Effect of Dilution Rate and Mg^{2+} Limitation on Toxic Shock Syndrome Toxin-1 Production by *Staphylococcus aureus* Grown in Defined Continuous Culture

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A toxic shock syndrome isolate of *Staphylococcus aureus* was grown in a chemostat, in a defined synthetic medium of six amino acids, glucose, two vitamins and salts. Steady states were achieved under limiting and replete Mg^{2+} conditions and at a range of relative specific growth rates. The biomass and toxic shock syndrome toxin-1 (TSST-1) were estimated at each condition. Under Mg^{2+} limitation the biomass and TSST-1 production rates were reduced compared to Mg^{2+} replete conditions. Optimal TSST-1 production occurred at 0.81 relative specific growth rate.

INTRODUCTION

Toxic shock syndrome (TSS), first described by Todd *et al.* (1978), is an acute illness with multi-organ involvement (Davis *et al.*, 1980; Shands *et al.*, 1980). The illness is frequently associated with young adult women during menstruation, and *Staphylococcus aureus* is the likely aetiological agent (Davis *et al.*, 1980; Shands *et al.*, 1980). Although other protein exocellular toxins of *S. aureus* may be involved, toxic shock syndrome toxin-1 (TSST-1) would appear to be a pathogenic determinant. This is supported by the *in vitro* and *in vivo* effects of TSST-1 (Parsonnet *et al.*, 1985; Schlievert *et al.*, 1981; De Azavedo & Arbuthnott, 1984; Igarashi *et al.*, 1984; Schlievert, 1983).

Davis *et al.* (1980) and Shands *et al.* (1980) showed that the menstrual TSS cases were associated with tampon use. Since then, one interest has been in determining the effect tampons might have on the physiology of *S. aureus* (Eady *et al.*, 1983; Holland *et al.*, 1985; Ingham *et al.*, 1985) and particularly on TSST-1 production. Schlievert & Blomster (1983) showed the importance of oxygen for TSST-1 production, which led to the proposition that the tampon effect was due to introduction of oxygen into the vagina (Schlievert *et al.*, 1984; Wagner *et al.*, 1984). More recently, Mills *et al.* (1985, 1986) have put forward a hypothesis that TSST-1 production by *S. aureus* is controlled by Mg^{2+} concentration, with the availability of this ion being altered by tampons. In all these experiments batch culture has been used with the disadvantages of nutrient, pH and oxygen concentration changes with incubation time. Even more significantly, this method of culture is unable to divorce the effects of both Mg^{2+} concentration and oxygen tension in the environment from specific growth rate. Consequently, the effects observed cannot be assigned with certainty to changes in Mg^{2+} concentration.

The purpose of this work was to determine the effects of Mg^{2+} replete and limiting conditions, and dilution rate on TSST-1 production by *S. aureus* in continuous culture, in highly defined conditions.

Abbreviations: TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin-1.

METHODS

Bacterial strain. *Staphylococcus aureus* strain FRI 1187 was used; it was kindly provided by Professor M. S. Bergdoll, Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, Wisconsin, USA.

Continuous culture. The organism was grown in an apparatus (LH Engineering) with a 1.0 l vessel, working volume 680 ml, and control modules for pH (7.4), temperature (37 °C) and stirring (900 r.p.m.). The defined synthetic medium, delivered by controlled flow, consisted of (l⁻¹): arginine, 0.36 g, aspartic acid, 1.46 g; cystine, 0.12 g; glutamic acid, 1.59 g; glycine, 5.18 g; proline, 1.44 g; glucose, 16.4 g; nicotinic acid, 11.94 mg; thiamine hydrochloride, 600 µg; (NH₄)₂SO₄, 12.7 g; KH₂PO₄, 1.34 g; Na₂HPO₄, 5.7 g; CaCl₂·2H₂O, 5 mg; CoCl₂·6H₂O, 2.4 mg; CuSO₄·5H₂O, 500 µg; MnSO₄·4H₂O, 5.6 mg; NaCl, 5.8 mg; ZnCl₂, 3.4 mg; FeCl₃·6H₂O, 2.7 mg; polypropylene glycol 1025 (BDH), 1 ml. All other chemicals were obtained from Sigma. The MgSO₄·7H₂O concentration was varied. The medium was sterilized by filtration. The culture was sparged with air at 2 l min⁻¹ and the oxygen tension monitored.

Samples were removed aseptically after the culture had adjusted to steady state and not before six changes of culture volume had occurred. Culture supernatant fluids were stored at -70 °C for subsequent analysis for TSST-1.

Estimation of biomass. The biomass values used for calculation of maximum specific growth rate ($\mu_{\max}$) and confirmation of Mg²⁺ limitation were estimated from their OD₆₀₀ in an SP6-450 spectrophotometer (Pye Unicam). In all other cases the biomass was determined directly by weight (Greenman *et al.*, 1981).

Determination of $\mu_{\max}$. This was estimated from the exponential growth of batch cultures of *S. aureus* in the continuous culture apparatus, with the nutrient flow rate at zero. $\mu_{\max}$ was determined before and after a change in steady state conditions.

Relative specific growth rate (μ_{rel}). The experiments using limiting (100 µM) and replete (1000 µM) Mg²⁺ media were done at 0.33 μ_{rel} . The dilution rate of the culture was adjusted to give a specific growth rate (μ) of 0.33 $\mu_{\max}$ for each condition.

Test for Mg²⁺ limitation. To confirm 100 µM-Mg²⁺ was growth limiting under the defined conditions, a single pulse of 0.15 g MgSO₄·7H₂O in 3.08 ml was injected into the steady state culture to bring the final concentration of Mg²⁺ to 1000 µM (replete level). Subsequent changes in the culture biomass were estimated, and the Mg concentration in the culture was determined, after filtration of the samples through 0.20 µm porosity membranes (Flow Pore D, Flow Laboratories), using an Instrumentation Laboratory 151 atomic absorption spectrophotometer at 285.4 nm. The sensitivity of the instrument was 0.6 µM-Mg. The Mg analysis was kindly performed by Professor A. D. Cove, Department of Animal Nutrition, University of Leeds, UK.

TSST-1 assay. Rapidly defrosted samples were assayed for TSST-1 using reversed passive latex agglutination. The assay was done in V-well microtitre plates (LIP Ltd), using the TST-RPLA kit (Oxoid). Serial doubling dilutions of the unknown and standard TSST-1 were prepared. The concentration of the unknown was calculated with reference to the standard. The agglutination film diameter was measured under a low power stereoscopic microscope with a graticule giving results in arbitrary units. The end-point was defined at 15 units and a negative control sample gave 5 units.

Glucose. Glucose in the culture was estimated by the glucose oxidase method (Sigma kit no. 510) on supernatant fluid.

Analysis of results. Results were expressed as production rate of biomass, g dry wt cells l⁻¹ h⁻¹ and specific production rate of TSST-1 as µg TSST-1 (mg biomass)⁻¹ h⁻¹. The mean and ±95% confidence limits were calculated and where appropriate significant differences in results were determined by Student's unpaired *t*-test.

RESULTS

Effect of Mg²⁺ limitation on TSST-1 production

The cell biomass (x) in continuous culture at steady state is predicted by the equation

$$x = Y \left[S_r - K_s \left(\frac{D}{\mu_{\max} - D} \right) \right] \quad (1)$$

where Y is the yield constant for the limiting nutrient, S_r the reservoir nutrient concentration, K_s the saturation constant, and D the dilution rate equal to μ . Consequently, when Mg²⁺ ions are pulsed into the vessel and all other factors are constant, there should be an immediate rise in biomass if Mg²⁺ is limiting. The results shown in Fig. 1 demonstrate this, proving Mg²⁺ was limiting growth at 100 µM. This was supported by the results for Mg present in the culture, 5.7 µM under limiting conditions and 188 µM under replete conditions.

Fig. 1. At the time indicated by the arrow a single pulse of Mg^{2+} was added to a steady state continuous culture previously operating at an Mg^{2+} concentration of $100 \mu\text{M}$. The Mg^{2+} concentration was immediately raised to $1000 \mu\text{M}$ (replete). The subsequent rise in biomass indicates that the culture at $100 \mu\text{M-Mg}^{2+}$ was limited by this ion.

Fig. 2. (a) Effect of growth rate on biomass production rate. (b) Effect of growth rate on TSST-1 specific production rate. The mean and $\pm 95\%$ confidence limits are shown.

At the same μ_{rel} the biomass production rates were 0.225 and $0.588 \text{ g l}^{-1} \text{ h}^{-1}$ ($P = 0.022$) respectively, for media containing 100 and $1000 \mu\text{M-Mg}^{2+}$. The results for TSST-1 specific production rate in steady state conditions at $0.33 \mu_{\text{rel}}$ with Mg^{2+} limiting ($100 \mu\text{M}$) and Mg^{2+} replete ($1000 \mu\text{M}$) were respectively, 17.22 ± 3.2 and $129.94 \pm 30.2 \mu\text{g TSST-1 (g dry wt cells)}^{-1} \text{ h}^{-1}$ ($P = 0.0039$). Throughout these experiments there was a positive dissolved oxygen tension.

Effect of dilution rate on TSST-1 production

The effect of increasing dilution rate on biomass production rate is shown in Fig. 2(a). Up to a specific growth rate of 0.18 h^{-1} there was a continual rise in biomass production rate and thereafter a large decrease approaching μ_{max} .

The results for TSST-1 specific production rate, for cells grown at different specific growth rates in Mg^{2+} replete medium, are given in Fig. 2(b). TSST-1 specific production rate was very

low at the lowest dilution rate used, remaining constant as the dilution rate increased until at a specific growth rate of 0.18 h^{-1} the rate of TSST-1 production was very high. At the highest specific growth rate tested the production rate returned to the medium range specific growth rate levels. All cultures had positive dissolved oxygen tensions.

DISCUSSION

Chemostat continuous culture offers the facility to examine the effects of a single environmental variable on the physiology of the organism, with all other factors kept constant. Furthermore, comparisons are validated by using the same relative specific growth rate for different conditions that would impose different specific growth rates on the organism (Evans, 1976; Tempest, 1976). This type of experimentation was used to determine the effects of limiting and replete Mg^{2+} on biomass and TSST-1 production. As expected Mg^{2+} limitation at $100\text{ }\mu\text{M}$ reduced the specific growth rate by 15% compared to that at $1000\text{ }\mu\text{M-Mg}^{2+}$, replete conditions. Cells grown at $0.33\text{ }\mu_{\text{rel}}$ produced less biomass and TSST-1 per unit time in Mg^{2+} limited medium. TSST-1 production rate was reduced in this condition even when the reduction in biomass was taken into consideration. These results are in contradiction to those of Mills *et al.* (1985, 1986) who, using 24 h batch cultures, found that media containing 20 and $40\text{ }\mu\text{M-Mg}^{2+}$ yielded substantially increased TSST-1 production whilst higher Mg^{2+} concentrations increased viable cell concentration and reduced TSST-1 production. In these experiments Mg^{2+} concentration affected the *S. aureus* growth rate. Schlievert (1985) did similar experiments but with both a different *S. aureus* isolate and culture medium; his results showed that Mg^{2+} concentration did not affect TSST-1 production. However, the concentrations of Mg^{2+} ranged from $650\text{ }\mu\text{M}$ to 13 mM , far greater than those used by Mills *et al.* (1986), or in this investigation. Experiments with *S. aureus* grown in batch culture, in synthetic medium in shaken flasks, have shown that the growth rate is reduced when the flask to medium volume ratio was below 20:1 (unpublished results). In the experiments of Mills *et al.* (1986) the flask to culture volume ratio was 2.5:1. Consequently, it would be expected that the growth rate at some time during culturing would be sub-maximal due to oxygen limitation. Another point of difference between this work and that of Mills *et al.* (1986) and Schlievert (1985) was the estimation of growth, both using viable cell count. Biomass was used in this study which overcomes problems inherent in viable cell counting and the change in cell mass caused by cells growing at different specific growth rates (Schaechter *et al.*, 1958). Consequently viable cell counts do not adequately define the cell mass producing the TSST-1 in different conditions which also impose changes of specific growth rate on the organism.

The results of changing specific growth rate by the dilution rate of the culture, with all other factors constant, demonstrates that specific growth rate is important with respect to the production of TSST-1. There was a peak of TSST-1 production rate at the specific growth rate approaching μ_{max} (0.18 h^{-1}), and this production rate was greatly reduced at 0.2 h^{-1} , the highest μ attainable in steady state. Therefore, the interpretation of this data is that Mg^{2+} itself is not a key factor but growth rate is. The results of Mills *et al.* (1986) may be interpreted similarly, that is in batch culture low concentrations of Mg^{2+} reduce specific growth rate. It follows that other ions and organic nutrients, when incorporated in media at concentrations resulting in a critical reduction in specific growth rate, may increase the production of TSST-1. This hypothesis is testable using the continuous culture model and will be the subject of further investigation.

Our results and those of Mills *et al.* (1986) support the hypothesis that glucose represses TSST-1 synthesis, since in batch culture TSST-1 appears after the first, glucose utilization, exponential phase of diauxic growth in $20\text{ }\mu\text{M-Mg}^{2+}$ concentration medium (present authors interpretation). In continuous culture at the specific growth rate tested nearest to μ_{max} the biomass concentration was decreased as predicted by the chemostat equation (1) and glucose was present in the culture vessel (27.7 mM), whilst at all other dilution rates used the culture glucose concentration was zero. The specific production rate of TSST-1 was reduced in this glucose excess environment.

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