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Cloning and sequencing of the gene encoding cytochrome *c*-551 from *Pseudomonas aeruginosa*

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The cytochrome *c*-551 gene from *Pseudomonas aeruginosa* was cloned by using two oligonucleotide probes, which had been synthesized based on the known primary structure of the protein. The restriction map of the cloned DNA and sequence analysis showed that the cytochrome *c*-551 gene is located 50 bp downstream of the nitrite reductase gene, which has recently been cloned and sequenced. DNA sequence analysis also indicated that cytochrome *c*-551 is synthesized in vivo as a precursor having an amino-terminal signal sequence consisting of 22 amino acid residues.

Cytochrome *c*-551; DNA sequence; Signal peptide; Nitrite reductase; (*Pseudomonas aeruginosa*)

1. INTRODUCTION

Cytochrome *c*-551 occurs in various *Pseudomonas* species [1] and plays an important role in the respiratory chain [2,3]. In *P. aeruginosa* grown anaerobically in the presence of nitrate, nitrite reduction (nitrite respiration) is effected by a respiratory chain composed of azurin, cytochrome *c*-551 and nitrite reductase [4,5]. The nitrite reductase [6] and azurin [7] genes have been cloned and sequenced, but the cytochrome *c*-551 gene has not yet been reported. Here we report cloning and sequencing of this gene by using two oligonucleotide probes synthesized based on the reported primary structure of the cytochrome [8].

2. MATERIALS AND METHODS

2.1. *Bacterial strains, vectors and media*

Pseudomonas aeruginosa PAO1161 and *E. coli* JM109 were cultivated at 37°C in L-broth or on YT-plates. pUC19 plasmid was used for cloning of the cytochrome *c*-551 gene. M13mp18 and mp19 phages were used for DNA sequencing. *E. coli* JM109 harbouring pUC19 or its derivatives were grown in the presence of ampicillin at 50 μg/ml. Derivatives of M13mp18 and mp19 were propagated in *E. coli* JM109 grown in 2×YT medium.

2.2. *Cloning*

Chromosomal DNA was isolated from *P. aeruginosa* according to the method of Marmur [9]. Two oligonucleotides were synthesized on a Beckman System 1 Plus DNA Synthesizer and purified by elution from a 20% polyacrylamide-7M urea gel. The purified oligonucleotides were radiolabeled by 5′-phosphorylation with [γ-^{32}P]ATP by polynucleotide kinase. Filter hybridization was carried out at 45°C. Other DNA techniques were carried out as described by Maniatis et al. [10].

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2.3. *DNA sequencing*

DNA fragments carrying the cytochrome *c*-551 gene were deleted by using Kilo-Sequence, Deletion Kit (Takara Shuzo Co., Kyoto, Japan). The isolation of single-strand DNA templates and dideoxy-DNA sequencing reactions were performed as recommended by United States Biochemical Corp., using Sequenase version 2.0. Considering the high GC content of the *P. aeruginosa* genome, 7-deaza-dGTP or dITP instead of dGTP were used for DNA sequencing.

3. RESULTS AND DISCUSSION

Two oligonucleotide probes, PA-1 and PA-2 (fig.1), were synthesized based on the known primary structure of *P. aeruginosa* cytochrome *c*-551 [8] and used for cloning of the cytochrome gene. The codon usage of *P. aeruginosa* [11] was taken into account in designing these probes. Southern blot analysis of various restriction fragments of *P. aeruginosa* chromosomal DNA indicated that 9-kb *Pst*I, 5.5-kb *Sph*I, 3.5-kb *Eco*RI and 1.5-kg *Sal*I fragments hybridized with both of the two probes (data not shown). The 9-kb *Pst*I fragment was recovered from agarose gel and inserted into the *Pst*I site of pUC19. The ligation mixture was then used to transform *E. coli* JM109. One positive clone out of 97

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                          7   8   9   10  11  12  13
Amino acid sequence     -Phe-Lys-Asn-Lys-Gly-Cys-Val-
possible anticodons      AAR TTY TTR TTY CCN ACR CAN
probe  PA-1          3' AAG TTC TTG TTC CCG ACG CA 5'

                          45  46  47  48  49  50  51
Amino acid sequence     -Ala-Gln-Arg-Ile-Lys-Asn-Gly-
possible anticodons      CGN GTY KCN TAZ TTY TTR CCN
probe  PA-2          3' CGN GTC GCN TAG TTC TTG CC 5'
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Fig.1. Oligonucleotide probes for the isolation of the cytochrome *c*-551 gene form *P. aeruginosa*. N = A, C, G, T; R = A, G; Y = C, T; K = G, T; Z = A, G, T.

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colonies was obtained by colony hybridization with probe PA-1.

The restriction map of the cloned 9-kb *Pst*I fragment is shown in fig.2. By southern blot analysis of various restriction fragments of the cloned DNA, the hybridization sites for probes PA-1 and PA-2 were located in the 1.5-kb *Sal*I fragment (fig.2) in agreement with the results of southern blot analysis of chromosomal DNA.

The 1.5-kb *Sal*I fragment containing the cytochrome *c*-551 gene was subcloned into phages M13mp18 and mp19. Deletion clones also constructed for sequencing. About two-thirds of the 1.5-kb fragment was sequenced according to the strategy shown in fig.2, and the nucleotide sequence thus determined is shown in fig.3.

As can be seen, the determined nucleotide sequence contains an open reading frame coding for a protein composed of 104 amino acid residues. The primary structure deduced for the protein is identical with that reported for *P. aeruginosa* cytochrome *c*-551 [8], except that the former has an amino-terminal extension of 22 residues. The amino acid sequence of this extension exhibits all the typical characteristics for procaryotic signal sequences [12]. It can thus be concluded that cytochrome *c*-551 is synthesized as a precursor and its amino terminal 22-residue signal sequence is cleaved during its export to the periplasm. In fact, it has been reported that cytochrome *c*-551 as well as nitrite reductase and azurin are located in the periplasm [5]. It is to be noted that the nitrite reductase [6] and azurin [7] genes also code for cleavable signal sequences. Since all the bacterial *c*-type cytochrome genes so far sequenced have been shown to code for signal sequences [13–18], it is likely that these *c*-type cytochromes are also exported to the periplasm.

The restriction map of the internal *Eco*RI-*Eco*RI (3.5 kb) region of the 9-kb *Pst*I fragment is identical with that of the 3.5-kb *Eco*RI fragment containing the nitrite reductase gene isolated by Silvestrini et al. [6]. Moreover, the nucleotide sequence of the 1.5-kb *Sal*I fragment in the upstream region of the cytochrome *c*-551 gene is exactly the same as that reported for the nitrite reductase gene [6]. Actually this region encodes the carboxyl-terminal portion of nitrite reductase (see fig.3). It is thus clear that the cytochrome *c*-551 gene is located only 50 nucleotides downstream of the nitrite reductase gene. Between the two genes there are an inverted repeat sequence and a putative Shine-Dalgarno sequence for ribosome binding [19]. Silverstrini et al. [6] have suggested that the inverted repeat sequence acts as a transcription terminator. However, since no promotor-like sequence can be found upstream of the putative Shine-Dalgarno sequence for the cytochrome *c*-551 gene, it is highly likely that both the nitrite reductase and cytochrome *c*-551 genes are transcribed as a single messenger RNA. There are two inverted repeat sequences downstream of the cytochrome *c*-551 gene. Transcription of the messenger RNA may be terminated at these sites. Inconsistent with this notion is the fact that in *P. aeruginosa* nitrite reductase is induced only in the presence of nitrate [3], whereas cytochrome *c*-551 is thought to be expressed in both aerobically and anaerobically grown cells. More work is still needed to understand the regulatory mechanism of the respiratory chain involved in nitrite respiration.

Analysis of the restriction map shows that the azurin gene [7] is not found in the 9-kb *Pst*I fragment obtained in this study.

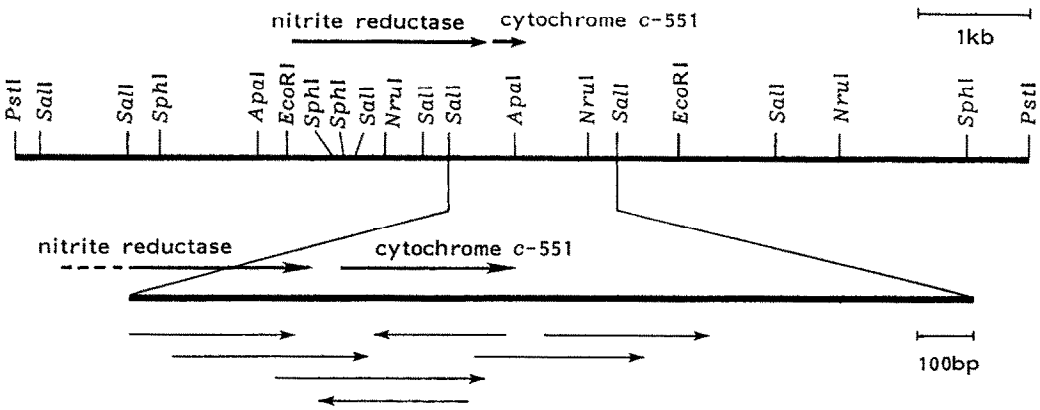

Fig.2. Restriction map and sequencing strategy for the gene encoding cytochrome *c*-551 from *P. aeruginosa*. The thick lines represent the DNA fragment containing the cytochrome *c*-551 gene. The upper arrows indicate the spans and transcriptional directions of the cytochrome *c*-551 and nitrite reductase genes. The lower arrows indicate the directions and lengths of sequence determinations.

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GTC GAC ACC ACC TTC AAC CCC GAC GCC AGG ATC AGC CAG AGC GTC GCG GTG TTC GAC   57
Val Asp Thr Thr Phe Asn Pro Asp Ala Arg Ile Ser Gln Ser Val Ala Val Phe Asp

CTG AAG AAC CTC GAC GCC AAG TAC CAG GTG CTG CCG ATC GCC GAA TGG GCC GAT CTC  114
Leu Lys Asn Leu Asp Ala Lys Tyr Gln Val Leu Pro Ile Ala Glu Trp Ala Asp Leu

GGC GAA GGC GCC AAG CGG GTG GTG CAG CCC GAG TAC AAC AAG CGC GGC GAT GAA GTC  171
Gly Glu Gly Ala Lys Arg Val Val Gln Pro Glu Tyr Asn Lys Arg Gly Asp Glu Val

TGG TTC TCG GTG TGG AAC GGC AAG AAC GAC AGC TCC GCG CTG GTG GTG GTG GAC GAC  228
Trp Phe Ser Val Trp Asn Gly Lys Asn Asp Ser Ser Ala Leu Val Val Val Asp Asp

AAG ACC CTG AAG CTC AAG GCC GTG GTC AAG GAC CCG CGG CTG ATC ACC CCG ACC GGT  282
Lys Thr Leu Lys Leu Lys Ala Val Val Lys Asp Pro Arg Leu Ile Thr Pro Thr Gly

AAG TTC AAC GTC TAC AAC ACC CAG CAC GAC GTG TAC TGA GACCCGCGTGCGGGGCACGCCCC  347
Lys Phe Asn Val Tyr Asn Thr Gln His Asp Val Tyr TER

                          -22 -21 -20 -19 -18 -17 -16 -15 -14 -13 -12 -11
GCACGCTCCCCCCTACGAGGAACCGTG ATG AAA CCG TAC GCA CTG CTT TCG CTG CTC GCC ACC  410
                            Met Lys Pro Tyr Ala Leu Leu Ser Leu Leu Ala Thr

-10  -9  -8  -7  -6  -5  -4  -3  -2  -1   1   2   3   4   5   6   7   8   9
GGC ACC CTG CTC GCC CAG GGC GCC TGG GCC GAA GAC CCC GAA GTG CTG TTC AAG AAC  467
Gly Thr Leu Leu Ala Gln Gly Ala Trp Ala Glu Asp Pro Glu Val Leu Phe Lys Asn

 10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25  26  27  28
AAG GGC TGC GTG GCC TGC CAT GCC ATC GAC ACC AAG ATG GTC GGC CCG GCC TAC AAG  524
Lys Gly Cys Val Ala Cys His Ala Ile Asp Thr Lys Met Val Gly Pro Ala Tyr Lys

 29  30  31  32  33  34  35  36  37  38  39  40  41  42  43  44  45  46  47
GAC GTC GCC GCC AAG TTC GCC GGC CAG GCC GGC GCC GAA GCG GAA CTC GCG CAG CGG  581
Asp Val Ala Ala Lys Phe Ala Gly Gln Ala Gly Ala Glu Ala Glu Leu Ala Gln Arg

 48  49  50  51  52  53  54  55  56  57  58  59  60  61  62  63  64  65  66
ATC AAG AAC GGC AGC CAG GGC GTC TGG GGC CCG ATC CCG ATG CCG CCG AAC GCG GTC  638
Ile Lys Asn Gly Ser Gln Gly Val Trp Gly Pro Ile Pro Met Pro Pro Asn Ala Val

 67  68  69  70  71  72  73  74  75  76  77  78  79  80  81  82
AGC GAC GAC GAG GCG CAG ACC CTG GCG AAG TGG GTC CTG TCG CAG AAA TGA ACGCCCC  696
Ser Asp Asp Glu Ala Gln Thr Leu Ala Lys Trp Val Leu Ser Gln Lys TER

TCCGGACTTCCGGCGCGCCGCCAGCCACGCCTTGTGGCTGGCCCTCGCGCTGACGTTCGCCTGTCCTCTTCCGGG  771

CCTGGCCGACGAGCATCCCGATGCCCGTCGCCAGGCCCAGTTGCGTCACCTGCTGTTGCAGGACTGCGGCTCCTG  846

CCACGGCCTGCGCCTGACCGGCGGCCTCGGCCCGGCGCTGACCCCCGAGGCCCTGCGCGGCAAGCCGCGCGAATC  921
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Fig.3. The DNA sequence of the gene encoding cytochrome *c*-551 from *P. aeruginosa*. The deduced amino acid sequence of cytochrome *c*-551 and the C-terminal portion of nitrite reductase is shown below. The numbers −22/−1 and 1/82 above correspond to the signal peptide and the mature protein of cytochrome *c*-551, respectively. The underlined sequence is the putative ribosome-binding site. The arrows underline the inverted repeat sequence.

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