INHIBITION OF SECRETION OF STAPHYLOCOCCAL ALPH TOXIN BY CERULENIN

F. A. K. SALEH AND J. H. FREER

Department of Microbiology, University of Glasgow, Glasgow G11 6NU, Scotland

SUMMARY. Secretion of α toxin by *Staphylococcus aureus* strain Wood 46 was preferentially inhibited by cerulenin, an antibiotic that stops fatty-acid synthesis by inhibiting β -keto acyl acyl carrier-protein synthetase. At the concentrations used, cerulenin had a negligible effect on cell growth and total protein synthesis, but reduced lipid synthesis by 50%. Extracellular and membrane-associated α toxin was absent in cultures treated with cerulenin, but toxin formation was resumed after either removal of the antibiotic or addition of exogenous fatty acids. The apparent absence of toxin precursor in membranes of inhibited cells favours inhibition at an earlier stage in toxin synthesis.

INTRODUCTION

In recent years, considerable attention has been focused on the mechanism of protein localisation and secretion in bacteria (Davis and Tai, 1980; Emr, Hall and Silhavy, 1980; Michaelis and Beckwith, 1982). Several observations suggest a link between phospholipid synthesis and the formation of toxins and enzymes in *Staphylococcus aureus* (Altenbern, 1977; Berkeley *et al.*, 1978).

Although the extracellular protein toxins of *S. aureus* have been extensively studied in terms of chemical structure and mode of action (Freer and Arbuthnott, 1982), there is almost no information about their mechanism of secretion or release from the cell. Because there are several toxins among the extracellular products of this organism that have an amphipathic structure and a high affinity for cell membranes and lipid-containing structures (Füssle *et al.*, 1981; Bhakoo, Birkbeck and Freer, 1982; Freer and Birkbeck, 1982), it is of interest to know whether or not these metabolites are secreted in the same manner as the more hydrophilic extracellular proteins of bacteria which use signal sequences in the primary translation products as a means of translocation through the plasma membrane (Silhavy, Benson and Emr, 1983).

The secretion of several exoproteins of gram-positive bacteria, including staphylococcal enterotoxin A (Berkeley *et al.*, 1978), staphylococcal enterotoxins B and C (Altenbern, 1977), staphylocoagulase (Engels and Kamps, 1981), penicillinase (Fishman, Rottem and Citri, 1978), levansucrase (Caulfield *et al.*, 1979), α amylase and protease (Paton, May and Elliott, 1980; Mäntsälä, 1982), has been shown to be sensitive to lipid inhibitors and membrane-perturbing agents. Therefore, we have used cerulenin — (2S) (3R)-2,3-epoxy-4-oxo-7,10-dodecadienoyl amide — an inhibi-

tor of Acyl-(acyl-carrier-protein):malonyl-(acyl-carrier-protein) C-acyltransferase (decarboxylating) (EC 2.3.1.41), a key enzyme in fatty-acid synthesis, in an attempt to determine the role of lipids in the secretion of the α toxin in *S. aureus*.

MATERIALS AND METHODS

Organism and growth conditions. *Staphylococcus aureus* strain Wood 46 (NCTC7121) was maintained by weekly transfer on horse-blood agar plates. Exponential-phase cultures were prepared by inoculating a haemolytic colony into 60 ml of yeast-extract diffusate medium (BS medium; Bernheimer and Schwartz, 1963) in a 250-ml Erlenmeyer flask which was incubated at 37°C on an orbital shaker to an optical density at 600 nm (OD 600) of 12.

Measurement of bacterial growth. OD 600 and cell dry weight were determined during growth in BS medium at 37°C with shaking during an incubation period of 16 h.

Erythrocytes. Lysis of rabbit erythrocytes was used as an assay for biologically active α toxin whereas lysis of cod erythrocytes was used to detect δ -lysin activity. Before use, erythrocytes were washed three times in phosphate-buffered saline (0.01M sodium phosphate in 0.015M NaCl, pH 7.0, PBS) and then packed at 600 *g* for 10 min before a 1.0% v/v suspension was prepared in the same buffer.

Haemolytic assay. Samples (50 μ l) of α toxin or culture supernate were serially diluted in 50 μ l of PBS in microtitration plates with U-shaped wells (Flow Laboratories, Irvine, Scotland). Rabbit erythrocytes (50 μ l of a 1% v/v suspension) were added to each well and the plates incubated at 37°C for 30 min before centrifugation for 30 s at 600 *g* in a MSE Mistral 6L centrifuge fitted with a plate-carrier adaptor. The end-point at 50% haemolysis was determined visually and the toxin dilution at which it was observed was taken as the titre.

Production and purification of toxin. Alpha toxin was purified according to a modification of the method of Cassidy and Harshman (1976) from culture supernates of strain Wood 46. Briefly, protein was precipitated from 15-h culture supernates by the addition of ammonium sulphate to 70% saturation. The precipitated protein was dissolved in phosphate buffer (KPS, potassium phosphate, 0.05M, pH 6.8) and dialysed against several changes of the same buffer for 6 h at 4°C. After dialysis, the protein was applied to a column of controlled-pore glass (BDH, Poole, England) according to the method of Cassidy and Harshman (1976). The peak eluting with 1.0M potassium phosphate, pH 7.5, contained the α toxin, which was dialysed against several changes of glycine 1% w/v before isoelectric focusing in a sorbitol gradient with a pH range of 3–10 on an LKB110 electrofocusing column (see LKB Application Note 219 for details).

Inhibition by cerulenin. Cerulenin (Sigma London Chemical Co. Ltd, Poole, England) was added to cultures as a sterile ethanolic solution (500 μ g/ml) or as a sterile aqueous solution (5–20 μ g/ml).

Effect of cerulenin on incorporation of lipid and protein precursors. The effect of cerulenin on the incorporation of lipid and protein precursors was determined during growth. In a typical experiment, 0.1 ml of an overnight broth culture was added to each of four Erlenmeyer flasks (250 ml) containing 60 ml of BS medium at 37°C. L-Phenyl [2,3-³H] alanine (444 KBq, 1.05 TBq/mmol, Amersham International plc, Amersham, England) was added to each of two flasks, and [U-¹⁴C] sodium acetate (4440 KBq, 2.19 GBq/mmol) was added to the remaining two flasks. After incubation for 10 min at 37°C with shaking (200 rpm), cerulenin was added to one of each pair of the flasks containing the labelled precursors, the remaining flask of each pair acting as a control. Duplicate samples (3 ml) were withdrawn at intervals and the OD 600 and the titre of α toxin in the cell-free supernate determined. The remainder of each sample was used for protein extraction as follows. Ice-cold trichloroacetic acid 10% w/v (TCA, 5 ml) was added to each sample (1 ml) and the mixtures allowed to stand for 10 min at 0°C before the precipitated proteins were filtered through membrane filters (0.45- μ m pore size; Oxoid Ltd, London, England) on an Amicon VFM-11 vacuum-filtration manifold (Amicon Corp., Lexington, MA, USA). The filters were washed twice with 5-ml volumes of ice-cold 10% TCA before being dried and the radioactivity counted in 5 ml of Triton:toluene scintillant (PPO, 4 g; POPOP, 0.1 g; Triton X-100, 500 ml; sulphur-free toluene, 1000 ml) in a Packard Tri-carb 3255 scintillation counter. Counts/min were corrected to disintegrations/min by the channels-ratio method by means of a quench-correction curve. Lipids were extracted by a modification of the method of

Bligh and Dyer (1959) described by Kates (1972). Each lipid extract was transferred to a scintillation vial, dried under a stream of N_2 and counted as described above.

Extraction and analysis of phospholipids. Total lipid extracts (see above) were applied to thin-layer plates coated with Silica gel G (Polygram SIL G, Macherey-Nagel and Co., W. Germany) and lipids separated by an ascending solvent with a volume-to-volume ratio of chloroform 80:methanol 18:acetic acid 12:water 5 (Minnikin and Abdolrahimzadeh, 1971). Individual phospholipids were visualised by reaction with the reagent of Dittmer and Lester (1964) and identified by comparing their mobility to standard phospholipids run in parallel.

Preparation of membranes. Total membranes were isolated from control and cerulenin-inhibited cells by a modification of the method of Salton and Freer (1965) in which lysozyme was replaced by lysostaphin as the muralytic agent. Briefly, cells were harvested after growth for 14 h and washed twice in 0.05M Tris-HCl buffer, pH 7.5, before resuspension to an OD 600 of 8 in the same buffer. Cell lysis, assessed by phase-contrast microscopy, was complete after incubation for 3 h at 37°C in the presence of lysostaphin (100 μ g/ml; Sigma) and DNAase (8 μ g/ml; Miles Laboratories Ltd, Slough, England). Membranes were harvested by centrifugation at 38 000 *g* for 45 min, and washed six times in Tris buffer (see above) before analysis.

Electrophoresis. Proteins were analysed by slab-gel polyacrylamide electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) in the discontinuous buffer system described by Laemmli (1970). Gels were fixed and stained overnight in an aqueous solution containing Coomassie blue 0.25% w/v, methanol 45% v/v and acetic acid 9% v/v and destained by diffusion in an aqueous solution of methanol 7.5% v/v and acetic acid 5% v/v.

FIG. 1.—Effect of cerulenin on growth and production of α toxin: — growth measured as OD600; --- titres of α toxin in culture supernate; ■ control culture; □ culture containing cerulenin 8.0 μ g/ml; ▲ culture containing cerulenin 20 μ g/ml.

Peptide mapping. Peptides of interest, separated by SDS-PAGE as described above, were excised from the gel after brief staining and destaining, and analysed by one-dimensional peptide mapping according to the method of Cleveland *et al.* (1977). Proteolysis was brought about by incorporating either trypsin, α chymotrypsin or V8 protease into the overlay buffer of the second-dimension gel. Samples including enzyme were transferred into the stacking gel by electrophoresis and proteolysis was allowed to proceed for 30 min at room temperature with the power switched off. Power was then reapplied and the peptides of the partial digests were stained on completion of electrophoresis by the silver staining method of Merrill, Goldman and van Keuren (1982).

Fatty-acid supplementation. Cultures were grown as described above either in the presence or absence of cerulenin (8 $\mu\text{g/ml}$) for 6 h at which time sodium salts of stearic or oleic acid were added to a final concentration of 8.3 $\mu\text{g/ml}$.

RESULTS

Toxin production

In agreement with previously published data (Duncan and Cho, 1971; Abbas-Ali and Coleman, 1977), α toxin was produced in a biphasic manner, the maximum rate of production occurring in exponential growth followed by a slower rate of production in late exponential and early stationary phase (fig. 1). Cell dry weight correlated directly

FIG. 2.—SDS-polyacrylamide gel-electrophoretic analysis of purified α toxin (mol. wt 34 000) which was freshly prepared (track 1) or which had been stored at 4°C in saturated ammonium sulphate for approximately 12 months (track 2). Arrow heads indicate positions of components at mol. wt 34 000, 27 000, 18 000 and 15 000 in descending order from the top of the figure.

with OD 600 (results not shown) and hence changes in OD 600 could be taken as directly reflecting changes in cell mass. Purified α toxin gave a heavily staining band of mol. wt 34 000 on SDS-PAGE with minor bands of mol. wt 27 000, 18 000 and 15 000 appearing as the toxin preparation aged at 4°C (fig. 2). These components corresponded to those reported by Dalen (1976), which appeared on storage of purified α toxin and probably reflected degradation products due to endogenous protease activity associated with α toxin. Peptide maps of all four components in the aged toxin preparation, including the component of mol. wt 34 000, showed a high degree of correlation with those of freshly isolated, authentic (mol. wt 34 000) α toxin (fig. 3). Membranes from control cells, but not from cerulenin-inhibited cells, had low but demonstrable haemolytic activity after disaggregation by treatment with ultrasound.

Toxin production in the presence of cerulenin

Concentrations of cerulenin up to approximately 12 μ g/ml of culture fluid had no effect on growth rate or final OD 600 of the culture but progressively inhibited the production of extracellular α toxin, with the titre falling from approximately 8000 HU/ml in control cultures (without cerulenin) to approximately 128 HU/ml at 8 μ g/ml and 64 HU/ml at 10 μ g/ml. Increasing the concentration of cerulenin to 20 μ g/ml

FIG. 3.—One-dimensional peptide maps in polyacrylamide gel (15%) in the presence of SDS (see *Methods*) of trypsin (track 1), and trypsin partial digests of excised bands from the aged α -toxin preparation shown in fig. 2. Track 2 = band of mol. wt 34 000, track 3 = band of mol. wt 27 000, track 4 = band of mol. wt 18 000 and track 5 = band of mol. wt 15 000. Trypsin was present in tracks 2–5 at the same level as in track 1. (Silver stain).

FIG. 4.—Incorporation of [¹⁴C]-acetate and [³H]-phenylalanine into control and cerulenin-inhibited cells. Growth of control cells (●—●) and cerulenin-inhibited cells (○—○) measured as OD 600; titre of α toxin in supernates of control culture (●---●) and culture containing cerulenin 10 μ g/ml (○---○); incorporation of [¹⁴C]-acetate (fig. 4a) or [³H]-phenylalanine (fig. 4b) into cells grown in the absence (control, ▲—▲) and in the presence (△—△) of cerulenin 10 μ g/ml.

resulted in a 50% inhibition of growth with complete inhibition of α -toxin secretion (fig. 1). Some variation in the degree of inhibition by cerulenin of α -toxin production was observed in different batches of medium. This variation appeared to be a function of the composition of the yeast extract diffusate and may reflect variation in the constituent fatty-acid components (see Altenbern, 1977).

Effect of cerulenin on lipid and protein synthesis

In conditions of cerulenin inhibition (10 μ g/ml) where production of α toxin was strongly inhibited (c. 95%), incorporation of [¹⁴C]-acetate into lipid-soluble material was inhibited by approximately 50% (fig. 4a), whereas incorporation of [³H]-phenylal-

TABLE
Optical density, α -toxin titre and lipid content of control and cerulenin-inhibited cells

Cultural conditions	OD600*	α -toxin titre (HU/ml)	Lipid content* (%)
Control	16.3 $\pm$ 0.28 (3)	8192	10.1 $\pm$ 0.9 (4)
Cerulenin (8 μ g/ml of culture)	15.6 $\pm$ 1.95 (3)	8 to 512	4.9 $\pm$ 1.1 (5)
Cerulenin (15 μ g/ml of culture)	11.9 $\pm$ 1.6 (3)	4 to 32	4.2 $\pm$ 1.0 (5)

* Mean values $\pm$ standard deviation (with the number of replicates in parentheses).

anine into TCA-precipitable material (an index of protein synthesis) was unaffected (fig. 4b). This level of cerulenin had no effect on growth rate or final OD 600. However, the content of lipid in the cell, expressed as a percentage of cell dry weight, was reduced to about half the level found in control cells (the table). No qualitative differences were detected in the types of phospholipid present in control and cerulenin-inhibited cells, the main phospholipids being phosphatidyl glycerol and diphosphatidyl glycerol (cardiolipin).

Effect of exogenous fatty acids

The effects of adding saturated or unsaturated fatty acids to cells under cerulenin inhibition was assessed by supplementing inhibited cultures with oleate (C18:1) and with stearate (C18:0). While these fatty acids had no effect on the growth or levels of α toxin in control cultures, the presence of oleate, but not stearate (result not shown), partially relieved the block in α toxin secretion (20% recovery of toxin production, fig. 5). The presence of these fatty acids had no effect on the haemolytic assay.

Effects of cerulenin on membrane proteins

Membranes of control cells showed a prominently stained band of mol. wt 34 000 which corresponded to the main component of authentic α toxin. This component

FIG. 5.—Effect of exogenous oleate on α -toxin production in cerulenin-inhibited cultures. Growth (●—●) and α -toxin titre (●—●) in control cultures; growth (○—○) and α -toxin titre (○—○) in the presence of cerulenin 8 μ g/ml of culture; and growth (□—□) and α -toxin titre (□—□) in the presence of cerulenin (8 μ g/ml of culture) and oleate (8.3 μ g/ml of culture).

FIG. 6.—SDS-polyacrylamide gel analysis of aged α toxin, total membrane, cytoplasmic and culture supernate fractions from control and cerulenin-inhibited cultures. Each track was loaded with *c.* 30 μ g of total protein. Tracks 1 and 12 = aged α toxin; tracks 2, 7 and 17 = mol. wt standards (arrow-heads indicate bovine serum albumin, mol. wt 66 000, ovalbumin, mol. wt 45 000, trypsinogen, mol. wt 24 000, β -lactoglobulin, mol. wt 18 400, reading from the top of the gel downwards). For meaning of numbers against arrow symbols see legend to fig. 7. Tracks 3, 4, 5 and 6 contain total membrane fractions from cells grown in the presence respectively of cerulenin 0, 5, 8, and 15 μ g/ml of culture. Tracks 8, 9, 10 and 11 contain supernates from the cultures containing the corresponding concentrations of cerulenin. Tracks 13, 14, 15, and 16 contain cytoplasmic fractions from cells grown in the presence of the corresponding cerulenin concentrations.

was unaffected by washing the control membranes three times in 1.0M NaCl, which would be expected to remove adsorbed toxin. The band corresponding to membrane-bound toxin was progressively reduced in intensity in membranes from cells grown in the presence of increasing concentrations of cerulenin. Its disappearance (in cells grown with cerulenin 5 μ g/ml) corresponded to the loss of α toxin haemolytic activity in the cell-free supernates of cerulenin-inhibited cultures. Control membranes contained three prominently staining bands which corresponded to peptides of mol. wt 32 000, 34 000 and 36 000, but only the band of mol. wt 34 000 (corresponding to the main α -toxin component) was lost under cerulenin inhibition (fig. 6). The components of mol. wt 32 000 and 36 000 were analysed by one-dimensional peptide mapping of trypsin, α -chymotrypsin or V8-protease partial digests, but both components differed significantly from the peptide maps of the α toxin of mol. wt 34 000. An example of the peptide maps obtained after α -chymotrypsin digestion is shown in fig. 7. Accompany-

FIG. 7.—One-dimensional peptide maps in acrylamide gel (15% w/v) of α -chymotrypsin partial digests (α chymotrypsin 0.2 μ g was added to each track except track 2). Tracks 1 and 9 = α chymotrypsin (0.2 μ g); track 2 = α toxin (mol. wt 34 000) without added enzyme; track 3 = band of mol. wt 34 000 from purified α -toxin preparation; track 4 = band of mol. wt 34 000 from membranes of cells grown in the absence of cerulenin; track 5 = band of mol. wt 36 000 from membranes of cerulenin-inhibited cells (cerulenin 8 μ g/ml of culture); track 6 = band of mol. wt 32 000 from membranes of cells grown in the absence of cerulenin; track 7 = band of mol. wt 32 000 from membranes of cells grown in the presence of cerulenin (cerulenin 8 μ g/ml of culture); track 8 = band of mol. wt 44 000 from membranes of cells grown in the presence of cerulenin (cerulenin 8 μ g/ml of culture). Numbers at arrows in fig. 6 correspond to the track number in fig. 7. (Silver stain).

ing the loss of the α -toxin band of mol. wt 34 000, a component of mol. wt 44 000 progressively increased in intensity in membranes from cerulenin-inhibited cells (fig. 6). An identical component was present in the culture supernates of control cells but neither of these two components showed close homology with the peptide maps of authentic α toxin of mol. wt 34 000 (fig. 7).

DISCUSSION

While cerulenin at sub-growth-inhibitory concentrations stopped the formation of extracellular α toxin, it reduced lipid synthesis by 50% only. Thus, there was no absolute correlation between lipid synthesis and toxin production in this instance, whereas Fishman *et al.* (1978) reported a requirement for lipid synthesis *de novo* in the secretion of penicillinase in *Bacillus licheniformis*. Paton *et al.* (1980) also found a relationship between lipid synthesis and secretion of protease and α amylase in *B. amyloliquefaciens*. It is not clear from these investigations whether the inhibitory effects of cerulenin on lipid synthesis, as measured by the incorporation of radiolabelled acetate, is directly responsible for the reduction in secretion of extracellular proteins, or whether the inhibition of exoprotein formation is a secondary effect of the antibiotic. The partial relief of the inhibitory effects of cerulenin by addition of exogenous fatty acids reported here and in previous studies (Fishman *et al.*, 1978; Paton *et al.*, 1980) does not resolve this question, because exogenous fatty acids may either provide an essential component for the secretion process or may have a

more indirect effect on the physical properties of the membrane (see Petit-Glatron and Chambert, 1981). Indeed, recent evidence by Jacques (1983) showed that the inhibition of lipid synthesis and secretion of glucosyltransferase by cerulenin in *Streptococcus salivarius* could be differentially relieved by Tween 80. The presence of this detergent largely eliminated the inhibitory effects of cerulenin on acetate incorporation, whereas the secretion of the exoenzyme was still inhibited. Also, inhibition of acetate incorporation and exoenzyme formation could be inhibited by dodecanoate, which has no known effects on lipid biosynthesis but does affect acetate uptake, as does cerulenin (Jacques, 1983).

In membranes from control cells, α toxin (mol. wt 34 000) was present as a prominent protein which was not released by washing with buffer of high ionic strength. No such component was evident in membranes from cerulenin-inhibited cells, nor was there any evidence of accumulation of a heavier precursor form in these membranes or in the cytoplasm. Our failure to detect such a precursor indicates that either a membrane-bound precursor is rapidly processed or degraded under conditions of cerulenin inhibition, or that no precursor form enters the membrane because toxin synthesis is blocked at an earlier stage. Mäntsälä (1982) showed that membrane-bound forms of several exoenzymes in *B. subtilis* were virtually absent in conditions of cerulenin inhibition, yet integral membrane-protein synthesis was unaffected. Recently, Tweten, Christianson and Iandolo (1983) detected two closely related membrane proteins in *S. aureus* which gave very similar peptide maps to authentic α toxin, but had several additional peptides. These membrane proteins probably represent precursor forms of the toxin that are processed very rapidly to mature toxin in normal cells but accumulate as a result of inhibition by dinitrophenol. No such precursor forms accumulated in the presence of cerulenin.

In elegant genetic studies, Kehoe *et al.* (1983) described the cloning and expression of the α -haemolysin determinant of *S. aureus* strain Wood 46 into *Escherichia coli* strain K-12, while Fairweather *et al.* (1983) reintroduced the cloned gene into *S. aureus* as well as into *B. subtilis*. Immunoprecipitation of cell lysates of both *E. coli* and *B. subtilis* showed the presence of two distinct gene products of mol. wt 33 000 and 34 000. It is not known whether or not these two polypeptides represent distinct gene products or one product in the processed and unprocessed form.

The absence of the precursor form of α toxin in membranes from cerulenin-inhibited cells may indicate a block in toxin formation at the level of transcription or translation. Depression of RNA synthesis by cerulenin has been reported in *B. subtilis* (Wille, Eisenstadt and Willecke, 1975), but the mechanism of inhibition was not clear. More recently, Müller *et al.* (1982) showed translation of cloned β lactamase in a cell-free wheat-germ system and in the presence of dog-pancreas microsomes the translation product was processed to authentic enzyme. These important experiments showed that cotranslational translocation of both prokaryotic and eukaryotic secretory proteins is by common mechanisms that make use of signal-recognition particle and docking proteins (Silhavy *et al.*, 1983). Direct effects of cerulenin on the physical properties of the membrane, as reported by Petit-Glatron and Chambert (1981) and Jacques (1983), may explain the absence of a membrane-bound precursor form of α toxin. Indeed, a range of membrane-modifying agents including aliphatic alcohols, phenethyl alcohol, benzyl alcohol and procaine affect the secretion of a wide variety of exoproteins in bacteria (Fishman, Rottem and Citri, 1980). If the

requirement for an active membrane-bound docking protein to relieve inhibition of translation by a signal-recognition particle proves to be a general feature of protein secretion in prokaryotes, agents such as cerulenin, which affect the physicochemical properties of the membrane by partitioning into the lipid domain of the membrane, may also act as inhibitors of exoprotein synthesis. Alteration of the environment of essential membrane components, such as docking protein, may render them inactive and thus explain the absence of accumulation of membrane-bound precursor forms.

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