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Note

A rapid procedure for the *in situ* assay of periplasmic, PQQ-dependent methanol dehydrogenase in intact single bacterial colonies

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ABSTRACT

Mechanistic details of methanol oxidation catalyzed by the periplasmically-located pyrroloquinoline quinone-dependent methanol dehydrogenase of methylotrophs can be elucidated using site-directed mutants. Here, we present an *in situ* colony assay of methanol dehydrogenase, which allows robotic screening of large populations of intact small colonies, and regrowth of colonies for subsequent analysis.

The pyrroloquinoline quinone (PQQ)-dependent and periplasmically-located methanol dehydrogenase (MeOHdH) of aerobic Gram-negative methylotrophs, catalyzes the oxidation of methanol to formaldehyde, transferring the electrons *via* the PQQ cofactor to cytochrome c_L (see Anthony, 1986). The cytochrome c_L transfers electrons to a cytochrome c_H (O'Keefe and Anthony, 1980), which subsequently reduces the membrane-bound cytochrome *c* oxidase utilizing oxygen as a terminal electron acceptor (see Anthony and Williams, 2003). The MeOHdH from *Methylobacterium extorquens* AM1 (Anthony and Zatman, 1964) used here, is well characterized and several crystal structures are available (Xia et al., 1992; Xia et al., 1996; Williams et al., 2005). The mechanism of methanol oxidation *via* PQQ has been shown to be exceedingly complex (Anthony et al., 1994; see Anthony and Williams, 2003), and many details are unclear. Although further resolution of the mechanistic details will require appropriate mutants, only a single site-directed mutagenesis study of the MeOHdH has been reported so far (Afolabi et al., 2001), and to date, no screening assay for the robotic screening of mutant MeOHdH populations has been reported. In this study, we present an *in situ* assay of MeOHdH that can be performed on small single colonies, with retention of colony viability to allow re-growth of interesting mutants for subsequent analysis.

Our *in situ* colony assay of MeOHdH is based upon the known observation that the outer membrane (OM) of Gram-negative bacteria exhibits a different sensitivity to detergent solubilization than the inner membrane (Nikaido and Nakae, 1979), depending upon the chemical nature of the detergent. Thus, it should be possible to find detergent solubilization conditions which permeabilise the outermost OM layers of a single colony, while leaving the localisation of the MeOHdH (which though water-soluble, is often weakly bound to the inner membrane) largely intact. In a weak solubilization event, the inner region of the colony should be unaffected, thus allowing the assay-positive colonies to be picked and re-grown for further analysis (see Fig. 1(A,B)).

M. extorquens AM1 was grown aerobically (shaker: 150 rpm, 2.5 cm throw) at 30 °C in medium employed by the Vorholt group (Peyraud et al., 2008) with 0.5% methanol as a sole C-source, to the late exponential phase. For the initial screening of conditions necessary to lyse the colony surface, the following experiment was performed (the "artificial colony" experiment). 1 ml aliquots of cell culture were pipetted into 1.5 ml Eppendorf tubes, and then centrifuged for 2 min at 16,000 × *g* at 4 °C, and the supernatant was discarded. In preliminary experiments, we screened the effectiveness of various detergents in their ability to solubilize the outer membrane with the concomitant maintenance of MeOHdH activity. Since different detergents have differing degrees of solubilization efficiency (Nikaido and Nakae, 1979), we used typical concentrations which have been employed for OM solubilization in the literature for the partial dissolution of the *M. extorquens* OM: 0.5% (v/v) Triton-X100 (Kent and Wisnieski, 1983), 1% (v/v) n-octyl-pentaoxyethylene (OPOE) (Cowan et al., 1992), 0.5% (w/v) Na-dodecylsulphate (SDS) (Kaneko et al., 1984), and 1% Sarkosyl (Filip et al., 1973; Aggeler et al., 1987) as well as 1% (w/v) Na-cholate (employed here only). Here, 100 µl of detergent solution was added to the cell pellet and incubated for 5–30 min. at 30 °C. Subsequently, the

Abbreviations: PQQ, Pyrroloquinoline quinone; MeOHdH, Methanol dehydrogenase; DCPIP, Dichlorphenolindophenol; PES, Phenazine methosulphate; NBTZ, Nitroblue tetrazolium; OPOE, n-Octyl-pentaoxyethylene; OM, Outer membrane

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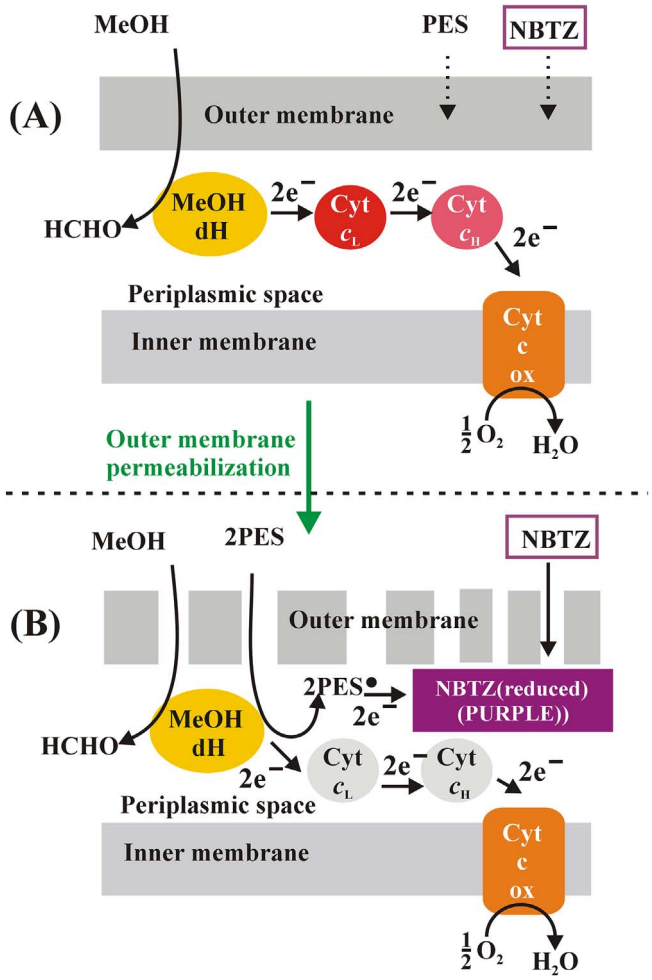

Fig. 1. The OM solubilization procedure used in this study. (A) Summary of the path of electron transfer from MeOH to the membrane-bound cytochrome *c* oxidase; (B) The intact OM is gently solubilized with detergent (OPOE - see text) while maintaining the integrity of the inner membrane. The solubilized OM is then accessible to the artificial electron acceptors PES and NBTZ, allowing a MeOHdH assay to be performed *in situ*, which is visualized by the accumulation of a purple precipitate of reduced NBTZ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

overlay solution was removed and then replaced with a MeOHdH assay solution (100 mM TrisHCl pH 9, 15 mM NH_4Cl, 1.1 mM phenazine ethosulphate (PES), 108 µM dichlorphenolindophenol (DCPIP) and 6.7 mM MeOH (modified after Anthony and Zatman, 1964) which allows reducing equivalents to be transferred from MeOH to the electron acceptor DCPIP *via* the membrane-impermeable electron mediator PES. Whereas DCPIP is intense blue in colour, $DCPIPH_2$ is colourless. Thus, after about 10 min further incubation at room temperature, the assay overlay solution immediately above the cell pellet becomes increasingly decolourized (see Fig. 2A), with the extent dependent upon the efficacy of detergent solubilization. In this initial screening (data not shown), the detergent OPOE proved to be highly effective and was then employed in subsequent experiments.

In the final optimization experiment within this series, 100 µl of 1.0% (v/v) OPOE was overlaid over the cell pellet in three of the Eppendorf tubes (prepared as above) without disturbing the pellet. The same volume of 2.0% (v/v) OPOE was added to a fourth Eppendorf tube. All of the overlaid pellets were incubated for 10 min. at room temperature and then the OPOE overlay was replaced by 100 µl of Na-phosphate buffer pH 7.1 for 1–2 min. Finally, the buffer was removed and 100 µl of NBTZ assay solution (100 mM TrisHCl pH 9.0, 15 mM NH_4Cl, 6.7 mM MeOH, 1.1 mM PES and 1 mM nitroblue tetrazolium

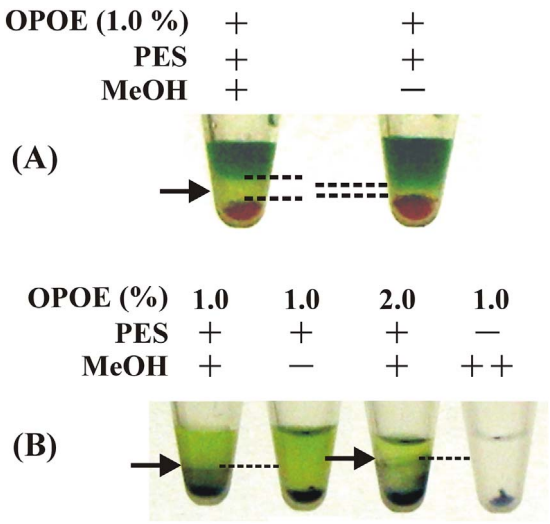

Fig. 2. (A) Solubilization of the cell pellet surface (cell pellet at the bottom of the tube) using OPOE and the DCPIP assay solution. The arrow indicates the region (dotted lines show limits) of DCPIP decolourization. The larger decolourization region in (A, left tube) is due to the MeOH dependence. (B) The NBTZ assay. The tubes 2 and 4 (reading from the left-hand side) confirm the MeOH and PES-dependence, respectively. The arrows indicate the extent of the positive purple region, corresponding to the amount of reduced NBTZ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(NBTZ)) added. In this second assay solution, NBTZ replaces DCPIP as the final electron acceptor. Whereas oxidized NBTZ is water-soluble and almost colourless, $NBTZH_2$ forms an intense purple precipitate in water, which in the assay tube appears as a coloured layer above the cell pellet. This coloured precipitate is completely stable (unlike the decoloured region of $DCPIPH_2$, which gradually diffuses away from the cell pellet over time), thus allowing a more sensitive assessment of solubilization activity (Fig. 2B).

The experiments shown in Fig. 2(A, B) confirm that the maximal reduction of the final electron acceptor (NBTZ or DCPIP) is both MeOH- and PES-dependent. 2% OPOE was optimal for rapid colour development. The “background” reduction (in the absence of MeOH) is due to electron transfer from other components of the electron transport chain (Fig. 1), which are still present and active in the partially solubilized colony. Control experiments which employed also KCN to inhibit the cytochrome oxidase did not eliminate the background reduction. Thus, for the assay of isolated cells, a control lacking MeOH should always be employed.

The NBTZ assay was adapted for single colonies of *M. extorquens*, growing on agar plates, as follows. Initially, (all steps were performed at room temperature) the plates were overlayed with 1 ml of 2.0% (v/v) OPOE and incubated for 5 min. The OPOE overlay solution was then decanted and replaced by 50 mM Na-phosphate buffer pH 7.1 and incubated for 1–2 min. Finally, the buffer was replaced with 2.0 ml of NBTZ assay solution and incubated for 5–20 min. Usually, all of the initially light pink colonies were observed to be purple (MeOHdH-positive) in colour within 5–8 min. (Fig. 3A).

We confirmed that the assay can distinguish colonies with differing degrees of NBTZ staining (which one expects in a site-directed mutagenesis experiment) by using colony populations obtained from fresh and aged cultures. Fig. 3A, and B (left panel) shows that every colony from a fresh culture shows approximately the same degree of staining. By comparison, populations obtained from aged cultures show differing degrees of NBTZ staining (Fig. 3B, right panel), even though the appearance of the unstained colonies appears to be identical (Fig. 3B, middle panel). The viability of both the assayed and control (no assay) colonies were tested by restreaking onto fresh plates followed by an incubation at 30 °C for 3–5 days. In all cases, growth was observed, although the assayed colonies were less viable than the