Production of Toxic Shock Syndrome Toxin 1 by *Staphylococcus aureus*
Requires Both Oxygen and Carbon Dioxide

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The effect of O2 and CO2 on expression of toxic shock syndrome toxin 1 (TSST-1) by *Staphylococcus aureus* was investigated under controlled growth conditions with continuous-culture techniques. To stimulate TSST-1 production, air and anaerobic gas were premixed before delivery to the culture vessel. At a growth rate—or mass doubling time (\(t_d\))—of 3 h, production of specific TSST-1 (expressed as micrograms per milligram of cell dry weight) was 5.9-fold greater at an O2 concentration of 4% than under anaerobic conditions. Increasing the O2 concentration to 11% did not result in a significant increase (\(P > 0.05\)) in the rate of toxin production over that during growth in 4% O2 but did result in a significant increase (4.9-fold; \(P < 0.001\)) in the rate of toxin production over that during anaerobic growth. At a \(t_d\) of 9 h, addition of 3.5% O2 resulted in a 7.6-fold increase in specific TSST-1 production. When room air was sparged through a culture growing at a \(t_d\) of 9 h, TSST-1 production increased significantly (by 3.4-fold) over that during anaerobic growth. When a growth environment of 4% O2—remainder N2 was studied, no increase in TSST-1 production was observed; this was also the case with 8% O2 at gas-flow rates of 0.1, 0.2, and 0.4 liters/min. In all experiments, production of biomass (expressed as milligrams of cell dry weight per milliliter) increased, indicating that O2 was metabolized by *S. aureus*. Addition of CO2 to the gas mix (4% O2, 10% CO2, 86% N2) resulted in a 5.1- to 6.8-fold increase in TSST-1 production over that during anaerobic growth and a 3.6-fold increase over that during growth in an environment of 4% O2—remainder N2. The *agr* mutant strain tested produced 6.1-fold more specific TSST-1 in a growth environment of 4% O2—10% CO2—86% N2 than during anaerobic growth. These data suggest that in this system, O2 alone does not trigger production of TSST-1; rather, both CO2 and O2 are required.

Staphylococcal toxic shock syndrome (TSS) is a relatively rare condition (0.06 cases per 100,000 people [2]) caused by one or more potent exotoxins produced by some strains of *Staphylococcus aureus* (4, 14, 32; P. M. Schlievert, Letter, Lancet 1:1149–1150, 1986). TSS is associated with a constellation of symptoms, including fever, rash, diarrhea, and the inability to maintain proper hemostasis. Severe cases often progress to multiple-organ involvement and desquamation of the skin over the entire body; some cases end in death (31). Of the 40% of TSS cases associated with menstruation (2), almost all are caused by TSS toxin 1 (TSST-1) (4, 14, 32; Schlievert, Letter, Lancet 1:1149–1150, 1986).

The association between menstrual TSS and tampon use has provoked considerable discussion regarding the possible role of tampons in menstrual TSS. Epidemiologic studies identified tampon brand and style as the most important risk factors for development of menstrual TSS (8, 21, 26, 28). Although some high-absorbency tampons appeared to increase the relative risk of TSS, epidemiologic studies did not resolve the issue of which specific tampon characteristic was associated with this increased risk. The possibilities cited included increased absorbancy, increased total surface area, chemical composition, and increased oxygen delivery via the tampon itself. In addition, alteration of the vaginal microflora during tampon use has received considerable attention. Previous in vivo studies from this laboratory indicated that tampons, regardless of fiber composition, neither alter the normal vaginal microflora during use nor provide a nidus for preferential growth of TSST-1-producing strains of *S. aureus* or other members of the vaginal microflora (18, 19, 20).

The concentrations of O2 and CO2, two components important to microbial growth, have been measured in the vagina before and after tampon insertion. In the undisturbed vaginal environment, O2 and CO2 levels (partial pressure) ranged from 0 to 3 mm Hg (0 to 0.5%) and from 50 to 60 mm Hg (6.5 to 7.8%), respectively (33). Immediately after tampon insertion, O2 and CO2 levels were 130 to 140 mm Hg (20.8 to 22.4%) and 10 to 15 mm Hg (1.3 to 2.0%), respectively. Later after insertion, the CO2 and O2 partial pressures approached preinsertion values at different rates: CO2 levels increased to preinsertion values within 20 to 90 min, while O2 concentrations did not fall to preinsertion levels within the 8-h observation period, remaining at >20 mm Hg or 3.2%. These observations suggest that substantial amounts of available O2 are present within the vagina for an extended period after tampon insertion. However, since the apparatus used for these measurements also resulted in the maintenance of a greater-than-normal total volume within the vagina, it was difficult to attribute the experimental observations to tampon insertion with any certainty, and the period of elevated O2 concentrations in the vaginas of test subjects may have been prolonged.

In vitro studies indicate that a number of environmental factors affect production of TSST-1, including magnesium concentration, oxygen concentration, growth rate, temperature, and pH (9, 13, 15, 16, 27, 29, 30, 34). However, it is still unclear whether any single factor affects TSST-1 production under controlled growth conditions that allow single variables to be altered. Most previous studies have used batch culture systems in which the growth rate, nutrient levels, and metabolite concentrations change during incubation. In such systems, alteration of one factor results in concomitant changes in other
factors associated with growth. But some studies have used the continuous-culture system, which allows the researcher to separate and define parameters that are interdependent during batch culture growth, such as growth rate, nutrient and product concentrations, and cell density. During continuous culturing, fresh medium is added to a culture at a fixed rate, and cells and medium are removed at a rate that maintains a constant culture volume. If, in addition, other growth environment parameters are held constant, the culture will reach a steady state at which there is no net change in production rates of metabolites or biomass. Once a steady-state growth has been achieved, the growth rate of the organism becomes independent of the concentration of nutrients. Continuous culture can be viewed as a technique for prolonging the exponential growth phase of a batch culture and for producing a population of cells growing indefinitely in an unchanging environment. Taylor and Holland used this type of system to evaluate the effects of various environmental factors on TSST-1 production; however, because they did not maintain a constant growth rate, it is unclear whether the effects noted for their experiments were due to changes in the environmental factors tested or were due to changes in the growth rate (29, 30). The purpose of the present study was to determine the effects of O2 and CO2 on production of TSST-1 in a controlled growth environment that included a constant growth rate.

MATERIALS AND METHODS

Bacterial strain, maintenance, and culture medium. S. aureus RN8846 is an S. aureus mutant strain generated from a TSST-1-producing parent, strain RN8465 (agr-), also isolated from a patient with menstrual TSS (provided by Richard Novick, New York University, New York, N.Y.). Strains were maintained by addition of sterile 1 N NaOH with the use of a pH meter (Cole Parmer) to determine the dissolved O2 concentration—were the same. A dissolved O2 probe and meter (Cole Parmer) were used to determine the dissolved O2 concentration produced by the gas mix being delivered to the second fermentor vessel. Samples (0.2 ml) were removed directly from the growth vessel of the second fermentor with a sidearm sampling port and were processed for confirmation of culture purity and measurement of CDW and TSST-1 concentrations.

Atmospheric O2. When O2 was delivered as a mixture of air and anaerobic gas mix to a culture growing at a t of 3 h, specific TSST-1 increased by 5.9-fold—i.e., from 2.6 ± 0.1 μg of TSST-1/mg of CDW in anaerobic conditions to 15.3 ± 2.5 μg/mg at an approximate atmospheric O2 concentration of 4% (P < 0.0001) (Table 1, experiment 1a). Biomass production increased 1.4-fold, which was determined not to be a significant increase (P > 0.05). At 11% atmospheric O2, there was a significant 4.9-fold increase in the amount of specific TSST-1 per unit of biomass and a 2-fold increase in biomass over that produced under anaerobic conditions (P < 0.0001) (Table 1, experiment 1b). Increasing the atmospheric O2 concentration from 4 to 11% did not result in significantly more specific TSST-1 or biomass (P > 0.05). At a slower growth rate (t of 6 h), specific TSST-1 increased by 7.6-fold and biomass by 2.0-fold under anaerobic conditions to an approximate atmospheric O2 concentration of 3.5% (Table 1, experiment 2). Delivery of 100% room air resulted in a significant, 3.4-fold increase in specific TSST-1 production (P < 0.005) and a 4.8-fold increase in biomass (P < 0.001) from anaerobic conditions to 21% atmospheric O2 (Table 1, experiment 3).

Analyzed gas mix of O2-N2. At a 9-h t0 and an O2 concentration of 4% (remainder N2), there was no significant change in specific TSST-1 levels (1.9-fold decrease; P > 0.05), while biomass increased 2.4-fold (P < 0.001) (Table 1, experiment 4). Likewise, there was no significant change in specific TSST-1 production when an 8% O2–92% N2 gas mix and increasing gas flow rates were used (Table 1, experiments 5, 6a, and 6b). Biomass production did increase 1.6-, 3.3-, and 4.6-fold for gas flow rates of 0.1, 0.2, and 0.4 liters/min (LPM), respectively (P < 0.001, except for experiment 5, for which the significance could not be calculated). There was a significant linear relationship between increasing biomass and increasing flow rate of the 8% O2–92% N2 gas mix (P < 0.0001). After the linear trend was accounted for, the difference among these values was still significant (P < 0.01).

Analyzed gas mix of O2-CO2-N2. In a gaseous environment of 4% O2–10% CO2–86% N2, the amounts of specific TSST-1 expression and biomass production were determined (Fig. 1). In all three experiments, there was a significant increase (P <
0.01) in biomass (by 2.0- to 2.6-fold) as well as in specific TSST-1 expression (by 5.1- to 6.8-fold) following exposure to the O₂-CO₂-N₂ analyzed gas mix. However, maximum biomass levels were not reached for 15 generations after the switch from anaerobic gas to the O₂-CO₂-N₂ mix. For specific TSST-1 expression, the lag before significant levels of expression were reached varied with the experiment; for the experiments depicted in Fig. 1A, B, and C, it was 9.4, 23.6, and 10.6 generations, respectively. The amount of biomass produced was the same for all three experiments (0.71 to 0.77 mg/ml), while the mean level of specific TSST-1 expression was not (3.5 ± 1.4, 10.2 ± 1.1, and 5.6 ± 0.7 µg/mg for Fig. 1A, B, and C, respectively). When an agr mutant strain was tested, there was a significant increase in both biomass (2.6-fold; P < 0.001) and specific TSST-1 expression (6.1-fold; P < 0.01) by 6.8 generations following exposure to the O₂-CO₂-N₂ analyzed gas mix (Table 1, experiment 8). In a similar experiment using the parent agr + strain (RN8465), there was an increase in both biomass (2.6-fold; P < 0.001) and specific TSST-1 expression (1.6-fold; P > 0.05).

Switch from O₂-N₂ analyzed gas mix to O₂-CO₂-N₂ analyzed gas mix. When the growth conditions were switched from O₂ and CO₂, specific TSST-1 increased by 3.6-fold—i.e., from 4.8 ± 0.02 µg/mg of CDW in a 4% O₂-96% N₂ gaseous environment to 15.3 ± 2.5 µg/mg in 4% O₂-10% CO₂-86% N₂ (P < 0.001). Biomass production did not significantly change. In a 4% O₂-96% N₂ gaseous environment, a CDW of 0.76 ± 0.02 mg/ml was produced; the switch to a gaseous environment of 4% O₂-10% CO₂-86% N₂ produced a CDW of 0.71 ± 0.02 mg/ml.

**DISCUSSION**

There are a number of ways air can be introduced into the vaginal environment, including through the tampon insertion process and as trapped air within the tampon. Our original aim was to determine how inclusion of O₂ introduced by tampon insertion affects *S. aureus* TSST-1 production by investigating whether O₂ as part of the gaseous environment of a continuous culture growth system resulted in increased specific TSST-1 production. Isolation and study of the effect of a single environmental variable, such as the concentration of dissolved O₂, on batch culture growth is difficult unless other variables are adequately controlled. An alternative to traditional batch culture methods is the use of the continuous-culture method, in which organisms are cultivated in a stable growth environment at a set growth rate. The growth rate can be changed without changing the amount of biomass in the system. The amounts of cell biomass produced during anaerobic growth were not statistically different (P > 0.05) between experiments at two growth rates (Table 1 and Fig. 1). These results demonstrate the separation between the growth rate and the biomass yield that can be achieved if continuous-culture methods are used. This type of system permits the study of factors that affect interactions of microfloral components or, as in the case of *S. aureus* TSST-1, production of virulence factors.

*S. aureus* MN8, growing at a t₅₀ of 3 h, did not produce significantly more specific TSST-1 when the level of O₂ (from air) was increased from 4 to 11%. When room air flowed through the system at 0.2 LPM, there was a significant increase in biomass compared to growth under anaerobic conditions and to oxygenated growth in experiments 1a, 1b, and 2, while TSST-1 production decreased in comparison to production at lower levels of O₂ (Table 1, experiment 3). When the growth rate was decreased (t₅₀, 9 h), the fold increase in specific TSST-1 was similar to that noted during growth at a t₅₀ of 3 h (Table 1, experiments 1a, 1b, and 2). These findings suggest that increased O₂ does not result in increased TSST-1 production and that the growth rate alone is not a factor in TSST-1 production.

In an attempt to more accurately study the effect of oxygen on TSST-1 production, an analyzed gas mix containing only O₂ and N₂ was used. Oxygen alone did not stimulate specific TSST-1 production but did stimulate a significant increase in biomass (2.39-fold at 4% O₂; Table 1, experiment 4). In light of the biomass increase (a result indicating O₂ metabolism), the lack of an increase in specific TSST-1 production cannot be attributed to insufficient O₂ transfer. In addition, increasing volumetric gas flow rates of 8% O₂ gas mix were associated with a significant trend of increasing biomass (Table 1, experiments 5, 6a, and 6b). These data indicate that oxygen alone, while capable of promoting increased biomass, does not trigger increased production of TSST-1 in this system. Hypothesizing
toxin expression or may act as a regulatory stimulus for toxin expression. It is important to note the lag in stimulation of specific TSST-1 production when an analyzed gas mixture of 4% O$_2$–10% CO$_2$–86% N$_2$ was used (Fig. 1). This lag in toxin production was not observed in other studies in which specific TSST-1 values increased or with strains RN8846 and RN8465. Within five generations after the addition of air to an anaerobic culture or CO$_2$ to a culture in an O$_2$-N$_2$ gaseous environment, the culture had already reached a new steady state and TSST-1 concentrations were maximal. Additional studies are under way to determine the cause of the lag in TSST-1 expression in this system.

The effect of CO$_2$ on TSST-1 production contrasts with that of O$_2$ and CO$_2$ on expression of other S. aureus virulence factors. In addition to exotoxins, such as TSST-1, S. aureus produces surface structures, such as teichoic acid and protein A, as well as several capsular polysaccharides (CPs), including CP5 (1). It has been shown that O$_2$ enhances CP5 production during exponential growth in batch culture (7, 11, 24) and that enhanced CP5 production is not due to respiratory activity alone (5). In batch culture studies in which increasing concentrations of O$_2$ were mixed with air, CO$_2$ levels as low as 1% inhibited expression of CP5 relative to expression in the presence of air alone, which has a CO$_2$ concentration of 0.1% (10). The authors noted that preliminary results from studies investigating the effects of CO$_2$ on CP8 expression were similar. In contrast, increased CO$_2$ levels (5%) did not affect the expression of protein A or teichoic acid. The effect of CO$_2$ alone on CP5 production was confirmed in studies using an O$_2$-N$_2$ gas mix. A 5% CO$_2$–20% O$_2$–75% N$_2$ gas mix inhibited CP5 expression relative to that measured for a gas mix without CO$_2$ (20% O$_2$, 80% N$_2$). In our studies, the positive effect of CO$_2$ on TSST-1 production was confirmed in a similar investigation. These experiments clearly demonstrate the importance of both O$_2$ and CO$_2$ in some S. aureus metabolic functions.

The human microflora encounters a variety of growth environments characterized by changing temperatures, nutrient concentrations, and gaseous atmospheres. These organisms have developed a number of regulatory responses by which to adapt to these changes. The mechanisms involved in the regulation of TSST-1 expression remain unclear. At least two global regulatory systems control expression of surface protein and exoprotein in S. aureus (including the expression of TSST-1): the regulatory loci agr and sar (3, 17, 23, 25). Our data demonstrate that a combination of O$_2$ and CO$_2$—but not O$_2$ alone—results in increased TSST-1 expression. The agr system regulates expression of some genes in S. aureus in response to the presence of O$_2$; an example is the positive regulation of CP5 expression (6). Preliminary results indicate that the agr system is not affected by changing CO$_2$ concentrations (10). The agr system also includes a cell-density-dependent regulatory mode that involves an octapeptide pheromone (12). Our studies show that the agr system is unlikely to be regulating TSST-1 production in this system, at least through a quorum-sensing signal. Specific TSST-1 production increased significantly while biomass remained unchanged when the gaseous environment of the culture was switched from 4% O$_2$ to 4% O$_2$–10% CO$_2$. In addition, when the biomass increased in response to the addition of O$_2$, specific TSST-1 production did not (Table 1, experiments 4 to 6b). Stimulation of specific TSST-1 production is not cell density dependent. Results from studies using an agr mutant, strain RN8846, (Table 1, experiment 8) also support the conclusion that the agr system is not regulating expression of TSST-1. Whether one of these systems or an as yet undescribed system regulates expression of TSST-1
is unclear. The nature of the relationship of TSST-1 production to CO₂ and O₂ will be the focus of additional research.

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REFERENCES