Histochemical Localization of Phosphatases in *Mycoplasma gallisepticum*¹

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Histochemical studies of adenosine triphosphatase and acid phosphatase activity were performed on *Mycoplasma gallisepticum*. The adenosine triphosphatase activity appears to be localized in the bleb and infrableb regions exclusively and is associated with the cell membrane; acid phosphatase activity is localized in the infrableb region and does not appear to be membrane-associated. These findings are consistent with data from biochemical studies of *Mycoplasma* cell fractions but, unlike them, reveal that adenosine triphosphatase activity is restricted to a particular part of the cell membrane.

The histochemical localization of enzymes in microorganisms could be done in a limited way with light microscope techniques. For example, Nickerson et al. (17) applied the Gomori technique to yeast and found that alkaline phosphatase activity appeared to be associated with Feulgen-positive structures. Electron microscopy has permitted more precise histochemical localization of enzymes in a variety of microorganisms (6, 18, 23–28). These, as well as biochemical studies (1, 4, 9, 12, 13, 16, 22), have demonstrated that a number of enzymes are associated with membranous structures, i.e., the cell membrane and mesosomes of large bacteria. It is of some interest to determine whether the smallest free-living organisms, the *Mycoplasma* spp., have similar associations of enzymes and membranes, since they have a very much reduced membrane area. They apparently lack mesosomes and have less than 0.1 the surface area of bacteria the size of *Escherichia coli*. *Mycoplasma gallisepticum* A5969 was chosen for this study, since it has been well characterized physiologically and chemically (15). The strain was obtained from Mark Tourtellotte, Department of Animal Diseases, University of Connecticut. The distribution of phosphatases in subcellular fractions of this strain of *Mycoplasma* has been studied by Pollack et al. (20), Munkres (unpublished data), and Rottem and Razin (21); therefore, these enzymes were chosen also for histochemical study.

**Materials and Methods**

The *Mycoplasma* strain was grown in tryptose broth supplemented with Difco PPLO Serum Fraction (15). An inoculum of 4.5 ml from a 24-hr stock culture was used per 2-liter culture, which was incubated statically at 37°C. The cells were harvested in exponential phase (about 24 to 30 hr) by centrifugation in a Lourdes refrigerated centrifuge for 10 min at 10,400 × g.

For histochemical studies, the cells were fixed in 3.13% glutaraldehyde buffered with 0.05 M sodium cacodylate (pH 7.2) and immediately centrifuged for 10 min at 10,400 × g. The cells were suspended in 0.2 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer (pH 7.2) at room temperature, and again centrifuged. The cells were then resuspended in a small amount of Tris buffer and added to the various incubating mixtures.

For the adenosine triphosphatase (ATPase) reaction, a modified Wachstein-Meisel method (30) was used. The incubating medium was buffered at pH 7.4 with Tris chloride, and incubation was carried out at room temperature for 1 hr. A modified Gomori method (19) was used for the nonspecific acid phosphatase reaction at pH 5.0. The cells were incubated for 1 hr at room temperature. Controls were carried out for all reactions by omitting substrate. The cells were not treated with ammonium sulfide after incubation for light microscopy.

After incubation, the cells were centrifuged for 10 min at 6,000 × g. The cells were resuspended in 0.2 M Tris buffer (pH 7.4) and recentrifuged. The pellets were then fixed for 1 hr at 4°C in 6.25% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium cacodylate. After washing overnight in 0.1 M sodium caco-

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The M. gallisepticum strain has at the end of the cell a budlike protrusion which is called the "bleb" (Fig. 1A and 1B). Of the 19 strains of Mycoplasma which have been observed at the electron microscope level (2, 5), M. gallisepticum is the only one with blebs. Other strains contain membrane-bound particles called "elementary bodies" (5) which are released upon lysis of the cell, but these have not been observed in M. gallisepticum. In an exponential culture, two blebs, one at either end of the cell, are often observed (Fig. 1B). The cell membrane of the bleb is continuous with that of the rest of the cell, and the bleb is not isolated from other parts of the cytoplasm by a membrane. The cytoplasm just below the base of the bleb is usually less electron-dense than is the rest of the cell (Fig. 2) and does not contain ribosomes or nuclear strands (11). This region is called the "infrableb."

Localization of both acid phosphatase and ATPase is seen at the electron microscope level by the reaction of lead with enzymatically released phosphates, which produces an electron-dense lead precipitate at the sites of enzyme activity. When adenosine triphosphate (ATP) is provided as the substrate at pH 7.4, the lead deposits are found in the bleb and, to a lesser extent, in the infrableb region (Fig. 3). In both the bleb and infrableb regions, the deposit is mainly localized at the inner surface of the cell membrane (Fig. 3 and 4). None is found within the membrane, at the exterior surfaces, or within any other parts of the cell. Figure 3 shows a cell with a single bleb, and Fig. 4 shows a cell with two blebs. Three-bleb cells are also seen occasionally. In all cases, distribution of the product of ATP hydrolysis was found to be similarly restricted to the blebs and was present in all blebs of incubated cells examined in the electron microscope.

Acid phosphatase is located exclusively in the infrableb region and is not restricted to proximity with the cell membrane (Fig. 5A and 5B). There appears to be no enzyme activity in the bleb itself. In contrast to the constancy of ATPase activity, a variation in acid phosphatase activity in the infrableb region, from very reactive to no detectable reaction, was observed. Perhaps this is related to the division cycle of the cells. Since this was an exponentially growing culture, it may be that double-bleb cells with no reaction were in stationary phase. It should be pointed out that, in sectioned material such as this, one cannot be sure that all the blebs of any particular cell are included in a section. Thus, when variations in enzymatic activity are observed in cells which appear to have only a single bleb, one may be observing both one- and multibleb cells.

**Discussion**

In recent studies on phosphatases of micro-organisms, Tonino and Steyn-Parve (26) found that nonspecific acid phosphatase (optimal activity at pH 3.0 to 4.0) in yeast is localized in the cell wall and that the pattern of distribution changes with age of the cells. Bennun et al. (3) described two kinds of ATPase in yeast: an intracellular ATPase which is structurally bound, and an acid ATPase which is located on the cell surface. Weinberg and Orton (31) also found evidence of an exocellular acid phosphatase in yeast. In similar studies of E. coli and a phosphate-constitutive mutant, Malamy and Horecker (10) and Neu and Heppel (16) reported a surface-bound alkaline phosphatase which, they proposed, functions in selective transport of substrates into the cell and in synthesis of cell wall components. Voelz (29) showed that the sites of ATPase activity vary among bacterial species. In E. coli, the activity is in the cytoplasmic membranes, cytoplasm, and nuclear region; in Mycoplasma xanthus, the activity is only in the cytoplasm. He attributed the diversity in localization sites to stage of development of the organisms.

Abrams (1) studied the release of bound ATP from Streptococcus faecalis membranes and postulated a regenerative type of cycle for ATP (in which ATP generated during glycolysis interacted with membrane ATPase). Cole and Hughes (4) found ATPase activity membrane-bound (pH optimum, 6.0) in Lactobacillus arabinosus. They concluded that ATPase was different from the ATPase associated with ion transport but was similar to that from mitochondrial membranes, although they did not exclude the possibility of involvement in cell membrane or cell wall synthesis.

Electron microscope studies by Done et al. (6) have shown that alkaline phosphatase in E. coli
is localized in the intermediate layer of the cell wall between the plasma membrane and the outer membrane.

In biochemical studies of *M. gallisepticum* cell fractions, Pollack et al. (20) and Munkres (unpublished data) found that 98% of the alkaline phosphatase was membrane-bound. Rottem and Razin (21), in a similar study of *Mycoplasma* cell fractions, showed that ATPase activity also is localized in the cell membrane. The histochemical demonstration that ATPase activity in *M. gallisepticum* is restricted to the bleb and infrableb membrane and is not external to the cell is in agreement with biochemical studies of cellular

![Image of Mycoplasma gallisepticum](http://jb.asm.org/)

**Fig. 1.** *Mycoplasma gallisepticum* from control blocks (substrate omitted from incubation mixture) showing typical structures: b = bleb region; i = infrableb region; and m = cell membrane. (A) X 154,800; (B) X 110,900.
fractions of *Mycoplasma* and offers refinement of localization not yet possible with fractionation procedures. It is not understood why ATPase is found only in the bleb and infrableb parts of the cell and is not uniformly distributed throughout the cell membrane; this may indicate that, in these minute cells, portions of the cell membrane are differentiated to perform specialized functions.

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**Fig. 2.** *Mycoplasma gallisepticum* fixed but not incubated. Some cylindrical ribosomal clusters have been cut in cross section (*r*, center of figure), whereas other clusters have been sectioned along the long axis (*r*, lower right). The nuclear material is visible as a filamentous area in each cell. × 125,000.
Fig. 3. Localization of ATPase activity (indicated by dense material) in bleb and infrableb regions. × 212,500.
that, in larger cells, are performed by specialized membranous structures such as mesosomes, mitochondria, and endoplasmic reticulum.

Acid phosphatase is localized within the infra-bleb region, with no indication that it is membrane-associated. The function of the bleb and infrableb region is not clearly understood, although it is known that the Mycoplasma cell

![Image of two cells with blebs](image)

**FIG. 4.** Cell with two blebs. ATPase activity is present in both bleb and infrableb regions. ×193,500.
forms a second bleb before dividing (14). The variability observed in acid phosphatase activity is perhaps related to cell division, but the function of the enzyme in this region remains unknown. It is hoped that further studies of synchronously dividing cells will be informative.

**Fig. 5.** Acid phosphatase localized in the infrableb region. (A) × 65,400; (B) × 65,400.

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LITERATURE CITED


