The Characteristics of Extracellular Protein Secretion by *Staphylococcus aureus* (Wood 46) and their Relationship to the Regulation of α-Toxin Formation

By BAHJAT ABBAS-ALI AND GEOFFREY COLEMAN

Department of Biochemistry, University Hospital and Medical School, Clifton Boulevard, Nottingham NG7 2UH

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**SUMMARY**

The progress of secretion of α-toxin and total extracellular protein by *Staphylococcus aureus* (Wood 46), grown aerobically at 37 °C, in a 3% (w/v) tryptone soya broth medium supplemented with vitamins was followed. Exoprotein was secreted at a high rate by intact bacteria during the exponential phase (to 9 h) and into the post-exponential phase. After 18 h, when exoprotein accounted for 33% of the total protein in the culture, no further exoprotein was secreted although the bacterial density continued to increase at a low rate beyond this time. During the phase of active secretion, α-toxin represented a constant proportion of total exoprotein, the differential rate of synthesis of which increased by a factor of four after the end of exponential growth. Concomitant with the increase in the differential rate of exoprotein formation there was a fourfold increase in the intracellular concentration of RNA precursor material.

**INTRODUCTION**

*Staphylococcus aureus* secretes proteins into its external environment. Up to 30 different protein components have been separated from the secreted material (Wadström, 1974). They include both degradative enzymes and toxins, of which the α-toxin is the most widely studied. This has haemolytic, dermonecrotic and lethal activities (Arbuthnott, 1970).

Over many years, attempts have been made to define the best culture medium for maximum formation of staphylococcal α-toxin. Among the factors found by earlier workers to be important for α-toxin production were CO₂ and O₂ tension, pH, carbohydrate concentration, the presence of particular amino acids and the addition of yeast extract. These studies, which are difficult to correlate due to the wide variety of cultural conditions and strains of the organism employed, have been summarized by Arbuthnott (1970) and Alouf & Raynaud (1970). A number of different patterns for the development of α-toxin activity have been proposed. The most recent examples of the various possibilities suggest that it may be formed either during exponential growth only (Kapral, Keogh & Taubler, 1965) or throughout the growth cycle, but mainly after the end of exponential growth (Duncan & Cho, 1971). Further, Arbuthnott (1970) argued that toxin biosynthesis is not simply related to growth since, under certain conditions, growth was accompanied by the production of little or no α-toxin (see, for example, Gladstone, 1938). More recently, Dalen (1973) suggested that α-toxin is formed in response to a specific inducer.

An almost infinite variation in the pattern of development of extracellular protein products might be possible. However, Coleman (1967) and Stormonth & Coleman (1974) showed that, in a bacillus species, the characteristic pattern of development of total exoprotein and...
its principal individual components were identical throughout the growth cycle. Subsequently, Coleman, Brown & Stormonth (1975) proposed a general mechanism for the regulation of those extracellular enzymes and toxins which are formed by bacteria at increased levels after the end of exponential growth.

It seemed possible that previous investigators had not found a common factor in their work due to their failure to perform differential plots and to study toxin formation in relation to the dynamics of growth and macromolecule synthesis. The present study was designed to overcome this criticism and to establish a background to an investigation of the mechanism of regulation of α-toxin formation in the widely used S. aureus strain Wood 46.

**METHODS**

**Organism.** *Staphylococcus aureus* strain Wood 46 was used.

**Growth conditions.** The growth medium contained: Tryptone Soya Broth (Oxoid), 3 % (w/v); trace metal ion solution (Coleman & Elliott, 1965), 0·2 ml l−1; vitamin solution (Stormonth & Coleman, 1974), 40 ml l−1; and MgSO₄, 0·8 mM. Batches of medium (50 ml) in 250 ml conical flasks were inoculated with bacteria taken from the surface of Tryptone Soya Agar (Oxoid) slopes by means of a platinum loop. The cultures were incubated at 37 °C in a gyratory incubator-shaker (model G25, New Brunswick Scientific Co.). Bacterial dry weight was determined turbidimetrically by the method of Stormonth & Coleman (1972).

**Estimation of intracellular nucleotides.** The bacteria from 40 ml of culture were harvested by centrifuging, washed once with 0·9 % (w/v) KCl, resuspended in 4 ml 0·5 M-perchloric acid at 0 °C and then allowed to stand at 0 °C for 30 min. The preparation was then centrifuged at 6500 g for 3 min at 0 °C, the supernatant fraction was retained and the perchloric acid treatment was repeated on the pellet. The supernatant fractions from the two extractions were pooled and the nucleotide content of the combined extracts was determined colorimetrically by the orcinol method of Schneider (1957).

**Estimation of RNA and DNA.** The pellet remaining after the extraction of the nucleotide pool was resuspended in 4 ml 0·5 M-perchloric acid and heated at 70 °C for 20 min. The preparation was then centrifuged at 6500 g for 5 min. The supernatant fraction was retained and the pellet was extracted once more with 0·5 M-perchloric acid. The supernatant fractions from the two extractions were pooled and the combined extracts were assayed for RNA by the orcinol method (Schneider, 1957) and for DNA by the diphenylamine method (Burton, 1956).

**Estimation of extracellular orcinol-reacting material.** The bacteria were removed from 5 ml of culture by centrifuging at 6500 g for 3 min, and an equal volume of cold 10 % (w/v) trichloroacetic acid was added to 4 ml of the supernatant fraction. The resulting preparation was allowed to stand for 30 min at 0 °C, and then centrifuged at 6500 g for 3 min at 0 °C. The orcinol-reacting material in the supernatant fraction was estimated as before.

**Cellular protein estimation.** The residue from the nucleic acid extraction was dissolved in 1 M-NaOH and the protein in the resulting solution was determined by the modified biuret method of Bürgi, Richterich & Briner (1967). Bovine serum albumin (Sigma) was included as an internal standard.

**Extracellular protein estimation.** Protein was precipitated from 5 ml of the culture supernatant fraction by adding an equal volume of 10 % (w/v) trichloroacetic acid. The preparation was allowed to stand for 15 min and then centrifuged at 6500 g for 5 min. The precipitate was redissolved in 1 ml 1 M-NaOH and the protein was determined as before.
Staphylococcal exoprotein

Fig. 1. Extracellular protein secretion (○) and bacterial density (●) throughout the growth cycle of S. aureus (Wood 46).

Fig. 2. Distribution of protein between cellular (○) and extracellular (●) phases during the growth of S. aureus (Wood 46). The results are expressed as percentages of the total protein produced by the culture after 24 h growth.

α-Toxin assay. The α-toxin content of culture supernatant fractions was estimated by the method of Bernheimer & Schwartz (1963). Reproducible results were obtained by using the blood of the same rabbit in all the assay mixtures.

RESULTS

Relationship between extracellular protein secretion and growth

The relationship between extracellular protein secretion and the growth of S. aureus (Wood 46), under aerobic conditions at 37 °C, was studied (Fig. 1). After an initial lag, exponential growth lasted until the culture has been incubated for 9 h. The rate of increase in bacterial density then declined until, after 30 h, no further growth was detectable. During exponential growth extracellular protein was secreted into the culture medium. The rate of secretion increased during the post-exponential phase of growth until 18 h, when no further increase in exoprotein was observed; at this stage it accounted for one-third of the total protein synthesized (Fig. 2). The differential rate of extracellular protein formation was biphasic, the rate in the post-exponential phase being four times that achieved during exponential growth (Fig. 3).

Progress of change of nucleic acid and nucleotide pool size

Total contents of DNA, RNA and ribonucleotide pool materials increased during the growth cycle (Fig. 4) and are consistent with high rates of increase during the exponential phase, up to 9 h, followed by a decline. The facts that there was no reduction in total intracellular nucleotides up to 24 h and that there was no detectable increase in orcinol-reacting material in the culture medium, suggest that the bacteria remained intact.

Coleman et al. (1975) drew attention to the possibility that the high levels of mRNA associated with high rates of exoprotein secretion by bacteria might be due, in part at least,
Fig. 3. Relationship between extracellular protein production and growth of *S. aureus* (Wood 46).

Fig. 4. Changes in RNA (○), DNA (●) and the ribonucleotide pool (△), and the increase in extracellular orcinol-reacting material (▲), during the growth of *S. aureus* (Wood 46).

Fig. 5. Change in intracellular ribonucleotides per unit of bacterial mass during the growth of *S. aureus* (Wood 46).

Fig. 6. α-Toxin and the total extracellular protein secreted during the growth of *S. aureus* (Wood 46).

to high substrate levels or increased nucleotide pools. The progress of the change of intracellular ribonucleotides per unit of bacterial mass was therefore determined (Fig. 5). During the transition from exponential to post-exponential conditions there was a fourfold increase in intracellular nucleotide concentration which declined significantly after 18 h when exo-protein formation ceased.
Staphylococcal exoprotein

Relationship between \( \alpha \)-toxin and total extracellular protein secretion

The \( \alpha \)-toxin content of a series of culture supernatant preparations, collected over the period of active exoprotein secretion, was determined, together with the total exoprotein (Fig. 6). \( \alpha \)-Toxin represented a constant portion of total extracellular protein throughout the bacterial growth cycle.

DISCUSSION

Under the culture conditions employed in this investigation extracellular protein was secreted by intact bacteria throughout the exponential phase and for a considerable period into the post-exponential phase of the growth cycle. When exoprotein secretion ceased it accounted for 33% of the total bacterial protein formed, suggesting that much of the bacterial protein synthetic machinery was concerned with this activity such that competition for precursors was possible. The fourfold increase in the differential rate of exoprotein formation observed after the end of exponential growth supported this idea and suggested a similarity to our model for regulation (Coleman et al., 1975). We suggested that an increased differential rate of exoprotein synthesis, after the end of exponential growth, could be accounted for by competition at the level of transcription. Two separate effects might be involved. The first is an increase in available RNA polymerase on 'switching off' ribosomal RNA synthesis, and the second an increase in substrate concentration resulting from rRNA turnover. The fourfold increase in intracellular nucleotide concentration which accompanied the fourfold increase in the differential rate of exoprotein formation may be significant here.

The formation of exoprotein ceased before cellular protein. This is somewhat similar to the pattern of \( \alpha \)-toxin secretion in the same organism noted by Duncan & Cho (1971), but they found that after reaching a maximum the \( \alpha \)-toxin activity was reduced by surface denaturation. We measured total exoprotein so that our measurements would be independent of surface denaturation. There was no loss of exoprotein over a period of at least 10 h. The reason why exoprotein synthesis stopped before cellular protein synthesis is not known but may be related to high bacterial densities.

The total exoprotein secreted precisely paralleled the extracellular \( \alpha \)-toxin activity suggesting a common mechanism for the regulation of most of the individual exoproteins. However, it does not exclude the possibility that minor extracellular components may be produced in response to specific stimuli.

Bearing in mind that, by analogy with a bacillus species (Coleman, 1967), modulation of the biphasic pattern of exoprotein production may result from changes in the nutritional status of the medium. We believe that a knowledge of the differential rates of synthesis of exoproteins is essential to the understanding of their characteristics of secretion by \textit{S. aureus}. Whilst it may be premature to make detailed comparisons, our results generally agree with those of Kapral et al. (1965), using \textit{S. aureus 182}, and Duncan & Cho (1971), with strain Wood 46. Previous workers have failed to take the differential rate of synthesis into account when considering the factors affecting toxin production. In particular, the suggestion that \( \alpha \)-toxin is formed in response to a specific inducer, histidine (Dalen, 1973), should be reassessed in the light of our findings.
REFERENCES


