INHIBITION OF FATTY ACID SYNTHESIS BY THE ANTIBIOTIC THIOLACTOMYCIN

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The antibiotic thiolactomycin inhibits the growth of *Escherichia coli* K-12 and *Pseudomonas aeruginosa* 507 (β-lactam supersensitive mutant). A micrograph of *E. coli* cells, which were grown at a sublethal concentration of thiolactomycin (20 μg/ml), revealed the morphological change with cell elongation. The effects of the antibiotic on syntheses of cellular constituents were studied by measuring the incorporation of labeled precursors into lipids and macromolecules. This antibiotic preferentially inhibited the incorporation of [^{14}C]acetate into fatty acids and lipids. Addition of both palmitate and oleate, but not of either fatty acid alone, reversed the growth inhibition of *P. aeruginosa* by thiolactomycin. These findings support the conclusion that the effects of thiolactomycin are due to a specific inhibition of fatty acid synthetase.

Thiolactomycin, an antibiotic of the structure of (4*S*)-(2*E*,5*E*)-2,4,6-trimethyl-3-hydroxy-2,5,7-octatriene-4-thiolide,[1,2] is active *in vitro* against many species of pathogens including Gram-positive, Gram-negative and anaerobic bacteria.[3,4] Its low toxicity in mice prompted us to investigate the selective action between bacteria and animal tissues. Recently we reported that this antibiotic inhibits fatty acid synthetase of *Escherichia coli* but has little effect on that of *Saccharomyces cerevisiae*, *Candida albicans* and rat liver.[5] Furthermore, we have surveyed the effect of thiolactomycin on fatty acid synthetases from various sources.[6] These results suggested that this antibiotic selectively inhibits type II fatty acid synthetases. The present paper reports that thiolactomycin, when inhibiting the growth of bacteria, blocks fatty acid synthesis in intact cells.

Materials and Methods

Materials

Thiolactomycin was prepared from culture fluid of *Nocardia* sp. No. 2-200.[1] Fresh ethanol solutions of the drug was prepared immediately before conducting each experiment. The final concentration of ethanol was always kept lower than 0.1%, and the same amount of ethanol was added to the control groups. [*Methyl*-^{3}H]thymidine (40 Ci/mmol), [5-^{3}H]uridine (29.7 Ci/mmol), [4,5-^{3}H]-leucine (50 Ci/mmol), *N*-acetyl D-[1-^{3}H]glucosamine (3 Ci/mmol) and [1-^{14}C]acetate (56.7 mCi/mmol) were purchased from the Amersham Co., Ltd. [^{3}H]Thymidine and *N*-acetyl D-[^{3}H]glucosamine were diluted with the corresponding non-labeled compound to 2.3 Ci/mmol and 1.3 Ci/mmol, respectively. [^{3}H]Uridine, [^{3}H]leucine and [^{14}C]acetate were added undiluted to the growing culture.

Growth of Cells

E. coli K-12 (strain YA 21) and *Pseudomonas aeruginosa* 507 (β-lactam supersensitive mutant) were

grown at 37°C in a nutrient medium consisting of beef extracts 0.7%, peptone 1.0% and NaCl 0.3%, pH 7.0. Starter cultures (5 ml in L-tubes) were grown from slants, and after 16 to 24 hours, inoculations (0.2~0.6 ml) were made into 10 to 30 ml of the nutrient medium. When necessary, the nutrient medium was supplemented with Triton X-100 (0.3%), palmitate and oleate (0.01% each). Fatty acids were added to the culture in the form of potassium salt solutions of oleate and palmitate in slightly alkaline aqueous ethanol (1: 3). Cell growth was followed by measuring the absorbance at either 620 nm or 550 nm.

Incorporation of [^{3}H]Thymidine, [^{3}H]Uridine, [^{3}H]Leucine and *N*-Acetyl D-[^{3}H]Glucosamine into Cellular Macromolecules

An overnight culture (0.2 ml) was transferred to 2.0 ml of the fresh medium, and the mixture was diluted with distilled water until the total volume of solution was 10 ml. When the culture density reached 0.1 at 550 nm, 10 μCi of radioactive precursors and thiolactomycin were added to the culture. At different times samples of cells (0.5 ml) were taken into cooled tubes containing 5 ml of 10% trichloroacetic acid and acid precipitable materials were collected on Whatman GF/C glass fiber disks. After washing with 5% trichloroacetic acid, followed by ethanol, the radioactivity on disks were counted in vials containing 10 ml of 2,5-diphenyloxazole (PPO)-toluene (4 g/liter) in a Packard Tri-Carb liquid scintillation counter.

Incorporation of [^{14}C]Acetate into Lipids and Fatty Acids

An overnight culture (0.2 ml) was transferred to 2.0 ml of the fresh medium, and the mixture was diluted with distilled water to the total volume of 10 ml as described previously. When the culture density reached 0.1 at 550 nm, [^{14}C]acetate (10 μCi) and thiolactomycin were added to the culture. After different times of growth, 0.5 ml-aliquots of the culture were put into cooled tubes containing 5 ml of 10% trichloroacetic acid, and cells were collected on Whatman GF/C glass fiber disks. After washing twice with 5% trichloroacetic acid, lipids were extracted from the cells with chloroform-methanol (2: 1). The chloroform-methanol extracts were evaporated to dryness, and the radioactivity was measured with a liquid scintillation counter. The lipid fraction was saponified with 15% KOH in methanol at 70°C for 1 hour, and the fatty acids were extracted with *n*-hexane after the mixture was acidified to pH 1 with 6 N HCl.

Results

Inhibition of Growth by Thiolactomycin

When thiolactomycin was added at zero time to the cultures of *E. coli* and *P. aeruginosa* cells, the growth was inhibited to a degree which depended on the concentration of the antibiotic in the growth medium (Fig. 1). The growth of *E. coli* was decreased by about 50% at a final thiolactomycin concentration of 20 μg/ml (Fig. 1A). *P. aeruginosa* was more sensitive than *E. coli*. The growth of *P. aeruginosa* was almost completely inhibited by a final thiolactomycin concentration of 0.5 μg/ml (Fig. 1B). A micrograph of *E. coli* cells which were grown at a sublethal concentration of thiolactomycin (20 μg/ml) revealed the morphological change with cell elongation (Fig. 2). These results suggested that it is impossible to build up a complete septum for cell division.

Thiolactomycin Effect on Synthesis of Cellular Constituents

The effect of thiolactomycin on DNA, RNA, protein, peptidoglycan and lipid syntheses was studied in order to ascertain whether the antibiotic interfered with any of these processes. An exponentially growing culture of *E. coli* was transferred to fresh medium and thiolactomycin and a labeled precursor were added when culture density reached 0.1 absorbance at 550 nm. Thiolactomycin (80 μg/ml, final concentration) inhibited the incorporation of [^{14}C]acetate into the total lipid and fatty acids by more than 95% as compared with the control (Fig. 3E and F). Under the same

Fig. 1. Effect of different concentrations of thiolactomycin on the growth of *E. coli* K-12 (A) and *P. aeruginosa* 507 (B).

A: ● 0 μg/ml, ▲ 20 μg/ml, ○ 50 μg/ml, □ 100 μg/ml. B: ● 0 μg/ml, ▲ 0.2 μg/ml, ○ 0.5 μg/ml.

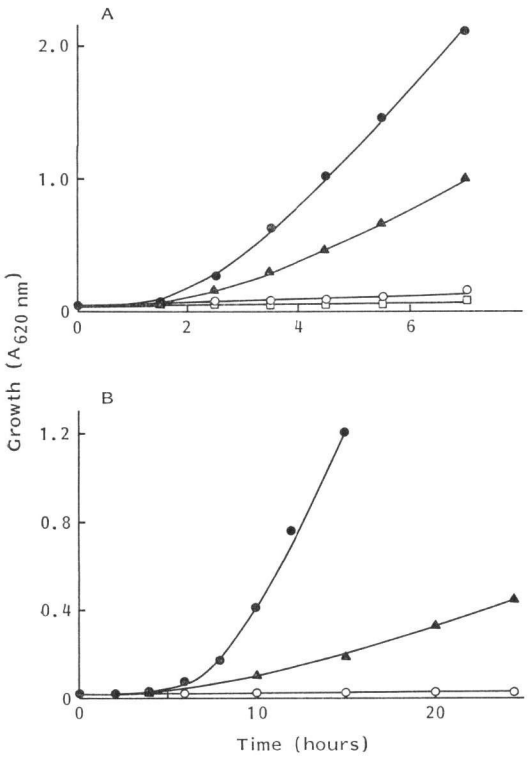


Table 1. Effect of fatty acid supplementation on the incorporation of [1-^{14}C]acetate into cellular fatty acids in the presence of thiolactomycin.

Supplement	Radioactivity in fatty acids (cpm)
None	12,200
Thiolactomycin	250
Palmitate and oleate	1,900
Palmitate, oleate and thiolactomycin	180

P. aeruginosa 507 was grown as described under Experimental. [^{14}C]Acetate (20 μCi) was added to the 12-hour culture (10 ml) and incorporation of radioactivity into fatty acids were measured after 1 hour of incubation. Thiolactomycin (0.5 μg/ml), palmitate (100 μg/ml) and oleate (100 μg/ml) were supplemented as indicated.

conditions, DNA, protein and peptidoglycan synthesis were reduced to 29~38% of the control levels and RNA synthesis was reduced to about 10% of the control level (Fig. 3A~D). Using an exponentially growing culture of *P. aeruginosa*, the effect of thiolactomycin on the incorporations of the labeled precursors into cellular constituents was also studied. These inhibition profiles were similar to the profiles which were obtained with *E. coli*. These results suggest that thiolactomycin primarily inhibits fatty acid synthesis.

Effect of Exogenous Fatty Acids on Thiolactomycin Inhibition

The inhibitory effects of thiolactomycin on growth of *P. aeruginosa* were reversed by supplimentation of cultures with palmitate and oleate (Fig. 4). Both palmitate and oleate were necessary for reversal of thiolactomycin inhibition. Addition of either fatty acid alone did not prevent the inhibitory

Fig. 2. Morphological change in *E. coli* K-12 caused by thiolactomycin.

E. coli K-12 was grown in the nutrient medium for 3 hours without thiolactomycin (A) and with 20 μg/ml thiolactomycin (B).

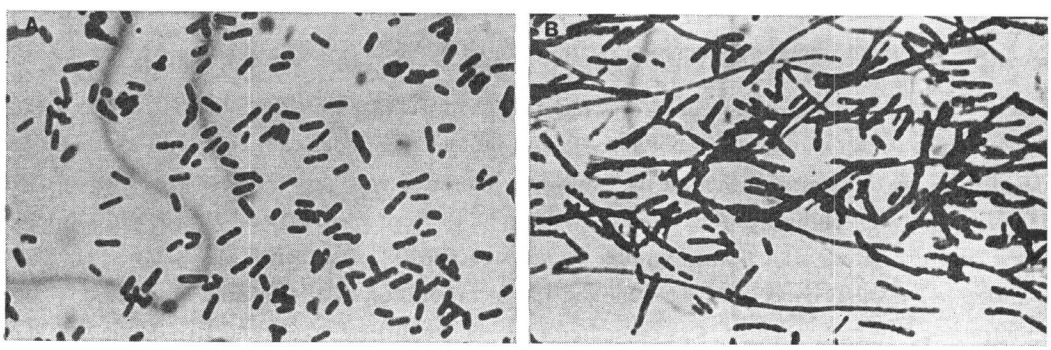
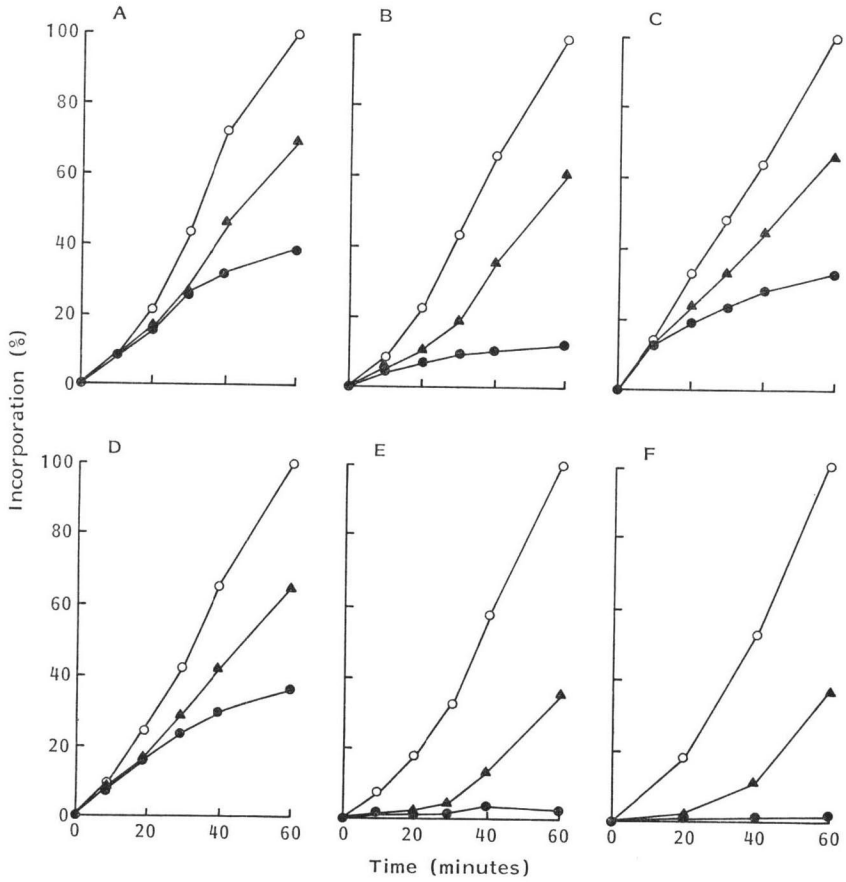


Fig. 3. Effect of thiolactomycin on synthesis of cellular constituents.

A: Incorporation of [^{3}H]thymidine into DNA. B: Incorporation of [^{3}H]uridine into RNA. C: Incorporation of [^{3}H]leucine into protein. D: Incorporation of *N*-acetyl [^{3}H]glucosamine into peptidoglycan. E: Incorporation of [^{14}C]acetate into lipid. F: Incorporation of [^{14}C]acetate into fatty acids.

○ Control; ▲ 10 μg/ml thiolactomycin; ● 80 μg/ml thiolactomycin.

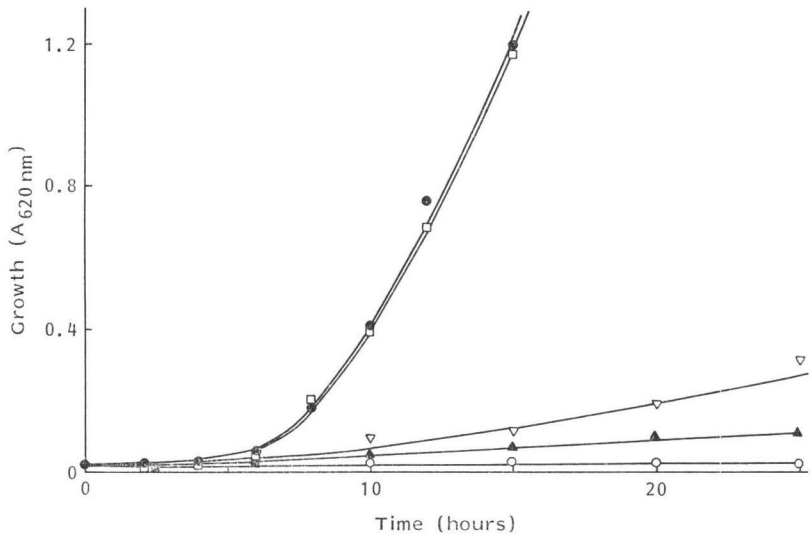


effect. When thiolactomycin inhibition was reversed by the addition of palmitate and oleate, virtually no incorporation of [^{14}C]acetate added to the medium into cellular fatty acids was observed (Table 1). Therefore, the relief of thiolactomycin inhibition by the combination of palmitate and oleate does not restore endogenous fatty acid synthesis and the possibility can be ruled out that the exogenous fatty acids prevents thiolactomycin from entering cell.

Discussion

The earlier *in vitro* studies[5,6] suggested that thiolactomycin inhibits type II fatty acid synthetases. The experiments in this paper were intended to ascertain that the fatty acid synthesis is a principal target of thiolactomycin *in vivo*. The time course of inhibition of various biosynthetic processes indicates that the fatty acid synthesis is primarily inhibited by this antibiotic. The supplement of both palmitate and oleate restores the thiolactomycin-inhibited growth of *P. aeruginosa*, whereas palmitate or oleate alone fails to overcome thiolactomycin inhibition. The ability of a given nutrient to reverse the inhibitory effects of an antibiotic is good evidence that the antibiotic blocks the cellular synthesis

Fig. 4. Reversibility of thiolactomycin inhibition in *P. aeruginosa* 507 by fatty acids.
Thiolactomycin 0.5 μg/ml, palmitate 100 μg/ml and oleate 100 μg/ml.
● Control; ○ + thiolactomycin; ▽ + thiolactomycin, palmitate; ▲ + thiolactomycin, oleate; □ + thiolactomycin, palmitate, oleate.



of the substance that overcomes the inhibition. The experiments shown in Table 1 were designed to know whether the reversal of the antibiotic effect by palmitate and oleate was due to utilization of the exogenous fatty acids for intracellular lipid synthesis or whether the fatty acids in the medium prevented the entry of the antibiotic into the cell. In the latter case, fatty acid synthesis from acetate might have continued in cells exposed to thiolactomycin. The results in Table 1 suggested that in cells exposed to thiolactomycin intracellular fatty acid synthesis from acetate is suppressed either in the presence or absence of exogenous fatty acids. In a control experiment without thiolactomycin, a marked reduction of cellular fatty acid synthesis from acetate was observed when the medium was supplemented with palmitate and oleate. This effect may be due to feedback inhibition of fatty acid synthesis by the fatty acids added to the medium. These results imply that fatty acid synthesis in *P. aeruginosa* is the principal target of thiolactomycin.

We also examined the relief of the growth inhibition of *E. coli* by the addition of palmitate and oleate to the medium. Unexpectedly, supplement of both palmitate and oleate did not reverse thiolactomycin inhibition. It should be noted that relatively high concentration of thiolactomycin (50 μg/ml) was necessary to obtain about 90% growth inhibition of *E. coli*. On the other hand, about 2.0 μg/ml thiolactomycin is enough to cause 90% inhibition of *E. coli* fatty acid synthetase *in vitro*.[5] Therefore, it is likely that such high concentration of the antibiotic causes some secondary effect except for that on fatty acid synthesis. However, the details remained unresolved.

Cerulenin is known to be a potent inhibitor of fatty acid synthetases isolated from various organisms[7] with the exception of the synthetase from a cerulenin-producing fungus, *Cephalosporium caerulens*.[8] This antibiotic, when inhibiting the growth of *E. coli*, specifically blocks fatty acid synthesis in this organism.[9] Cerulenin specifically inhibits the activity of β-ketoacyl thioester synthetase (condensing enzyme).[7] The action mechanism of thiolactomycin seems to be different from that of cerulenin. Thiolactomycin selectively inhibits the type II fatty acid synthetases[5,6] and type I synthetases are insensitive to this antibiotic.

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