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Pyrroloquinoline Quinone-Dependent Cytochrome Reduction in Polyvinyl Alcohol-Degrading *Pseudomonas* sp. Strain VM15C

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A polyvinyl alcohol (PVA) oxidase-deficient mutant of *Pseudomonas* sp. strain VM15C, strain ND1, was shown to possess PVA dehydrogenase, in which pyrroloquinoline quinone (PQQ) functions as a coenzyme. The mutant grew on PVA and required PQQ for utilization of PVA as an essential growth factor. Incubation of the membrane fraction of the mutant with PVA caused cytochrome reduction of the fraction. Furthermore, it was found that in spite of the presence of PVA oxidase, the membrane fraction of strain VM15C grown on glucose without PQQ required PQQ for cytochrome reduction during incubation with PVA. The results provide evidence that PVA dehydrogenase couples with the electron transport chain of PVA-degrading bacteria but that PVA oxidase does not.

Pseudomonas sp. strain VM15C, a polyvinyl alcohol (PVA)-degrading bacterium, which is a component strain of a symbiotic mixed culture (6, 11), requires pyrroloquinoline quinone (PQQ) as an essential growth factor for PVA utilization (7, 8, 14). PQQ acts as a vitamin for the strain and as a coenzyme for a novel PVA-oxidizing enzyme, PVA dehydrogenase (9), which is also the first example of a secondary alcohol dehydrogenase among quinoprotein enzymes (2). However, the involvement of PQQ in PVA oxidation does not conclusively demonstrate that PQQ is an essential growth factor for PVA utilization, because the strain has another type of PVA-oxidizing enzyme, PVA oxidase (10, 12, 13), as do other PVA-degrading bacteria (5, 15). PVA oxidase does not utilize PQQ as a coenzyme, but the two enzymes have similar substrate specificities (9). PVA dehydrogenase utilizes artificial electron acceptors such as phenazine ethosulfate (PES) but not O_2 (9). PVA oxidase also utilizes artificial electron acceptors, besides O_2, and shows PVA dehydrogenase activity (9). PVA dehydrogenase is a membrane-bound enzyme, and PVA oxidase is also partly present in the membrane of this bacterium (9, 10, 13).

However, the requirement for PQQ as an essential growth factor by the strain indicates that PVA dehydrogenase may play a significant role which PVA oxidase does not play.

In the previous study (9), we found that PVA oxidation by the membrane fraction of the strain led to cytochrome reduction of the fraction. This phenomenon could not be assumed to result from PVA dehydrogenation by PVA dehydrogenase because of the presence of PVA oxidase and its PVA-dehydrogenating activity in the membrane. Determination of whether one or both of the enzymes couple with the electron transport chain may allow a better understanding of the role of PQQ as an essential growth factor. In this report, we show that only PVA dehydrogenation by PVA dehydrogenase causes cytochrome reduction in the membrane fraction of this PVA-degrading bacterium. A number of quinoprotein enzymes have been reported since the discovery of PQQ in 1979 (2), and the physiological roles of PQQ and quinoprotein enzymes have become of much interest in recent years.

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MATERIALS AND METHODS

Microorganisms and growth conditions. *Pseudomonas* sp. strain VM15C and a mutant lacking PVA oxidase, *Pseudomonas* sp. strain ND1, which was obtained as described below, were maintained on nutrient agar slants and used throughout this work. The basal medium was the same as that described previously (6). PVA, glucose, and peptone (Difco Laboratories, Detroit, Mich.) were used as sole carbon sources at 5 g/liter. The medium containing PVA was supplemented with PQQ (Mitsubishi Gas Chemical, Tokyo, Japan) at 10 ng/ml. YP medium was composed of (per liter of deionized water) 2.5 g of peptone, 2.5 g of yeast extract (Difco), 1.25 g of NaCl, 1 ml of the vitamin mixture (6), and 15 g of agar (Nissui Seiyaku, Tokyo, Japan), at pH 7.0. Cultivation was carried out as described previously (6).

Mutagenesis and selection of a PVA oxidase-lacking mutant. Washed cells of strain VM15C grown in 200 ml of the peptone medium for 3 days were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml) in 50 mM potassium phosphate buffer (KPB, pH 7.5) at 30°C for 30 min. After being washed, the mutagenized cells were cultivated in 200 ml of the peptone medium for 3 days and then plated on YP medium. PVA oxidase-lacking mutants were searched for by the following procedure. After cultivation for 7 days, colonies that grew on a plate were replicated on a filter paper (diameter, 8 cm) by pressing the filter paper onto the surface of the plate culture. The filter paper was put into a petri dish, and then 0.75 ml of 50 mM phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.5) saturated with 4-heptanol was spread over the filter with a pipette. 4-Heptanol acts as a substrate for PVA oxidase (9) and induces the lysis of cells of strain VM15C (Shimao, unpublished data). After incubation of the filter paper at room temperature for 3 min in the closed petri dish, 0.75 ml of the PVA oxidase assay mixture, containing peroxidase (90 U/ml; type I, Sigma Chemical Co., St. Louis, Mo.), 4-aminoantipyrine (0.05 mg/ml), and phenol (0.3 mg/ml) in 50 mM phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.5), was spread over the filter paper, and then the filter paper was further incubated in the closed petri dish at 30°C for 30 to 60 min.

After this enzymatic staining, PVA oxidase-active colonies turned red due to H_2O_2 produced by oxidation of 4-heptanol by the enzyme. A PVA oxidase-lacking mutant, strain ND1, was isolated from a colony, a replicated colony

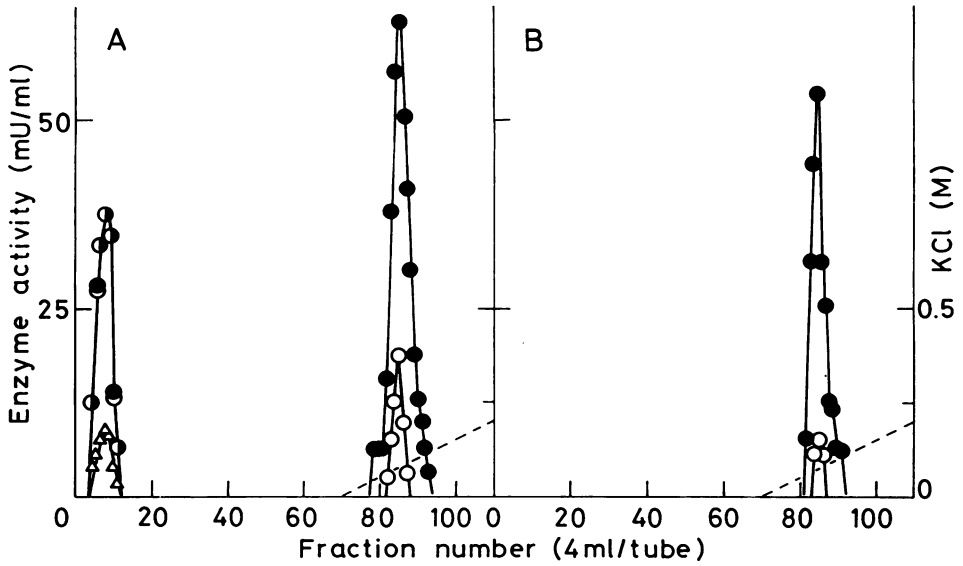

FIG. 1. DEAE-Sephacel chromatography of cell extracts of *Pseudomonas* sp. strain VM15C (A) and the mutant, ND1 (B). Chromatography was carried out as described in the text. PVA oxidase activity (△) was determined without preincubation. PVA dehydrogenase activity was assayed after preincubation with 0 (○) or 1 (●) μM PQQ for 30 min. Activities not detected are not plotted in this figure. The KCl concentration is shown (— — —).

which did not turn red. Strain ND1 was confirmed to be essentially the same as parent strain VM15C in various characteristics, such the kinds of carbon sources utilized for growth, pigment production, and the requirement for growth factors.

Preparation of cell extracts and membrane fractions. Cell extracts were prepared by ultrasonic disruption as described previously (9). Membrane fractions were obtained by centrifugation (100,000 × *g*, 60 min) from cell extracts prepared from fresh cells, washed once with 50 mM KPB (pH 7.5), and then immediately used for spectrophotometric difference analyses.

Chromatography of cell extracts. All operations were performed at 5°C. A cell extract was treated with 2% Triton X-100 in 50 mM KPB (pH 7.5) with gentle stirring for 15 h to solubilize membrane-bound protein in the cell extract and then dialyzed against 10 mM Tris hydrochloride (pH 8.5) containing 0.1 % Triton X-100. The dialysate, containing 50 mg of protein, was applied to a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column (1.6 by 10 cm) equilibrated with the same buffer. The column was washed with the equilibration buffer, followed by elution with a linear concentration gradient of KCl in the buffer.

Enzyme assays. PVA oxidase activity was determined from the rate of H_2O_2 formation after PVA oxidation by the enzyme, and PVA dehydrogenase activity was measured, with PES as the electron acceptor, as described previously (9). When PVA dehydrogenase activity was assayed, enzyme preparations were preincubated with or without additional PQQ (final concentration in the assay mixture, 1 μM) for 30 min as described previously (9).

Analyses. Difference spectra of membrane fractions were obtained with a Shimadzu model UV-210A spectrophotometer. Protein was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as the standard. Growth and PVA concentration were measured as described previously (6).

Materials. PVA (Wako Pure Chemical Industries, Osaka, Japan) was washed with methanol in a Soxhlet extractor. PVA with a degree of polymerization of 500 was used for cultivation, and PVA with a degree of polymerization of 1,500 was used for enzyme assays and difference spectrophotometry.

RESULTS

PVA dehydrogenase activity of the mutant. In cell extracts and culture supernatants of strain ND1 grown on PVA, glucose, and peptone, no PVA oxidase activity leading to H_2O_2 formation was detected. On the other hand, cell extracts showed the following PVA dehydrogenase activities (in milliunits per microgram of protein) with (+PQQ) or without (−PQQ) additional PQQ: cells grown on PVA, 5.5 (−PQQ) and 22.2 (+PQQ); cells grown on peptone, 1.7 (−PQQ) and 14.3 (+PQQ); and cells grown on glucose, 0 (−PQQ) and 7.5 (+PQQ). The PVA dehydrogenase activity of cell extracts was dependent on the presence of additional PQQ. In an assay without additional PQQ, the PVA dehydrogenase activity of a cell extract was low or not detectable.

However, PVA oxidase can utilize PES as an electron acceptor and shows PVA dehydrogenase activity. To confirm the actual absence of PVA oxidase in the mutant, cell extracts of strain VM15C and the mutant grown on PVA were subjected to DEAE-Sephacel chromatography, and the elution patterns were compared (Fig. 1). After chromatography of the cell extract of the parent strain, PVA oxidase was eluted in fractions 5 to 11 and PVA dehydrogenase was eluted in fractions 79 to 93. On the other hand, the cell extract of strain ND1 showed no PVA oxidase or dehydrogenase activity in fractions corresponding to those in which the PVA oxidase of strain VM15C was eluted. The PVA dehydrogenase activity of the mutant was found in fractions corresponding to those in which the PVA dehydrogenase of the parent strain was eluted. The PVA dehydrogenase activity in these fractions was also dependent on additional PQQ. These results showed that mutant ND1 lacks PVA oxidase but possesses PVA dehydrogenase.

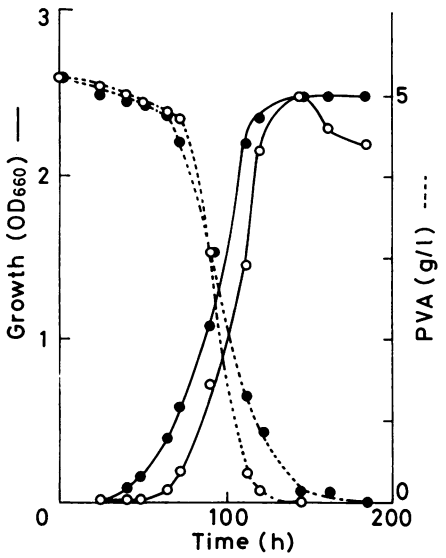

FIG. 2. PVA utilization by *Pseudomonas* sp. strain VM15C and the mutant, ND1. Strains VM15C (○) and ND1 (●) were cultivated in 200 ml of PVA medium supplemented with PQQ at 10 ng/ml, at 30°C, with reciprocal shaking.

From the facts mentioned above, the PVA dehydrogenase activity which cell extracts of the mutant showed in the absence of additional PQQ can be concluded to have been due to the holoenzyme of PVA dehydrogenase and not to PVA oxidase. The highest holo-PVA dehydrogenase activity was found in the cell extract of the mutant grown on PVA. This was due to addition of PQQ to the medium. On the other hand, in the cell extract of strain ND1 grown on glucose without PQQ, PVA dehydrogenase was present exclusively as the apoenzyme. The small amount of holo-PVA dehydrogenase activity detected in the cell extract of strain ND1 grown on peptone without PQQ is thought to have been due to a trace amount of PQQ contained in the carbon source (1).

Growth of the mutant. The fact that strain ND1 could grow on PVA in spite of the absence of PVA oxidase shows that PVA oxidase is not essential for the growth of the wild-type strain, VM15C, on PVA. PQQ was also required by the mutant as an essential growth factor for PVA utilization (data not shown). To evaluate the role of PVA oxidase, PVA utilization by the mutant was compared with that by strain VM15C (Fig. 2). The specific growth rate of strain ND1 (0.061/h) was somewhat lower than that of strain VM15C (0.085/h). The rate of PVA decrease in the ND1 culture was also somewhat lower than that in the VM15C culture. However, the maximum growth of strain ND1 (OD_{660}, 2.60) in the stationary phase was almost the same as that of strain VM15C (OD_{660}, 2.59). Furthermore, the relationship between growth and PVA degradation in Fig. 2 shows that the cell yield (growth per amount of PVA degraded) in the exponential phase of strain VM15C was much lower (about 50%) than that of the mutant. A part of the PVA oxidase of strain VM15C is excreted into the culture supernatant (10, 12). The lower cell yield of the strain in the exponential phase may be understood to result from transient accumulation of a degradation product (PVA oligomer) formed after extracellular degradation, in which PVA oxidase participates. Thus, it can be thought that PVA oxidase contributed to extracellular PVA degradation and stimulated PVA utilization to a small extent in the culture of strain VM15C.

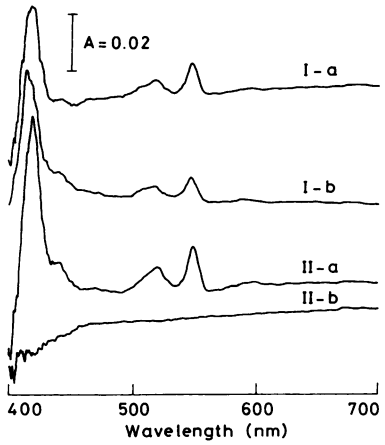

FIG. 3. Cytochrome reduction of the membrane fractions of *Pseudomonas* sp. strain VM15C and the mutant, ND1. (I) Membrane fraction prepared from ND1 cells grown on PVA with PQQ at 10 ng/ml for 4 days. No PVA oxidase activity was detected in the fraction. PVA dehydrogenase activity was 168 mU (without PQQ) and 393 mU (with PQQ) per mg of protein. (II) Membrane fraction prepared from VM15C cells grown on glucose without PQQ for 4 days. PVA oxidase activity in the membrane fraction was 25 mU/mg of protein. PVA dehydrogenase activity was 134 mU (without PQQ) and 843 mU (with PQQ) per mg of protein. These membrane fractions were preincubated with (a) or without (b) PQQ for 30 min and then further incubated with PVA for 5 min in 50 mM KPB (pH 7.5) at 30°C. The difference spectra were obtained with a Shimadzu model UV-210A. The membrane fractions not incubated with PVA were used as references. The final concentrations of protein, PVA, and PQQ were 1.9 mg/ml, 7.5 mg/ml, and 1 μM, respectively.

PQQ-dependent cytochrome reduction of the membrane fraction. The membrane fraction prepared from cells of strain ND1 grown on PVA medium showed cytochrome reduction during incubation with PVA (Fig. 3, I-a and I-b). The cytochrome reduction must result from PVA dehydrogenation by PVA dehydrogenase because of the absence of PVA oxidase in the mutant. The cytochrome reduction of the membrane occurred without additional PQQ for preincubation of the membrane (Fig. 3, I-b), but this was due to the presence of active holo-PVA dehydrogenase in the fraction, as described above. Coupling of PVA dehydrogenation by PVA dehydrogenase with cytochrome reduction was further confirmed with the membrane fraction of the mutant grown on glucose without PQQ, which required PQQ for cytochrome reduction of the membrane fraction during incubation with PVA (data not shown). This fact, together with the results for PVA dehydrogenase activity in cell extracts, also showed the absence of holo-PVA dehydrogenase in the membrane of cells grown on glucose without PQQ.

On the other hand, the membrane fraction of strain VM15C grown on glucose without PQQ also required PQQ for cytochrome reduction during incubation with PVA, in spite of the presence of PVA oxidase (Fig. 3, II-a and II-b). Although the membrane fraction showed PVA dehydrogenase activity in the absence of additional PQQ (see the legend to Fig. 3), this activity can be deduced to be due to PVA oxidase because, as in strain ND1 grown on glucose, the PVA dehydrogenase of the membrane fraction should be present essentially as the apoenzyme in the absence of additional PQQ. The PQQ-dependent cytochrome reduction of the membrane therefore showed that PVA oxidase does not couple with the electron transport chain in addition to

coupling of PVA dehydrogenase with the electron transport chain.

The peaks with absorption maxima at approximately 550 and 520 nm in the difference spectra (Fig. 3, I-a, I-b, and II-a) can be assigned as α and β absorption peaks characteristic of a reduced *c*-type cytochrome, respectively. A broad, slight peak was also observed at about 600 nm in the difference spectra (Fig. 3, I-a and II-a). This peak may be attributed to cytochrome *c* oxidase, although further study is needed for its assignment. Such a peak was not obvious in the case of the membrane fraction of the mutant grown without additional PQQ (Fig. 3, I-b); however, this may be due to insufficient reduction of the cytochrome system of the membrane fraction, as indicated by the fact that the difference spectrum showed a γ absorption peak at a shorter wavelength (414 nm) than those of the others (418 nm).

DISCUSSION

This report presented evidence that PVA dehydrogenase couples with the electron transport chain in a PVA-degrading bacterium, *Pseudomonas* sp. strain VM15C, but that PVA oxidase does not. This evidence indicates a further possibility that ATP synthesis is coupled with PVA dehydrogenation by PVA dehydrogenase. Quinoprotein dehydrogenases have often been observed to be coupled with respiratory chains (2–4). On the basis of the above evidence and possibility, it can be proposed that PQQ functions to take up energy generated after PVA dehydrogenation by PVA dehydrogenase as a coenzyme, and through the bioenergetic mechanism it acts as an essential growth factor for PVA utilization. PVA oxidase was shown not to be essential for PVA utilization. The role of the latter enzyme is thought to be acceleration of PVA utilization through involvement in extracellular PVA degradation, as shown by the somewhat faster growth and PVA degradation rates of strain VM15C than of mutant ND1 (Fig. 2).

The difference spectra of the membrane fractions showed that reduction of cytochrome *c* occurs with PVA dehydrogenation. Whether electron transport from PVA dehydrogenase to the cytochrome *c* is direct or requires other electron carriers remains to be elucidated. PVA dehydrogenase did not utilize ubiquinones as electron acceptors (9). It may be possible, as in the case of a quinoprotein enzyme (3), that PVA dehydrogenase utilizes cytochrome *c* as the primary electron acceptor in vivo.

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