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Effect of Cerulenin on *Streptococcus faecalis* Macromolecular Synthesis and Cell Division

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The antibiotic cerulenin has been used to study macromolecular synthesis and cell division in *Streptococcus faecalis*. The data suggest that lipid and lipoteichoic acid synthesis as well as cell number increase are affected prior to any observable effects on overall mass increase or DNA, RNA, protein, or peptidoglycan synthesis. Treatment with cerulenin of cultures growing at various rates and analysis of the subsequent cell divisions indicate that the antibiotic may block a cell cycle event that precedes the completion of chromosome replication by about 10 min.

Cleveland et al. (4, 5) have shown that autolytic activity in *Streptococcus faecalis* is inhibited both in cell-free extracts and in intact cells by lipoteichoic acid (LTA) as well as by certain native lipids. This suggests that certain lipids and/or LTA may function in the directional regulation of wall synthesis necessary for septum formation during the normal growth and division cycle of these streptococci (7, 8). It has been proposed by Shockman et al. (15) that such regulation would occur through inhibition of autolytic activity associated with chromosome termination, i.e., during the final 25 to 30 min before division of *S. faecalis*.

We have employed the antibiotic cerulenin, an inhibitor of fatty acid synthesis in bacteria (19), to block the synthesis of lipids and LTA in *S. faecalis* ATCC 9790 in an effort to delineate their functions in the divisional process of these bacteria.

MATERIALS AND METHODS

Growth of cells. *S. faecalis* ATCC 9790 was grown in a chemically defined medium (14) at 37°C. Culture turbidity was followed spectrophotometrically on a Coleman spectrophotometer model 14 at 675 nm. The absorbance was corrected for deviations from linearity (18), yielding adjusted optical density units (AOD). Cultures were maintained in balanced growth for at least 8 to 10 mass doublings before an experiment.

Determination of cell numbers. Culture samples (0.1 ml) were fixed for 30 min on ice in 0.4 ml of 7.4% formaldehyde in distilled water. Dilutions for counting were made with 0.9% NaCl that had been previously filtered through a nitrocellulose filter (0.22-μm pore size, 47-mm diameter; Millipore Corp., Bedford, Mass.). Cell numbers were obtained with an Electrozone/Celloscope particle counter (30-μm-diameter orifice; Particle Data Corp., Elmhurst, Ill.).

Determination of macromolecular synthesis. Cultures were grown for at least five to seven generations in the presence of [^{14}C]- or [^{3}H]glycerol (0.5 μCi/ml, 8 μg/ml), [^{14}C]thymidine (1 μCi/ml, 15 μg/ml), [^{3}H]lysine (0.5 μCi/ml, 30 μg/ml), or [^{14}C]uracil (0.5 μCi/ml, 30 μg/ml) before sampling. Isotopes were purchased from New England Nuclear Corp., Boston, Mass. Total trichloroacetic acid-precipitable isotope incorporation was determined by placing 0.5-ml samples in 4.5 ml of 10% trichloroacetic acid on ice for a minimum of 30 min, filtering through a Reeve-Angel 984-H glass-fiber filter (Hurlbert Paper Co., South Lee, Mass.), and washing the filters twice with 2 ml of ice-cold 10% trichloroacetic acid and once with 2 ml of 95% ethanol. The filters were treated with 0.5 ml of a 90% solubilizer solution (NCS; Amersham/Searle, Arlington Heights, Ill.) for 2 h at 55°C, and 5 ml of a toluene base scintillator was added to each vial. All radioactive samples were counted in a Mark I liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.), and disintegrations per minute were calculated by using an external standard. The efficiencies were approximately 60% for ^{14}C and 25% for ^{3}H. In dual-label experiments, data were corrected for overlap of ^{14}C into the ^{3}H counting channel (10 to 15%) by using external standard channel ratios for each sample.

For LTA determination, 2-ml samples of glycerol-labeled cultures were washed twice with 5 ml of cold medium containing 80 μg of unlabeled glycerol per ml and then centrifuged at 2,000 rpm for 15 min. The pellet was extracted with 2 ml of 45% phenol at 65 to 70°C for 30 min by the procedure of Wicken and Knox (21). The phases were separated, and the organic phase was washed once again with 1 ml of doubly distilled water; the aqueous phases were then pooled. To 0.2 ml of this aqueous extract was added 5 ml of Formula 947 (New England Nuclear Corp.), an aqueous base scintillator for counting.

Lipid content was estimated in glycerol-labeled cultures by extraction of a lyophilized culture sample (0.5 ml) with 1 ml of methanol at either 65 or 95°C

for 30 min in tightly capped, screw-cap glass tubes with Teflon inserts by a modification of the procedure of Toennies et al. (17). The tubes were then cooled, 2 ml of chloroform and 0.6 ml of 2 M KCl were added, and the tubes were stirred overnight. On the next day, the tubes were centrifuged to separate the aqueous and organic phases, and the upper aqueous phase was removed with a Pasteur pipette and discarded. A 1-ml amount of methanol-water (1:1, vol/vol) was added to the organic phase as a wash. This mixture was agitated for an additional 2 h at room temperature and centrifuged to separate the phases, and the aqueous phase was removed and discarded. A 1-ml amount of this chloroform extract was placed in a scintillation vial and gently heated to evaporate the chloroform. The residue was digested in 0.5 ml of 90% solubilizer as described above, and, finally, 5 ml of a toluene base scintillator was added before counting.

The amount of [^{3}H]lysine incorporated into peptidoglycan was determined in 0.5-ml samples by the method of Boothby et al. (2).

Treatments with cerulenin and mitomycin C. Cerulenin was the generous gift of S. Omura. The antibiotic was dissolved in 95% ethanol to a concentration of 1 mg/ml. A second batch of cerulenin was commercially obtained from Makor Chemicals, Ltd., Jerusalem, Israel. A stock solution (10 mg/ml in 95% ethanol) was prepared and stored at −70°C. Working stocks (1 mg/ml) were prepared and stored as above. Exponentially growing cultures (100 to 300 AOD_{675}) were treated with the antibiotic, or an equivalent volume of 95% ethanol was added to the controls (either 0.002 or 0.005 ml/ml of culture). Mitomycin C (Nutritional Biochemical Corp., Cleveland, Ohio) was added to cultures to a final concentration of 0.5 μg/ml.

RESULTS

Exponentially growing cultures were treated with concentrations of cerulenin from 1 to 8 μg/ml, and both cell number and culture turbidity were measured. Figure 1A shows the data for both cell number and culture turbidity expressed as the relative increase from the time of addition of cerulenin to up to 75 min after the addition. The data suggest that at a concentration of 2 to at least 8 μg/ml, cell numbers were more profoundly affected than was culture turbidity. The effect of the antibiotic on cell division processes was maximal at 5 μg/ml, as evidenced by a flattening out of the curve beyond this point. Concentrations of 2 μg/ml, the lowest concentration affecting cell growth significantly, and 5 μg/ml, the lowest concentration giving maximal effects on cell numbers, were chosen for further study.

Antibiotic effects on lipid and LTA synthesis. Pieringer and Ambron (12) have shown that radioactively labeled glycerol is incorporated predominantly into the lipids and LTA of *S. faecalis* and, therefore, can be used as an index of the amount of lipid and LTA synthesized in a population of cells. Cultures were labeled continuously for at least five generations before sampling with either [^{14}C]- or [^{3}H]glycerol. Duplicate samples were taken at regular intervals and extracted for either lipid or cellular LTA. Total cellular glycerol incorporation (data not shown) was also determined by the precipitation of the label in ice-cold 10% trichloroacetic acid. Figure 2 shows that incorporation of the label into lipid halted within 10 min with an antibiotic treatment of 5 μg/ml. With 2 μg/ml, both syntheses appeared to continue but at decreasing rates for as long as 90 min.

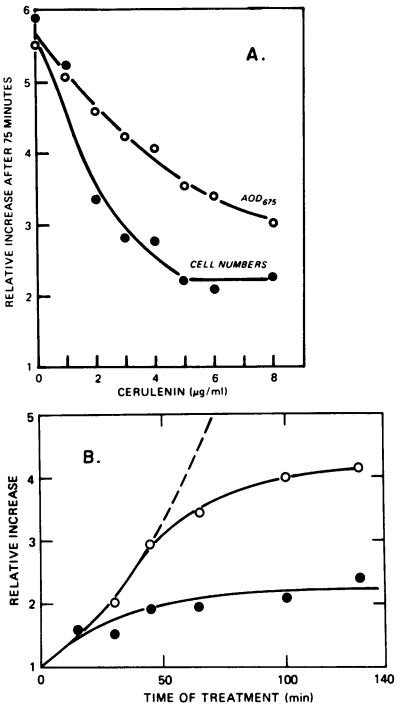

FIG. 1. *Effect of cerulenin on culture turbidity and cell numbers. (A) Determinations of cell numbers (●) and culture turbidity (AOD_{675} [○]) were made at the initiation of antibiotic treatment and after 75 min. The antibiotic was added at a cell density of 80 μg/ml (1.2 × 10^8 cells per ml). (B) Determinations of cell numbers (●) and culture mass (AOD_{675} [○]) were made after the addition of 5 μg of cerulenin per ml. The ratio of the value obtained at the time indicated to the zero time value is expressed. Dashed line indicates the exponential increase of untreated cultures.*

Effects on DNA, RNA, protein, and peptidoglycan syntheses. Figure 3A shows the results obtained for these determinations expressed as relative increases for cultures treated with 2 μg of cerulenin per ml versus time of treatment. The data indicate that although

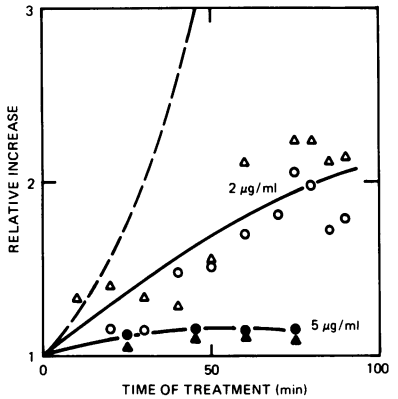

FIG. 2. *Effect of cerulenin on net lipid and LTA synthesis. Samples of cultures grown in the presence of [^{14}C]- or [^{3}H]glycerol were extracted for lipids or LTA after the initiation of antibiotic treatment. The ratio of the glycerol incorporation into lipid (1.5 × 10^{4} dpm/ml) or LTA (6 × 10^{3} dpm/ml) is expressed as the relative increase. Symbols: ○, lipid with 2 μg of cerulenin per ml; △, LTA with 2 μg of cerulenin per ml; ●, lipid with 5 μg of cerulenin per ml; ▲, LTA with 5 μg of cerulenin per ml; - - -, exponential increase of untreated cultures.*

there seems to be an effect on all of these syntheses, nonetheless, there was little or no effect for at least the first 50 min of treatment. Thereafter, the synthetic processes continued at a decreased, but still substantial, rate for at least 100 min of treatment. With 5 μg of cerulenin per ml, DNA and protein syntheses continued at the control rate (dashed line) for approximately 60 min and then began to level off. RNA and peptidoglycan syntheses were affected both earlier (i.e., after 40 min of treatment) and to a greater extent, exhibiting a final increase of 200% as opposed to about 350% for DNA and protein.

Effects of cerulenin on cell division. Cerulenin (5 μg/ml) was added to cultures dividing with varying doubling times between 30 and 63 min, and the increase in cell numbers was determined 2 h later (Table 1). The results of a typical experiment at a fast growth rate are shown in Fig. 1B. With decreasing doubling times, there was a progressive increase in residual divisions after antibiotic addition. Assuming that each cell past a critical point in the cell division cycle proceeds through the next division and that all other cells do not, one can calculate the timing of this critical point in the cell division cycle from the residual divisions by using the exponential growth equation $N/N_0 = e^{-\alpha t}$, where α is $\ln 2/T_D$ in hours. The calculated time, t, until cessation of division was 43 min (Table 1) and appears to be independent of growth rate. If a cell cycle event is truly being affected by the antibiotic, then we might expect to obtain the same value for t regardless of growth rate (3, 7).

Cultures were also treated separately with mitomycin C (0.5 μg/ml), which will only allow those cells that have completed chromosome replication to divide, and can, therefore, be used to approximate the timing of chromosome termination within the cell division cycle (3, 7). The value obtained for mitomycin C-treated

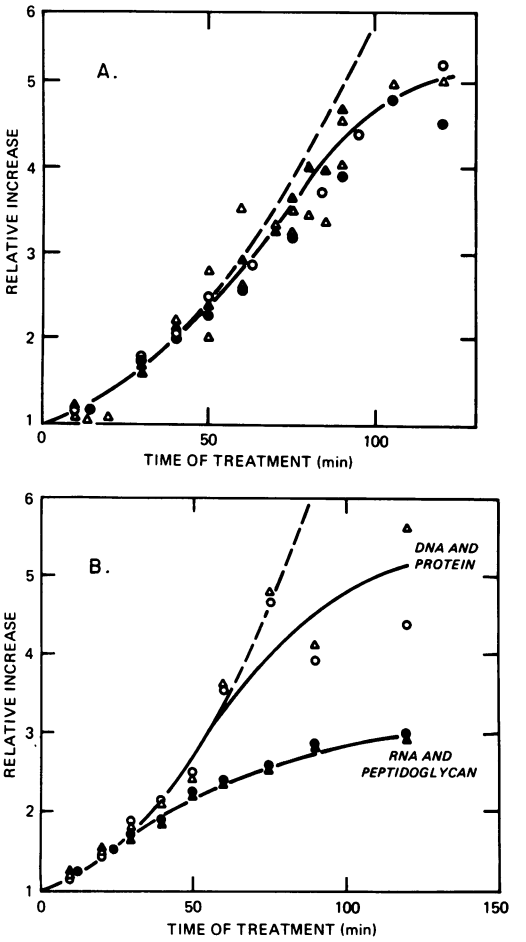

FIG. 3. *Effect of cerulenin on DNA, RNA, protein, and peptidoglycan syntheses. (A) 2 μg of cerulenin per ml; (B) 5 μg of cerulenin per ml. Samples were taken of cultures grown in the presence of the appropriate isotope after the addition of cerulenin. Macromolecular incorporation was determined, and the ratio of the value obtained for each parameter at the time indicated to the zero time value (2.6 × 10^{3} dpm/ml for [^{3}H]thymidine into DNA; 1.9 × 10^{4} dpm/ml for [^{14}C] uracil into RNA; 1.8 × 10^{4} dpm/ml for [^{3}H]lysine into protein; 4.6 × 10^{3} dpm/ml for [^{3}H]lysine into peptidoglycan) is expressed as the relative increase on the ordinate. ○, DNA; ●, RNA; △, protein; ▲, peptidoglycan; - - -, exponential increase of untreated culture.*

TABLE 1. *Effect of cerulenin on cell division*

Doubling time[a] before antibiotic addition (min)	Cerulenin (5 μg/ml)		Mitomycin C (0.5 μg/ml)	
	N/N_0[b]	t (min)	N/N_0[b]	t (min)
63	1.60	43		
60	1.60	41		
59			1.73	37
55	1.61	38		
50	2.23	58	1.65	36
43	2.02	45	1.81	38
41			1.66	30
41	1.86	37	1.74	28
38	2.26	45	1.89	35
38			1.92	35
32	2.34	39	1.95	31
31			1.91	29
30.5			2.16	34
30			2.24	35
30	2.42	39	2.0	31
Mean		43		33

[a] To vary the growth rate of these cultures, glutamine was omitted from the medium, and glutamate was added at various concentrations (15 to 300 μg/ml) (P. Lancy, Ph.D. thesis, Temple University, Philadelphia, Pa., 1976).

[b] N/N_0 is the ratio of cell numbers 2 h after treatment (N) to cell numbers at the time of treatment (N_0).

cultures was also relatively constant, with a mean of 33 min before cell division.

DISCUSSION

Cerulenin affected *S. faecalis* cell division to a much greater extent than culture mass (Fig. 1), suggesting that cell division might be intimately linked more with the primary target of the drug than with other specific macromolecular syntheses. No observable effects on the synthesis of DNA, RNA, protein, or peptidoglycan were seen at the time when cell division was found to be inhibited, i.e., within 30 to 40 min with 5 μg/ml. The synthesis of lipid and LTA, however, was inhibited before the observable inhibition of cell division (Fig. 1B). These data raised the possibility that de novo synthesis of either one or both of these classes of molecules may be a primary factor required for the normal process of division to occur.

Cultures at a variety of growth rates were treated with 5 μg of cerulenin per ml. An analysis of residual divisions after the initiation of antibiotic treatment indicated that cerulenin affected an event about 43 min before the cessation of division and approximately 10 min before the termination of chromosomal replication as determined by treatment of similar cultures with mitomycin C.

Transient increases in lipid synthesis have been reported in the late stages of the cell division cycle in bacteria (6, 11). Also, observations in many other bacterial species (1, 9, 10, 13, 16, 20) are consistent with a primary lipid involvement in the cell division process. The present study suggests that cerulenin, which blocks de novo lipid and LTA synthesis, can block a crucial cell division event.

If lipid and/or LTA function as in vivo autolytic inhibitors, then prevention of their synthesis with cerulenin might not permit inhibition of autolytic activity. Shockman et al. (15) have proposed that inhibition of autolytic activity is necessary for septum formation and, therefore, for cell division in streptococci. The timing of the cerulenin-sensitive event just before or during the completion of chromosome replication would be consistent with the hypothesis that such autolytic inhibition would be associated with the onset of septum formation (7). The effects of cerulenin at a concentration of 5 μg/ml on cell morphology and autolytic behavior are currently being investigated.

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