal volume of preheated (55°C) TB medium. The culture was then allowed to grow for an additional 3 h at 42°C before harvesting.

2.2.2. IPTG induction

Strain TG1 carrying pUG vectors (pUG1, 2, 4 or 5) was inoculated 1:100 from overnight cultures. Growth was followed and when A_{550} reached 1.0, IPTG was added to a final concentration of 0.3 mM. 3 h after induction, cells were collected. Usually 8 g wet cells were obtained from 1 l culture.

2.3. *DNA techniques*

Gene cloning and DNA extractions were carried out using standard methods [10] with minor modifications. DNA sequence determinations were according to Chen and Seeburg [11] and gel electrophoresis as described by Olsson et al. [12].

2.4. *Protein purification*

Azurin was purified from 1-l cultures of strains expressing the protein.

2.4.1. Periplasmic fraction

3 h after induction cells were harvested by centrifugation (2800 × *g*) for 10 min at 4°C. The pellet was resuspended in 1/10 vol. (100 ml) saccharose solution (20% saccharose, 0.3 M Tris-HCl, pH 8.1, 1 mM EDTA), incubated for 10 min at room temperature and centrifuged (6300 × *g*) for 10 min at 4°C. The supernatant was decanted off and cells resuspended in the residual liquid by vortex-mixing. Ice-cold 0.5 mM $MgCl_2$ (100 ml) was added and cells resuspended thoroughly with a pipette followed by 10 min incubation on ice before centrifugation (17700 × *g*) for 20 min at 4°C. The supernatant (100 ml) is the periplasmic fraction.

2.4.2. Acid protein precipitation

To the clear, yellowish supernatant 1/10 vol. (10 ml) of 0.5 M ammonium acetate (pH 4.0) was added. The pH was adjusted to 4.1 with concentrated acetic acid. After 30 min incubation at room temperature, precipitated proteins were collected by centrifugation (4400 × *g*) for 10 min. The supernatant (containing azurin) was saved for further purification. The following steps were performed at 0–5°C.

2.4.3. CM52 ion-exchange chromatography

The supernatant was pumped (flow rate 450 ml/h) through a 5 × 5 cm column of Whatman CM52 ion-exchange cellulose equilibrated with 0.05 M ammonium acetate (pH 4.1). Azurin and most of the bulk proteins were bound to the top of the column. Azurin was eluted by 0.05 M ammonium acetate (pH 5.1). To the fractions expected to contain azurin, 2 μl/ml of 0.5 M $CuSO_4$ was added. Test tubes containing azurin then turn blue.

2.4.4. CM52 ion-exchange chromatography pH gradient

The pooled top fractions from the broad azurin peak were adjusted to pH 4.1 with concentrated acetic acid before being pumped (flow rate 150 ml/h) through a 2.5 × 15 cm column of Whatman CM52 ion-exchange cellulose equilibrated with 0.05 M ammonium acetate (pH 4.1). Azurin was eluted by a 0.05 M ammonium acetate pH gradient from pH 4.1 to 9.0. The azurin fractions eluted at pH 4.75.

2.4.5. Concentration

The top fractions from the broad azurin peak were dialysed (Spectrapor MWCO 6000–8000) vs solid PEG 20 until the volume was reduced to less than 10 ml.

2.4.6. Sephacryl S-100 gel filtration

The azurin concentrate was loaded on a 2.5 × 115 cm column of Sephacryl S-100 (Pharmacia, Uppsala), equilibrated with 0.05 M ammonium acetate (pH 5.1) and then eluted (flow rate 5.8 ml/h) using the same buffer. Azurin was detected during the purification steps by its blue colour, PAGE-Western blotting and the absorbance at 625 nm.

2.5. *Protein electrophoresis*

Protein extracts were analysed by SDS-PAGE [13] using the Mini Protean II system (Bio-Rad).

2.6. *Mass spectrum analyses*

Plasma desorption mass spectrometry was carried out using a Bio-Ion instrument [14].

2.7. *Amino acid sequence analysis*

Amino acid sequences were determined on an Applied Biosystems model 470A protein/peptide sequencer equipped with an on-line detection system (Applied Biosystems model 120A phenylthiohydantoin analyzer). The apparatuses were operated according to the manufacturer's instructions and the sequencer resulted in yields of approx. 90–92%. The initial yield was not determined.

2.8. *EPR analyses*

X-band EPR spectra were recorded on a Bruker ER 200D-SRC ESR spectrometer equipped with an Oxford Instruments EPR-9 helium-flow cryostat at 20 K.

2.9. *Immunological techniques*

Rabbit anti-azurin serum was obtained by immunization of white rabbits according to Löwenadler et al. [15], with minor modifications.

Western blotting of protein gels was carried out as follows: proteins separated on SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane and treated with rabbit anti-azurin serum (1:50 in PBS) followed by biotinylated anti-rabbit antibodies (1:500) and avidin-conjugated horseradish peroxidase (0.8 mg/ml) (Sigma). The membrane was finally developed using 4-chloro-1-α-naphthol as electron donor.

Purification of serum and antibodies was carried out as follows: azurin (3 mg) was coupled to 1 g of CNBr-activated

Sepharose 4B [16]. Anti-azurin serum (2 ml) was diluted with 2 × PBS-Tween to 4 ml and loaded on the gel. The gel was washed with 10 ml PBS-Tween followed by 10 ml PBS. Bound antibodies were eluted with 1 M propionic acid and neutralized with solid Tris. Fractions containing protein were pooled, desalted on a PD-10 column (Pharmacia) and eluted with PBS. The protein concentration was 0.8 mg/ml. ELISA quantification of protein concentrations was carried out using polyvinyl chloride U-bottomed microtiter plates (Dynatech). 100 μl affinity-purified antibody (5 μg/ml) was used per well. The highest concentration of purified azurin used was 200 ng/ml. The protein mixture from the periplasmic preparation was diluted at least 1:10000 in PBS. Alkaline phosphatase was conjugated to antibodies using standard methods [17] and diluted 1:1000 in PBS-Tween before use.

3. RESULTS

3.1. *Subcloning of the azurin gene*

A 1284 bp *Pst*I restriction fragment carrying the azurin structural gene has previously been subcloned into plasmid pUC19 [4]. An *Sty*I restriction site is located just upstream of the azurin ribosome-binding site (position 472). Using this site, filling in the overhang and then cutting with *Bam*HI (in the vector part), we were able to subclone this fragment into the runaway-replication vector pCP3 (cut with *Eco*RI, filled-in and cut by *Bam*HI), transforming strain KK2174 (carrying the temperature-sensitive cI repressor on a plasmid). The resulting plasmid is termed pUG3.

This cloning procedure restored the *Eco*RI site and the azurin gene could be further subcloned into plasmids pUC18 and pUC19 simply by cutting with *Eco*RI and *Hin*dIII (which cuts in the polylinker part of plasmid pCP3), transforming strain TG1. The resulting plasmids are denoted pUG4 and pUG5, respectively (fig.1).

3.2. *Expression of azurin in* E. coli

We compared the expression of azurin from all vector constructs using both Western blotting of protein gels (fig.2) and ELISA quantification (table 1). From these experiments one can conclude that the highest expression was obtained with the pUG4 vector and that the amount of azurin in the periplasmic fraction corresponds to nearly 30% of the total protein content in the periplasmic space.

3.3. *Purification of azurin expressed in* E. coli

Bacterial strain TG1 with the pUG4 vector was used for purification of azurin in large amounts.

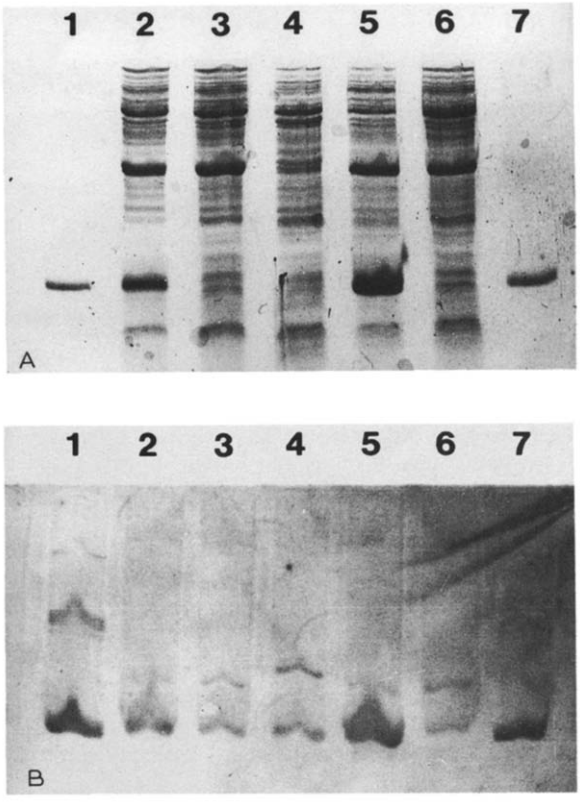


Fig.2. Electrophoretic analysis of azurin expressed in *E. coli*. (A) SDS-PAGE: (lanes 1,7) azurin; (lanes 2–6) periplasmic fraction of pUG1–pUG5. (B) Western blotting of gel from A.

Best yield was obtained in 1-l shaking cultures. Purification is rapid and easy using only standard methods [18] (fig.3 and table 2). Pure recombinant

Table 1

ELISA quantification of azurin expressed in different host/vector combinations

	Host/vector combination				
	TG1 pUG1	TG1 pUG2	KK2174 pUG3	TG1 pUG4	TG1 pUG5
Culture volume (l)	1.0	1.0	1.0	1.0	1.0
Wet mass (g)	7.7	9.1	6.6	8.6	7.8
Periplasmic volume (ml)	99	99	98	101	99
Azurin concentration (μg/ml)	45.0	6.7	18.7	290	0.0
Azurin (total)	4.5	0.7	1.8	29.3	0.0

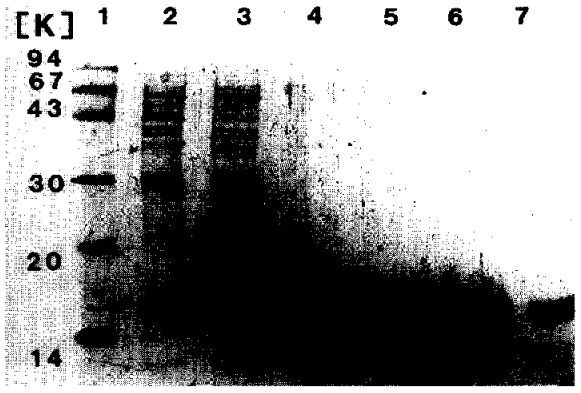


Fig.3. Electrophoretic analysis of fractions from different steps in the purification of azurin expressed in *E. coli*. (Lane 1) Molecular mass markers (Pharmacia LMW); lanes 2–7 refer to the respective sections 2.4.1–2.4.6.

azurin is obtained in 57% yield, corresponding to 16 mg/l starting culture.

3.4. *Characterization of purified azurin*

Purified azurin was characterized by various methods. Mass spectral analysis and N-terminal amino acid sequencing (20 steps) showed that correctly processed azurin (13959 ± 14 Da) was expressed in *E. coli*. The EPR spectra (fig.4) show all the characteristics of a type I copper center, and are identical to those of *P. aeruginosa* azurin. The purified protein has a $625^{ox}/280$ ratio of 0.53.

Table 2

Purification of azurin expressed in *Escherichia coli*/pUG4

	Step no.					
	1	2	3	4	5	6
Volume (ml)	101	111	79	54	9	20
Azurin concentration (mg/ml)	0.29	0.29	0.32	0.41	2.68	0.83
Protein concentration (mg/ml)	1.06	0.90	0.65	0.56	3.02	0.80
Azurin/protein	0.27	0.32	0.49	0.73	0.89	1.04
Azurin (total) (mg)	29.3	32.2	25.3	22.1	24.1	16.6
Azurin (relative) (%)	100	110	86	76	82	57

Steps 1–6 refer to the corresponding sections 2.4.1–2.4.6. Azurin concentration was measured with ELISA. Protein concentration was estimated via the BCA method (Pierce). As standards BSA was used in steps 1 and 2 and pure azurin (*Pseudomonas aeruginosa*) for steps 3–5

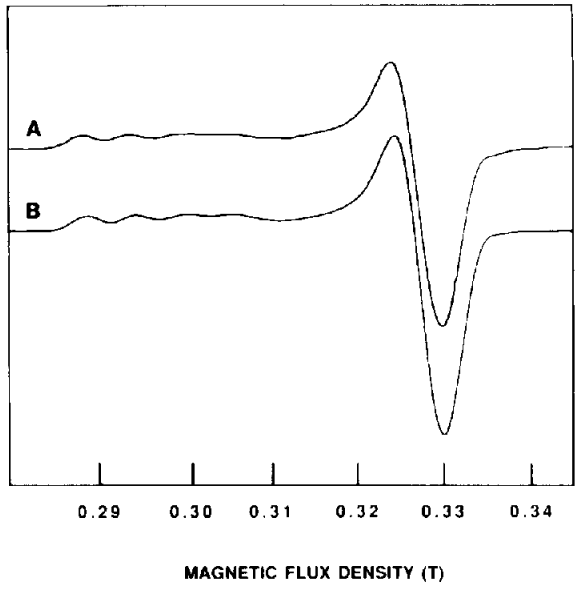


Fig.4. ESR spectra of purified azurin from (A) *P. aeruginosa* and (B) *E. coli*.

4. DISCUSSION

We have subcloned the azurin gene from *P. aeruginosa* to an *E. coli* plasmid expression vector and the azurin produced in *E. coli* is apparently processed correctly. Our subcloning in plasmid pUC18 placed the azurin gene under the inducible *lac* promoter. The subcloning was carried out by using a restriction site just upstream of the azurin ribosome-binding site. The sequence around the ribosome site is in this construction nearly optimal [19]. The azurin protein is expressed as a precursor protein and exported to the periplasmic space where it can account for up to 50% of the total protein content, depending on the growth and cell lysis conditions. By using this system we have been able to purify large amounts of azurin from *E. coli*. The amount of pure azurin (per g cells) obtained is at least twice that previously reported for azurin purified from *P. aeruginosa* [18]. Purified azurin has been subjected to different analyses and as judged according to these criteria, is indistinguishable from the protein purified from *P. aeruginosa*. We expect that other copper proteins can be expressed in our system and such studies are now underway. Our studies concerning the structure and function of small blue copper proteins are

currently in progress with site-directed mutagenesis of residues important for the function of the protein and especially the function of the copper site. We shall approach the expressing of these azurin mutant proteins by using the expression system we have described here.

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