Environmental factors affecting toxic shock syndrome toxin-1 (TSST-1) synthesis

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Summary. The production of toxic shock syndrome toxin-1 (TSST-1) was studied in batch and continuous culture of *Staphylococcus aureus* strain 1169 in a carbohydrate-free chemically defined medium (CDM). In continuous culture oxygen- and arginine-limitation were required for steady-state TSST-1 synthesis. Aeration suppressed toxin synthesis. The amount of TSST-1 per mg dry weight (specific toxin) at dilution rates from 0.05 to 0.15 h⁻¹ was inversely proportional to the dilution rate. Protease activity increased with increasing dilution rates. In batch culture, TSST-1 began to accumulate in the medium towards the end of the exponential phase of growth, after a critical cell mass was attained. Maximum specific toxin production was observed in medium with an initial pH between 6.5 and 7.0. Growth and toxin synthesis took place in anaerobic conditions when CDM was supplemented with pyruvate and uracil. The Mg⁺⁺ concentration had no effect on the specific toxin in anaerobic conditions. In aerobic conditions, specific toxin increased c. 23-fold as the Mg⁺⁺ concentrations increased to 0.4 mM. Further increases in the Mg⁺⁺ concentration resulted in a reduction in specific toxin.

Introduction

Toxic shock syndrome (TSS) was first described by Todd *et al.* (1978) who defined criteria to distinguish this multisystem illness from other similar diseases. Although the pathogenesis of TSS remains unclear, it is often associated with strains of *Staphylococcus aureus* capable of producing toxic shock syndrome toxin-1 (TSST-1), and an illness very similar to TSS has been described in some animal models (Scott *et al.*, 1983; Arko *et al.*, 1984; Reeves *et al.*, 1984; Crass and Bergdoll, 1986). Factors affecting TSST-1 synthesis have been the subject of few studies and are still largely unknown (Schlievert and Blomster, 1983; Mills *et al.*, 1985; Schlievert, 1985). All previous investigations have used staphylococci grown in batch culture which has several disadvantages. Batch cultures are closed systems in which the environment is constantly changing because of the depletion of nutrients and accumulation of end products. Furthermore, batch cultures are time-dependent; the cells and their products from the exponential phase of growth may

not be the same as those from the stationary phase of growth. These problems are avoided in continuous culture where the growth rate is controlled by the rate at which a growth-limiting nutrient is added to the culture.

TSST-1 synthesis has hitherto been examined with staphylococci grown in nutrient excess and, consequently, with a rapid generation time. In comparison, the growth rate of micro-organisms in mammalian tissues has been shown to be very slow (Maw and Meynell, 1968; Polk and Miles, 1973) which suggests that, *in vivo*, the growth rate is limited by the availability of substrates. This study was designed to determine the effect of a controlled environment on TSST-1 synthesis. The effects of anaerobiosis, pyruvate, and magnesium were also examined in batch culture.

Materials and methods

Bacteria

S. aureus strains 1169 and 033 were obtained from M. S. Bergdoll (Food Research Institute, University of Wisconsin, WI, USA) and R. J. Arko (Sexually Transmitted Diseases Laboratory Program, Centers for Disease Control, Atlanta, GA, USA) respectively. With chemically defined medium (Catlin, 1973), we found that strain 1169 required arginine and proline for growth. *Bacteroides*

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fragilis strain 860009 was provided by V. R. Dowell (Centers for Disease Control, Atlanta, GA).

Medium and growth conditions

The chemically defined medium (CDM) described by Reeves *et al.* (1984) was used in all experiments. The medium was sterilised by filtration (prefilter and 0.22- μ m pore size filter; Millipore, Bedford, MA, USA). Aerobic batch cultures were grown in 250-ml Klett flasks, each containing 50 ml of CDM. The flasks were incubated at 37°C in a rotary shaking water bath at 150 rpm.

Anaerobic cultures were grown in 50-ml tubes fitted with loose metal caps; each tube contained 10 ml of CDM. After inoculation, the medium was flushed for 1 min with N₂ 95%, CO₂ 5% which had been sterilised by filtration. The tubes were then placed in an anaerobic jar with a gas-generating sachet (Gas Pak; BBL Microbiology Systems, Cockeysville, MD, USA) and incubated at 37°C on a rotary shaker at 150 rpm. *B. fragilis* was included in these experiments as a control for anaerobic conditions.

Continuous culture

Initial experiments indicated that growth depended on the availability of amino acids. Thus, the CDM used for continuous culture was modified to contain one eighth the amount of amino acids present in the original medium. The concentration of arginine was such as to be the growth-rate-limiting nutrient. Staphylococci were grown in model C-30 and C-32 chemostats (New Brunswick Scientific Co., New Brunswick, NJ, USA) equipped with 350-ml or 1350-ml culture vessels, respectively. Dissolved oxygen (DO) was monitored with a 900 series dissolved oxygen electrode (New Brunswick Co.). Before inoculation, the medium was saturated with oxygen by adjusting the air flow to 400 ml/min and the impeller speed to 800 rpm. Inocula consisted of a suspension of *S. aureus* strain 1169 dispensed in 1-ml volumes and stored at -70°C. After thawing, the suspension was used to inoculate the chemostat. The culture was kept at 37°C for 10 h with an air flow of 400 ml/min and an impeller speed of 400 rpm. After 10 h, the nutrient pump was started and the impeller speed increased to 700–800 rpm. Foaming was controlled by the addition of Antifoam C emulsion (Sigma Chemical Co., St Louis, MO, USA) to the growth medium before filter sterilisation. The final dilution of the antifoam C in the medium was 1 in 10⁶. To ensure the attainment of a steady state, staphylococcal cultures were allowed to remain at each dilution rate until at least 2 volumes of medium had passed through the culture vessel.

Toxin assay

Culture supernates were obtained after centrifugation at 5000 *g* for 10 min at 4°C. The culture supernates were concentrated 25- or 50-fold by ethanol precipitation

(Reeves *et al.*, 1984). The concentration of TSST-1 was determined by a radial immunodiffusion assay with goat antitoxin diluted 1 in 30 (Mancini *et al.*, 1965). The gel diffusion plates were incubated at room temperature and read after 24 h. The goat antitoxin was provided by M. W. Reeves (Centers for Disease Control). Reference toxin standards containing 12.5, 25, and 50 μ g of TSST-1/ml were prepared by diluting purified TSST-1 in phosphate-buffered saline, pH 7.2. A reference curve was plotted for each experiment. The purified TSST-1 was obtained from J. Kirkland (Procter and Gamble Co., Cincinnati, OH, USA).

SDS-polyacrylamide gel electrophoresis (PAGE)

The proteins in culture supernates were precipitated with 10% trichloroacetic acid. The precipitates were collected by centrifugation and resuspended in one-fifteenth of the original sample volume of final sample buffer. These samples were electrophoresed by the procedure of Laemmli (1970), modified by the addition of 70 mM NaCl (final concentration) to the separating gel (Mietzner *et al.*, 1984). The final acrylamide concentration was 12%. Gels were stained with Coomassie blue R250 (Miles Laboratory, Elkhart, IN, USA) 0.125% in ethanol 40% and acetic acid 10%. Acetic acid 10% v/v was used to destain the gels.

Western blot analysis

After electrophoresis, proteins were transferred on to nitrocellulose by the method of Towbin *et al.* (1979). TSST-1 was detected after incubation with goat antitoxin diluted 1 in 100. Bound antibody was detected by the method of Blake *et al.* (1984) with rabbit anti-goat IgG, conjugated to horseradish peroxidase (Miles Laboratories). The developing reagent was 4-chloro-1-naphthol (Sigma).

Amino acid analysis

Amino acids were determined by the method of Spackman *et al.* (1958), with a Beckman Model 121 automatic amino acid analyser. Samples were pretreated with trichloroacetic acid (final concentration 10%) to precipitate proteins.

Protease assay

Protease activity was measured with Hide Powder Azure (Calbiochem, La Jolla, CA, USA) as substrate. The reaction mixture consisted of 20 mg of Hide Powder Azure, 400 μ l of the sample to be tested, and 4–6 ml of 0.05 M tris-hydroxymethyl amino methane buffer, pH 7.8 (Rinderknecht *et al.*, 1968). Tubes were incubated at 37°C in a shaking water bath. At 15-min intervals, samples were removed from the water bath, placed in ice, and passed through a membrane filter. The optical density of each filtrate was determined at 595 nm.

Miscellaneous determinations

The total protein was measured in culture supernates, obtained from the chemostat, by the method of Lowry *et al.* (1951). Egg white lysozyme was used as a standard. Dry weights were determined as previously described (Morse *et al.*, 1974).

Results

Kinetics of TSST-1 synthesis

S. aureus strains 1169 and 033 were grown in batch culture in CDM, pH 6.5. Samples were removed at hourly intervals for the determination of turbidity, dry weight and TSST-1. The results (fig. 1) show that TSST-1 was not detected until the

Fig. 1. Kinetics of TSST-1 synthesis by *S. aureus* strain 1169 during batch culture in CDM.

middle of the exponential growth phase; strain 033 exhibited identical kinetics of toxin synthesis (data not shown). TSST-1 was detected after cultures attained a dry weight of about 250 μg/ml. Specific toxin rapidly increased as cultures entered the stationary growth phase; it continued to increase during the early stationary phase, at which point cell mass had attained its maximum value. Little additional toxin was synthesised during the late stationary phase.

The differential rate of TSST-1 synthesis by *S. aureus* strain 1169 continued to increase after a critical cell mass was attained (fig. 2). This rapid increase corresponded to the end of the exponential growth phase. The pH increased from 6.5 to 6.8 during exponential growth.

S. aureus strain 1169 synthesised about twice as much TSST-1 as strain 033. Strain 1169 was, therefore, used in subsequent experiments.

Fig. 2. Differential rate of TSST-1 synthesis during growth of *S. aureus* strain 1169 in CDM.

Effect of aeration on specific TSST-1

The effect of aeration was examined by growing strain 1169 in volumes of growth medium from 10 to 50 ml, in 300-ml nephelometer flasks, with constant agitation at 150 rpm. Samples were removed from each flask when growth reached the maximum stationary phase, and TSST-1, pH and dry weight were measured. The results (fig. 3) indicated that specific toxin increased by c. 200% with increasing medium volume, that is with decreasing oxygen availability. This was accompanied by a 17% decrease in the cell yield, and a decrease in the final pH from 7.6 to 7.2.

Effect of initial pH on TSST-1 synthesis

Specific toxin was significantly affected by the initial pH of the medium (table I). Bacterial growth

Fig. 3. Effect of aeration on TSST-1 synthesis by *S. aureus* strain 1169 grown in 300 ml nephelometer flasks containing volumes of CDM from 10 to 50 ml, with constant agitation at 150 rpm.

Table I. Effect of initial pH of chemically defined medium on TSST-1 synthesis of *S. aureus* strain 1169

Initial pH	Final pH (17 h)	Corrected cell yield (mg)*	TSST-1 ($\mu\text{g}/\text{mg}$ dry weight)
4.0	4.1	0	0
5.0	5.0	0	0
6.0	7.1	0.444	12.2
6.5	7.4	0.418	18.4
7.0	7.6	0.460	18.5
7.5	7.9	0.652	9.5
8.0	8.2	0.490	10.6

*Dry weight after growth for 17 h minus dry weight at time 0.

or toxin synthesis was not observed in medium with an initial $\text{pH} \leq 5.0$. The highest cell yield was observed in medium with an initial pH of 7.5 but the highest specific toxin was observed in medium with an initial pH between 6.5 and 7.0. There was no obvious relationship between the final pH of the medium and the specific toxin.

Effect of anaerobiosis, pyruvate, and magnesium on TSST-1 synthesis in batch culture

S. aureus strain 1169 grew anaerobically when CDM was supplemented with 40 mM pyruvate and 0.1 mM uracil. Pyruvate concentration was varied to determine whether it affected TSST-1 synthesis. Fig. 4 shows that the cell yield increased by *c.* 30% as the pyruvate concentration increased from 10 to 50 mM but specific toxin remained relatively unaffected.

Fig. 4. Effect of pyruvate concentration on TSST-1 synthesis by *S. aureus* strain 1169 grown anaerobically.

Complete CDM contained 0.7 mM Mg^{++} . To study the effect of Mg^{++} on TSST-1 synthesis, the Mg^{++} source was omitted from the medium. Atomic absorption spectroscopy indicated that omitting the Mg^{++} source resulted in a 26-fold reduction in Mg^{++} to 0.027 mM. The Mg^{++} concentration had no effect on the specific toxin in anaerobic conditions (fig. 5) but in aerobic condi-

Fig. 5. Effect of Mg^{++} concentration on TSST-1 synthesis by *S. aureus* strain 1169 grown in aerobic and anaerobic conditions: □ = aerobic conditions; ● = anaerobic conditions; ▲ = aerobic conditions and CDM supplemented with 40 mM pyruvate and 0.1 mM uracil.

tions, specific toxin increased *c.* 23-fold with increasing Mg^{++} concentrations up to a concentration of 0.4 mM; higher concentrations resulted in a 90% reduction in specific toxin. In aerobic conditions, the addition of 40 mM pyruvate and 0.1 mM uracil to the CDM somewhat modified the effect of Mg^{++} on specific toxin. The specific toxin increased *c.* 3.5-fold as the Mg^{++} concentration was increased to 0.8 mM; higher concentrations reduced the specific toxin by 25%.

Growth and TSST-1 synthesis in continuous culture

S. aureus strain 1169 was grown in continuous culture in arginine-limiting conditions. A steady state was attained at various dilution rates and the dry weights at each of these dilution rates were determined (table II). Washout occurred at dilution rates greater than 0.45 h^{-1} . The results presented in table II indicate that the highest specific toxin was observed when generation times were increased. During these experiments, the dissolved oxygen concentration remained between 50 and 60% of saturation. When the dissolved oxygen level was adjusted to 80–100% of saturation, growth occurred but no TSST-1 was detected (data not shown). This supported previous results obtained

Table II. Effect of dilution rate on the synthesis of TSST-1, protease and total extracellular protein by *S. aureus* strain 1169

Dilution rates (h ⁻¹)	Dry weight (mg/ml)	Total extracellular protein (µg/ml)	Extracellular protein (µg/mg dry weight)	TSST-1 (µg/mg dry weight)	Relative protease activity*/mg dry weight
0.05	0.380	272	716	4.47	24.2
0.08	0.355	208	586	2.25	240.6
0.11	0.295	240	814	2.71	338.9
0.15	0.160	233	1456	0	437.5

* Relative protease activity expressed as a percentage of the highest activity observed.

when the effect of aeration on TSST-1 synthesis was examined in batch culture.

SDS-PAGE analysis of samples obtained at the dilution rates given in table II revealed that numerous proteins were present, and there appeared to be both qualitative and quantitative differences between samples obtained at various dilution rates. The gel was electroblotted and the proteins transferred to nitrocellulose as described above. A band corresponding to TSST-1 was apparent after reaction with goat anti-TSST-1, as described previously. Fig. 6 shows that the anti-serum reacted specifically with a protein band corresponding to an apparent mol. wt of c. 22 000. The intensity of the bands (fig. 6) supports the data presented in table II. The absence of higher mol. wt species that react with the goat anti-TSST-1 suggests that TSST-1 precursors were not produced in significant amounts in these conditions.

The effect of dilution rate on TSST-1 synthesis was not a general phenomenon applicable to other staphylococcal extracellular proteins. For example, protease activity as a function of dry weight increased with increasing dilution rates (table II).

Discussion

Several investigators have used continuous culture to study extracellular protein synthesis by *S. aureus* (Engels *et al.*, 1980; Engels and Kamps, 1982). Results have indicated that several extracellular enzymes were produced optimally when *S. aureus* was grown under oxygen-limitation (Jarvis *et al.*, 1973; Carpenter and Silverman, 1974). Our results demonstrated that *S. aureus* strain 1169, grown in continuous culture with arginine-limitation, produced TSST-1 only when oxygen availability was decreased. A similar relationship between oxygen and TSST-1 synthesis was observed when this strain was grown in batch culture.

The dilution rate has been shown previously to have a varied effect on the synthesis of staphylo-

Fig. 6. Western blot of culture supernates of *S. aureus* strain 1169 grown in continuous culture and probed with goat anti-TSST-1. Lanes A–D: dilution rates 0.15, 0.11, 0.08 and 0.05, respectively.

coccal extracellular proteins. Jarvis *et al.* (1975) examined *S. aureus* strain S6 grown in continuous culture with Mg⁺⁺ limitation and observed that enterotoxin B, lipase, and deoxyribonuclease increased with increasing dilution rates; in contrast, lysozyme decreased. The synthesis of TSST-1 was also affected by the dilution rate. Specific toxin

decreased as the dilution rate increased; no TSST-1 was detected by immunodiffusion at a dilution rate of $\geq 0.15 \text{ h}^{-1}$. However, Western-blot reactions with goat anti-TSST-1 indicated that low levels of TSST-1 were produced at a dilution rate of 0.15/h. In contrast, the relative protease activity and total extracellular protein, expressed as functions of dry weight, increased 18- and 2-fold, respectively, with increasing dilution rates.

Although the relative protease activity increased substantially with increasing dilution rates, the absolute protease activity remained low (data not shown). This suggested that the decrease in specific toxin observed with increasing dilution rates was not the result of the proteolytic degradation of TSST-1.

The kinetics of TSST-1 synthesis were determined during batch culture. The results supported previous findings (Schlievert, 1985) which indicated that TSST-1 began to accumulate in the medium towards the end of the exponential phase of growth. An examination of the differential rate of toxin synthesis revealed that TSST-1 increased rapidly after a critical cell mass had been attained. This observation, together with the finding that specific toxin increased with decreased availability of oxygen, suggested that the critical cell mass was required to decrease the dissolved oxygen concentration sufficiently to permit optimal toxin synthesis. Other staphylococcal exoproteins such as coagulase (Engels *et al.*, 1980), enterotoxin B, and nuclease (Carpenter and Silverman, 1974) were also produced optimally in oxygen-limited conditions.

The vagina is basically an anaerobic environment (Wagner *et al.*, 1984). The insertion of a tampon was reported to change temporarily the vaginal micro-environment from anaerobic to aerobic (Wagner *et al.* 1984). The subsequent decrease in oxygen tension may create a suitable micro-environment for TSST-1 synthesis. However, our data demonstrating that TSST-1 synthesis also occurred in anaerobic conditions suggest that factors other than oxygen tension were involved. The specific toxin produced in aerobic conditions in batch cultures was only 4-fold higher than that produced in anaerobic conditions. The results concerning the effect of anaerobiosis on TSST-1 synthesis differed from those obtained by Schlievert and Blomster (1983) who reported a substantial increase in TSST-1 in aerobic conditions. This discrepancy may have resulted from differences in the media used in these studies. For example, uracil and pyruvate were added to CDM because they are required for the growth of *S. aureus* in anaerobic conditions (Richardson, 1936). Schlievert and

Blomster (1983) used a complex medium containing glucose; they showed that glucose decreased TSST-1 synthesis. Furthermore, metabolism of glucose in anaerobic conditions resulted in the production of lactic acid and a concomitant decrease in the pH (Friedman, 1939).

The initial pH of the medium markedly affected the synthesis of TSST-1. Maximal specific toxin was observed in medium with an initial pH between 6.5 and 7.0; no growth occurred at $\text{pH} \leq 5.0$. The pH of the vagina is approximately 4.5 (Rakoff *et al.*, 1944) and increases to about 7 during menstruation (Beller and Schweppe, 1979). Thus, the pH of the vagina during menstruation is optimal for synthesis of TSST-1.

The effect of Mg^{++} on TSST-1 synthesis has been the subject of several investigations and remains an area of controversy. Mills *et al.* (1985) reported that the aerobic synthesis of TSST-1 was controlled by the concentration of Mg^{++} , and that the greatest concentration of TSST-1 was observed when the concentration of Mg^{++} was low. Schlievert (1985), was unable to confirm this observation. We have examined the effect of Mg^{++} concentration on specific toxin during aerobic and anaerobic growth in batch culture in CDM. Our results in aerobic conditions are consistent with those of Mills *et al.* (1985). Even though we determined that the optimum Mg^{++} concentration was two-fold higher than that reported by Mills *et al.* (1985), a similar effect of Mg^{++} on TSST-1 synthesis was observed. Mills *et al.* (1985) omitted iron and manganese from their chemically defined medium. Both of these cations were present in CDM and this may account for the differences between our findings and those of Mills *et al.* (1985). It is possible that iron or manganese or both may also be involved in the regulation of TSST-1 synthesis. However, further studies are needed on the effect of different metals on TSST-1 synthesis. The concentration of Mg^{++} had no effect on the anaerobic synthesis of TSST-1 in medium supplemented with pyruvate and uracil. To verify that the addition of pyruvate and uracil was not responsible for the differences between the results obtained during growth in aerobic and anaerobic conditions, specific toxin was estimated during aerobic growth in CDM supplemented with pyruvate and uracil. The addition of pyruvate and uracil modified the effect of Mg^{++} on specific toxin. Morse and Baldwin (1973) observed that pyruvate repressed enterotoxin B synthesis in aerobic conditions. The inhibitory effect of pyruvate may depend on the concentration of Mg^{++} .

In a recent communication, Mills *et al.* (1986)

addressed the differences between their results and those of Schlievert. Like Mills *et al.* (1986), we feel that our results are *in-vitro* observations and that their validity *in vivo* should be assessed in relation to other environmental factors.

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