LOCALIZATION OF ACID AND ALKALINE PHOSPHATASES IN *Myxococcus coralloides* D

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Myxococcus coralloides produces two different phosphatases, one acid and the other alkaline. Both enzymes were localized by physical and biochemical techniques. Spheroplasts from *M.coralloides* released 20-30% of the phosphatase activities. Osmotic shock or treatment with high $MgCl_2$ or LiCl concentrations did not produce a greater releasing. Cytochemical localization situated the phosphatases in the outer membrane and the periplasmic space. Separation of the cytoplasmic membrane and outer membrane of the cells by sucrose gradient centrifugation showed that phosphatases are located primarily in the outer membrane.

Introduction

Myxococcus coralloides is a gliding bacterium that produces a great variety of enzymes, such as amylases, nucleases and phosphatases (Gonzalez *et al.* 1990; Martinez-Cañamero *et al.* 1991; Farez-Vidal *et al.* 1992). It is well known that various enzymes of gram-negative bacteria exist and function outside of the cytoplasmic membrane; hydrolytic enzymes are most frequently found in the periplasm (Nikaido & Vaara 1985). The existence of a number of proteins in the outer membrane has been firmly established, but relatively few of these proteins are known to have hydrolytic activities (Lugtenberg & Van Alphen 1983). *M.coralloides* produces two extracellular phosphatases and two cell-bound phosphatases, one acid and the other alkaline in both cases. 90% of these activities correspond to cell-bound phosphatases (Gonzalez *et al.* 1989). Acid and alkaline phosphatase production by *M.coralloides* has been reported during its vegetative growth and during its life cycle (Gonzalez *et al.* 1987; 1989). Moreover, we have published research on the changes in the phosphatase activities during *Myxococcus xanthus* development (Gonzalez *et al.* 1991). The localization of phosphatases can be useful in the study of the changes that occur in the development of myxobacteria; thus Weinberg and Zusman (1990) and our results (Gonzalez *et al.* 1991) have shown that phosphatase activities should make useful biochemical markers for development stages.

To pursue this line of inquiry, we report here on the localization of the cell-bound phosphatases in *M.coralloides*.

Materials and Methods

BACTERIAL STRAINS, MEDIA AND GROWTH CONDITIONS

Myxococcus coralloides strain D is a bacterium from our laboratory stock. Vegetative cells of this myxobacterium were used in this study. It was grown in TT liquid medium, containing (g/l): Trypticase Peptone-BBL, 7,5; $MgSO_4.7H_2O$, 1; in 10 mmol/l potassium phosphate buffer pH 6.5. The cultures were incubated at 28°C in an orbital shaker at 200 rev/min.

ANALYTICAL, ENZYME ASSAYS AND PREPARATION OF CELL EXTRACTS

Protein was assayed by Bradford's method (Bradford 1976). Lipopolysaccharide was estimated by determining the amount of 2-keto-3-deoxyoctonate (KDO) in the membrane fractions. KDO was determined by the thiobarbituric acid method as modified by Osborn *et al.*(1972). Phosphatase assays have previously been described (Gonzalez *et al*. 1987). The preparation of the cell-free extract, cold osmotic shock, $MgCl_2$ or LiCl treatment and differential centrifugation of cell-free extract were carried out as described by Von Tigerstrom (1980) and Von Tigerstrom & Stelmaschuk (1985). NADH-dehydrogenase (NADH-DH) and Glucose-6-phosphate-dehydrogenase (G6P-DH) were determined according to Orndorff and Dworkin (1980).

CYTOCHEMISTRY

Cells from exponentially growing cultures (100 ml, 0.3-0.4 optical density at 650 nm) were washed twice by suspension and centrifugation in 10 ml 10 mmol/l Tris-maleate buffer (pH 5.0 for acid acid phosphatase reaction or pH 8.0 for alkaline phosphatase reaction). Thus, cells were suspended in 10 ml of the following solution: 5% (w/v) sucrose, 3 mmol/l sodium glycerophosphate, 3 mmol/l p-nitrophenolphosphate in 10 mmol/l Tris-maleate buffer (pH 5.0 or pH 8.0). After 30 min at 28°C, the cells were centrifuged and resuspended in the same buffer containing 3 mmol/l lead citrate. After 15 min, cells were

washed in the same buffer without lead citrate and then prepared for electron microscopy examination according to Voelz and Ortigoza (1968).

SPHEROPLASTS AND MEMBRANE ISOLATION AND FRACTIONANTION

The preparation of spheroplasts and the membrane isolation and fractionation were attempted according to Orndorff and Dworkin (1980).

Results and Discussion

Spheroplasts of *M.coralloides* were obtained after a long incubation with lysozyme-EDTA-sucrose at low temperature (12 h at 4°C). In *M.coralloides* which had been subjected to sphaeroplasting, about 95-100% of the cell population is converted to spheroplasts, but only 20-30% of the acid and alkaline phosphatase activities were detected in the supernatant, which contains the periplasmic space (Table 1). Cold osmotic shock of *M.coralloides* cells released only 10-15% of the phosphatase activities. The treatment with high concentrations of $MgCl_2$ or LiCl (200 mmol/l) removed only 5-15% of the phosphatase activities.

Moreover, after differential centrifugation of a cell-free extract (Table 1), the acid phosphatase was sedimented (about 70%) in the 48000 × g pellet, which contained membranes and cell wall fragments. 80% of alkaline phosphatase activity was also sedimented. Results indicated that only 20-30% of the phosphatases are free in the periplasm. Glucose-6-phosphate dehydrogenase was used as a soluble marker enzyme. All these results indicated that *M.coralloides* phosphatases are primarily associated with the envelope fractions of the cells by hydrophobic, rather than ionic, interaction.

The acid phosphatase cytochemical reaction production was confined to the periplasmic space as well as the outer membrane. The reaction for alkaline phosphatase was also found in the periplasm and outer membrane. Under the conditions examined, cytochemical reactions were also observed on the cell surface (outer surface of the outer membrane). This deposition of the

reaction product also appeared in *Capnocytophaga spp.* (Poirier & Holt 1983), and it could possibly be related to the excreted *M.coralloides* phosphatases (Gonzalez *et al.* 1990).

To determine the location of the enzymes within the cell envelope, the cytoplasmic membrane and outer membrane were separated. The total membrane fraction from EDTA-lysozyme-trated cells was separated into two major fractions by isopycnic density centrifugation (Table 2). The isolated fractions (named I and II) were subjected to further analysis. The enzyme activities, proteins and KDO concentrations are shown in Table 2. NADH-DH was used as a marker enzyme for the cytoplasmic membrane and the KDO as a marker of the outer membrane. The separation of the outer and cytoplasmic membrane was similar to that reported for *M.xanthus* (Orndorff & Dworkin 1980). Acid and alkaline phosphatase activities were associated with fraction II (90% of detected activity). This fraction also contained the KDO. Therefore, the phosphatases are associated with the outer membrane of *M.coralloides*, but not with the cytoplasmic membrane. These results verify the cytochemical results and are in agreement with the cell-fractionation results.

Phosphatases from other gliding bacteria are also cell-envelope associated enzymes. Thus, *M.xanthus* phosphatases have been reported to be present on the external surface of the cytoplasmic membrane and in the periplasm (Voelz & Ortigoza 1968); in *Capnocytophaga spp.*, the phosphatases are associated with the outer membrane and the periplasm (Poirier & Holt 1983); and in *Lysobacter enzymogenes*, the alkaline phosphatase is located in the outer membrane (Von Tigerstrom & Stelmaschuk 1985).

The cell envelope from myxobacteria is involved in its development (Shimkets 1989), and have atypical properties (Orndorff & Dworkin 1980). Myxobacteria degrade complex macromolecules and even lyse other microorganisms and the products of the degradation are utilized as nutrients. The high levels of phosphatases in vegetative cells of *M.coralloides* (Gonzalez *et al.* 1987) and its cell localization, which we have described here, favour the view that the phosphatases have a

nutritional role in the vegetative cells, providing orthophosphate from the phosphorylated metabolites found in the medium.

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Table 1. Phosphatase activities from *M.coralloides* after differential centrifugation of cell-free extract and the effect of sphaeroplast formation on the release of these enzymes.

Fraction	AcP[1]	AlP[2]	G6P-DH[3]
Total cell-bound	9.15	5.12	0.30
Cell-free extract	8.86	4.70	0.31
48000 x g supernatant	2.31	0.94	0.30
48000 x g pellet	6.20	3.76	0.00
105000 x g supernatant	0.98	0.21	0.25
105000 x g pellet	1.21	0.63	0.02
Sphaeroplast formation			
supernatant	2.81	0.86	ND[4]
pellet	6.55	3.44	ND

These values are the means of three representative experiments. Activities are expressed as specific activities (U/mg protein). [1] Acid phosphatase activity, [2] Alkaline phosphatase activity, [3] Glucose-6-phosphate dehydrogenase activity, [4] Not determined.

Table 2. Chemical and enzymatic analysis of isolated membrane fractions from *Myxococcus coralloides*. Membrane fractions were obtained from a sucrose isopycnic density centrifugation.

Fractions[1]	AcP[2]	AlP[3]	NADH-DH[4]	Protein[5]	KDO[6]
1	-*	-	0.02	0.11	<0.1
2	-	-	0.03	0.12	<0.1
3	-	-	0.08	0.24	<0.1
4	-	-	0.10	0.35	<0.1
5	-	-	0.16	0.52	<0.1
6	-	-	0.15	0.42	<0.1
7	0.2	-	0.06	0.15	0.10
8	0.4	0.6	0.05	0.28	0.17
9	1.8	1.2	0.03	0.62	0.36
10	5.0	2.3	0.02	1.12	0.48
11	5.6	3.6	0.02	1.35	0.35
12	4.6	2.0	-	0.82	0.25
13	1.8	1.6	-	0.43	0.21
14	1.0	0.4	-	0.21	0.12
15	0.3	-	-	0.10	<0.1
I (3-6)	-	-	0.12	0.41	<0.1
II (9-13)	5.4	3.2	0.02	0.83	3.23

These values are the means of three representative experiments. Enzyme activities are expressed in U/ml. [1] Fractions 3-6 and 9-13 were pooled for further analysis and were designed as fractions I and II, respectively. [2] Acid phosphatase activity. [3] Alkaline phosphatase activity. [4] NADH-dehydrogenase activity. [5] Relative values of proteins (absorbance at 280 nm) are expressed for fractions 1-15; for fractions I and II, protein values are expressed as mg/ml. [6] Relative values of 2-keto-3-deoxyoctonoate (absorbance at 548 nm) are expressed for fractions 1-15; for fractions I and II, KDO values are expressed as µg/ml. * Not detected.