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Unique choline-containing phosphoglycolipids in *Mycoplasma fermentans*

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1. Introduction

It is a privilege to participate in this memorial issue for Robert (Bob) Bittman and to recall with gratitude his kindness and warm hospitality during my visits to his laboratory at the chemistry department of Queens College of New York. Through the passage of time, his friendship stood true. Bob embodied the qualities we hope to see in our colleagues, love of science, enthusiasm and optimism. I first met Bob in the summer of 1976 when he visited my laboratory in Jerusalem. We discussed the many gaps in our knowledge of sterols function in cell biology. I mentioned to Bob that for examining sterol function in biological systems, sterol auxotrophs, either natural or those of cells raised under restrictive conditions might yield relevant information. I brought to his attention that native sterol-requiring organisms are the mycoplasmas, bacteria bounded by a single membrane, the plasma membrane. At that time, we successfully adapted a sterol-requiring mycoplasma to grow with very little cholesterol (Rottem et al., 1973b), providing us with a useful sterol auxotroph. Bob liked the idea and we established a collaborative study that lasted five years (1978–1983) during which nine papers were published. Throughout the following years, Bob advice and ideas were invaluable. Bob's presence will be missed both as an invaluable scientist and as a friend.

Mycoplasmas (class *Mollicutes*) are the smallest and simplest self-replicating bacteria (Razin et al., 1998). These microorganisms lack a rigid cell wall and are bound by a single membrane, the plasma membrane. Wall-less bacteria were first described 100 years ago and now over 210 species, widely distributed among humans, animals, insects and plants are known. The lack of a cell wall is used to distinguish these microorganisms from ordinary bacteria and to include them in a separate class named *Mollicutes*. Phylogenetically, the *Mollicutes* are related to Gram-positive bacteria from which they developed by genome reduction (Maniloff, 1996). Therefore, the *Mollicutes* are not at the root of the phylogenetic tree but are most probably late evolutionary products. Most human and animal mycoplasmas are *Mycoplasma* species of the family *Mycoplasmataceae*.

2. Mycoplasmas require free fatty acids and sterols for growth

Owing to their extremely small genome (0.58–2.20 Mb compared with the 4.64 Mb of *Escherichia coli*), these organisms have limited biosynthetic capabilities and depend on the host or growth medium for the supply of many nutrients (Razin et al., 1982). Thus, mycoplasmas are incapable of fatty acid biosynthesis and depend on the host or growth medium for their supply. The dependence of mycoplasmas on an exogenous supply of fatty acids has been one of their greatest advantages as models for membrane studies (Kornspan and Rottem, 2012; Rottem, 1980). The ability to introduce controlled alterations in mycoplasma membrane lipids, simply by controlling the composition and content of fatty acids in the growth medium, has been used most effectively in elucidating membrane lipid organization and function in the membrane (Rottem, 1980).

In addition to long-chain fatty acids, most mycoplasmas require a sterol for growth, a nutritional dependence not found elsewhere among prokaryotes (McElhaney, 1993). Plant and animal sterols

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meet this requirement, and so do certain sterol derivatives, provided they contain the cholesterol ring system (A/B *trans*), an un-substituted equatorial hydroxyl group, and a branched aliphatic side chain eight or more carbon atoms in length (Razin et al., 1982). For some *Mycoplasma* species, the sterol specificity is surprisingly broad, and various modified cholestane derivatives satisfy, if weakly, the sterol requirement (Dahl, 1993). When grown in a serum-supplemented growth medium, the main sterol found in mycoplasmas is unesterified cholesterol, despite the presence of excessive amounts of esterified cholesterol in medium. None of the mycoplasmas tested so far are capable of sterol synthesis (Bittman, 1993; Razin et al., 1982). The low levels of esterified cholesterol incorporated from the growth medium are not required for growth and appear to form lipid droplets or pockets in the membrane (Melchior and Rottem, 1981).

The total dependence of mycoplasmas on an external supply of a sterol has been utilized to introduce controlled alterations in the sterol composition and content of the membranes, thus facilitating the analysis of the effects of sterols on membrane properties and on cell growth. The successful adaptation of sterol-requiring mycoplasmas to grow with very little cholesterol provided us with a useful model system (Rottem et al., 1973b). Adaptation was achieved by serial transfers of the mycoplasma in a lipid-depleted growth medium containing decreasing concentrations of cholesterol. The adapted strain grew more slowly than the native strain, and the adapted cells were more fragile. In *Mycoplasma mycoides var. capri,* the cholesterol/phospholipid molar ratio decreased from 0.9 in the native strain grown with 10 μg/ml cholesterol to 0.2 in the adapted strain grown on 0.5 μg/ml of cholesterol, with the total phospholipid content of the cell membrane remaining unchanged. The most remarkable difference between the membranes of the two strains was that only in the cholesterol-poor membranes was it possible to demonstrate a thermal-phase transition (Rottem et al., 1973a). Differential-scanning calorimetry revealed an endothermic phase transition centered at about 25 °C in membranes of the adapted strain, whereas no transition was discernible in the cholesterol-rich membranes of the native strain. Other techniques, such as fluorescence polarization and freeze fracturing further confirmed these findings.

The experiments, carried out with the cholesterol-poor *M. mycoides* subsp. *Capri,* provided perhaps the first clear-cut evidence with membranes of growing cells to support the hypothesis promoted by Rothman and Engelman (1972) that cholesterol functions as a regulator of membrane fluidity, maintaining an intermediate fluid condition during changes in growth temperature or following alterations in the fatty acid composition of membrane lipids. Why are mycoplasmas the only prokaryotes dependent on cholesterol for growth? One would tend to associate this requirement with the lack of a cell wall in mycoplasmas, which is undoubtedly the single most important property distinguishing the mycoplasmas from all other prokaryotes. It has long been suggested that cholesterol increases the tensile strength of the cell membrane of mycoplasmas, thus facilitating their survival and growth without the protection of a rigid cell wall. It can be argued that the ability of mycoplasmas to incorporate large quantities of cholesterol into their membranes compensates for their inability to regulate membrane fluidity by preferential fatty acid synthesis. How then can some mycoplasmas (the *Acholeplasma* species) grow without cholesterol? The ability of these organisms to synthesize saturated fatty acids (Rottem, 1980) supports the suggestion that the *Acholeplasma* retain at least part of the mechanism for regulating membrane fluidity through changes in the chain length of the synthesized fatty acids. Moreover, *A. laidlawii* was shown to be capable of selective incorporation of exogenous fatty acids to maintain a relatively constant fluidity of membrane lipid. It is not clear yet whether the sterol-requiring *Mycoplasma* species have this ability. Studies carried out with *M. arginini* (Razin et al., 1998) showed that at 37 °C, this organism preferentially incorporated palmitate from a mixture of palmitate and oleate added to the growth medium. In this case, the incorporation of large quantities of cholesterol into the membrane may be necessary to prevent the membrane from becoming too viscous.

3. The polar lipids of mycoplasmas

Mycoplasmas have long resisted detailed analyses because of complex nutritional requirements, poor growth yields, and a paucity of useful genetic tools. A detailed lipid analysis of a variety of *Mycoplasma* species revealed that the lipid fraction contains 35–50% neutral lipids, mainly unesterified cholesterol incorporated from the growth medium, and 50–65% polar lipids (Bittman, 1993; Rottem, 1980). The polar lipids of mycoplasmas, located exclusively in the plasma, are primarily phosphoglycerolipids (Bittman, 1993). The phospholipid (PL) composition is rather simple, comprising of a few *de novo* synthesized PLs, mainly phosphatidylglycerol (PG) and cardiolipin (CL). As mycoplasmas are unable to synthesize or modify long-chain fatty acids, this organism depends on an exogenous supply of fatty acids in the growth medium. Thus, mycoplasmas could be metabolically labeled with [^{3}H] palmitate or [^{3}H] oleate. In addition to the *de novo* synthesized phospholipids, many *Mycoplasma* species incorporate phosphatidylcholine (PC) and sphingomyelin (SPM) from the growth medium (Rottem, 1980). The SPM in all *Mycoplasma* species analyzed so far appears to be incorporated unchanged from the growth medium, whereas the PC in some species is a disaturated PC, differing from the 1-saturated, 2-unsaturated PC found in the growth medium (Rottem and Markowitz, 1979). In these species, the disaturated PC is synthesized by the insertion of a saturated fatty acid at position 2 of lysophosphatidylcholine (lyso-PC), derived from exogenous PC of the growth medium, by what appears to be a deacylation-acylation enzymatic sequence (Rottem and Markowitz, 1979). The ratio of SPM to PC in *mycoplasmas* is higher (0.8–2.6) than that found in the growth medium (∼0.4). Analyzing the SPM to PC ratios reported in various *Mycoplasma* species whose lipids were thoroughly analyzed and genomes were completely sequenced revealed that whereas in several *Mycoplasma* species *such as M. penetrans and M. hyorhinis* the SPM to PC ratio was very high (∼2.6), in *M. fermentans* and *M. gallisepticum,* the ratio was much lower (Kornspan et al., 2014). Interestingly, the genomes of *M. hyorhinis* and *M. penetrans* (GenBank: CP002669.1 and NC_004432.1 respectively), unlike the genome of *M. fermentans* (GenBank: CP001995.1) encode a CL synthetase (GenBank: NP_757669.1) and accordingly possess a high CL to PG ratio in their polar lipid fraction (3.35 and 2.29, respectively). It is tempting to assume that since CL in the presence of divalent cations tends to form inverted hexagonal phase structures and the balance between lipids forming lamellar and hexagonal structures must be kept within certain limits, the increased capacity of CL containing *Mycoplasma* species to incorporate exogenous lipids, mainly SPM, is a consequence of a regulatory attempt aiming to preserve the bilayer stability, maintaining the properties of a permeability barrier.

Most of the genes associated with the classical PG biosynthesis employing CDP-diacylglycerol (Raetz and Dowhan, 1990) were detected in mycoplasmas, whereas the CL synthase gene (*cls*) was detected only in some of the species. Analysis of the 16 *Mycoplasma* species deposited in the GenBank database so far revealed that 10 species contained *cls* and six species did not (Kornspan et al., 2014). Lipid analyses of the *Mycoplasma* species containing *cls* revealed a higher level of exogenously incorporated phospholipids with a preferential incorporation of SPM, whereas lower amounts of