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The Blue Copper Protein Gene of *Alcaligenes faecalis* S-6 Directs Secretion of Blue Copper Protein from *Escherichia coli* Cells

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The gene encoding a blue copper protein (a member of the pseudoazurins) of 123 amino acid residues, containing a single type I Cu^{2+} ion, was cloned from *Alcaligenes faecalis* S-6. The nucleotide sequence of the coding region, as well as the 5′- and 3′-flanking regions, was determined. The deduced amino acid sequence after Glu-24 coincided with the reported sequence of the blue protein, and its NH_2-terminal sequence of 23 residues resembled a typical signal peptide. The cloned gene was expressed under the control of the *tac* promoter in *Escherichia coli*, and the correctly processed blue protein was secreted into the periplasm. The blue protein produced in *E. coli* possessed the activity to transfer electrons to the copper-containing nitrite reductase of *A. faecalis* S-6 in vitro.

Blue copper-containing proteins with relatively low molecular weights have been found in various bacteria. These proteins contain a single type I Cu^{2+} ion, giving an intense blue color due to strong absorption at around 600 nm in the oxidized state (1). These proteins isolated from *Pseudomonas aeruginosa* (9) and *Alcaligenes denitrificans* (18) are called azurins, whereas other blue copper proteins, such as amicyanin from *Pseudomonas* strain AM1 (3) and pseudoazurin from *Achromobacter cycloclastes* (2), possess markedly different amino acid sequences. Although these proteins seem to be involved in the electron transport systems of bacterial cells, their physiological roles have remained mostly unknown.

A blue protein with a M_r of 12,000 from a denitrifying bacterium, *Alcaligenes faecalis* S-6, has been noteworthy since its role as an electron carrier for the denitrifying enzyme has been identified (14). This blue protein is easily reduced with ascorbate and other reducing agents, and the reduced protein transfers electrons to a copper-containing nitrite reductase of the same organism, catalyzing reduction of NO_2^- to NO under anaerobic conditions in vitro. When the reduced protein and the nitrite reductase are incubated in the presence of air, the enzyme catalyzes the reduction of molecular oxygen to H_2O_2, which causes suicidal inactivation of the enzyme itself. Thus, the blue protein seems to play a dual physiological role not only as an essential electron carrier but also as a regulatory factor of the anaerobic nitrate respiration system. The 123-amino-acid sequence of the blue protein (8) shows close homology with pseudoazurins from *Achromobacter cycloclastes* (65% amino acid identities) (2). Linkage of pseudoazurin with copper-containing nitrite reductase has also been reported with *Achromobacter cycloclastes* (16). The present paper deals with the cloning and sequencing of the *A. faecalis* S-6 blue protein gene, along with its expression in *Escherichia coli*. The results revealed that the blue protein is synthesized with an NH_2-terminal signal sequence and secreted into the periplasm in *E. coli*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Alcaligenes faecalis* S-6 was used as the source of the blue protein gene for cloning, and *E. coli* HB101 (*hsd20 recA13 ara proA2 lacY1 gal2K rpsL20 xyl mtl supE*), *E. coli* C600 (*hsdR hsdM leu thr thi supE*), and *E. coli* JM105 [Δ(*lac pro*) *thi rpsL endA sbcB15 hsdR4* F′ *traD36 proAB lacI*^{q} Δ*lacZM15*] were used as hosts. The plasmid pYEJ001 (Pharmacia P-L Biochemicals, Milwaukee, Wis.) was used as a cloning vector, and plasmids pBR322, pUC18, and pDR540 (Pharmacia P-L Biochemicals) (6) were used as expression vectors.

Media. *A. faecalis* was cultured in NBAN (nutrient broth-acetate-nitrate) medium anaerobically at 30°C. *E. coli* strains were cultured in L broth aerobically at 37°C. The concentration of ampicillin used for selection was 50 μg/ml. To express the cloned blue gene under the control of the *tac* promoter, 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added.

Enzymes and chemicals. Nitrite reductase and blue protein were prepared from *A. faecalis* S-6 as described previously (13, 14). Restriction enzymes, the large (Klenow) fragment of DNA polymerase I, T4 DNA ligase, T4 polynucleotide kinase, bacterial alkaline phosphatase, and a DNA sequencing kit for the dideoxy method were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). An in vitro packaging kit was obtained from Amersham International. The labeled compounds, [γ-^{32}P]ATP and [α-^{32}P]dCTP, were obtained from New England Nuclear Corp. (Boston, Mass.). DEAE-Toyopearl 650M and CM-Toyopearl 650M were obtained from Toyo Soda Mfg. Co.

Synthesis of the oligonucleotide probes. Three kinds of mixed oligonucleotide probes were synthesized by using a Beckman DNA synthesizer (system 1 plus) and purified by preparative polyacrylamide gel electrophoresis. The synthesized probes were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Construction of a genomic library for *A. faecalis*. Chromosomal DNA of *A. faecalis* S-6 prepared by the method of Saito and Miura (21) was partially digested with *Sau*3A1 to give fragments larger than 30 kilobases (kb). The fragments

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                    6                          10
Amino acid          His Met Leu Asn Lys Gly
mRNA          5'CAU AUG CUN AAU AAA GG3'
                  C           C   G
Probe 17A     3'GTA TAC GAN TTA TTT CC5'
                  G           G   C

                    45                         51
Amino acid          Lys Asp Met Ile Pro Glu Gly
mRNA          5'AAA GAU AUG AUU CCN GAA GG3'
                  G   C       C
Probe 20B-1   3'TTT CTA TAC TAA GGN CTT CC5'
                  C   G       G

                    80                         86
Amino acid          His Tyr Ala Met Gly Met Ile
mRNA          5'CAU UAU GCN AUG GGU AUG AU3'
                  C   C       C
Probe 20B-2   3'GTA ATA CGN TAC CCA TAC TA5'
                  G   G       G
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FIG. 1. Mixed oligonucleotide probes used and their corresponding amino acid and mRNA sequences in the blue protein (7). The amino acid numbers from the NH_2 terminus of the blue protein are indicated by italic type.

were treated with bacterial alkaline phosphatase and then ligated to the left and right arms of cosmid pJB8 DNA prepared as described by Ish-Horowicz et al. (12). The ligated DNA was packaged in bacteriophage particles by using an in vitro packaging kit and used to infect *E. coli* HB101. A total of approximately 6,000 Amp^r colonies was obtained from 5 μg of DNA.

Cloning of the blue protein gene. The genomic library of *A. faecalis* S-6 in *E. coli* was screened by colony hybridization using the synthetic oligonucleotide probes according to the method of Wallace et al. (24). The temperatures for the hybridization and washing were 43°C and room temperature, respectively. Small-scale preparation of recombinant plasmid DNAs from transformants was performed by the boiling method as described by Holmes and Quigley (7). Transformation of *E. coli* with plasmid DNA was performed by the method of Norgard et al. (17).

Southern blot analysis. Transfer of DNA fragments from agarose gel to nitrocellulose paper was performed as described by Southern (23).

DNA sequencing. Specific restriction fragments of the cloned DNA were ligated into the appropriate M13 vector mp18 or mp19 and sequenced by the chain-terminating dideoxy method (22).

Fractionation of extracellular, periplasmic, and cytoplasmic proteins in *E. coli*. Fractionation was performed by the method of Cornelis et al. (5). Cells of *E. coli* and *A. faecalis* S-6 were harvested at the early stationary phase and washed twice with 10 mM Tris hydrochloride buffer (pH 7.5) containing 25% sucrose. The washed cells were suspended in the same buffer containing 25% sucrose and 1 mM EDTA and incubated with shaking for 10 min at room temperature. After centrifugation at 7,000 × *g* for 10 min, the cells were quickly and vigorously suspended in ice-cold water. The suspension was further shaken for 10 min at 4°C and centrifuged at 9,000 × *g* for 10 min. The pelleted cells were suspended in 10 mM Tris hydrochloride buffer (pH 7.5) and disrupted by sonication. The extracellular enzyme fraction was defined as the sum of the activities in the culture supernatant, the two washes, and the supernatant after EDTA treatment. The periplasmic fraction was defined as the activity found in the supernatant after treatment with cold water. The cytoplasmic fraction was defined as the activity found in the supernatant after sonication.

Determination of the NH_2-terminal amino acid sequence of the blue protein. The blue protein produced in *E. coli* was purified (14). The NH_2-terminal amino acids of the protein were determined in a Beckman 890D protein-peptide sequencer. Approximately 20 nmol of the protein was subjected to 10 cycles of automated Edman degradation, and the phenylthiohydantoin derivatives of the released amino acids were analyzed with a high-performance liquid chromatograph equipped with a Shodex protein WS-803 column (Showa Denko, Tokyo, Japan).

Assay of enzyme activities. To measure the degree of inactivation of nitrite reductase by the blue protein, 1,400 U of the nitrite reductase was incubated with various amounts of the blue protein in 200 μl of 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM ascorbate at 30°C for 30 min. Residual enzyme activity was measured according to the method of Kakutani et al. (14). Activity of β-lactamase was measured by iodometry (20).

RESULTS

Cloning of the blue protein gene. For identification of the blue protein gene, we synthesized three kinds of 17- and 20-mer mixed oligonucleotide probes, 17A, 20B-1, and 20 B-2, corresponding to the hexa- and heptapeptides in the amino acid sequence of the blue protein (Fig. 1). The cosmid library of the *A. faecalis* genes in *E. coli* was screened with the labeled synthetic probes. Fourteen colonies which hybridized with all three probes were detected among 6,000 Amp^r colonies. A large plasmid containing a 40-kb *Sau*3A1 fragment in pJB8 was recovered from one of the colonies and designated pAB1. On the other hand, *A. faecalis* chromosomal DNA was digested with various restriction enzymes and analyzed by Southern blot hybridization with the synthetic probes. A 4.8-kb *Eco*RI fragment was found to hybridize with all three probes. Therefore, pAB1 was digested with *Eco*RI, the 4.2- to 5-kb fragments were isolated electro-

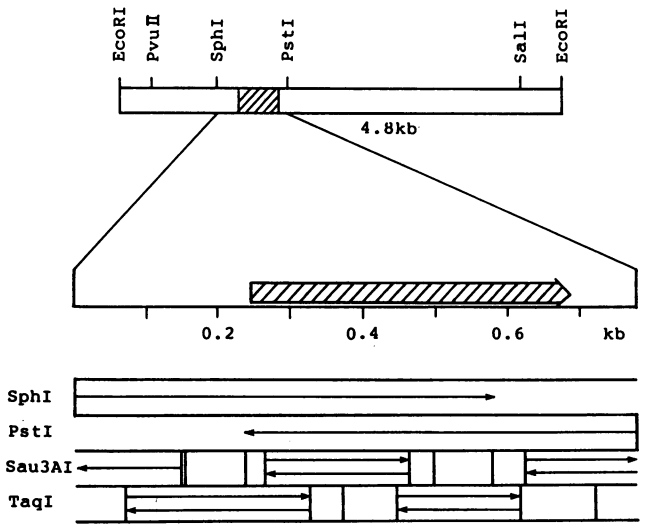

FIG. 2. Restriction map of the insert DNA in pAB101 and sequencing strategy. The hatched area shows the open reading frame.

FIG. 3. Nucleotide and deduced amino acid sequences of the blue protein gene. The overlined sequences correspond to the probes used for cloning. The underlined sequence is the determined NH_2-terminal sequence of the blue protein produced in *E. coli*. SD, Shine-Dalgano sequence. An inverted repeat structure is indicated by the facing arrows. The regions which show a high degree of homology with the *ntrA*-dependent promoters are boxed. Arrowhead Cleavage site of the signal peptide as determined with the purified enzyme.

phoretically, and these were subcloned into the *Eco*RI site of pYEJ001. A recombinant plasmid, pAB101, was recovered from a colony detected by colony hybridization with the synthetic probes.

DNA sequencing of the cloned blue protein gene. The 4.8-kb *Eco*RI insert of pAB101was digested with various restriction enzymes, and the resulting fragments, separated by agarose gel electrophoresis, were examined with the labeled probes. The *Sph*I-*Pst*I fragment of about 780 base pairs (bp) was found to hybridize with the three probes.

This fragment was cloned into phage M13 mp18 and sequenced according to the strategy shown in Fig. 2. A single open reading frame of 438 bp was found to encode a polypeptide of 146 amino acid residues (Fig. 3). The amino acid sequence after Glu-24, deduced from the nucleotide sequence, was completely identical with that of the blue protein previously reported (8). The NH_2-terminal sequence of the open reading frame from Met-1 to Ala-23 was similar to a typical signal sequence containing a basic NH_2-terminal segment, followed by a stretch of hydrophobic residues.

Two possible ribosome binding sequences were shown to be present 4 and 10 bp upstream from the initiation codon. Considering the space to the initiation codon, the latter GGAGA sequence may be functional. No sequence homologous with the typical *E. coli* consensus promoter was found in the upstream region. However, nucleotides 71 to 78 and 87 to 91 were found to show a high degree of homology with the promoters of genes whose transcription requires the *ntrA* gene product (10), except that the space between the two sequences is 8 bp, which is longer than the 4 bp in the usual *ntrA*-dependent promoters. An inverted repeat structure with $\Delta G = -10.2$ kcal/mol was found between the putative *ntrA*-dependent promoter and the ribosome-binding sequence.

Expression of the blue protein gene in *E. coli*. The 783-bp *Sph*I-*Pst*I fragment containing the coding sequence for the blue protein was inserted between the *Sph*I and *Pst*I restriction sites on pUC19 downstream from the *lac* promoter with the correct orientation. The resulting expression plasmid pAB201 was introduced into *E. coli* JM105, and the transformant was grown in the presence of IPTG. A very faint protein band corresponding to the blue protein was observed in the cell extract by sodium dodecyl sulfate (SDS)-gel electrophoresis, although no activity of the blue protein in inactivating *Alcaligenes* nitrite reductase was detected in either the cell extract or the culture medium. It seemed probable that expression of the blue protein gene from the *lac* promoter was inhibited by the inverted repeat DNA sequence upstream of the coding sequence described above. Therefore, the *Sph*I-*Pst*I fragment cloned into the M13 mp18 phage DNA was recovered by double digestion with *Hin*dIII and *Sal*I, and the inverted repeat sequence was removed by partial digestion with *Sau*3A1. The resulting 550-bp *Sau*3A1-*Sal*I fragment was ligated to the large *Pst*I-*Sal*I fragment of pBR322 and a small *Pst*I-*Bam*HI fragment of pDR540 containing the *tac* promoter (6). The constructed plasmid, pAB301, carrying the blue protein coding sequence downstream from the *tac* promoter, was introduced into *E. coli* C600, and the transformant was grown aerobically in L broth containing IPTG at 37°C. A protein comigrating with the authentic blue protein from *A. faecalis* S-6 was detected in the cell extract by SDS-gel electrophoresis (Fig. 4).

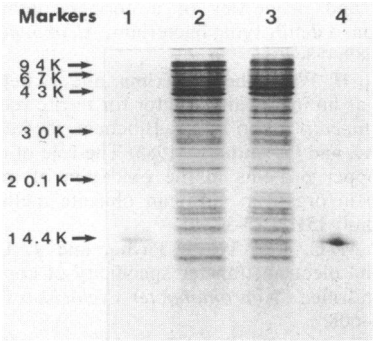

FIG. 4. SDS-polyacrylamide gel electrophoresis of the blue protein in *E. coli* C600(pAB301). Gels were stained with Coomassie brilliant blue. Lane 1, Purified blue protein from *A. faecalis* S-6; lane 2, sonicated crude extract of *E. coli* C600; lane 3, sonicated crude extract of *E. coli* C600(pAB301); lane 4, purified blue protein from *E. coli* C600(pAB301).

Inactivation of the *Alcaligenes* nitrite reductase was also observed upon incubation with the extract. The amount of the blue protein produced in *E. coli* cells was estimated to be 3.9% of total soluble proteins. Fractionation of the *E. coli* cells revealed that the blue protein was present mainly in the periplasmic fraction and partially in the extracellular medium (Fig. 5), whereas most of the β-lactamase activity of the host was localized in the periplasm (data not shown).

Properties of the blue protein produced in *E. coli*. The blue protein produced and accumulated in the periplasm of *E. coli* C600(pAB301) was purified by successive chromatographies, using DEAE-Toyopearl, CM-Toyopearl, and Sephadex G75 columns, to give a single band upon SDS-polyacrylamide gel electrophoresis. The purified protein from the Sephadex column showed a distinct blue color with absorption maxima at 277, 282, 450, 593, and 750 nm, respectively (data not shown), which were identical with those of the authentic preparation (14). The A_{593}/A_{277} ratios of the *E. coli* and *Alcaligenes* blue proteins were 0.493 and 0.509, respectively.

The NH_2-terminal sequence of the *E. coli* blue protein was directly determined to be Glu-Asn-Ile-Glu-Val-His-Met-Leu-Asn-Lys-, identical with that of the *Alcaligenes* blue protein, thus confirming that the signal peptide was correctly processed in *E. coli*.

The purified *E. coli* blue protein from *E. coli* C600 (pAB301) was incubated with the *Alcaligenes* nitrite reductase in the presence of ascorbate, and the degree of inactivation of the reductase was measured. The extent of the inactivation by the blue protein produced by *E. coli* was the same as that by the *Alcaligenes* blue protein (data not shown).

Localization of the blue protein in *A. faecalis* S-6. Cells of *A. faecalis* S-6 grown in NBAN medium under anaerobic conditions were fractionated, and localization of the blue protein was analyzed by SDS-gel electrophoresis (Fig. 5). Taking into account the applied amounts of samples in the gel, we estimated that approximately 55% of the total blue protein was localized in the periplasmic fraction. No blue protein was found in the extracellular medium.

DISCUSSION

In the present study, we isolated the gene of the blue protein (pseudoazurin) of *A. faecalis* S-6 and determined its nucleotide sequence. The open reading frame encoded a typical NH_2-terminal signal peptide sequence consisting of 23 amino acid residues. This finding suggests that the *Alcaligenes* blue protein is a secreted protein. In fact, the cloned gene in the *E. coli* host caused secretion of the blue protein into the periplasm, and approximately half of the blue protein in the cells of *A. faecalis* was localized in the periplasmic fraction. In addition, approximately 10% of the total nitrite reductase activity in this organism was also detected in the periplasm fraction (data not shown). It thus seems probable that the nitrite reductase system is localized in the periplasm of *A. faecalis* S-6. Recently, the gene for azurin was cloned from *P. aeruginosa* (4). Its nucleotide sequence also encodes an NH_2-terminal signal sequence of 19 amino acid residues. Periplasmic localization of amicyanin was also demonstrated in some methylotrophic bacteria (11, 15). Most of the bacterial blue copper proteins might be secreted through the cytoplasmic membrane and play as electron carriers in the periplasm.

A sequence similar to the *ntrA*-dependent promoter after an inverted repeat structure was present upstream from the coding sequence of the blue protein. The blue protein plays an essential role as an electron carrier for nitrite reductase, and the enzyme activity is induced by nitrate or nitrite in *A. faecalis* S-6 under anaerobic conditions (13). It therefore seems possible that these sequences are involved in characteristic regulation of the blue protein gene as a component of the anaerobic nitrate respiration system in this organism.

The blue protein produced in *E. coli* cells inactivated the *Alcaligenes* nitrite reductase in the presence of reducing agents in vitro. This indicates that the blue protein is correctly processed and folded in *E. coli* cells so that it can express its native activity of transferring electrons to nitrite reductase.

The tertiary structure of the blue protein of *A. faecalis* S-6 has recently been elucidated by X-ray crystallography (19). By using the cloned gene and the *E. coli* expression system reported here, it should become possible to produce modified blue protein by site-directed mutagenesis, thus providing information on structure-function relationships.

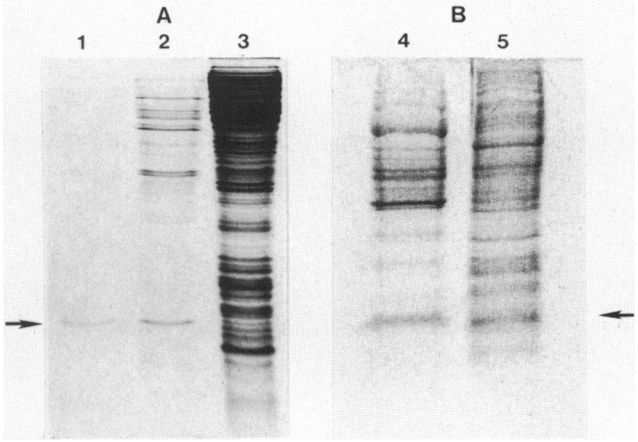

FIG. 5. Localization of blue protein in cells of *E. coli* C600(pAB301) (A) and *A. faecalis* S-6 (B). Arrows indicate positions of the authentic blue protein. Cells in the extracellular medium (lane 1), periplasmic fraction (lanes 2 and 4), and cytoplasmic fraction (lanes 3 and 5) were used. Each lane in A contains the fraction equivalent to the same amount of *E. coli* cells. In B, the periplasmic fraction in lane 4 contains 3.3 times the equivalent of the cytoplasmic fraction in lane 5.

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