

Cloning of the Na⁺-translocating NADH-quinone reductase gene from the marine bacterium *Vibrio alginolyticus* and the expression of the β -subunit in *Escherichia coli*

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Abstract The Na⁺-translocating NADH-quinone reductase purified from the marine bacterium *Vibrio alginolyticus* is composed of three subunits, α , β and γ . From the N-terminal amino acid sequences of each subunit and its polypeptide fragment obtained by partial digestion with V8 protease, oligonucleotides corresponding to forward and reverse primers for each gene (*NQR A*, *B* and *C*) encoding the α , β and γ subunit, respectively, were synthesized. Using these primers, a part of each gene was amplified from the chromosomal DNA of *V. alginolyticus* by a PCR method, and the PCR products were used for the cloning of the *NQR* gene in λ phage. Among the subclones selected by probe C, the expression of the β -subunit as a gene product was detected in *Escherichia coli* membranes by activity staining and Western blotting.

Key words: Na⁺ pump; NADH-quinone reductase; Cloning of *NQR* gene; Expression of β -subunit; *Vibrio alginolyticus*

1. Introduction

Gram-negative marine and moderately halophilic bacteria possess a unique Na⁺-translocating NADH-quinone reductase complex as a first segment of the respiratory chain [1–5]. The enzyme complex purified from the marine bacterium *Vibrio alginolyticus* is composed of three subunits, α , β and γ [6]. The β -subunit, containing FAD as a cofactor, reacts with NADH and reduces ubiquinone-1 (Q-1) to ubisemiquinone, by a one-electron transfer pathway [1]. In the presence of the γ -subunit, the α -subunit, containing FMN as a cofactor, further reduces ubisemiquinone to ubiquinol. The latter reaction is strictly dependent on Na⁺ and the coupling site of Na⁺ translocation. To further investigate the reaction mechanism and the mode of energy coupling in the enzyme complex, attempts have been made to clone a gene encoding the Na⁺-translocating NADH-quinone reductase complex (*NQR*) from *V. alginolyticus*. This paper reports the cloning of the *NQR* gene and the expression of the β -subunit in *Escherichia coli*.

2. Materials and methods

2.1. Materials

The λ DASHII/*Bam*HI vector kit and Gigapack II Gold (Stratagene) were used for the cloning of *NQR* gene. The TA cloning kit (Invitrogen) was used for the amplification of PCR products. The DIG DNA labeling kit (Boehringer-Mannheim Biochemica) was used for DNA labeling with digoxigenin-dUTP, and the detection of hybrids was performed with a colorimetric method.

2.2. Analytical methods

N-Terminal amino acid sequences were determined with a protein sequencer 477A (Applied Biosystems, Inc.). Oligonucleotides were synthesized with a Cyclone Plus DNA Synthesizer (MilliGen Biosearch Co.). For the detection of NADH dehydrogenase activity, membrane fractions were solubilized with 1.0% Liponox DCH [1] and the extracts were subjected to PAGE (7.5% gel) in the presence of 0.1% Liponox DCH. The gels were incubated at room temperature in the reaction mixture containing 0.2 mM NADH, 0.1 mM menadione, 0.5 mM 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

and 0.1 M Tris-HCl (pH 7.5). Detection of the β -subunit was also performed by Western blot analysis using the antibody for the β -subunit, and alkaline phosphatase-conjugated anti-rabbit goat IgG (Tropix) as the second antibody. The antibody for the β -subunit was purified from the antiserum for the *NQR* complex by the method described in [7].

2.3. Bacterial growth and preparation of membrane fractions

E. coli TG1 cells bearing a plasmid were grown in LB medium containing 100 μ g/ml ampicillin and 0.3 mM isopropyl β -D-thiogalactopyranoside (IPTG). Cells suspended in a medium containing 25 mM Tris-HCl (pH 7.0), 5 mM MgSO₄, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride were broken by a French press (Aminco) at 14,000 psi, and then membranes were separated by ultracentrifugation.

2.4. Cloning of the *NQR* gene from *V. alginolyticus*

Cloning, subcloning and restriction mapping were performed as described in [8]. Genomic DNA from *V. alginolyticus* was partially digested with *Sau*3AI and size-fractionated by sucrose-density gradient centrifugation. The fragments from 10 to 20 kbp were ligated into λ DASHII arms previously digested with *Bam*HI, and then packaged. λ clones containing the *NQR* gene were selected by plaque hybridization using digoxigenin-labeled A, B, and C probes. The selected λ clones were digested with restriction enzymes and the fragments were ligated to pUC118 and 119.

3. Results and discussion

3.1. Preparation of probes for the *NQR* gene

The *NQR* complex purified from *V. alginolyticus* is composed of three subunits, α , β , and γ . Each subunit was separated by SDS-PAGE, and its N-terminal amino acid sequence was analyzed. Each subunit was also digested with V8 protease, and the N-terminal amino acid sequences of the separated polypeptide fragments were determined. Then, oligonucleotides corresponding to forward and reverse primers for each gene (*NQR A*, *B*, and *C*) encoding the α , β , and γ -subunits, respectively, were synthesized (Table 1). Using these primers, a part of each gene was amplified from the chromosomal DNA of *V. alginolyticus* by a PCR method. The PCR products obtained from the combination of each forward and reverse primers described in Table 1 corresponded to 307 (A), 611 (B), and 521

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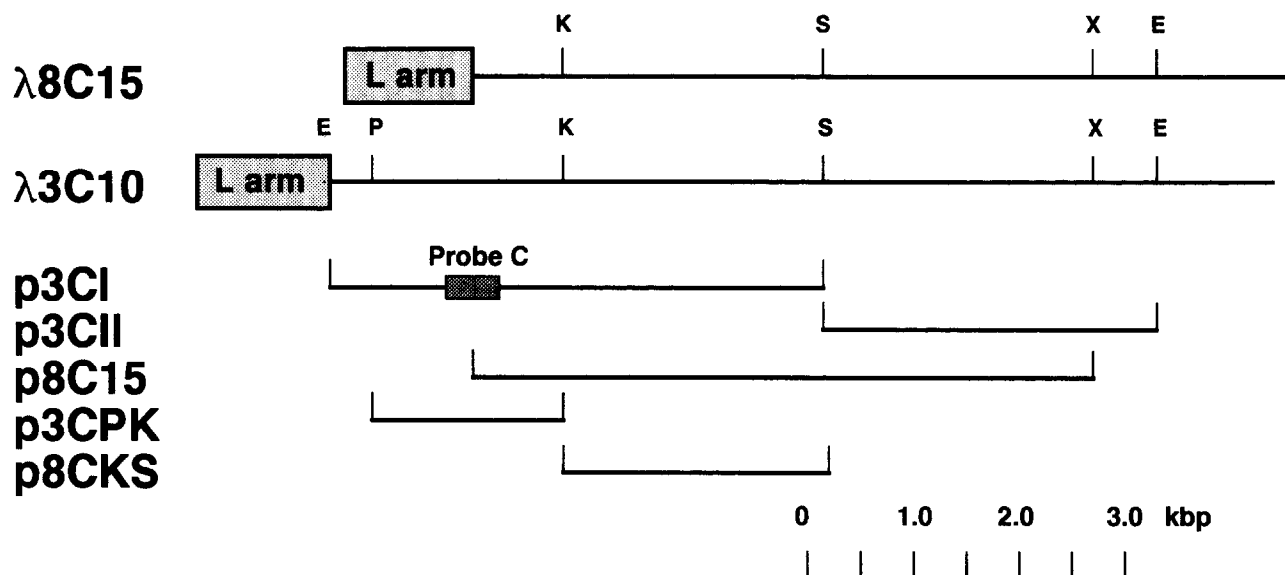


Fig. 1. Restriction maps and subcloning of the *NQR* gene selected by probe C. L arm, the left arm of λ phage; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xho*I.

(C) bp, which were presumed to be a part of the *NQR A*, *B*, and *C* gene, respectively. These PCR products were used for the cloning of the *NQR* gene as described in section 2.4.

3.2. Cloning of the *NQR* gene and expression of the β -subunit

When each subunit is separated from the complex, the β -subunit has a unique NADH dehydrogenase activity by itself [1], and thus its activity is easily detected by a staining method on PAGE. The α - and γ -subunits, however, manifest no apparent enzyme activities by themselves and reconstitution to the complex is required for the detection of their activities [6]. Therefore, we first attempted to clone the *NQR B* gene. Using probe B labeled with digoxigenin, we could not find out any gene fragment that expressed a unique NADH dehydrogenase activity specific for the β -subunit. Thus, the probe B prepared in this experiment was inappropriate for the cloning of the *NQR B* gene for unknown reasons. However, among the clones selected by probe C we could detect the activity of the β -subunit in the gene products.

Fig. 1 shows the restriction maps of the gene fragments selected by probe C. λ 8C15 and λ 3C10 contained about 13 and 18 kbp, respectively, of genomic DNA from *V. alginolyticus*, and the cloning sites overlapped, as judged from the restriction map. These λ clones were digested with restriction enzymes and the fragments were ligated to pUC118 and 119 (Fig. 1). Each plasmid was transformed into *E. coli* TG1 and then the expression of the β -subunit was analyzed by activity staining and Western blotting. As shown in Fig. 2A, NADH dehydrogenase activity corresponding to the β -subunit (arrow a) was detected with p3CI (lane 3), p8C15 (lane 2) and p8CKS (lane 6), but not with p3CII (lane 4) and p3CPK (lane 5). Since the activity bands pointed out by arrow b were not detected in membranes from *V. alginolyticus* (lane 1), they were apparently derived from *E. coli* membranes. Indeed, activity band a, but not b, specifically reacted with the antibody for the β -subunit (Fig.

2B). These results clearly indicate that the *NQR B* gene encoding the β -subunit exists downstream of the *NQR C* gene.

Although *E. coli* was grown in the presence of IPTG in Fig. 2, IPTG showed no stimulating effect for the production of the β -subunit. Thus, the *lac* promoter is not functioning in the expression of the β -subunit. At present, the sequencing of the whole *NQR* gene is underway to identify the promoter region and each structural gene.

Table 1

N-Terminal amino acid sequences and synthesis of oligonucleotides

α -subunit (<i>NQR A</i>)	
Met Ile Thr Ile Lys Lys Gly Leu Asp ---	
5'- ATG ATI ACI ATI AAA AAA GGI TTI GA -3'	
(G) (G) (C)	
Leu Phe Gly Leu Ala Met Pro ---	
3'- AI AAG CCI GAI CGI TAC GG -5'	
(A) (A)	
β -subunit (<i>NQR B</i>)	
Val Asn Phe Pro Gly Tyr Met ---	
5'- GTI AAC TTC CCI GGI TAC ATG -3'	
(T) (T) (T)	
Met Gly Leu Asn Tyr Ala Ile Leu Asn Glu Thr ---	
3'- TTG ATG CGI TAI GAI TTG CTT TG -5'	
(A) (A) (A) (A) (C)	
γ -subunit (<i>NQR C</i>)	
Ala Ser Asn Asn Asp Ser Ile Lys Lys Thr Leu ---	
5'- AAT AAT GAT TCI ATI AAA AAA AC -3'	
(C) (C) (C) (G) (G) (G)	
Glu Thr Pro Gly Leu Gly Gly ---	
3'- TT TGI GGI CCI GAI CCI CC -5'	
(C) (A)	

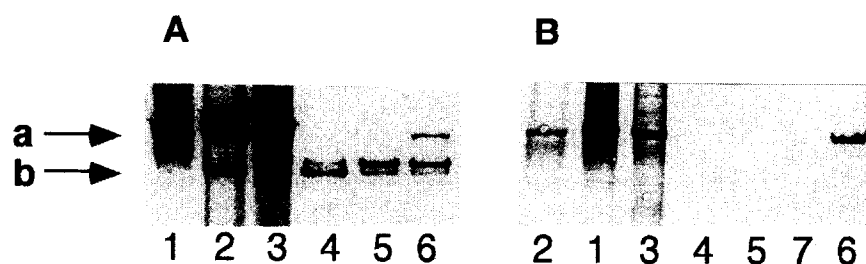


Fig. 2. Detection of the β -subunit in membrane fractions from *E. coli* containing subcloned plasmids. Detection was performed as described in section 2.2 after native PAGE. (A) Activity staining; (B) Western blotting. Lane 1, soluble extracts of membranes from *V. alginolyticus*. Other lanes are soluble extracts of membranes from *E. coli* containing p8C15 (lane 2), p3CI (lane 3), p3CII (lane 4), p3CPK (lane 5), p8CKS (lane 6), or pUC119 (lane 7).

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References

- [1] Hayashi, M. and Unemoto, T. (1984) *Biochim. Biophys. Acta* 767, 470–478.
- [2] Unemoto, T. and Hayashi, M. (1989) *J. Bioenerg. Biomembr.* 21, 649–662.
- [3] Unemoto, T. and Hayashi, M. (1993) *J. Bioenerg. Biomembr.* 25, 385–391.
- [4] Unemoto, T., Tokuda, H. and Hayashi, M. (1990) in: *The Bacteria*, Vol. XII, *Bacterial Energetics* (Kruschwitz, T.A., Ed.) pp. 33–54, Academic Press, New York.
- [5] Unemoto, T., Akagawa, A. and Hayashi, M. (1993) *J. Gen. Microbiol.* 139, 2779–2782.
- [6] Hayashi, M. and Unemoto, T. (1987) *Biochim. Biophys. Acta* 890, 47–54.
- [7] Bisson, R. and Schiavo, G. (1986) *J. Biol. Chem.* 261, 4373–4376.
- [8] Sambrook, J., Fritsch, F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.