Proc. Natl. Acad. Sci. USA
Vol. 75, No. 9, pp. 4107–4109, September 1978
Biochemistry

Sterol requirement of *Mycoplasma capricolum*

(growth response/sterol structure specificity/membrane structure)

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Contributed by Konrad Bloch, May 22, 1978

ABSTRACT **Mycoplasmas require an external source of sterol for growth. For *Mycoplasma capricolum* this requirement is met not only by cholesterol but also by the methylcholestane derivatives lanosterol, cycloartenol, 4,4-dimethylcholesterol, and 4β-methylcholestanol. Cholesteryl methyl ether and 3α-methylcholestanol serve equally well as sterol supplements. None of the growth-supporting sterol derivatives tested was metabolically modified. The unusual acceptance of diverse cholestane derivatives by a mycoplasma species contrasts with the structural attributes thought to be necessary for sterol function in eukaryotic membranes.**

Mycoplasma species [order Mycoplasmatales; family Mycoplasmataceae (1)] require an external sterol source for growth (2, 3), a nutritional dependence not found elsewhere among prokaryotes. Plant and animal sterols meet this requirement, and so do certain sterol derivatives, provided they contain the cholestane ring system (A/B *trans*), an unsubstituted equatorial hydroxyl group, and a branched aliphatic side chain eight or more carbon atoms in length (4, 5). Similar structural features appear to be necessary for regulatory functions (e.g., solute permeability and viscosity) of sterols in biological or artificial membranes (6). We describe here the growth response of *Mycoplasma capricolum* to a variety of previously untested sterols and biogenetically related substances. Our results show that the "sterol" specificity for *M. capricolum* growth is surprisingly broad.

EXPERIMENTAL

M. capricolum (California Kid strain 14, ATCC 27342) was kindly supplied by M. W. Grabowsky. Cells were grown on modified Edward medium (1). The PPLO-serum fraction was omitted and replaced by fatty acid-poor bovine serum albumin (Sigma, 5 mg/ml). Other additions were palmitic acid (5 μg/ml) and elaidic acid (6.5 μg/ml). Sterols and other test compounds were dissolved in ethanol and added to the growth medium in the quantities indicated in the figure legends. Cells were grown in air in a 37° water bath without shaking. Growth was determined by measuring the optical density of the cultures at 640 nm. Initially the pH values of the culture media were 8.0, they declined with time as a function of cell density. Typically, in a culture grown on 10 μg of cholesterol per ml the optical density rose from 0.04 to 0.42 and the pH fell concurrently to 6.2. In some experiments (not shown) the Difco heart infusion broth, Difco Bacto-peptone, and the Difco yeast extract components of the modified Edward medium were delipidated by extraction with chloroform/methanol, 2:1 by volume. Delipidation did not significantly affect the growth response of mycoplasma to the various test compounds. For isolation of unsaponifiable fractions, cells were harvested at mid to late logarithmic phase by centrifugation at 12,000 × *g* for 15 min at 4°. Resuspended pellets were washed once with 0.25 M NaCl, recentrifuged, and extracted with chloroform/methanol (2:1). Lipid extracts were saponified with 3 M KOH (1 hr at 70), and the nonsaponifiable materials were extracted with diethyl ether. Extracts were analyzed by gas–liquid chromatography on a Perkin–Elmer instrument (model 900) equipped with a 6-foot (1.8-m) column of OV-17 (Supelco) at 260°. All test compounds were checked before use by gas–liquid chromatography and found to be 99% pure. The following compounds were received as gifts: 4β-methylcholestan-3β-ol from T. A. Spencer; 4,4-dimethylcholesterol (4,4-dimethylcholest-5-en-3β-ol) from R. B. Woodward; cycloartenol, euphol, and euphenol from G. Ourisson; and β-amyrin from E. J. Corey. 3α-Methylcholestan-3β-ol (7) and cholesteryl methyl ether (8) were synthesized by A. K. Lala.

RESULTS

M. capricolum, like other mycoplasmas, fails to grow on sterol-deficient media. Cholesterol satisfies this requirement, as it does for all mycoplasma species tested (2, 3). We describe here the growth response of *M. capricolum* to a number of sterols differing structurally from cholesterol in nuclear substitutions and various other details. Test compounds were of several types. One group included lanosterol, 4,4-dimethyl- and 4-monomethylsterols, known intermediates in cholesterol biosynthesis. They were chosen *inter alia* because alkyl-cholestane derivatives comprise the major sterols found in the methanotroph *Methylococcus capsulatus*. This organism is the only prokaryote known with certainty to synthesize sterols (9, 10). *M. capricolum* grew well on media containing the methylcholestane derivatives, though somewhat more slowly than in the presence of cholesterol (Fig. 1*A*). When the unsaponifiable fraction of cells cultured on lanosterol was analyzed by gas–liquid chromatography, only the unchanged sterol was detected. Hence growth in this instance is not due to metabolic demethylation or other modifications of the trimethylcholestane supplement. The lanosterol isomer cycloartenol, the first cyclic precursor of plant sterols (11), was equally effective for mycoplasma growth (Fig. 1*B*). This sterol was also recovered unchanged from mycoplasma cells. On the other hand, *M. capricolum* failed to grow on media supplemented with euphol or euphenol (24,25-dihydroeuphol) (Fig. 1*B*), stereoisomers of lanosterol having opposite configurations at C_{13}, C_{14} (C/D ring fusion) and at C_{17} (side chain attachment). β-Amryin, a representative pentacyclic triterpene, supported mycoplasma growth moderately (Fig. 1*C*). In this fully cyclized squalene derivative, rings A, B, and C are fused normally, i.e., as in cholestane. The acyclic hydrocarbon squalene and squalene 2,3-epoxide did not sustain mycoplasma growth (results not shown).

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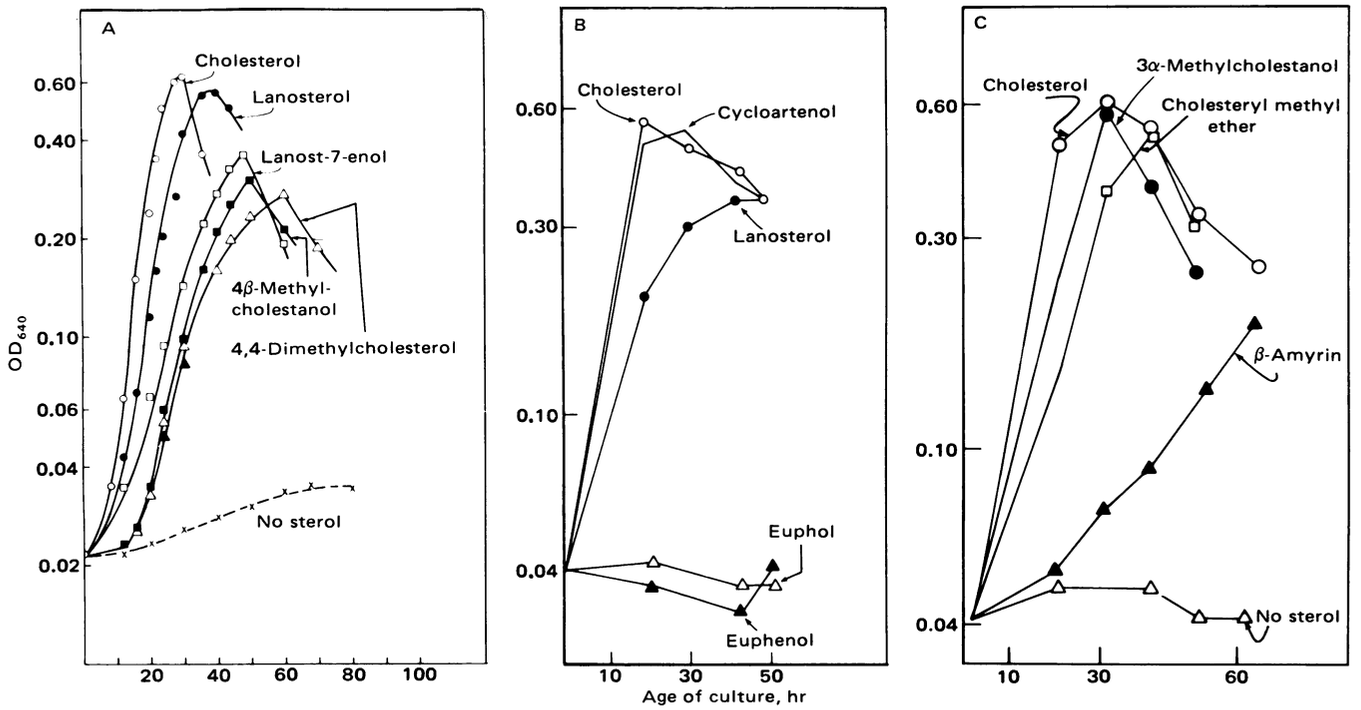

FIG. 1. Growth of *M. capricolum* on various sterols at 37°. Sterol concentrations in the culture media were 5 μg/ml for *A* and 10 μg/ml for *B*. The concentrations of cholesterol, sterol derivatives, and β-amyrin were 10 μg/ml for *C*.

Exploring other structural features commonly regarded as essential for sterol function in membranes, we have tested cholesteryl methyl ether, a sterol lacking a free hydroxyl group. In the presence of the methyl ether derivative cells grew nearly as rapidly and to about the same optical density as on cholesterol (Fig. 1*C*). Unchanged methyl ether was recovered from the cells. Excellent growth was also obtained with 3α-methylcholestanol (Fig. 1*C*). The two last-mentioned cholestane derivatives do not occur in nature.

DISCUSSION

From the examples given it is clear that variously modified cholestane derivatives satisfy the sterol requirement for *M. capricolum* growth. At least three such modifications appear to be tolerated:

(*i*) A free 3-hydroxyl group is not needed; cells grow well on cholesteryl methyl ether. By contrast, eukaryotic membranes contain only free sterols. Moreover, cholesteryl methyl ether is only poorly incorporated into lecithin vesicles (12). It is widely believed (13), though not documented experimentally, that a free hydroxyl group and hence the potential for hydrogen bonding is essential for sterol positioning and functioning in the membrane bilayer. Whether *M. capricolum* cells are exceptional in this regard remains to be investigated.

(*ii*) *M. capricolum* grows well on lanosterol, 4,4-dimethylcholesterol, and a 4-monomethylsterol and leaves these structures intact. On the other hand in eukaryotes, metabolic removal of the methyl substituents at C_4 and C_{14} appears to be mandatory for sterol function in the phospholipid bilayer, judging from the fact that the dominant sterols of animal, plant, and fungal membranes lack these methyl substituents. The nutritional requirements of eukaryotic sterol auxotrophs support this contention. Thus, lanosterol, unlike cholesterol, fails to support larval growth of the sterol-requiring hide-beetle *Dermestes vulpinus* (14). Similarily, lanosterol replaces ergosterol only poorly as a growth factor for anaerobic yeast (15). Most strikingly, a mutant line of Chinese hamster ovary cells that is defective in oxidative demethylation and accumulates lanosterol is not viable; such cells lyse rapidly and die (16).

Molecular models suggest why lanosterol may be unsuitable for eukaryotic membrane function as conventionally assigned to sterols (17). The axial 14α-methyl group of lanosterol is exposed and for that reason may obstruct packing between the otherwise planar α face of the sterol ring system and adjacent phospholipid acyl chains. Such steric interference would explain the observation that lanosterol, unlike cholesterol, changes the glucose permeability of lecithin vesicles only slightly (18). Similarly, the ^{13}C NMR spectra of lanosterol-containing lecithin vesicles indicate that this sterol is much less immobilized in the membrane than cholesterol (19). Thus, lanosterol demethylation involving the removal of bulky α-face substituents can be viewed as beneficial metabolic processes that improve or optimize van der Waals interactions of lipid components in the membrane bilayer. Clearly, such structural modifications are not essential for sterol utilization by the prokaryotic mycoplasma cell. The positive growth response obtained with 3α-methylcholestanol reinforces this conclusion. An axial methyl substituent at C_3 would be expected to have the same weakening effect on hydrophobic interactions as the 14α-methyl group of lanosterol. Both disturb the planarity of the sterol α face. Indeed, 3α-methylcholestanol, when incorporated into lecithin vesicles (18), neither lowers glucose permeability nor raises microviscosity.

(*iii*) The ability to grow on β-amyrin, albeit more slowly, broadens the "sterol" specificity of *M. capricolum* still further. This pentacyclic triterpene lacks the flexible aliphatic side chain characteristic of membrane sterols.

In general, the changes in fluidity or permeability caused by sterols in artificial membranes (liposomes or lecithin vesicles) seem to mirror faithfully the behavior of sterols in membranes of eukaryotic origin (6). For the mycoplasma strain used here, a sterol-requiring prokaryote, such artificial systems appear to be inadequate models. Compounds that are nonfunctional in

membranes by current criteria nonetheless satisfy the "sterol" requirement of a mycoplasma strain. To rationalize this response we suggest, without specifying any physiological consequences, that insertion of bulky polycyclic squalene derivatives may separate otherwise interacting phospholipid molecules without necessarily altering membrane fluidity. In accord with this hypothesis, NMR spectroscopy reveals that lanosterol, for example, although relatively mobile in lecithin bilayers, nevertheless causes separation of phospholipid head groups, as does cholesterol (19). Such spatial effects caused by insertion of bulky and rigid molecules may represent a more primitive and perhaps the only significant function of polycyclic squalene derivatives in certain prokaryotic membranes. In this regard we note that pentacyclic terpenes of the hopane series have been isolated from a halophilic bacterium (20) and from *Acetobacter* species (21, 22). This suggests a wider occurrence than hitherto supposed of squalene-derived cyclization products in prokaryotes.

We thank Krishna Lala for technical assistance and Dr. A. K. Lala for some gas–liquid chromatography analyses and helpful discussions. This work was supported by grants from the National Institutes of Health, the National Science Foundation, and the Eugene Higgins Fund of Harvard University.

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