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Gliding Mycoplasmas Are Inhibited by Cytochalasin B and Contain a Polymerizable Protein Fraction

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Studies are presented on the effect of cytochalasin B (CB) on the growth of five Mycoplasma species, three Acholeplasma species, and one Spiroplasma species. The three gliding mycoplasma species (M gallisepticum, M pneumoniae and M pulmonis are the only mycoplasmas inhibited by CB. These are the only prokaryotes reported to be inhibited by CB. This suggested that these three mycoplasmas might have some sort of cytoskeletal structure. A protein fraction has been isolated from M gallisepticum which polymerizes in 0.6 M KCl and depolymerizes when KCl is removed. This fraction contains a major 58,000-dalton protein, a 46,000-dalton protein, and a minor 87,000-dalton protein.

Key words: mycoplasma, cytochalasin B, actin-like protein, cytoskeleton

The mycoplasmas are a group of prokaryotes which lack cell walls; each cell is bounded by a single lipoprotein cell membrane (see, for example, Maniloff and Morowitz [1] and Razin [2]). These are the smallest free-living cells, with genomes of only (0.5–1.0) $\times 10^9$ daltons of DNA. From a comparison of mycoplasma and bacterial 16S rRNA oligonucleotides (see Maniloff et al [3]; C. R. Woese, J. Maniloff, and L. B. Zablen, manuscript in preparation), it has been concluded that the mycoplasmas are close to one of the clostridia sublines. Therefore, mycoplasmas are not related to some simpler prokaryote which was ancestral to eubacteria; instead, mycoplasma arose from the gram-positive eubacteria. The only reported exception is a thermophilic acidophilic mycoplasma which belongs to the archaebacteria, not the eubacteria.

Among the more than 50 reported mycoplasma species, three species are different because their cells have polar subcellular structures and exhibit gliding motility [4]. These three species, all of which are respiratory pathogens in different animal hosts, are Mycoplasma gallisepticum, M pneumoniae, and M pulmonis.

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Studies on the subcellular polar structures and molecular events in the cell cycle have focused on M gallisepticum. These cells are pear-shaped with a polar bleb structure, and prior to division cells are elongated with bleb structures at both ends [5]. Some micrographs show a bleb area in the cytoplasm near a polar bleb structure [6], indicating that each new bleb might be assembled at a preexisting one. The polar bleb can be separated from the cell by sonication, isolated, and characterized [6–9]. These studies showed that the blebs contain the sites of DNA replication and that the chromosome origin remains attached to a bleb throughout the cell cycle. Studies with synchronized cells showed that the M gallisepticum chromosome is replicated once per cell generation and that there is a gap in DNA synthesis during cell division [8].

These data led us to suggest that the M gallisepticum bleb structures might function as a primitive "mitotic-like" apparatus for chromosome organization and segregation [9]. If one of the nascent chromosomes were attached to a new bleb (made at a preexisting one and, hence, near the DNA origin attachment site), then bleb migration to form the opposite pole of the cell for division would assure chromosome segregation. Since each cell may only contain a single genome copy [10], this cell cycle could have evolved to assure segregation of the two daughter chromosomes before cell constriction and division.

Such a cell cycle requires a mechanism for the intracellular movement of subcellular structures. To investigate this, the effect of several drugs on M gallisepticum was examined [11]. Colchicine and vinblastine had no effect on cell growth, but cytochalasin B (CB) inhibited M gallisepticum cell division. The data indicated that CB blocks at two points in the cell cycle: at the time of bleb structure formation and at the time of cell division [11].

We report here studies to see what other mycoplasma species might be inhibited by CB and the isolation of a polymerizable protein fraction from M gallisepticum.

CYTOCHALASIN B STUDIES

The effect of CB on the growth of five Mycoplasma species, three Acholeplasma species, and one Spiroplasma species was examined. Typical dose-response curves are shown in Figure 1. Some species were slightly inhibited at higher ethanol concentrations. However, only M gallisepticum, M pneumoniae, and M pulmonis were inhibited by CB. The inhibition of M gallisepticum, but not of A laidlawii or M capricolum, confirms recently reported results [11], although different A laidlawii and M capricolum strains were used in this and the previous study. Hence, the three mycoplasma species having polar subcellular structures are inhibited by CB.

CHARACTERIZATION OF POLYMERIZING PROTEIN FRACTION

The isolation protocol was based on those used to isolate actin-like proteins (see, for example, Pollard and Weihing [19]). However, n-butanol was used instead of acetone, since n-butanol has been reported to solubilize mycoplasma membranes more effectively than acetone [20]. Preliminary experiments indicated that a better yield of mycoplasma polymerizable protein was obtained with n-butanol than with acetone (data not shown).

M gallisepticum cells from a 10- to 12-liter culture were harvested by centrifugation (10,000g for 10 min at 4°C) and washed once with 200 ml 0.01 M Tris–0.001 M EDTA buffer (pH 7.5). The cell pellet was resuspended in 50 ml depolymerizing buffer (0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.5 mM Tris, pH 7.6) and freeze-thawed (–196°C to

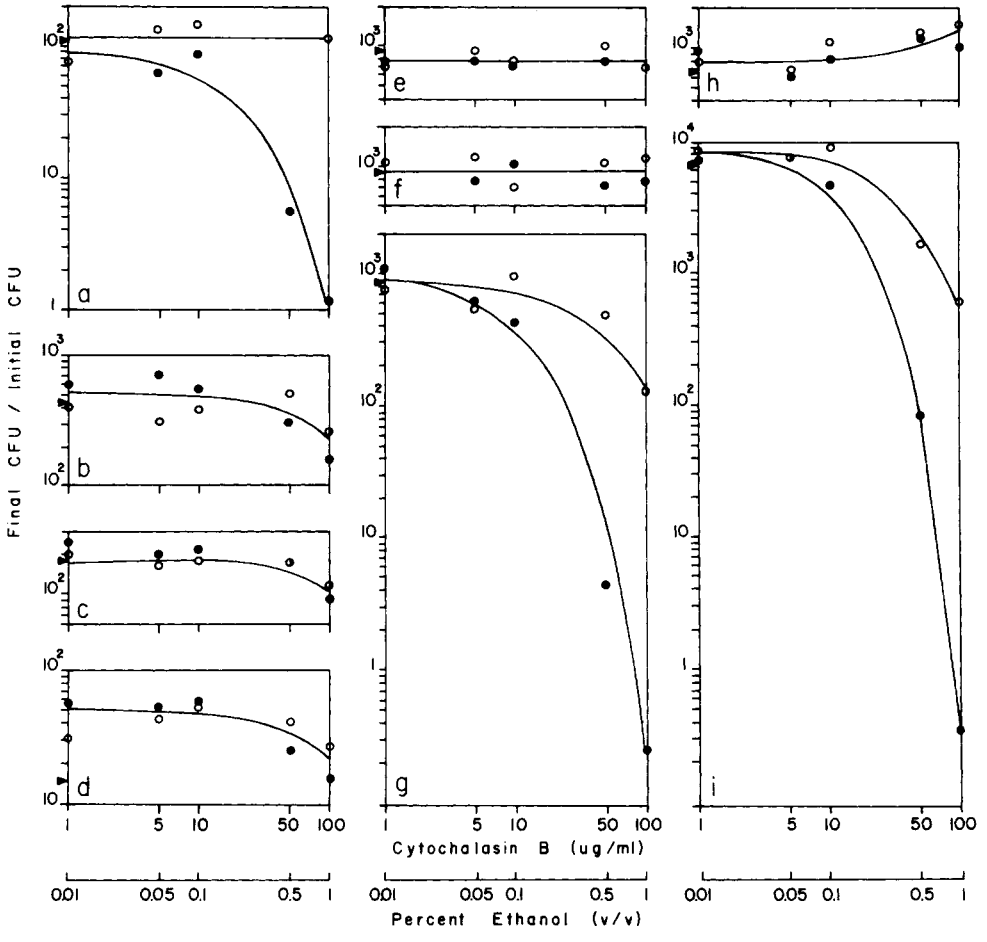

Fig. 1. Effect of CB on mycoplasma growth. CB was dissolved in ethanol and diluted in growth media. Mycoplasmas were added to tubes containing medium plus CB (•) and, as controls, to tubes containing medium plus ethanol concentrations equivalent to those in the CB-containing tubes (○). At each concentration growth is expressed as the number of colony-forming units (CFU) assayed after a 2- 4-day incubation, divided by the number of CFU assayed at the start of the experiment. The arrow on each ordinate shows growth in a control tube containing no CB or ethanol. a) M gallisepticum strain A5969 [12]; b) A modicum strain PG-49; c) A axanthum strain S-743; d) S citri strain Maroc; e) M capricolum strain Kid [13]; f) M arginini strain G230; g) M pneumoniae strain FH; h) A laidlawii strain JA1 [14]; i) M pulmonis strain PG-34. The strains without literature citations were supplied by Dr. J.G. Tully (NIAID, Bethesda, Maryland), except S citri, which was supplied by Dr. R.F. Whitcomb (US Department of Agriculture, Beltsville, Maryland. b,h) Grown on tryptose broth medium [15]; g) grown on a mycoplasma broth base medium [16]; d) grown in a PPLO broth medium [17, 18]. All others grown in a mycoplasma broth base medium [10].

40°C) twice. An equal volume of n-butanol was added and the suspension was stirred for 4–6 h at 4°C. The suspension was filtered through Whatman No. 3 paper and dried. The butanol powder was resuspended in 20 ml depolymerizing buffer. The solution was stirred for 16 h at 4°C and centrifuged (48,000g for 2 h at 4°C) to remove cellular debris. The supernatant was removed and centrifuged again (120,000g for 2 h at 4°C) to remove other slower-sedimenting material. Protein in this supernatant was polymerized by the addition of KCl to a final concentration of 0.6 M. The solution was stirred gently for 16 h at 4°C and then centrifuged (120,000g for 2 h at 4°C). The pellet was resuspended in 3 ml polymerizing buffer (depolymerizing buffer containing 0.6 M KCl, pH 7.6) and allowed to disperse overnight at 4°C. The solution was dialyzed for 3 days at 4°C against 1 liter depolymerizing buffer. The protein was then polymerized again by the addition of KCl to a final concentration of 0.6 M.

The final solubilized protein after two cycles of polymerization-depolymerization was termed polymerizable protein fraction. The yield from 10–12 liters of culture was 300–600 μg protein. SDS-polyacrylamide gel electrophoresis in 10% acrylamide slab gels [21] of this protein revealed three bands: a major band at 58,000 daltons, a band with a little less material at 46,000 daltons, and a faint band at 87,000 daltons (data not shown).

The polymerization-depolymerization kinetics of the protein fraction were followed by viscometry. The addition of KCl (to a final concentration of 0.6 M) to the protein fraction in depolymerization buffer caused an increase in the specific viscosity from 0.05 to 0.13 in about 4 h (Fig. 2a). The viscosity increase was less if the protein fraction was mixed with both KCl (0.6 M final concentration) and $MgSO_4$ (0.001 M final concentration) (Fig. 2b). No increase in viscosity was seen with $MgSO_4$ alone (data not shown).

Dialysis of the KCl polymerized protein against depolymerizing buffer, to reduce the KCl concentration, caused a decrease in viscosity (Fig. 2c). However, after 72 h dialysis the specific viscosity had not returned to its original depolymerized value of 0.05. Addition of KCl, to 0.6 M final concentration, caused this material to polymerize again (Fig. 2d).

DISCUSSION

The three mycoplasma species, which have terminal structures and exhibit gliding mobility, are the only mycoplasmas found to be inhibited by cytochalasin B. These three (M gallisepticum, M pneumoniae, and M pulmonis) are the only prokaryotes reported to be inhibited by CB. Previous studies have shown that the effect on M gallisepticum is not due to an inhibition of the uptake of glucose or macromolecule precursors [11], indicating that CB may be affecting processes dependent on some sort of microfilament structure.

With regard to the antibacterial action of the cytochalasins, cytochalasins B, D, and E have been reported to have no effect on gram-positive and gram-negative bacteria [22–24]. Cytochalasin A (5–25 μg/ml) has been reported to inhibit the growth of gram-positive bacteria (probably by inhibiting solute transport), but not to inhibit gram-negative bacteria [25]; The latter observation conflicts with a report that cytochalasin A (25 μg/ml) causes the lysis of Escherichia coli [23].

An actin-like protein has been reported to have been isolated from M pneumonia [26]. However, the M pneumoniae results need to be reevaluated in terms of the more recent data that the actin isolation procedure applied to E coli leads to the isolation of the translation elongation factor EF-Tu, and that EF-Tu has properties similar to actin [27–29]. Also, the M pneumoniae experiments [26] followed an isolation protocol described for platelet actin, which leads to a protein fraction that polymerizes in low salt and depoly-

merizes in high salt [30]. Opposite properties have been reported for muscle actin and all other nonmuscle actins (see, for example, Pollard and Weihing [19] and Korn [31]). Recently, it has been shown that the polymerization behavior of platelet actin can be explained by the salt dependence of its critical concentration [32]; when this is taken into account, it was found that platelet actin polymerization was similar to that of other nonmuscle actins. Hence, the polymerization behavior of the M pneumoniae protein needs reexamination as well.

The studies described here were begun to investigate possible mechanisms for the movement of M gallisepticum polar structures during the cell cycle and for DNA segregation. The CB inhibition results were consistent with the idea that there might be some sort of microfilament structure in M gallisepticum and led to experiments to isolate these structures. A protein fraction has been isolated from M gallisepticum which polymerizes in 0.6 M KCl and depolymerizes when KCl is removed. This fraction contains a major 58,000-dalton protein, a 46,000-dalton protein, and a minor 87,000-dalton protein. Experiments are in progress to further characterize this protein fraction.

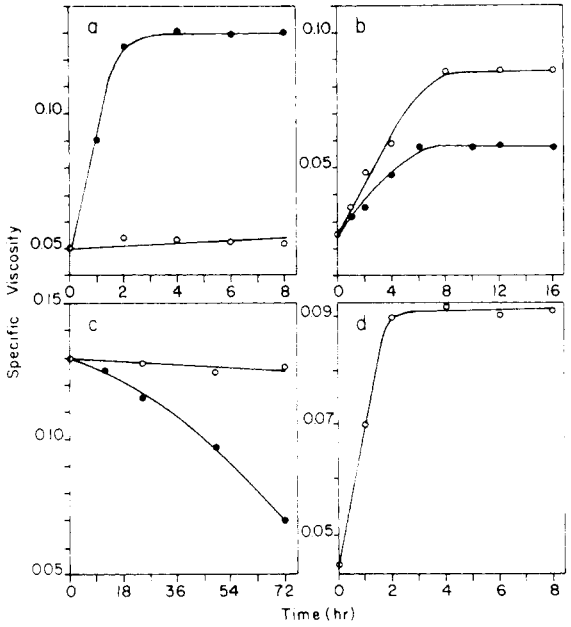

Fig. 2. Viscosity of M gallisepticum polymerizable protein fraction. Viscosity was measured at room temperature (22°C) using an Ostwald-type viscometer, having a minimum volume of 2 ml and a flow time of about 90 sec for buffer solutions. The specific viscosity is the flow time of the protein divided by the flow time of the buffer, minus unity. a) Protein (300 μg/ml final concentration) in depolymerizing buffer was mixed with KCl (0.6 M final concentration) at room temperature and viscosity was measured as a function of time (•). A control protein sample received no KCl (○). b) The protocol was as for (a), except that one sample received 0.6 M KCl (○) and the other received 0.6 M KCl plus 0.001 M $MgSO_4$ (•). The final protein concentration was 200 μM/ml. c) Protein (300 μm/ml) in polymerizing buffer was dialyzed against depolymerizing buffer at 4°C and viscosity was measured as a function of dialysis time (•). A control polymerized protein sample was dialyzed against polymerizing buffer (○). d) Protein depolymerized as described in (c) was mixed with KCl; final concentrations were 0.6 M KCl and 200 μg protein/ml. Viscosity was measured as a function of time after KCl addition.

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