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Anaerobically induced expression of the nitrite reductase cytochrome *c*-551 operon from *Pseudomonas aeruginosa*

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The nitrite reductase gene (*denA*) and the cytochrome *c*-551 gene (*denB*) are located only 50 bp apart from each other in the *Pseudomonas aeruginosa* chromosome. We report evidence that these two genes are co-transcribed as an operon only under anaerobic (denitrifying) conditions. The nucleotide sequence of the promoter (regulatory) region of the operon is highly AT-rich and contains a sequence closely resembling the consensus FNR binding site in *E. coli*.

Nitrite reductase; Cytochrome *c*-551; Promoter; FNR binding site; *Pseudomonas aeruginosa*

1. INTRODUCTION

Nitrite reductase (cytochrome cd_1: EC 1.9.3.2) of *Pseudomonas aeruginosa* [1] is a soluble redox protein occurring in the periplasm [2]. It catalyzes the reduction of nitrite to nitric oxide [3] and thus plays an important role in anaerobic respiration (denitrification). The activity of this dissimilatory enzyme has been reported to be expressed only when the cells are grown anaerobically in the presence of nitrate [4]. Coyne et al. [5] have also immunochemically shown that the enzyme protein is synthesized exclusively in denitrifying cells. It is further known that cytochrome *c*-551, a small redox protein, acts as the direct electron donor for nitrite reductase [6]. We have recently cloned a gene cluster encoding both nitrite reductase and cytochrome *c*-551 and shown that the two genes are located only 50 bp apart from each other [7]. Here we report that the two genes are transcribed as an operon from an oxygen-regulated promoter. We also propose to call the nitrite reductase and cytochrome *c*-551 genes *denA* and *denB*, respectively.

2. MATERIALS AND METHODS

P. aeruginosa PAO1161 [8] and *E. coli* JM109 [9] were grown at 37°C in YT-broth (5 g yeast extract, 8 g trypton and 5 g NaCl/l, pH 7.3) or on YT-plate. For anaerobic cultivation, 100 mM $NaNO_3$ or 20 mM $NaNO_2$ was added to the medium in sealed flasks and air in the headspace was replaced by N_2 gas. When necessary, the following antibiotics were added to the medium: ampicillin (50 μg/ml), kanamycin (25 μg/ml) and streptomycin (10 μg/ml) for *E. coli*; carbenicillin (300 μg/ml) and streptomycin (500 μg/ml) for *P. aeruginosa*. Transfer of plasmids from *E. coli* to *P. aeruginosa* was performed by the triparental mating method using pRK2013 as a helper plasmid [10]. Pseudomonas isolation agar (Difco) was used for isolation of *P. aeruginosa* transformants. pTS1045 (IncQ, Ampicillin^r, Streptomycin^r, *xylE*) was used to probe the promoter activity of DNA fragments. If a functional promoter is ligated to the upstream of *xylE* in this vector, the activity of catechol 2,3-dioxygenase (C230), the *xylE* gene product, can be expressed [11]. Protein was determined by Bio-Rad method using bovine serum albumin as a standard. DNA sequencing was performed as described [7]. Other DNA manipulations were carried out as described by Sambrook et al. [9].

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3. RESULTS AND DISCUSSION

3.1. *Identification of promoter region of the nitrite reductase gene*

Various restriction fragments of the upstream region of the nitrite reductase gene (*denA*) were ligated to the promoter probe vector pTS1045. The constructed plasmids, pHA301 through 306 (see Fig. 1), were transferred to *P. aeruginosa*. The promoter activities of the ligated fragments were then examined under both aerobic and anaerobic conditions by measuring the activity of C230, the *xylE* gene product. As shown in Table I, under aerobic conditions, no or little C230 activity was expressed in the cells harbouring any of the constructed plasmids even in the presence of nitrate or nitrite. On the other hand, under anaerobic conditions the cells carrying pHA301, pHA302, pHA304 or pHA306, but not pHA303 or pHA305, expressed high C230 activities when grown in the presence of nitrate or nitrite. These results indicated that the nitrite reductase gene is transcribed only under anaerobic conditions and the promoter and regulatory region of this gene is located in the *Apa*I–*Eco*RI fragment (see Fig. 1). It was

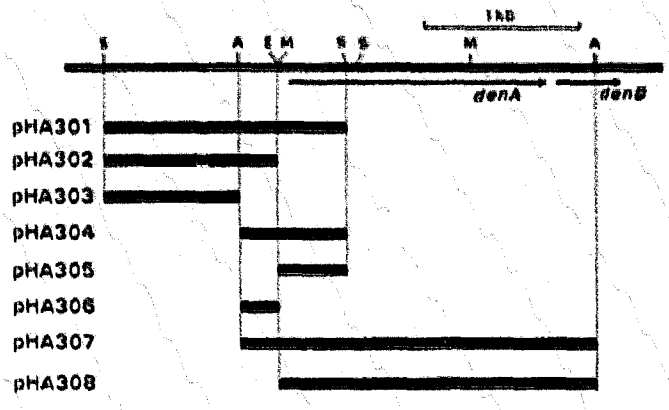

Fig. 1. Fragments used for C230 assay. The plasmids pHA301-308 were constructed by ligating these restriction fragments containing for *denA* and/or its flanking regions to the upstream of *xylE* of the promoter probe vector pTS1045. A, *Apa*I; E, *Eco*RI; M, *Sma*I; S, *Sph*I.

Table I

C230 activity in cell extract of *P. aeruginosa* PAO1161 containing pTS1045 and pHA301-308

	C230 activity (U/mg protein)				
	aerobic			anaerobic	
	YT	NO_3^- 100 mM	NO_2^- 20 mM	NO_3^- 100 mM	NO_2^- 20 mM
pTS1045	1.61	0.99	1.19	1.70	0.98
pHA301	1.47	1.91	2.09	20.94	25.77
pHA302	1.67	1.42	2.46	29.78	28.66
pHA303	1.62	1.58	2.86	2.21	2.27
pHA304	1.59	1.42	3.35	18.66	25.22
pHA305	1.17	1.16	1.43	1.10	1.22
pHA306	1.43	1.62	2.08	20.00	21.25
pHA307	1.32			10.12	
pHA308	1.20			1.69	

also suggested that the activity of this promoter (regulator) region is sensitive to molecular oxygen.

3.2. *Identification of promoter region of the cytochrome c-551 gene*

In the chromosome of *P. aeruginosa*, the cytochrome *c*-551 gene (*denB*) is located 50 bp downstream of *denA* and no promoter-like sequence can be found in this 50-bp region [7,12], suggesting that these two genes are transcribed as an operon. To confirm this possibility, two fragments were prepared, ligated to pTS1045 (pHA307 and pHA308, see Fig. 1), transferred to *P. aeruginosa*, and their promoter activities were measured by C230 assay. pHA307 contains the *denA* promoter region, in addition to the *denA* coding region, 50-bp non-coding region, and about half of the *denB* coding region, whereas pHA308 lacks the *denA* promoter region. As shown in Table I, the cells harbouring pHA308 did not express C230 activity even under anaerobic conditions and in the presence of nitrate, indicating that the promoter for *denB* is not located in pHA308. On the other hand, the cells carrying pHA307 expressed C230 activity when grown anaerobically in the presence of nitrate. The growth of the latter cells was, however, very poor under anaerobic conditions, probably because of a large amount of nitrite reductase expressed from the multicopy plasmid. The C230 activity of the cells harbouring pHA307 grown anaerobically was also considerably lower than that expressed in the cells carrying pHA304. This low expression was probably due to the poor growth. In any case, it was concluded that *denB* is transcribed from the *denA* promoter; in other words, *denA* and *denB* are transcribed as an operon.

3.3. *Nucleotide sequence of the promoter (regulator) region of* denAB

We determined the nucleotide sequence of the *denA* promoter region (*Apa*I-*Eco*RI fragment). As shown in Fig. 2, this region is highly AT-rich as compared to the average GC content of *P. aeruginosa* DNA (67–68%) [13]. This high AT content may be necessary for the binding of regulatory elements. In this region there is a sequence that is closely similar to the consensus FNR binding site (A-A-TTGAT--A-ATCAAT---) of *E. coli* [14]. FNR (regulatory protein for fumarate and nitrate reduction) is a regulator for anaerobiosis of *E. coli* and activates the expression of proteins such as nitrate reductase, which are required for anaerobic respiration [15]. Azurin, a blue copper protein, is able to act as an alternative electron donor for nitrite reductase [6], and a putative FNR binding site is also located in the upstream region of the azurin gene of *P. aeruginosa*

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ApaI
GGGCCCTTGA GCAATACCGG CAGGCCGTGC CGCCAGGCGC GCTCGAAGAC TTCGATCTCA     60
TGGCCGGTGG CCTCGTAGAA GGGTGTCGCG TCCCGCATGT CCTACTCCTG CGCTAGGGAT    120
                                    ORF1      rbs
TAGGACCGCA CGCTATTCAC AGTTGGAAGG TGCCACAAGC GCAAAGCAAC GCAATCTTGA    180
                                                        EcoRI
TTCCGGTCAA GCAAGGGTAA AGACCCTGCT TCTATGATC CTTTCGCGCC ATGAATTC       238
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Fig. 2. Nucleotide sequence of the promoter (regulatory) region of *denAB* (*Apa*I-*Eco*RI fragment). The sequence resembles to the consensus FNR binding site of *E. coli* is boxed. The arrow underline an open reading frame extending to the opposite direction of *denA* (ORF1). The putative ribosome binding site for ORF1 is underlined.

[16]. The occurrence of an FNR-like element in this organism has been suggested by Lodge et al. [17]. It seems that the regulatory mechanism for anaerobiosis in *P. aeruginosa* resembles that in *E. coli*. We also found that upstream region of *denA* has an anaerobically inducible promoter activity in the opposite direction (data not shown). This region further contains an open reading frame (ORF1 in Fig. 2), shortly after which a putative ribosome binding site is located. It is likely that ORF1 encodes an activator for the transcription of the *denAB* operon, as in the case of *trpI-trpBA* in *P. aeruginosa* [18].

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