Inhibition of De Novo Fatty Acid Synthesis by the Antibiotic Cerulenin in Bacillus subtilis: Effects on Citrate-Mg\(^{2+}\) Transport and Synthesis of Macromolecules

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Inhibition of de novo fatty acid biosynthesis by the antibiotic cerulenin in Bacillus subtilis stopped de novo synthesis of neutral lipids and phospholipids. The bacteria ceased growing but remained completely viable. Addition of 12-methyltetradecanoic acid and palmitic acid to the culture medium of cerulenin-treated cells restored growth of the bacteria, albeit at a reduced rate. Although the de novo synthesis of all lipid components of the membrane was blocked, citrate-Mg\(^{2+}\) transport activity remained inducible, and induced cells did not lose this transport activity when treated with cerulenin. Shortly after the addition of cerulenin, the rate of ribonucleic acid synthesis dropped rapidly and was followed by a slower decrease in the rate of protein synthesis. The rate of deoxyribonucleic acid synthesis remained almost unaffected. The rapid decrease of ribonucleic acid synthesis in cerulenin-treated cells might be due to the inhibition of de novo fatty acid biosynthesis or it might be due to a secondary effect of cerulenin in B. subtilis cells.

Since biological membranes consist mainly of lipid and protein components, it would be interesting to determine whether a common regulation exists in the living cell for the biosynthesis of lipids and membrane proteins. This problem can be approached by experimental interruption of either the biosynthesis of lipids or of membrane proteins. Unfortunately, to date it has not been possible to block specifically the biosynthesis of all membrane proteins without simultaneously affecting the biosynthesis of cytoplasmic proteins, too. In contrast to the distribution of proteins in both cytoplasm and membranes, almost all lipids of a bacterial cell are components of the membrane (2). Thus, biochemical or genetic interference with lipid biosynthesis would be expected to have direct effects on membrane biosynthesis. Bacterial mutants with blocks at different steps of lipid synthesis have been used to explore this problem: Escherichia coli mutants auxotrophic for unsaturated fatty acids (8, 18), mutants of Bacillus subtilis (12, 13, 25), E. coli (9), and Staphylococcus aureus (19) auxotrophic for glycerol, and temperature-sensitive mutants of E. coli that do not synthesize phospholipids at the restrictive temperature (6, 7, 18). On the other hand, specific inhibitors of lipid biosynthesis do not necessarily require either the use of special mutants or any shift of growth temperature that may also affect the lipid composition of the preexisting membrane. 3-Decynoyl-N-acetylcysteamine is a specific inhibitor of the biosynthesis of unsaturated fatty acids in E. coli (10, 18, 20, 22). Robbins and Rotman reported that methylgalactoside transport in E. coli is inhibited by halting unsaturated fatty acid biosynthesis with this inhibitor (22).

Recently a new antibiotic, cerulenin, has been shown to block specifically the condensing enzyme (acyl coenzyme: acetyl coenzyme A C-acyl transferase; EC 2.3.1.16) of fatty acid chain elongation and thereby lipid biosynthesis in E. coli (6, 24). After addition of cerulenin to a culture of growing E. coli cells, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis was reduced by 25%, whereas protein synthesis was not affected (6). Specific membrane functions such as transport activities have not been studied under these conditions. We attempted to answer the following two questions by using cerulenin to stop total fatty acid biosynthesis in B. subtilis.

(i) Is it possible to prevent the accumulation of free fatty acids under conditions whereby de novo phospholipid biosynthesis is blocked in these bacteria? We had previously shown that glycerol deprivation in a glycerol-requiring B. subtilis mutant immediately stopped lipid synthesis but allowed free fatty acids to accumu-
late (15, 25). The induction of citrate-Mg\textsuperscript{2+} transport activity, however, was not influenced under these conditions (25). Therefore, one could argue that the continued production of free fatty acids allowed the continued integration of the transport protein into the membrane. To exclude this possibility, we wished to follow the induction of citrate-Mg\textsuperscript{2+} transport activity in the absence of de novo fatty acid and lipid synthesis.

(ii) Does the inhibition of lipid synthesis by cerulenin influence the rate of macromolecular synthesis in B. subtilis? Glaser et al. (5) had reported that macromolecular synthesis ceased after shifting a temperature-sensitive E. coli mutant to the restrictive temperature, which stopped phospholipid biosynthesis. Unfortunately, these experimental conditions were accompanied by a rapid decrease in viability of the E. coli mutant. On the other hand, cerulenin-treated B. subtilis cells remain perfectly viable and thus offer an independent system for answering the second question.

In this paper we show that addition of cerulenin to the culture medium stopped fatty acid synthesis in B. subtilis and, as a consequence, both the accumulation of free fatty acids and net phospholipid synthesis ceased. Induction of the citrate-Mg\textsuperscript{2+} transport system was found to be delayed. This was probably due to the rapid decrease in the rate of RNA synthesis in cerulenin-treated cells.

**MATERIALS AND METHODS**

**Bacteria.** For most experiments B. subtilis B42 was used. This strain has been isolated and characterized by Mindich (13, 14). B42 is a derivative of B. subtilis 168 and is auxotrophic for indole, histidine, and glycerc. In addition it lacks a nicotinamide adenine dinucleotide-independent L-α-glycerol phosphate dehydrogenase (EC 1.1.2.1). Because of this double mutation in glycerol metabolism, B42 can neither synthesize nor degrade glycerol. The cells, however, are able to use glycerol for de novo lipid synthesis and presumably for teichoic acid synthesis.

The fatty acid supplementation studies were carried out with B. subtilis SB26. This is a methionine- and tryptophan-requiring derivative of the 168 strain. B. subtilis SB26 is presumably identical with B. subtilis 60015 (4). SB26 was chosen for the fatty acid supplementation studies because it grows better than B42 in the presence of Brij35.

**Media.** B. subtilis B42 was grown in a minimal salts medium supplemented with amino acids (AAM medium). A similar medium was previously used for studies of citrate-Mg\textsuperscript{2+} transport in B. subtilis B42 (25). Composition of the medium (per liter) was: K\textsubscript{2}HPO\textsubscript{4}, 14.0 g; KH\textsubscript{2}PO\textsubscript{4}, 6.0 g; MgSO\textsubscript{4}•7H\textsubscript{2}O, 200 mg; MnSO\textsubscript{4}, 20.0 mg; glycerc, 40.0 mg; ammonium sulfate, 2.0 g; sodium glutamate, 1.5 g; arginine, 28.9 mg; histidine, 30.0 mg; lysine, 61.2 mg; glycine, 2.7 mg; alanine, 19.2 mg; valine, 35.7 mg; isoleucine, 31.5 mg; leucine, 61.5 mg; proline, 63.6 mg; phenylalanine, 1.8 mg; serine, 25.4 mg; threonine, 37.0 mg; tyrosine, 1.8 mg; methionine, 20.4 mg; cysteine, 21.6 mg; asparagine, 12.9 mg; and tryptophan, 37.5 mg. The amino acids, except glutamate, tryptophan, and proline, were stored frozen as a 100-fold-concentrated solution.

The supplementation studies were carried out in glucose minimal medium (medium NG1) (27).

**Chemicals.** Cerulinen [(2S), (3R)\textsuperscript{2},3-epoxy-4-oxo-7,10-dodecadienoylamide)] (1) was a gift of S. Ômura, Kitasato Institute, Tokyo, Japan. At the beginning of this work, cerulinen was stored as a stock solution at 2 mg/ml in ethanol at 4 C. Aliquots of this solution were added to a shaking culture in order to rapidly disperse the antibiotic. Later we used stock solution (12 mg/ml) of cerulinen prepared in warm water (40 C) (S. Ômura, personal communication). 2-Fluoro-L-erythro-\textsuperscript{[3,4,5,6-\textsuperscript{14}C]}citrate had been synthesized by Peter Oehr in this laboratory (19). 12-Methyltetradecanoic methyl ester was obtained from Applied Science Laboratories (State College, Pa). Myristic acid, palmitic acid, and stearic acid were purchased from Sigma Chemical Co.; Brij 35 was bought from Calbiochem (Lucerne). All isotopes were obtained from Amersham Buchler (Braunschweig), luciferase (plus luciferin) from Boehringer (Mannheim), and diisobutylketone from Fluka AG (Neu Ulm). All other chemicals were purchased from Merck (Darmstadt).

**Culture conditions.** The bacteria were plated on tryptose blood agar (Difco) and incubated for 20 h at 37 C. Fresh single colonies were used for inoculation of medium in side-arm Erlemeyer flasks (10-fold volume of the medium). The cultures were incubated in a gyrotory water bath shaker (New Brunswick) at 37 C and 150 rpm. Growth of the cultures was monitored by measuring the turbidity (Klett-Summerson photoelectric colorimeter, no. 54 filter) against a water blank. Under these conditions 50 Klett units (KU) corresponded to about 1.5 x 10\textsuperscript{10} cells/ml.

**Viability.** A logarithmically growing culture of B. subtilis B42 was divided at about 40 KU into two cultures of 6 ml each. After addition of cerulinen (20 \mu g/ml) to one of the cultures, the cells were grown at 37 C. Aliquots of 0.05 ml were removed at 10-min intervals and diluted 3 x 10\textsuperscript{fold} in AAM medium without glycerol supplemented with 5 g of glucose per liter. A 0.1-ml amount of the dilution was plated on Tryptose blood agar. The plates were incubated for 20 h at 37 C before counting the colonies.

**Size distribution.** Half of a logarithmically growing culture was treated with cerulinen (20 \mu g/ml). The second half served as a control. At different times, 0.1-ml aliquots of the cultures were removed and diluted in 10 ml of saline-formaldehyde solution (9 g of NaCl and 2.9 ml of 35% formaldehyde solution per liter). For measuring the size of the cells, we used a detector similar to that in a Coulter counter, equipped with a 30-\mu m pore. The signal was traced by a pulse amplifier and a digital discriminator and then taken up by a 512 channel memory. The output was plotted.

Fatty acid and lipid synthesis. Analysis of free fatty acids were carried out as previously described (25). Phospholipids were identified by two-dimensional paper chromatography according to the method of Lillich and White (12). Cerulenin was added to the cells at a final concentration of 20 μg/ml. For supplementation with fatty acids, . subtilis SB26 cells were grown in NG1 medium in the presence of 0.075% Brij 35. Stock solutions of fatty acids (50 μg/ml) were dissolved in 0.01 N KOH, neutralized with HCl, and autoclaved. Aliquots of these stock solutions were added to cultures of . subtilis SB26. The final concentrations are indicated in the legend of Fig. 2. The methylester of 12-methyltetradecanoic acid was hydrolyzed to the free acid as previously described (26).

Citrate-Mg++ transport. Citrate-Mg++ transport was measured with the citrate analogue 2-fluoro-l-erythro-[3,4,5,6-C]citrate, which is not significantly metabolized in . subtilis cells. Details have been described by Oehr and Willecke (19). To inhibit protein synthesis during uptake studies, the assay medium contained chloramphenicol (50 μg/ml).

Rate of RNA synthesis. This experiment was started with cells growing exponentially in AAM medium at about 30 KU. Aliquots of the cultures were removed, and incorporation of [2-14C]uracil (specific activity, 26 mCi/mmol) was followed for 3 min at 37 C. To withdraw and incubate samples from two simultaneously growing cultures, the water bath shaker had to be stopped every 1 to 2 min. The reaction was terminated by addition of trichloroacetic acid (final concentration, 5%). After the addition of acid the samples were kept in ice for at least 20 min and subsequently filtered through Sartorius membrane filters (25 mm, 0.45-μm pore size). The filters were washed with 5 ml of ice-cold trichloroacetic acid (5%) and dried at 100 C. Radioactivity was measured in 4 ml of scintillation fluid (29) and counted in a liquid scintillation spectrometer (Packard Tri-Carb, model 2420). All data were corrected by subtracting the radioactive background of proper controls.

Uracil uptake. Samples (1.6 ml) were withdrawn from a logarithmically growing culture of . subtilis B42, filtered, and washed with 5 ml of 0.1 M tri-s(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.2. The bacteria were resuspended in 0.8 ml of Tris buffer, and 0.6 ml of this suspension was incubated with 0.2 μCi of [14C]uracil (25 mCi/mmol) for 1 min at 37 C. Then the suspension was rapidly filtered and washed with 5 ml of Tris buffer. Radioactivity was measured as described above.

DNA and protein synthesis. The rates of DNA and protein synthesis were measured as RNA synthesis. Aliquots of logarithmically growing cultures were incubated with [methyl-3H]thyminde (2 μCi; specific activity, 520 mCi/mmol) for 3 min at 37 C. Protein synthesis was determined with cells grown in proline-free AAM medium. The incorporation of L-[3H]proline (2 μCi; 60 mCi/mmol) into trichloroacetic acid-precipitable material was followed in aliquots for 3 min at 37 C. Radioactivity was measured as usual.

ATP content. The determination of adenosine triphosphate (ATP) was carried out according to the modified method of Klofat et al. (11). The cells were grown in AAM medium to about 35 KU. At different times, 0.5 ml of the culture and 0.5 ml of formic acid solution (0.45 M formic acid, 2 × 10^-3 M sodium ethylenediaminetetraacetic acid) were rapidly mixed and kept in ice for 15 to 30 min. Aliquots (0.5 ml) of this mixture were diluted with 4.5 ml of 0.1 M TES-NaOH buffer [N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid], pH 7.4. Luciferase solution (containing luciferin), 0.1 ml, was added with an automatic syringe (Fisons LFA 20) to 0.6 ml of the diluted aliquots in a 1-ml cuvette. The luminescence was measured by the SKAN XP 2000 spectrophotometer (Skan AG, Basel) and recorded on a Philips recorder, model 8000. Each experiment was calibrated by using purified ATP. To measure the level of intracellular ATP, aliquots (0.5 ml) of the cultures were filtered and resuspended in 0.5 ml of formic acid mixture plus 0.5 ml of AAM medium. Cells treated with 1% toluene were used as a zero control.

RESULTS

Mindic (15) had previously shown that . subtilis B42 accumulated large amounts of free fatty acids when the bacteria were deprived of glycerol. This accumulation of free fatty acids in glycerol-deprived cells did not take place in the presence of cerulenin (Fig. 1). Furthermore, the content of free fatty acids decreased in cerulenin-treated cells grown in glycerol-containing medium. In other experiments we compared the incorporation of [14C]acetate into phospholipids and neutral lipids of . subtilis B42 cells growing in the presence or absence of cerulenin. We found that de novo synthesis of phospholipids and neutral lipids was stopped in the presence of cerulenin even when glycerol was present. The relative amounts of the main phospholipids were not significantly changed under these conditions (data not shown). We conclude from these results that the arrest of de novo fatty acid biosynthesis by cerulenin caused a quick termination of de novo lipid synthesis in these bacteria.

Addition of cerulenin (20 μg/ml) to the culture medium of logarithmically growing . subtilis cells stopped growth within half the generation time of control cells (see Fig. 4A). During this time the average size of cells, which gradually ceased growing in the presence of cerulenin, became significantly smaller than the average size of control cells growing in the absence of cerulenin. (Experimental details are described under Materials and Methods.) Evidently, the cells react toward the shortage of lipids, i.e., essential components of the membr-
branched, by decreasing the mean size of all daughter cells formed in the presence of cerulenin.

If the primary action site of cerulenin is the block of fatty acid biosynthesis, it should be possible to restore growth of cerulenin-treated bacteria by supplying exogenous long-chain fatty acids in the medium. *B. subtilis* SB26 grew slowly in the presence of cerulenin when supplemented with a mixture of 12-methyltetradecanoic acid and palmitic acid in the medium (Fig. 2). The growth rate under these conditions, however, was very slow when compared with that of cells growing in the absence of cerulenin. This was probably due to the decreased rate of RNA synthesis in the presence of cerulenin (see below). Straight-chain fatty acids in the medium did not restore growth. Citrate-Mg$^{2+}$ transport could be induced under conditions that blocked de novo fatty acid synthesis. The time necessary for reaching the same level of induction was three times longer in cerulenin-treated cells than in control cells induced in the absence of cerulenin. This delayed induction could be due to the decreased rate of RNA synthesis in cerulenin-treated cells. Cerulenin did not change the level of citrate-Mg$^{2+}$ transport in fully induced cells (Fig. 4).

**Fig. 1.** Incorporation of $[^{14}C]$acetate into free fatty acids in *B. subtilis* B42. The cells were grown in AAM medium, filtered, and washed. The filter was cut into four pieces. One piece each was suspended at zero time in the following media: glycerol-containing medium in the presence of cerulenin (closed circles), glycerol-containing medium in the absence of cerulenin (open circles), glycerol-free medium in the presence of cerulenin (closed triangles), and glycerol-free medium in the absence of cerulenin (open triangles). All media contained $[^{1-14}C]$sodium acetate (2.5 μCi/ml; 0.5 mCi/mmole). Portions of the cells were withdrawn at indicated times, and the lipids were extracted and analyzed after thin-layer or paper chromatography (25). The results of both chromatographic methods were identical.

**Fig. 2.** Supplementation of cerulenin-treated *B. subtilis* B26 with different long-chain fatty acids. The cells were grown in glucose minimal medium containing 0.075% Brij 35. A logarithmically growing culture was divided and the different components were added at zero time. All cultures except the control received 30 μg of cerulenin per ml. The cerulenin-treated cultures were supplemented with mixtures of either 12-methyltetradecanoic acid (8 μg/ml) and palmitic acid (8 μg/ml) (closed circles) or mixtures of myristic acid (2 μg/ml), palmitic acid (12 μg/ml), and stearic acid (2 μg/ml) (open triangles). The third cerulenin-treated culture (closed triangles) was not supplemented with fatty acids. The control culture (open circles) was growing in the absence of cerulenin but in the presence of 12-methyltetradecanoic acid (8 μg/ml) and palmitic acid (8 μg/ml).
In the presence of cerulenin. All ATP was found inside the cells. This result excluded the possibility that ATP leaking out of the cerulenin-treated cells might have caused the decrease in the rate of RNA synthesis.

**DISCUSSION**

*B. subtilis* cells remain totally viable for several hours when de novo synthesis of fatty acids (and lipids) is blocked by cerulenin. This is an experimental advantage over other methods which had been used to block fatty acid and lipid synthesis in bacteria and which led to considerable cell death (5, 8, 22). We used the antibiotic cerulenin to prevent accumulation of free fatty acids in glycerol-deprived *B. subtilis* B42 cells. These bacteria stop de novo synthesis of phospholipid but accumulate large amounts of fatty acids without synthetic activity (data not shown in order to improve the clarity of Fig. 5).

Once the transport system is functionally integrated in the membrane, its activity is apparently not influenced when de novo fatty acid and lipid synthesis is stopped.

Finally, we asked whether addition of cerulenin to the culture medium influenced macromolecular synthesis in *B. subtilis*. The rate of RNA synthesis decreased by about 90% within 10 min after addition of cerulenin (Fig. 5). The rate of protein synthesis decreased more slowly (Fig. 5). The rate of DNA synthesis was reduced by only 10% after 40 min in the presence of cerulenin (data not shown in order to improve the clarity of Fig. 5). The control cultures also showed a slight decrease in the specific rates first of RNA and later of protein synthesis. Particularly the rate of RNA synthesis in *B. subtilis* cells was most sensitive with regard to limited oxygen supply. Under our experimental conditions the rate of RNA synthesis decreased by about 20% when the water bath shaker was stopped about every 1 to 2 min to withdraw samples. In further experiments, we determined that the ATP content of the cells did not change in the presence of cerulenin.
amounts of fatty acids when glycerol is removed from the culture medium. Growth of *B. subtilis* in the presence of cerulenin could be restored by supplementation with 12-methyltetradecanoic acid and palmitic acid. This confirms under in vivo conditions that the primary action site of cerulenin is the chain elongation reaction of fatty acid biosynthesis. A mixture of long straight-chain fatty acids did not restore growth. This result supports our conclusion from studies of a branched-chain fatty acid requiring *B. subtilis* mutant (26) that methyl-branched fatty acids are essential components for the structural integrity of *B. subtilis* membranes.

There is some controversy in the literature about whether inhibition of fatty acid or de novo lipid synthesis affects the inducibility of transport systems in bacteria. Fox and co-workers (3, 9, 28) reported that de novo lipid synthesis is necessary for induction of lactose transport in *E. coli*. Robbins and Rotman (22) concluded that the methylgalactoside transport system needs unsaturated fatty acid synthesis for induction and maintenance of activity (22). On the other hand there is strong evidence from several laboratories suggesting that induction of β-galactoside transport is independent of simultaneous de novo phospholipid synthesis (5, 18, 20). In an earlier report we had found normal inducibility of citrate-Mg²⁺ transport in *B. subtilis* after inhibition of de novo phospholipid synthesis but in the presence of accumulating free fatty acids (25). In this paper we show that after cerulenin treatment of *B. subtilis* cells, citrate-Mg²⁺ transport is induced with some delay and to lower activity than in control cells. The activity of fully induced cells was not affected by cerulenin. Since termination of de novo phospholipid synthesis by glycerol deprivation did not change the timing of induction of citrate-Mg²⁺ transport (25), the results shown in Fig. 3 seemed to suggest that simultaneous de novo fatty acid synthesis might be required for normal induction. When we measured the effect of cerulenin treatment on the rates of macromolecular synthesis, however, a rapid decrease in the rate of RNA synthesis was seen (Fig. 5). The rates of protein and, in particular, DNA synthesis were much less affected by cerulenin treatment of the bacteria. Thus, the delayed induction of citrate-Mg²⁺ transport was most likely caused by the reduced rate of RNA synthesis under these conditions.

Our results recall to mind the report by Glaser et al. (5), who also observed a rapid decrease of the rate of RNA synthesis by shifting a temperature-sensitive *E. coli* mutant defective in glycerol-3-phosphate acyltransferase (EC 2.3.1.15) to the restrictive temperature. The authors found that this decrease was directly followed by a drastic decline of DNA and protein synthesis. They interpreted their findings as a regulatory effect of de novo phospholipid biosynthesis on macromolecular syntheses. The experiments by Glaser et al. were carried out under conditions that resulted in a drastic loss of viability shortly after the decrease of macromolecular syntheses. This loss of viability makes it difficult to generalize the conclusions of these authors. In contrast to these observations, studies of glycerol-deprived *B. subtilis* (13, 25) or *S. aureus* mutants (21) and oleate-starved *E. coli* mutants (8) showed no or very little decline of RNA synthesis.

There are several possibilities for explaining the reduced rate of RNA synthesis in cerulenin-treated *B. subtilis* cells. (i) The uptake of uracil might be reduced during treatment with cerulenin. According to a control experiment (data not shown), the uracil uptake remained stable. (ii) The rate of RNA synthesis dropped because of a decrease in energy supply. This did not seem to be the case since the internal ATP level was not reduced in cerulenin-treated cells. (iii) The ratio of the utilizable intracellular and
exogenous uracil might have changed under our experimental conditions (23). This possibility was not excluded.

It remains to be clarified whether the rapidly reduced rate of RNA synthesis in cerulenin-treated *B. subtilis* cells is caused by the arrest of de novo fatty acid biosynthesis. Alternatively, cerulenin may directly act on RNA synthesis in *B. subtilis* independently of its inhibitory effect on fatty acid chain elongation.

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