GROWTH INHIBITION OF HALOBACTERIUM CUTIRUBRUM BY CERULENIN, A POTENT INHIBITOR OF FATTY ACID SYNTHESIS

Craig Dees and James D. Oliver

From the Department of Biology, University of North Carolina at Charlotte
Charlotte, N. C. 28223

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SUMMARY: The polar lipids of Halobacterium cutirubrum are known to consist exclusively of diether derivatives of glycerol, and do not contain fatty acids. However, cerulenin, a specific and potent inhibitor of fatty acid synthesis, was shown to inhibit the growth of this organism. Protection from growth inhibition was demonstrated when fatty acids of 18 carbons were added to the growth medium, but not when palmitic or palmitoleic acids were used. Cerulenin appears to affect synthesis of all polar lipids in this organism while relative levels of protein and nucleic acids were not significantly affected. Growth inhibition by cerulenin supports the conclusion that the fatty acid synthetase system present in H. cutirubrum is necessary for lipid biosynthesis, despite the fact that fatty acids are not structural components of the lipids of this bacterium. A pathway is proposed to account for these observations.

INTRODUCTION

The extremely halophilic bacteria are a novel group of organisms characterized by their requirement of high salt concentrations for growth and viability (17). The internal salt concentrations for some ions are as much as 1500 times that found in the growth medium. Potassium, for example, has been shown to be 4.57 M inside the cell, while at only 0.03 M in the medium (7). These bacteria are also unusual in that they have been shown to possess novel polar lipids. Kates (12) has shown the lipid phosphatides of Halobacterium cutirubrum to consist exclusively of diether derivatives of sn-1-glycerol phosphate, in contrast to the diacyl derivatives of sn-3-glycerol phosphate found in other bacteria. Since fatty acids are not structural components of the polar lipids, the finding of only trace amounts of fatty acids in these bacteria was not unexpected (18). A functioning fatty acid synthetase system has been demonstrated in H. cutirubrum, although the enzymes are inhibited by the high concentrations of sodium and potassium found in these cells (16). It has been suggested that this inhibition of the

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fatty acid synthetase system is evidence that H. cutirubrum evolved from an organism with much lower salt requirements (3).

To date, no studies have been made on the role of the fatty acid synthetase system in the halobacteria. The antibiotic cerulenin, a potent and specific inhibitor of this enzyme complex (5,8,15,20), was used to clarify the importance of fatty acid biosynthesis to membrane lipid biosynthesis in H. cutirubrum.

MATERIALS AND METHODS

Culture Conditions

H. cutirubrum (obtained from Morris Kates, University of Ottawa) was grown in a complex medium (17) at 39°C with vigorous shaking and no light. Cerulen (Makor Chemicals, Jerusalem, Israel) was made up as a stock solution of 2 mg/ml as described by Wille et al. (20). Aliquots of the antibiotic were added to cultures in the early log phase of growth. Fatty acids were emulsified in Tween 40 (Sigma Chemicals, St. Louis, Mo.).

Analytical Procedures

Growth was monitored through optical density determinations with a Bausch and Lomb Spectronic 20 spectrophotometer. All colorimetric determinations were made with a Beckman Model 25 spectrophotometer.

Synthesis of nucleic acids was determined by the incorporation of [^{14}C]uracil and [^{3}H]thymidine (ICN, Irvine, Calif.) Nucleic acids were extracted from aliquots of cells taken at various time intervals during log phase growth by methods described previously (13). Scintillation counting was performed on a Picker Nuclear Liquimat 220. Protein was determined by the method of Lowry et al. (14) on the pellet left after extraction of nucleic acids. Lipids were extracted essentially by the method of Bligh and Dyer (2). Total lipid phosphorus was assayed according to Ames and Dubin (1) and lipid carbohydrate by the method of Dubois et al. (6). Synthesis of lipids was also monitored through the incorporation of [^{14}C]glycerol (ICN) into lipid structures. Total activity in the extracted lipids was determined by taking an appropriate aliquot for scintillation counting. The remaining sample was fractionated by thin-layer chromatography using chloroform: 90% acetic acid: methanol (30:20:4, v/v) as the driving solvent. Spots were visualized by autoradiography and then removed to vials for scintillation counting.

RESULTS

H. cutirubrum exhibited a generation time averaging 6.5 hours when grown in the complex medium. Cerulenin, when added to early log phase cultures, markedly inhibited the growth of H. cutirubrum (Figure 1). Inhibition of growth was seen to increase in proportion to the amount of antibiotic in the culture medium. Similarly, the time required for the effects of the

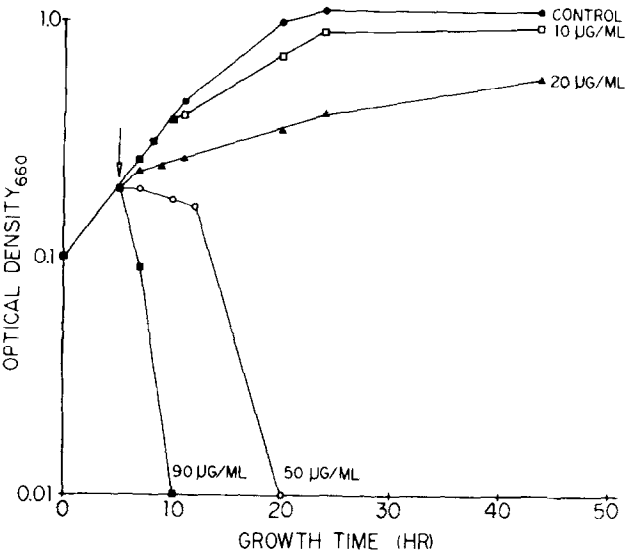

Figure 1. Growth inhibition of H. cutirubrum by various concentrations of cerulenin. Point of addition of antibiotic is indicated by the arrow.

antibiotic to be noted by optical density measurements was dependent on the amount of antibiotic. At the highest concentrations of cerulenin examined (50 and 90 µg/ml), cell lysis was evident. At lower concentrations, the effects were not observed until 5 hours post addition of the antibiotic. The delay in growth inhibition seen at the lower concentrations corresponds to approximately one generation time.

In an attempt to elucidate the specific mechanism of growth inhibition by cerulenin in H. cutirubrum, the relative amounts of certain cell components in cerulenin-treated cultures were measured and compared to those found in control cultures during log phase growth. When cerulenin was added to the culture medium at a concentration of 10 µg/ml, optical densities of the cerulenin and control cultures were equal for approximately one generation (Figure 1), indicating the number of cells present in each culture was approximately equal during this time period. Relative levels of protein, nucleic acids, and lipid could thus be compared through this interval. The levels of

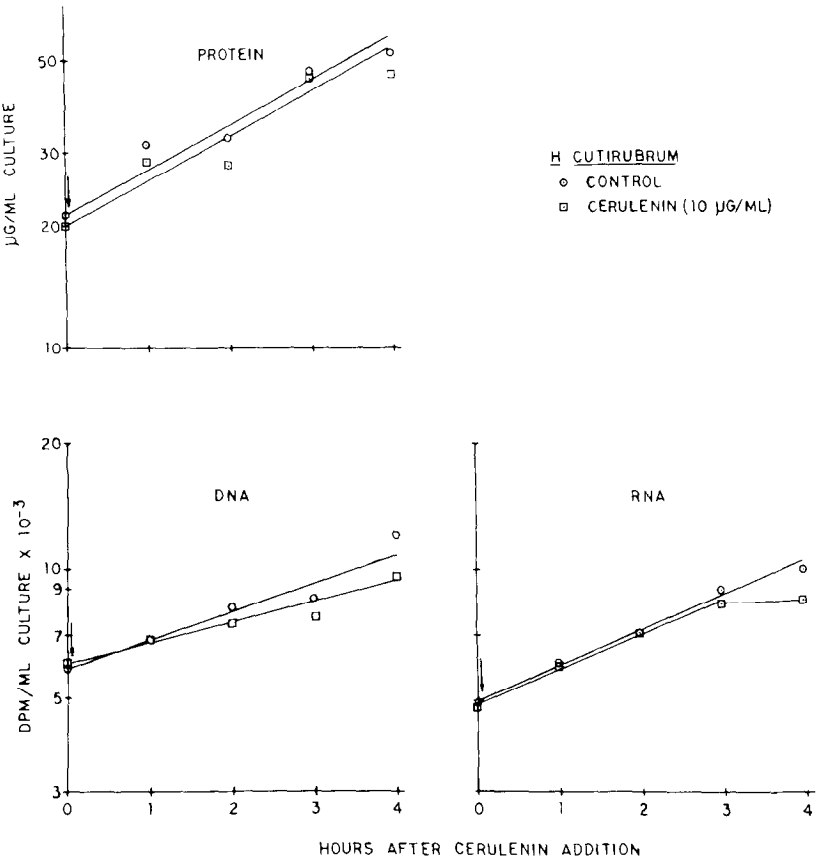

Figure 2. Effect of cerulenin addition (arrows) on levels of protein, DNA and RNA.

protein, DNA, and RNA during the first four hours after cerulenin addition were found to be relatively unchanged (Figure 2). However, a dramatic and immediate decrease in level of lipid phosphorus, followed by a decrease in lipid carbohydrate, was noted after the addition of cerulenin (Figure 3). These observations were confirmed through the addition of [^{14}C]glycerol to early log cultures. Figure 4 shows that the uptake of [^{14}C]glycerol into total lipids of cerulenin-treated cultures ceases approximately 1 hour after antibiotic addition. The activity found in the various lipid fractions after thin-layer chromatography did not differ significantly from those found in control cells, nor did any lipid component appear to be selectively affected. This might be expected as

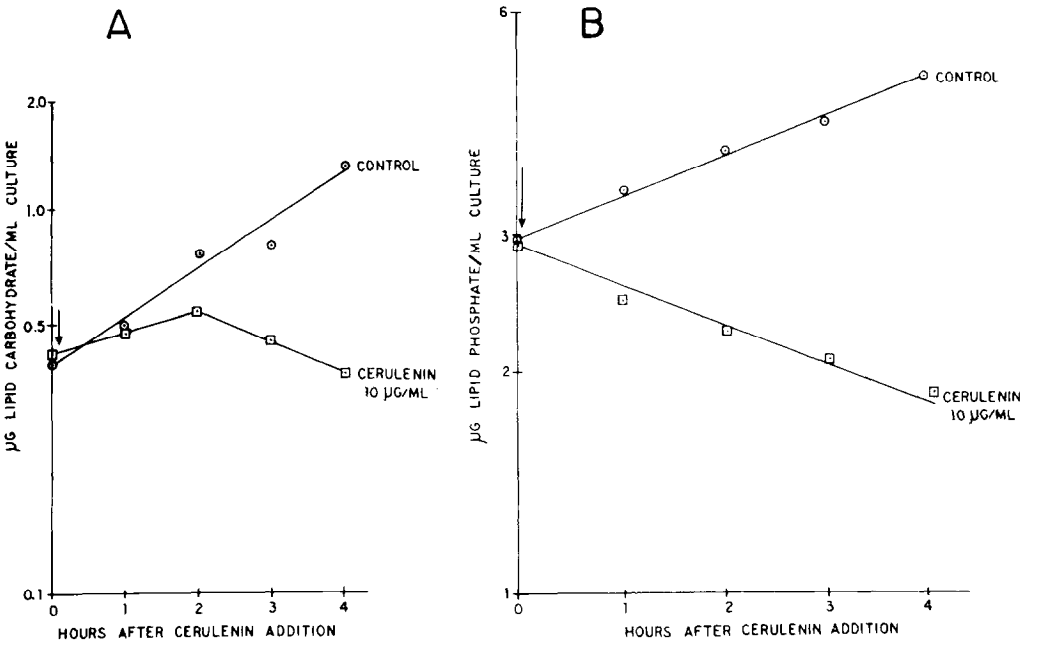

Figure 3. Effect of cerulenin addition (arrows) on levels of lipid carbohydrate (A) and lipid phosphate (B)

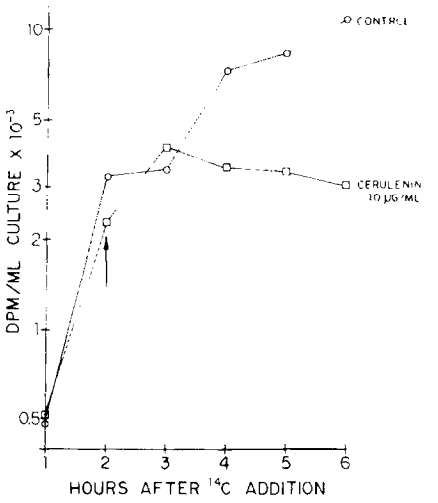

Figure 4. Effect of cerulenin addition (arrow) on incorporation of [^{14}C] glycerol into total lipids of H. cutirubrum. Labeled glycerol was added at 0.5 μCi/ml culture 2 hours prior to cerulenin addition.

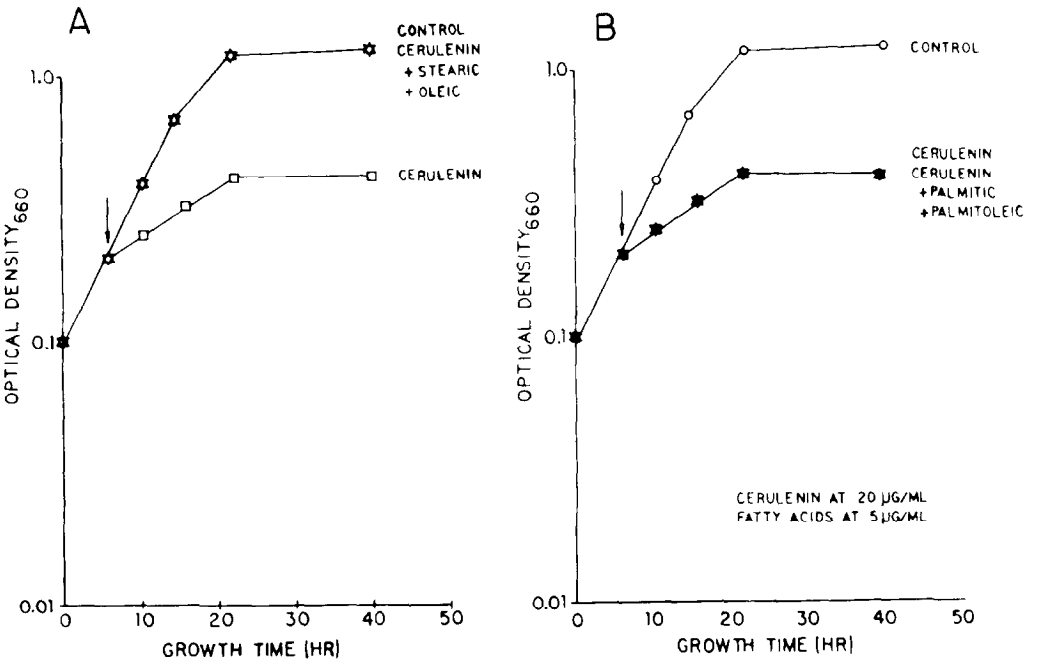

Figure 5. Protection against cerulenin-induced growth inhibition by fatty acids. Cerulenin was added (arrows) at a concentration of 20 µg/ml, and fatty acids at 5 µg/ml. In each case control cultures contained the corresponding fatty acids and emulsifier. (A) Effect of C18 fatty acids. (B) Effect of C16 fatty acids.

the duration of this portion of the study corresponded to less than one cell doubling.

Various fatty acids were added to the culture medium concurrent with the addition of cerulenin in an attempt to prevent growth inhibition by the antibiotic. Figure 5 shows that fatty acids of chain length 18 added at a concentration of 5 µg/ml of culture medium protected the cells from cerulenin inhibition. Fatty acids of chain length 16 did not provide protection at the same concentration. The presence of a double bond in the fatty acid did not influence the chain length effect. Palmitic acid (99% pure) at 100 µg/ml of culture provided partial protection, although this is likely due to contamination with fatty acids of chain length 18.

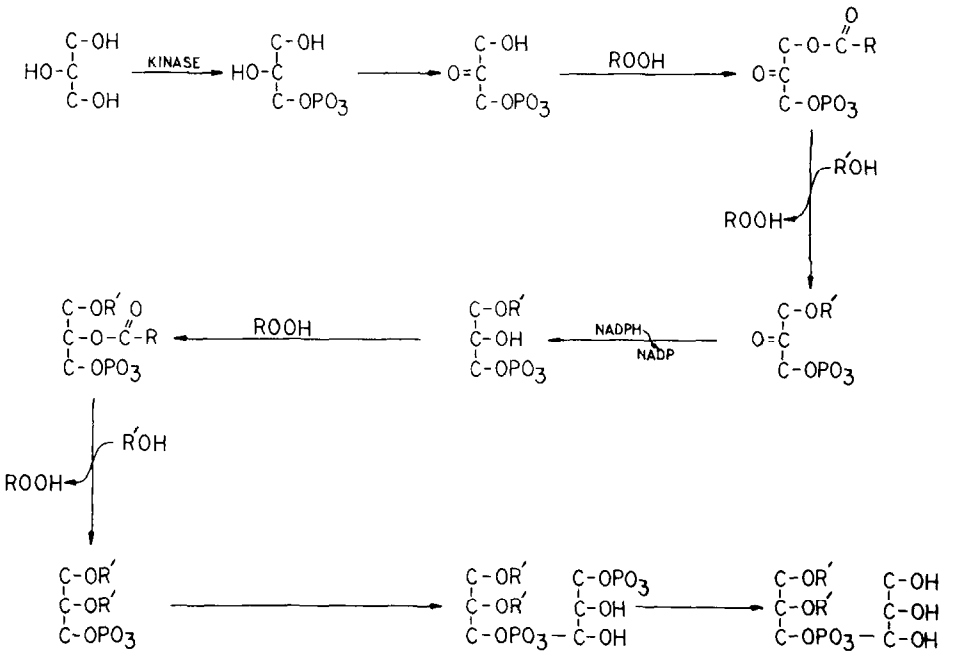

Figure 6. Proposed pathway for the biosynthesis of the di-O-alkyl phospholipids occurring in H. cutirubrum. See text for details.

DISCUSSION

We conclude from these studies that cerulenin is acting directly on the fatty acid synthetase complex in H. cutirubrum. Both de novo biosynthesis, as well as chain elongation, appear to be affected. Such a finding is consistent with the known activity of the antibiotic in numerous other organisms (5,8,15,20). We also conclude that a functioning fatty acid synthetase system is essential for the biosynthesis of lipids in H. cutirubrum, despite the fact fatty acids are not structural components of the lipids found in this bacterium. That the fatty acid synthetase system is greatly inhibited by the high salt concentrations existing intracellularly does not pose a problem, in that only trace levels of fatty acids appear to be required for lipid biosynthesis. We postulate, therefore, that before an ether bond can be formed, a fatty acid of 18 carbons must be esterified to the glycerol backbone. Fatty acids of 16 carbons are not effective since they are unable

to reverse cerulenin inhibition of growth. The esterfied fatty acid would be replaced by the long chain fatty alcohol phytanol, or its pyrophosphate derivative, for the formation of the diether found in this bacterium. Such a pathway is similar to that reported in neoplastic cells by Snyder (19) and Hajra (10,11) in which a monoacylated precursor reacts with a long chain fatty alcohol in the formation of a monoether lipid. This sequence of events would likely be repeated at the C-2 position, possibly with a stereochemical reduction of the keto group at C-2 to provide the sn-1-glycerol phosphate configuration found in H. cutirubrum (Figure 6).

It is also of interest to note that cell growth continues after the addition of cerulenin at low levels, while lipid synthesis is rapidly shut down. Cell division, or at least cell enlargement, appears to continue for approximately 6 hours (one generation). Further studies are currently underway in our laboratory to examine the morphology of these cells after cessation of lipid synthesis, and to characterize the acyl intermediate we postulate to exist transiently prior to the formation of the novel dialkyl ether lipids in H. cutirubrum.

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