

Localization of Periplasmic Redox Proteins of *Alcaligenes faecalis* by a Modified General Method for Fractionating Gram-Negative Bacteria

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A lysozyme-osmotic shock method is described for fractionation of *Alcaligenes faecalis* which uses glucose to adjust osmotic strength and multiple osmotic shocks. During phenylethylamine-dependent growth, aromatic amine dehydrogenase, azurin, and a single cytochrome *c* were localized in the periplasm. Their induction patterns are different from those for the related quinoprotein methylamine dehydrogenase and its associated redox proteins.

Aromatic amine dehydrogenase (AADH) from *Alcaligenes faecalis* is induced during growth on phenylethylamine (19). It catalyzes the oxidative deamination of primary amines (12) and subsequent electron transfer to azurin, a type I blue copper protein (13). AADH is the second enzyme (7) known to contain the tryptophan tryptophylquinone (TTQ) cofactor (17). The other TTQ enzyme is a methylamine dehydrogenase (MADH), which has been isolated from various sources (8, 11, 15, 16, 20, 22). MADH is localized in the periplasmic space and donates electrons to periplasmic *c*-type cytochromes via another copper protein, amicyanin (9, 10). With a newly developed fractionation method, AADH, azurin, and a single *c*-type cytochrome were localized in the periplasm of *A. faecalis*. Their induction patterns were also characterized and compared with those of MADH and the analogous periplasmic redox proteins with which it interacts.

A. faecalis was cultured aerobically at 30°C with β -phenylethylamine as the sole carbon source (7). It was not previously possible to efficiently fractionate *A. faecalis* with conventional lysozyme-osmotic shock methods (18, 23) that generate spheroplasts, using sucrose to adjust osmotic strength. We hypothesized that the problem was that the exclusion size of the outer membrane porin of *A. faecalis* is too small to allow easy diffusion of sucrose (14). Use of glucose rather than sucrose allowed efficient fractionation of the cells. Glycine and NaCl were also tested as osmotic agents and found to be better than sucrose but not as good as glucose. Furthermore, whereas a single osmotic shock is typically used, with *A. faecalis* use of a second osmotic shock increased the release of the periplasm from about 15 to 75%. This allowed us to localize the redox proteins that are associated with phenylethylamine-dependent growth. A flowchart describing the new modified fractionation method is shown in Fig. 1. Purifications of AADH (7) and azurin (5) from the periplasmic extract were as described previously.

A large majority of the total AADH and azurin was found in the periplasmic fraction (Table 1). Malate dehydrogenase, a cytoplasmic marker, was essentially absent from the osmotic shock supernatant and found primarily in the soluble fraction

after disruption of spheroplasts. The single soluble cytochrome *c* that was identified (discussed later) was also localized in the periplasm.

Whereas cytoplasmic dehydrogenases typically transfer electrons to the respiratory chain via NADH, periplasmic dehydrogenases often transfer electrons to the respiratory chain via *c*-type cytochromes. Heme staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell fractions of phenylethylamine-grown *A. faecalis* revealed several heme-containing proteins (Fig. 2). Reducing agents were included in the sample buffer because they substantially diminish the peroxidase activity of noncovalent hemoproteins, making this strain reasonably specific for *c*-type cytochromes (6). One 13-kDa cytochrome is clearly localized in the periplasm. The other heme-staining species are primarily associated with the membrane. We previously identified two cytochromes in whole-cell sonicated extracts of *A. faecalis* that could accept electrons from AADH via azurin *in vitro* (5). These appear to correspond to the 13- and 25-kDa cytochromes. The latter was most likely released from the membrane into the soluble fraction in the earlier study by the prolonged sonication which was employed to completely disrupt cells.

Nearly 100% of the 13-kDa cytochrome appears in the periplasmic fraction, whereas only 74% of the AADH activity

TABLE 1. Localization of proteins in *A. faecalis*

Cell fraction ^a	% Total recovery (<i>n</i> = 4)			
	AADH ^b	Azurin ^c	Cytochrome ^d	Malate dehydrogenase ^e
Periplasm	73.9 ± 2.5	76.5 ± 3.1	100	7.5 ± 0.9
Cytoplasm	24.3 ± 2.1	24.5 ± 0.3	0	89.8 ± 3.1
Membrane	1.6 ± 0.2	ND ^f	0	2.6 ± 0.1

^a Cells were fractionated as described in Fig. 1.

^b The steady-state assay for AADH activity was performed as described previously (12).

^c The amount of purified azurin was quantitated using the extinction coefficient $\epsilon_{600} = 4,000 \text{ M}^{-1} \text{ cm}^{-1}$ (13).

^d Cytochrome *c* levels were quantitated by performing densitometry of the heme-stained proteins after SDS-PAGE.

^e The assay of malate dehydrogenase was performed as described previously (1).

^f ND, it was not possible to determine what azurin if any remains associated with the membrane fraction as it is a soluble protein that can be detected only spectrophotometrically.

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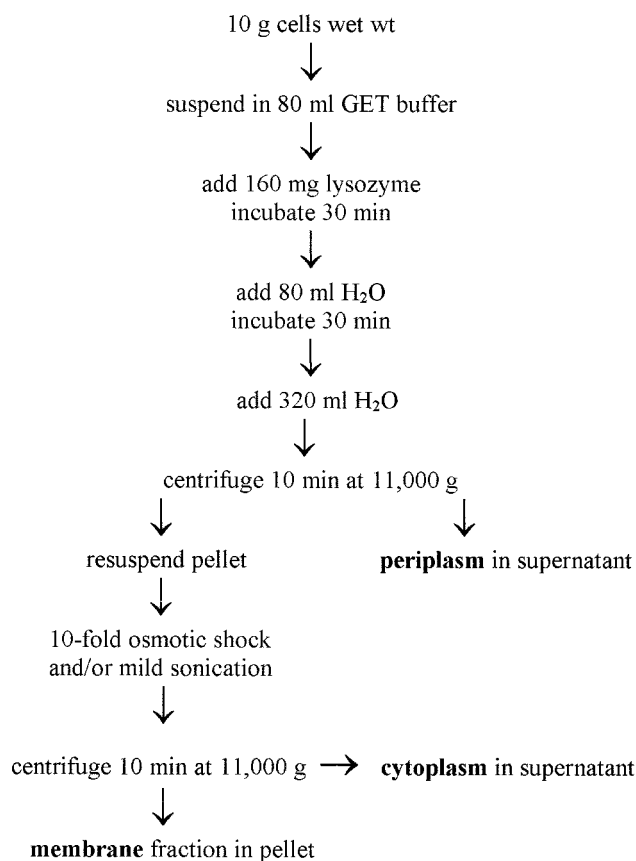


FIG. 1. Flowchart for optimal fractionation of *A. faecalis*. All procedures were performed at 30°C. Cells were resuspended in 0.5 M glucose plus 1 mM EDTA in 0.2 M Tris-HCl, pH 7.4 (GET buffer), to a concentration of 1 g (wet weight) of cells per 8 ml of buffer. Lysozyme, which was first suspended in water, was then added to a concentration 1 mg/ml. Phenylmethylsulfonyl fluoride (a protease inhibitor) was added to 100 µg/ml. Under conditions when some release of cytoplasmic components was suspected, 20 mM MgCl₂ plus 10 µg of DNase I per ml were added the supernatant to reduce viscosity due to release of DNA. Disruption of spheroplasts that were separated from the periplasm was accomplished by a 10-fold osmotic shock followed by mild ultrasonication (30 W of power for 10 min).

is present (Table 1, Fig. 2). This could be due to the relative size of the two proteins, 13 kDa for the cytochrome and 125 kDa for AADH. If only partial degradation of the cell wall occurs during lysozyme treatment, the smaller protein may be more readily released. Interestingly, only 77% of azurin (13.4 kDa) is released. This may mean that AADH and azurin are associated in the periplasm prior to release from the cell.

To identify which of these proteins are specifically induced by phenylethylamine, cells were also grown on Luria-Bertani (LB) medium and fractionated. No AADH activity was detected in these cells. Azurin was produced by these cells, but the level of azurin was only 28% of that produced by phenylethylamine-grown cells. Levels of the 13-kDa periplasmic cytochrome and the membrane-associated cytochromes were similar in cells grown on LB and on phenylethylamine (data not shown).

The induction pattern of periplasmic redox proteins in *A. faecalis* in the presence of phenylethylamine is different from the induction pattern of periplasmic redox proteins in *Paracoccus denitrificans* in the presence of methylamine. Like AADH, MADH is synthesized by *P. denitrificans* only when the substrate amine is present as the sole carbon source (11). The

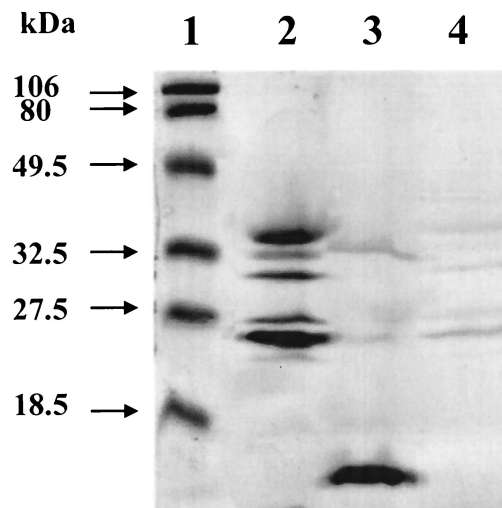


FIG. 2. Localization of *c*-type cytochromes of *A. faecalis*. Samples were subjected to SDS-PAGE on a 15% polyacrylamide gel. An approximately equivalent amount of each cell fraction on a per-cell-weight basis was loaded onto the gel. The gel was specifically stained for heme-containing proteins as previously described (6). Lane 1, Bio-Rad prestained molecular weight markers; lane 2, membrane fraction; lane 3, periplasmic fraction; lane 4, cytoplasmic fraction.

synthesis of amicyanin is also observed only with methylamine present as the sole carbon source (9). MADH and amicyanin genes are known to be under the control of the same promoter (3, 21). In contrast, while azurin levels are greater during growth on phenylethylamine, it is also synthesized in its absence. This suggests that in *A. faecalis* either the genes for AADH and azurin are not under control of the same promoter or multiple genes for azurin are present, one constitutive and one inducible. In *P. denitrificans*, three periplasmic *c*-type cytochromes are present during growth on methylamine, a constitutive cytochrome *c*-550 and two inducible cytochromes, *c*-551i and *c*-553i (10). Cytochrome *c*-551i is the most efficient acceptor of electrons from MADH and amicyanin *in vitro* (10). These inducible cytochromes *c* do not react directly with the membrane-bound respiratory chain but transfer electrons to it via cytochrome *c*-550 (4). In *A. faecalis*, when AADH is induced by phenylethylamine, no additional cytochromes *c* are induced and the level of the periplasmic 13-kDa cytochrome is unchanged. Thus, in *A. faecalis*, phenylethylamine-derived electrons are likely donated from AADH and azurin to the respiratory chain via the single periplasmic cytochrome *c*. Its physiologic role may be like that of cytochrome *c*-550 in *P. denitrificans* (2), which mediates electron transfer to cytochrome oxidase from multiple periplasmic dehydrogenases.

In the past, the characterization of proteins of *A. faecalis* as periplasmic was primarily inferred from the presence of signal sequences in their genes. Sequences of the genes for AADH and these *A. faecalis* cytochromes have not been reported. The new modified lysozyme-osmotic shock method described in Fig. 1 allows efficient fractionation of *A. faecalis* and will now allow a detailed study of the physiologic redox reactions associated with the dissimilation of aromatic amines, as well as better characterization of other periplasmic proteins and metabolic processes.

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