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Conjugation-dependent recovery of the Na^+ pump in a mutant of *Vibrio alginolyticus* lacking three subunits of the Na^+ pump

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The Na^+ pump-deficient mutant, Nap1, of *Vibrio alginolyticus* was found to lack three subunits of Na^+-dependent NADH:quinone oxidoreductase complex. Although a spontaneous Na^+ pump positive revertant did not appear from Nap1, transconjugants that recovered both the Na^+ pump activity and the subunits were isolated from Nap1 conjugated with the wild type. Moreover, the wild type was found to contain two different sizes of plasmids. These results suggest the possibility that the Na^+ pump is encoded by a plasmid.

Na^+ pump; NADH oxidase; Conjugation; Plasmid; (Marine bacterium)

1. INTRODUCTION

The respiratory Na^+ pump of *Vibrio alginolyticus* generates an electrochemical potential of Na^+ in the presence of a proton conductor, CCCP [1,2], and thus enables the cells to grow on CCCP-containing media [3]. Strains Nap1 and Nap2 were isolated as CCCP-sensitive mutants and found to lack the Na^+ pump activity due to a defect in the NADH:quinone oxidoreductase segment of NADH oxidase [4,5]. The Na^+ pump activity of the NADH oxidase was confirmed in isolated membrane vesicles [6] and reconstituted proteoliposomes [7]. Examinations of respiratory activity further revealed that NADH-dependent reduction of ubiquinone to ubiquinol (NQR activity) in the wild type occurs via two pathways, NQR-1 and NQR-2. Both mutants were found to retain NQR-2 activity but lack the activity of NQR-1, which requires Na^+ and is sensitive to HQNO [5,8]. NQR-1 was recently purified to near homogeneity and shown to contain three subunits, α, β and γ [9]. Although Nap2 has a mutation in the β-subunit conferring Ag^+-sensitive NADH dehydrogenase [8], Nap1 seems to lack the activities of not only β but also other subunits. This paper shows that Nap1 lacks three subunits of NQR-1 but recovers all of them by conjugation with the wild type.

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; octylthioglucoside, *n*-octyl-β-D-thioglucopyranoside; Nal, nalidixic acid; Rm, rifamycin

2. EXPERIMENTAL

2.1. *Bacteria*

The following strains of *V. alginolyticus* were used: 138-2, wild type; Nap1 and Nap2 [4], Na^+ pump-defective mutants; Nap2R [5], a spontaneous revertant of Nap2; 138-2Nal^r and Nap1Rm^r, antibiotic-resistant derivatives of respective strain. All strains were grown on a complex medium [3].

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2.2. *Conjugation*

Cultures of donor strain 138-2Nal^r and recipient strain Nap1Rm^r were mixed and filtered through a Millipore HA filter. The filter was incubated at 37°C for 3–5 h on the complex agar medium to allow conjugation. The cells were resuspended and plated onto the complex agar medium containing 5 μM CCCP and 320 μg Rm/ml at pH 8.5. CCCP^r Rm^r transconjugants showing Nal^s phenotype on a plate containing 8 μg Nal/ml were selected and purified.

2.3. *Selective solubilization of NQR-1*

Membranes prepared as in [10] were resuspended at 5 mg protein/ml in 20 mM Tris-HCl, pH 7.5, containing 3% sodium cholate. After 10 min incubation on ice, the membranes were recovered by centrifugation at 30000 × g for 30 min and treated for 10 min on ice with 20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1% Liponox DCH, a non-ionic detergent [11]. The Liponox-washed membranes were collected and solubilized with 20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 25 mM NaCl and 0.5% octylthioglucoside (Wako, Japan) for 10 min on ice. After centrifugation, the supernatant was saved as the NQR-1 fraction.

2.4. *Respiratory activities*

Reduction of ubiquinone-1 to ubiquinol-1 by NADH:quinone oxidoreductase was determined as in [12]. NADH dehydrogenase was assayed in the presence of 0.1 mM menadione as an electron acceptor as in [8]. In order to inactivate NQR-2 [5,8], 0.2% Liponox was included in assay mixtures.

2.5. *Other methods*

Membrane proteins were analysed by SDS-polyacrylamide gel electrophoresis as in [13]. NQR-1 was partially purified from the wild type as in [9]. Plasmids were isolated by the method in [14], and analysed by 0.7% agarose gel electrophoresis as described in [15].

3. RESULTS AND DISCUSSION

3.1. *Subunit compositions of Na^+-motive NQR-1*

It was reported that cholate dissolves about 20% of membrane proteins without dissolving NQR-1 [16]. Moreover, we found that Liponox in the presence of 10 mM Mg^{2+} was also effective to remove impurities. When membranes treated with cholate and then Liponox plus Mg^{2+} were dissolved with 0.5% octylthioglucoside, about 80% of NQR-1 activity was solubilized with only about 15% of membrane proteins. The octylthioglucoside extracts of wild type, Nap1, Nap2 and Nap2R were analysed by SDS-gel electrophoresis with a partially purified NQR-1 as a control (fig.1). Although identification of NQR-1 subunits on the gel of total membrane proteins was difficult (lane a), these subunits were significantly concentrated in the octylthioglucoside extract and easily identified (lanes b, d and e). All the strains except Nap1 contained three subunits of NQR-1. It was shown that incubation of Nap2 membrane proteins with the wild type β-subunit led to the reconstitution of

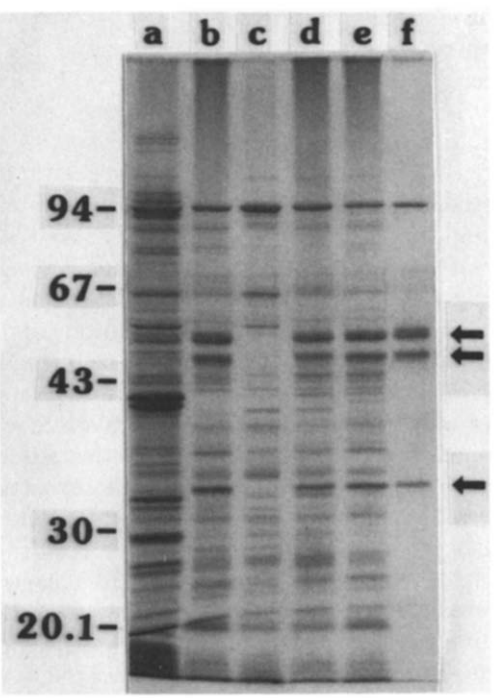

Fig.1. Selective solubilization and identification of NQR-1 subunits. Octylthioglucoside extracts prepared from 138-2 (lane b), Nap1 (c), Nap2 (d) and Nap2R (e) were analysed by 0.1% SDS-10% polyacrylamide gel electrophoresis. Partially purified NQR-1 (f) and total membrane proteins (a) of 138-2 were also examined for comparison. Arrows indicate from top to bottom α-, β- and γ-subunits of NQR-1. Positions of molecular mass markers are shown on the left in kDa.

NQR-1 activity [8]. Moreover, Nap2R was spontaneously obtained from Nap2 at a frequency of about 10^{-7} [5]. These observations and the result shown in fig.1 indicate that Nap2 has a point mutation and synthesizes inactive β-subunit. In marked contrast, Nap1 was found to lack all the subunits of NQR-1. Such a finding immediately explains why NQR-1 activity was not reconstituted from the wild type β-subunit and Nap1 membrane proteins [8].

3.2. *Isolation of Na^+ pump positive transconjugants*

When Nap1 was inoculated on a plate containing CCCP at pH 8.5, tiny colonies appeared at a frequency of about 10^{-7} as reported [5]. However, it was later found that, unlike Nap2R, these colonies were not true Na^+ pump positive revertants and lacked NQR-1 activity. On the other hand, when Nap1Rm^r was mated with 138-2Nal^r, CCCP^r transconjugants (Rm^r, Nal^s) appeared at a frequency of 10^{-4} donor cells. Growth of these transconjugants on the CCCP-containing plate was distinguishably better than that of apparent CCCP^r colonies, which appeared when Nap1 alone was plated. Moreover, the transconjugants were found to recover the Na^+ pump activity (not shown). Respiratory activities of membranes isolated from the donor, recipient and transconjugants were examined in the presence of Liponox. Since Liponox inactivates NQR-2 and its NADH dehydrogenase but not NQR-1 and its NADH dehydrogenase [5,8], Nap1 membranes showed little activity whereas 138-2 membranes retained the activities of Ag^+-sensitive NADH dehydrogenase and Na^+-dependent, HQNO-sensitive NQR-1 (table 1). These properties in all the transconjugants tested were essentially the same as those of the wild type. A typical result obtained with one of the transconjugants (TCR-3) is shown in table 1. Furthermore, SDS-gel electrophoresis of the octylthioglucoside extract revealed that TCR-3 recovered all the subunits of NQR-1 (not shown).

These results establish that NQR-1 is composed of three subunits and essential for the Na^+ pump activity.

3.3. *Examination of plasmids in* V. alginolyticus

The results described above indicate that a genetic determinant for NQR-1 (Na^+ pump) is transmitted from the wild type to Nap1 by conjugation. It seemed therefore likely that a plasmid exists in *V. alginolyticus* and is involved in conjugal gene transfer. Lysates of the wild type were analysed by agarose gel electrophoresis to examine the presence of plasmid. As shown in fig.2, two different sizes of plasmid were detected. From *Eco*RI restriction fragments (not shown), the sizes of plasmids were calculated to be about 100 and 6.5 kb. Since the plasmid of about 6.5 kb is too small as a self-transmissible plasmid, conjugation seems to be mediated by the larger plasmid. The simplest explanation for the mutation of Nap1 is that this strain lacks a plasmid encoding the Na^+ pump. However, our preliminary experiments showed that both plasmids are present in Nap1. Therefore, it seems likely that the larger plasmid in Nap1 is mutated, which results in the deficiency of

Table 1

NADH dehydrogenase and NADH-dependent ubiquinol formation by membranes prepared from 138-2Nal^r, Nap1Rm^r and Na^+ pump positive transconjugant TCR-3

Assay	Salt (0.2 M)	Inhibitor	Activity (units/mg protein)		
			138-2Nal^r	Nap1Rm^r	TCR-3
NADH dehydrogenase	NaCl	–	9.30	0.15	7.92
	NaCl	1 μM $AgNO_3$	1.53	0.15	1.31
Ubiquinol formation	KCl	–	0.06	0.02	0.14
	NaCl	–	1.09	0.01	1.03
	NaCl	5 μM HQNO	0.05	0.03	0.17

All the assays were performed in the presence of 0.2% Liponox as described in the text

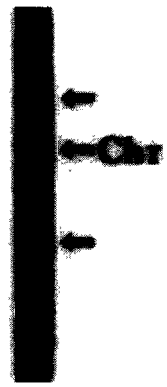

Fig.2. Agarose gel electrophoresis of DNA from *V. alginolyticus* 138-2Nal^r. Positions of two plasmids and chromosomal DNA (Chr) are indicated by arrows. The gel was run from top to bottom.

Na^+ pump expression. At present, however, it is not completely excluded that the genetic determinant for the Na^+ pump is on a chromosome or smaller plasmid and transmitted from the wild type to Napl by mobilization of the larger plasmid. Further genetic examination is necessary to clarify these points.

It was recently found that not only *Vibrio* species [17] but also other marine bacteria retain analogous Na^+-motive NADH:quinone oxidoreductases (in preparation). The plasmid found in *V. alginolyticus* may be a genetic reason for the wide distribution of Na^+ pumps in marine bacteria.

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REFERENCES

[1] Tokuda, H. and Unemoto, T. (1981) Biochem. Biophys. Res. Commun. 102, 265–271.
[2] Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014.
[3] Tokuda, H. and Unemoto, T. (1983) J. Bacteriol. 156, 636–643.
[4] Tokuda, H. (1983) Biochem. Biophys. Res. Commun. 114, 113–118.
[5] Tokuda, H. and Unemoto, T. (1984) J. Biol. Chem. 259, 7785–7790.
[6] Tokuda, H., Udagawa, T. and Unemoto, T. (1985) FEBS Lett. 183, 95–98.
[7] Tokuda, H. (1984) FEBS Lett. 176, 125–128.
[8] Asano, M., Hayashi, M., Unemoto, T. and Tokuda, H. (1985) Agric. Biol. Chem. 49, 2813–2817.
[9] Hayashi, M. and Unemoto, T. (1986) FEBS Lett. 202, 327–330.
[10] Tokuda, H. (1986) Methods Enzymol. 125, 520–530.
[11] Unemoto, T., Hayashi, M. and Hayashi, M. (1981) J. Biochem. 90, 619–628.
[12] Unemoto, T. and Hayashi, M. (1979) J. Biochem. 85, 1461–1467.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Kieser, T. (1984) Plasmid 12, 19–36.
[15] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning, pp.149–172, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[16] Hayashi, M. and Unemoto, T. (1984) Biochim. Biophys. Acta 767, 470–478.
[17] Udagawa, T., Unemoto, T. and Tokuda, H. (1986) J. Biol. Chem. 261, 2616–2622.