RESEARCH ARTICLE

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CLONING AND EXPRESSION OF PSEUDOAZURIN GENE IN *ESCHERICHIA COLI* DH5α

ABSTRACT:

Pseudoazurin (PAz) is a blue copper protein that functions as an electron carrier in numerous microorganisms. In several denitrifying bacteria PAz serves as the electron donor to nitrite reductase (NiR) in the anaerobic respiration system. *Achromobacter cycloclastes* IAM 1013 was grown at 30*ºC* in LB medium and genomic DNA was isolated (using the GENOME kit from BIOgene). The genomic DNA was used as a template in polymerase chain reactions (PCR) in which the PAz gene was amplified using the following two primers: ccatggtgaatgcaatcaagag (forward primer) and ccatggctagaagtcgcttagt (reverse primer). The primer sequences were based on the DNA sequence of *Achromobacter cycloclastes* IAM 1013 PAz and were designed in such a way to include the signal sequence for the protein. The amplified fragment was cloned into PET 15b vector which digested with Nco1.Polyacrylamide gel electrophoresis was used to characterize the extracted protein.

KEY WORDS:
Cloning, Expression, Pseudoazurin, Gene; Protein.

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INTRODUCTION:

Pseudoazurin (PAz) belongs to a family of blue (type 1) copper proteins that act as electron-transfer (ET) agents in the respiratory chains of denitrifying bacteria and various plants (Alberts *et al*., 1994; Burley and Petsko 1988). In numerous microorganisms, PAz serves as an electron donor to nitrite reductase (NiR) that carries out the reduction of NO_2 to NO under anaerobic conditions (Alberts *et al*., 1994).

Pseudoazurin (PAz) is a blue copper protein that functions as an electron carrier in numerous microorganisms (Zumft, 1997). In several denitrifying bacteria PAz serves as the electron donor to nitrite reductase (NiR) in the anaerobic respiration system. The structures of PAz have been determined by x-ray crystallography, the protein contains eight B-strands, which form two B-sheets giving an overall B-sandwich structure, additionally, Paz possesses two α-helices which are located at its C terminus (Inoue *et al.,* 1999).The single copper atom is located approximately 5A beneath the protein surface has a distorted tetrahedral geometry. The copper atom is coordinated by the N atoms of His 40 and His 81, the thioether sulfur of Met86 and thiolat sulfur of cys78. A methionine residue [Met16] is located within the Van der Waals contact of Cu coordinated His81 residue in all the PAz structures (Inoue *et al.,* 1999).

The oxidized copper center of PAz displays a number of unusual properties, including an intense absorption centered around 594 nm (e = 3700 M^{-1} cm^{-1}) arising from (Cys)S, Cu(II) charge transfer (CT), relatively high redox potential (260 mV), and a distinctive rhombic EPR spectrum with a small hyperfine coupling constant (55 G). Up to this date, several X-ray crystal structures of pseudoazurins from various sources, including *Achromobacter cycloclastes* (Inoue *et al.* 1999), *Alcaligenes faecalis* (Yanagisawa *et al*., 2003), Methylobacterium extorquens (Zumft, 1997), and Thiosphaera pantotropha (Adman *et al.,* 1989) were reported.

Denitrification is an important biological process in which inorganic nitrate and nitrite are converted into gaseous nitrogen in a number of steps which involve reductases for nitrate, nitrite, nitric oxide and nitrous oxide. Several of

these enzymes are supplied with electrons by copper proteins and or *c*-type cytochromes (Alberts *et al.,* 1994). Pseudoazurins are small copper-containing redox proteins present in some denitrifying bacteria. In their oxidized state, pseudoazurins and other cupredoxins are characterized by a strong peak in their absorption spectrum near 600 nm and a very small hyper®ne coupling constant in their EPR spectrum (Burley and Petsko, 1988). These shuttle proteins transport electrons from various donors to acceptors within the bacterial periplasm. In some bacteria, the synthesis of pseudoazurin is enhanced under denitrifying conditions, and a role for pseudoazurin as an electron donor for soluble nitrous oxide reductase (Abdelhamid *et al.,* 2007), copper-containing nitrite reductase, cytochrome *cd*" nitrite reductase and membrane-bound nitric oxide reductase (Burley and Petsko, 1988) has been proposed. Pseudoazurin may be involved in a wide range of electron-transfer reactions with a number of structurally very different donors and acceptors, suggesting that the interaction between redox proteins is only pseudo-specific (Yanagisawa *et al.,* 2003). The crystal structure of the oxidized form of this protein has been determined by Yanagisawa *et al.* (2003). This unique report may indicate that this pseudoazurin crystallizes as a dimer in the asymmetric unit, whereas all other pseudoazurins of known structure are monomeric (Inoue *et al.,* 1999). Interestingly, the reduced form of the *T. pantotropha* pseudoazurin is monomeric, suggesting the intriguing possibility that a change in molecular mass might control the redox properties of the protein (Yanagisawa, 2003). In common with other pseudoazurins, the fold of this polypeptide chain follows the eight-stranded Greek-key motif with a type I copper site. In contrast with the related plastocyanins, however, there are two a-helices located towards the C-terminus of the molecule (Alberts *et al.*, 1994).

Further understanding of the structure and roles of pseudoazurin from *T. pantotropha* requires substantial amounts of protein that can only be obtained by cloning and efficient expression of the *paz*S gene (yields of only 0.6 mg) are possible with the naturally expressed protein (Burley and Petsko, 1988). The present paper reports studies that have achieved this aim, together with an analysis of the basis for expression of pseudoazurin in its natural host only under anaerobic conditions (Vakoufari *et al.*, 1994).

MATERIAL AND METHODS:

Escherichia coli DH5α was grown at 37ºC in LB medium (LB medium 4950 µl, 10 mg / ml ampicllin 50 µl) for 16 h. then *E. coli* was harvested by centrifugation (3000 rpm for 5 min at 4ºC).

Plasmid DNA Purification was performed by the QIAprep Spin Miniprep Kit and a Microcentrifuge (QIAGEN, USA).breifly, this procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt.

Extraction of gene from agarose gel:

The gel with the sPAz gene was cut, crushed and centrifuged at 15,000 rpm for 5 min. at 4ºC. Neutral phenol (in Tris-HCl pH 8.0) with the same volume, and then, this solution stored at -80ºC for 30 min. then centrifuged for 10 min. The mix was dissolved at room temperature. The PCI added to the filtrate up 500µl, and centrifuged for 5 min. 3 M sodium acetate of 1/10 multiple and 2-propanol of same volume was added to the supernatant solution and centrifuged for 20 min. the solution was thrown then 500 µl of 70% ethanol were added and centrifuged for 20 min. at the same steps. The precipitation was dissolved under the vacuum dry, and then 10 µl water was added.

Fragmentation of DNA by restriction enzyme Nco1:

pET plasmid and DNAs fragment were prepared for cloning by digestion with restriction enzyme Nco1 that generate compatible termini. The optimum digestion could be carried out simultaneously with enzyme. At the end of the restriction analysis reaction, a small aliquot of the restricted DNA (3 µl) was analyzed by 0.8% agarose gel electrophoresis to ensure that both fragment and plasmid DNAs were converted to the right linear sizes.

Ligation and transformation:

After digestion the restricted DNA was purified by standard ethanol precipitation. The purified DNA was dissolved in TE buffer (pH, 8.0) at a concentration of ~ 100 µg /ml.

The gene ligated into PET 15b vector as an expression vector. The resultant vector was used for transformation to *Escherichia coli* DH5α by electroporation (Xcell, Bio-Rad).

The plasmid DNA was extracted using Qia plasmid miniprep kit. The primers used were (F) and reverse (R) primer was designed for polymerase chain reactions (PCR). Primer pairs: 5'-caaggacggcgcggtcgttttcgagccgg-3' (F) and 5'-ccggctcgaaaacgaccgcgccgtccttg-3' (R); the resultant recombinant vectors were used for transformation of *Escherichia coli* DH5α by electroporation (Xcell, Bio-Rad). Transformed cells were cultured for 16 h in the presence of ampicllin at 37ºC, and 10 ml precultured medium was transferred to 600 ml medium and cultured until the optical density reached 0.6–0.8 (approximately 3 h), then it was cultured in the presence of 2 mM $CuSO_4 \cdot 5H_2O$ and 1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) for 16 h at 37ºC.

Protein isolation and purification:

Transformed cells were harvested by centrifugation and washed with 0.8% NaCl solution. The harvested cells were suspended in 5 mM Tris – HCl buffer (pH 7.0) containing

DNase, RNase and phenylmethylsulfonyl fluoride were disrupted by sonication in an ice bath. After removal of debris by centrifugation, the recombinant proteins in the supernatant were dialyzed in 20 mM phosphate buffer (pH 6.0), and purified according to previous methods of Abdelhamid *et al*. (2007) based on Kohzuma *et al*. (1995), with slight modifications. Protein purity was checked by sodium dodecyl sulfate electrophoresis on 12.5% (w/v) polyacrylamide gels, and the gels were stained with Coomasie brilliant blue. Introduction of the desired mutation in the proteins was confirmed by the measurement of molecular weight with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Autoflex, Bruker Daltonics). Protein concentrations were determined by the bicinchoninic acid protein assay kit (Pierce where Copper content was determined for each protein with an inductively coupled plasma atomic emission spectrophotometer; a 1:1 concentration ratio of copper ion to protein was confirmed in each case. The molar extinction coefficients of the variant proteins (in 100 mM phosphate buffer) were obtained on the basis of the copper ion concentration using a Hitachi U-3310 spectrophotometer at 25ºC.

RESULTS:

Figure 1 shows the PCR product of Pseudoazurin gene from *Achromobacter cycloclastes* obtained by digestion of cloned PET plasmid with Nco1 restriction enzyme.

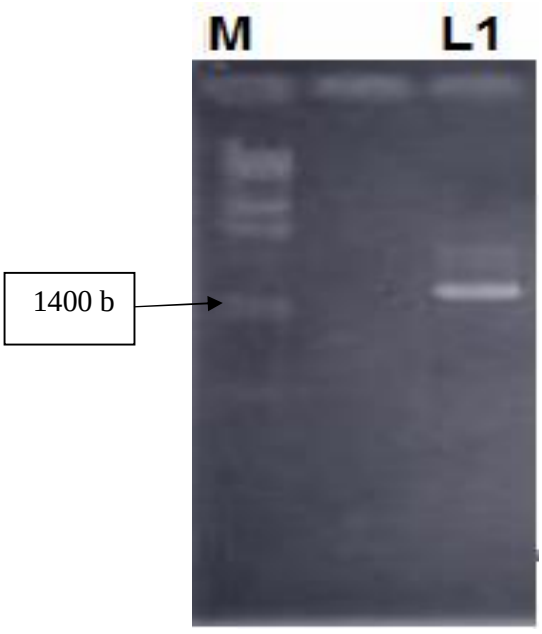

Fig. 1. PCR product of pseudoazurin gene showing single band at 1.4 Kb cleared by marker (M) and lane 1 represent the gene (L1).

Cloning of pseudoazurin gene:

The ligated product of 1.4kb pseudoazurin fragment digested with Nco I , and ligated into pET15b vector (Fig. 2).

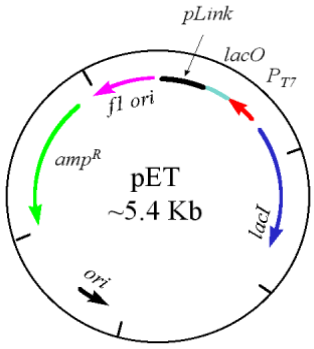

Fig. 2. The pET Vector. This plasmid contains a drug resistant marker for ampicillin resistance (amp^R), the lacI gene, the T7 transcription promoter, the lac operator region 3' to the T7 promoter, and a polylinker region (pLink). Also, there are two origins of replication - one is the f1 origin which enables the production of a single stranded vector under appropriate conditions, and the other is the conventional origin of replication.

Plasmid DNA was isolated from transformed cells of *E. coli* DH5α and restricted with Nco1 and run on agarose gel. Nco1 cut at specific one site on the plasmid and cut at one site on the gene, so the Nco1 has one recognition site on the vector and one site on the pseudoazurin gene. The digestion of recombinant plasmid with Nco1 was resulted into two fragments at the right size. Similarly restricted enzyme Nco1 digest the plasmid on specific site and digest the gene on specific site, so digestion of plasmid with insert gives two bands (Fig. 3).

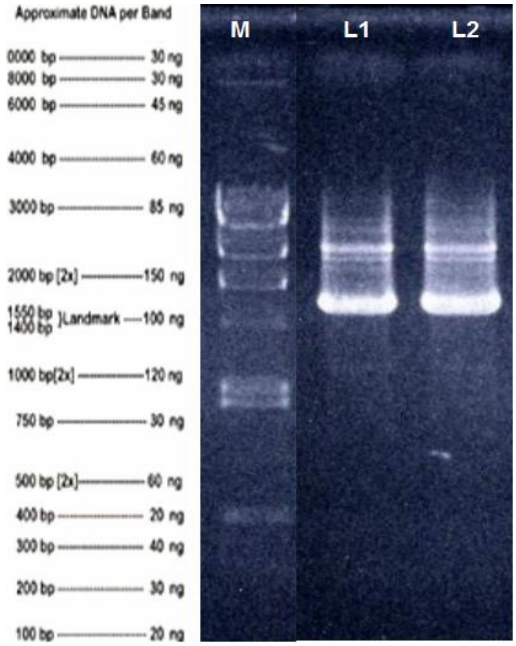

Fig. 3. plasmid pET15b cute with Nco1 and gives two bands one represents the pseudoazurin gene and other band represent the plasmid band (L1 and L2) and (M) maker.

Electrophoretic soluble protein fractionations:

Protein purity was checked by electrophoresis on 12.5% (w/v) SDS-polyacrylamide gel, and the gels were stained

with Coomasie brilliant blue. Protein concentrations were determined by the bicinchoninic acid protein assay kit (Pierce). Copper content was determined for each protein with an inductively coupled plasma atomic emission spectrophotometer; a 1:1 concentration ratio of copper ion to protein was confirmed in each case. The molar extinction coefficients of the variant proteins (in 100 mM phosphate buffer) were obtained on the basis of the copper ion concentration using a Hitachi U-3310 spectrophotometer at 25ºC.

Protein pattern by SDS PAGE for bacterial strains under this study was investigated. The lane showing the protein marker pattern; the band that appeared at molecular weight 14.4 kDa represents the pseudoazurin protein, was found in the strain. It is obvious from the result recorded that the lane of the marker and lane of protein band at molecular weights a 14.4 KDa represents the pseudoazurin band, Bacterial strain protein pattern showed sharply condensed protein band.

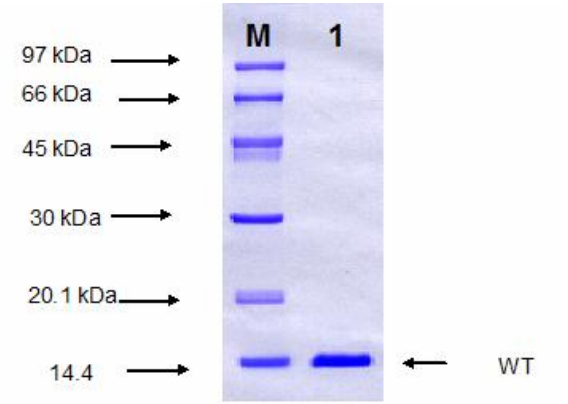

Fig. 4. SDS- polyacrylamide gel showing the over expressed recombinant protein pseudoazurin (14.4KDa) from cloning in *Escherichia coli* DH5α. M: protein marker; 1: extracted protein.

DISCUSSION:

Cloning of the *nitrate reductase* gene revealed the deduced amino acid sequence of a subunit. NIR from *Achromobacter cycloclastes* was recently crystallized and its three dimensional structure has been already determined by X-ray crystallographic analysis at 2.3 A resolution (Godden *et al.,* 1991). This analysis revealed that *Ach. cycloclastes* NIR is a trimer composed of three identical subunits. Three type I copper atoms occur in the complex (one in each subunit) while three type II copper atoms are bound in the spaces between the subunits.

The significant identity in the sequence strongly suggests that NIR of *A. faecalis* also takes a similar trimeric form. In *Ach. Cycloclastes* NIR, four amino acid residues (His-95, Cys-136, His-145, and Met-150) serve as the ligand for the type I copper with a distorted tetrahedral arrangement and three histidines, His-100, His-135, and His-306, and a water molecule are associated with the type II copper atom with a regular tetrahedral arrangement. All these residues are conserved in the sequence of the NIR of *A. faecalis*. These residues are good candidates for site directed mutagenesis studies to elucidate the mechanism of the catalytic reaction and of electron-transfer in copper-containing NIRs. Although the expression plasmid pNIR5O 1 was constructed to express the *nir* gene under the control of the *lac* promoter, large amounts of the NIR proteins were produced at 37ºC even in the absence of IPTG. The expression of the *nir* gene at the higher temperature may result from the combination of these two factors. When the *nir* gene was expressed at 37ºC, the NIR protein was produced in the cell as enzymatically inactive inclusion bodies. But at 26.5ºC, the protein was produced in soluble forms in the cytoplasmic fraction.

Furthermore, at 20ºC NIR was partially secreted into the periplasmic space. It has been reported that human interferon-a2, which forms inclusion bodies in *E. coli* at 37ºC, took a soluble form when the cells were grown at a lower temperature (Schein and Noteborn, 1988*)* and that the processing of subtilisin E of *Bacillus subtilis* fused to the *E. coli* OmpA. Signal peptide was greatly affected by the growth temperature during its secretion into the periplasm of *E. coli* (Ikemura *et al.,* 1987*).* These results support the idea that a lower rate of protein synthesis at a lower temperature may allow the NIR protein to be folded correctly in the cytoplasm and may facilitate the secretion to the periplasmic space. The FNR protein is a transcription factor for a number of genes expressed only during anaerobic growth of *E. coli* cells (Spiro and Guest, 1990*).* In the genes positively regulated by FNR, the FNR-binding sequences are present about *25* bp upstream of the *-10* consensus sequence, while the - *35* consensus sequence is not found. Such a feature is also found in the *nir* gene. This is also true for the pseudoazurin gene of this organism, which encodes the electron donor to NIR (Yamamoto *et al.,* 1987*).* Since FNR is detected at the same concentration in the cells during either aerobic or anaerobic growth (Unden and Guest, 1985*),* this protein is assumed to take an active form only in the absence of oxygen to bind a specific sequence in the promoters of FNR-dependent genes. Previous works revealed the necessity of anaerobic growth for the efficient production of both NIR and pseudoazurin in *A. faecalis* (Kakutani *et al.,* 198l). We may assume that the FNR-like sequences located upstream of the *nir* and pseudoazurin structural genes are recognized by the FNR protein presumably present in *A. faecalis* and serve to enhance the

transcription of these genes under anaerobic growth conditions. *E. coli* possesses NADH-dependent NIR, which may account for the low NIR activity even in the untransformed *E. coli* cells; the expression of the *nirB* gene encoding the NIR is regulated not only by oxygen but also by nitrite (Cole, 1968; Jayaraman *et al.* 1987). The production of *E. coli* NIR was stimulated by two- to three folds by the addition of nitrite. Although several studies to reveal the nucleotide sequence responsible for the nitrite induction of the *nirB* gene of *E. coli* have been carried out, it has not yet been identified precisely (Jayaraman *et al*., 1988; Bell *et al*., 1990). Similar induction of the NIR production by nitrite was also found in *A. faecalis* (Kakutani *et al.,* 1981), suggesting the presence of a similar mechanism of induction by nitrite. However, comparison of the nucleotide sequences upstream of the *nir* gene from *E. coli* and *nirB* gene from *A. faecalis* does not lead to identification of the homologous sequence possibly responsible for the nitrite induction. Further studies to reveal the mechanism for enhancing the expression of the *nir* and pseudoazurin genes are apparently required.

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استزراع وتعبير جين البسيدوازورين في بكتيريا ايشرشيا كولاى

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ترجع أهمية بروتين البسيدوازورين إلى انه يعمل كحامل للالكترونات في العديد من الكائنات الدقيقة ويسمى بالبروتين النحاسي الأزرق تم فيها استخلاص المادة الوراثية DNA من بكتريا اكروموباكتر سيكلوكلاستس الحاملة للجين المسئول عن إنتاج بروتين البسيدوازورين وتم تصميم برا يمر للحصول على ألجين المطلوب عن طريق جهاز تفاعل البلمرة المتسلسل (PCR) وتحميله على بلازميد ونقله إلى بكتريا ايشرشيا كولاى(بكتريا الامعاء)، ثم تم تنمية البكتيريا وعزل البلازميد وتم تقطيعه بإنزيمات القطع Nco1 وتم تحميل ألجين على بلازميد أخر وهو بلازميد تعبيري ونقله إلى بكتيريا اشريشيا كولاى ونميت البكتيريا المحتوية على البلازميد الذي به ألجين المسئول عن إنتاج بروتين البيسيدوازورين وتم عمل بروتين بروفيل عن طريق التفريد الكهربي (Electrophoresis) وتم فصل البروتين و تنقيته كثيرا إلى أن وصلنا إلى باند واحدة وهى التي تمثل البروتين المطلوب وذلك من خلال الوزن الجزيئي للباند الموجودة وهذا يدل على انه تم التعبير عن ألجين المنقول إلى بكتريا الايشريشيا كولاى كما هو واضح من النتائج.

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