Oxygen and Carbon Dioxide Regulation of Toxic Shock Syndrome Toxin 1 Production by Staphylococcus aureus MN8

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Toxic shock syndrome toxin 1 (TSST-1) is a pyrogenic toxin superantigen produced by many pathogenic strains of Staphylococcus aureus. TSST-1 stimulates the proliferation of both T cells (5) that display particular Vβ elements in their T-cell receptors (11). TSST-1 is associated with staphylococcal toxic shock syndrome (TSS) and is considered to be the cause of nearly all cases of menstrual TSS and at least 50% of nonmenstrual cases (3). TSS is a severe multisystem condition characterized by high fever, rash, hypotension, and skin desquamation. TSST-1 has also been implicated in recallitang, erythematous, desquamating syndrome, which affects AIDS patients (6). TSST-1 is produced by organisms present in 60 to 70% of patients with Kawasaki syndrome, an illness that shares many features with TSS and that is typically seen in children under 4 years of age (12). The role of TSST-1 in Kawasaki syndrome remains controversial, however.

Numerous studies have demonstrated the importance of oxygen tension in the regulation of TSST-1 production by S. aureus. Excess aeration of cultures, as well as complete anaerobiosis, resulted in repression of TSST-1 production, while microaerobic environments appeared to stimulate toxin expression (15, 21, 24). It has been suggested that elevated vaginal oxygen levels associated with the insertion of a tampon stimulate the production of TSST-1 (17). Wagner et al. (23) demonstrated that the vaginal environment is normally anaerobic and that insertion of a tampon dramatically increased the oxygen level on the vaginal mucosal surface. Following tampon insertion, oxygen levels slowly declined throughout the observation period of 8 h.

The response of S. aureus, however, to the full spectrum of physiologically relevant oxygen tensions has never been completely and carefully examined in cultures that mimic physiological conditions in vivo. What oxygen levels result in maximum toxin levels and what oxygen levels effectively shut off toxin production have remained unanswered questions. In the study described here, we examined the production of TSST-1 by S. aureus in the presence of a range of oxygen levels relevant to in vivo conditions and discuss their implications for antistaphylococcal chemotherapy. We also characterize the response of S. aureus to various oxygen levels in the presence or absence of carbon dioxide. We introduce the use of thin-film culture to examine the response of S. aureus to oxygen and carbon dioxide.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The S. aureus strain used in this study, MN8, was isolated from the vagina of a TSS patient (4). Strains were grown in beef heart medium prepared as described previously (4) with 1% glucose-phosphate buffer (60 g of glucose, 40 g of NaHCO3, 40 g of NaCl, 30 g of Na2HPO4, and 4 g of L-glutamine in 1 liter of H2O) in either batch or thin-film cultures. For batch cultures, 300 ml of medium in 2-liter flasks was inoculated with S. aureus to achieve an initial density of 107 CFU/ml. Batch cultures were incubated at 37°C for 24 h. After removal of the cultures from the chambers, the cells were washed and were centrifuged at approximately 500 × g for 5 min to remove insoluble debris in the preparation for determination of TSST-1 concentrations by enzyme-linked immunosorbent assay (ELISA).

For thin-film cultures, 1 ml of medium containing 107 S. aureus CFU was placed on the bottom of polystyrene petri dishes (100 by 15 mm; Fisher Scientific, Pittsburgh, Pa.) and was held in place with squares (4 by 4 cm) of polyethylene mesh. The thin-film cultures were placed in sealed, humidified Plexiglas cell culture chambers (as described by Mishell and Mishell [13]; internal dimensions, 20 by 26 by 7.5 cm). The chambers had inlet and exit ports for flushing with the indicated gas mixtures before sealing. The thin-film cultures were incubated at 37°C for 24 h. After removal of the cultures from the chambers, the cells were
resuspended by agitation in 3 ml of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). Two milliliters of this suspension was immediately treated with 8 ml (4 volumes) of ethanol, and the mixture was incubated at room temperature overnight to precipitate the toxin. As described above, removal of cells prior to ethanol treatment was not necessary for quantitative toxin detection. The mixture was centrifuged at 500 × g for 10 min, the supernatant was removed, and the pellet was desiccated to remove excess liquid. The pellets were resuspended in 1 ml of phosphate buffer solution containing 1.0% fetal calf serum and 0.05% PTF, and centrifuged at 20,000 × g for 1 min to remove insoluble cell debris in preparation for determination of TSST-1 concentrations by ELISA.

**RESULTS**

Both batch and thin-film cultures were exposed to an atmospheric oxygen range of 0 to 21% (vol/vol) on the basis of the observation that vaginal oxygen concentrations are normally nearly anaerobic and will not exceed ambient oxygen concentrations (21%) upon insertion of a tampon (23).

**Batch cultures.** Batch cultures were incubated under continuous gas flow at 37°C until both the cultures had passed postexponential phase and the maximum rate of toxin production corresponded to densities of 1 × 10^8 CFU/ml. A representative growth curve and a time course of TSST-1 production are shown in Fig. 3. We have chosen to express toxin levels as the amount of TSST-1 produced per milliliter of culture, as the effect on the host will primarily be determined by total toxin levels and not the total amount of toxin produced per cell. With the exception of the batch culture grown in the presence of 0.5% oxygen, however, all cultures demonstrated nearly identical growth patterns and reached similar cell densities.

**Thin films.** Staphylococci grown in thin-film cultures formed a visible, opaque film covering the area (4 by 4 cm) of the petri dish. Without carbon dioxide, the production of TSST-1 by *S. aureus* in these cultures increased as oxygen levels were increased from 0 to 21% (Fig. 5). Cell densities were highly similar in these cultures, with an average of 3.6 × 10^9 CFU/ml. In the presence of carbon dioxide, TSST-1 production began to level off in the presence of approximately 8% oxygen and remained high in the presence of oxygen at up to 21% (Fig. 5). Cell densities ranged from 2.3 × 10^8 CFU/ml under anaerobic conditions to 5.8 × 10^9 CFU/ml in the presence of 21% oxygen.
gen. TSST-1 production was significantly enhanced in the presence of carbon dioxide (approximately 1 log unit).

**DISCUSSION**

TSS emerged as a recognized disease in the late 1970s and early 1980s. Early in the study of TSS, a correlation between the use of certain types of tampons and incidence of the disease was demonstrated (19). Numerous studies have attempted to determine the nature of this association. In 1983, our laboratory proposed that the presence of oxygen in the vaginal environment played a pivotal role in the induction of TSST-1 production by *S. aureus* (17). Several studies have shown that elevated oxygen, carbon dioxide, and protein levels, along with a relatively neutral pH, are required for TSST-1 production by *S. aureus* (8, 17, 24). Todd et al. (22) showed that altering any of these conditions greatly reduced the amount of TSST-1 synthesized by *S. aureus* in vitro and, furthermore, that these conditions are present in patients who experience TSS. Previous studies have not thoroughly examined the role of oxygen in the regulation of TSST-1 production, however, across the entire range of oxygen concentrations relevant to in

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**FIG. 1.** TSST-1 production by *S. aureus* MN8 in batch cultures flushed with gas mixtures containing various oxygen concentrations balanced with nitrogen. (A) Time course measurements of TSST-1 production in each batch culture. (B) Concentration of TSST-1 in each batch culture at 6 h. Data are means ± standard errors of the means of triplicate ELISA readings for each time point. Toxin production was measured in at least two independent batch cultures at each oxygen concentration, with similar results.
vivo conditions. In this study, we approximate in vivo conditions using a high-protein medium (beef heart) at neutral pH and controlled levels of carbon dioxide and oxygen. We fully characterize TSST-1 production by *S. aureus* exposed to the full range of oxygen concentrations expected to occur in vivo (0 to 21% [vol/vol] oxygen) in the presence and absence of carbon dioxide.

Both oxygen and carbon dioxide exhibit controlling effects on TSST-1 production in batch and thin-film cultures. In both culture types, anaerobiosis led to a reduction in the level of
toxin production to nearly undetectable levels (Fig. 1B, 2B, and 5). Similarly, batch cultures incubated without carbon dioxide in the presence of oxygen levels above 6% exhibited greatly reduced levels of toxin production (Fig. 1B). In contrast, the presence of carbon dioxide in either the batch or the thin-film cultures greatly increased the level of TSST-1 production (Fig. 2B and 5). This is consistent with previous studies that showed that both elevated oxygen and elevated carbon dioxide levels

FIG. 3. Growth curve and time course measurement of TSST-1 production by S. aureus MN8 representative of cultures incubated in the presence of oxygen balanced with nitrogen. The data shown here are for the batch culture incubated in the presence of 6% oxygen balanced with nitrogen. All cultures, with the exception of the culture incubated in the presence of 0.5% oxygen, exhibited similar growth patterns and toxin production profiles. TSST-1 production data are means ± standard errors of the means of triplicate ELISA readings for each time point.

FIG. 4. Growth curve and time course measurement of TSST-1 production by S. aureus MN8 representative of cultures incubated in the presence of oxygen balanced with nitrogen and 7% carbon dioxide. The data shown here are for the batch culture incubated in the presence of 8% oxygen balanced with nitrogen and 7% carbon dioxide. All cultures, with the exception of the culture incubated in the presence of 1% oxygen, exhibited similar growth patterns and toxin production profiles. TSST-1 production data are means ± standard errors of the means of triplicate ELISA readings for each time point.
were required for significant toxin production (8, 22). (TSST-1 production remained high even in the presence of normal atmospheric levels of oxygen [21%] in the presence of carbon dioxide. This suggests that previous studies that demonstrated an inhibitory effect of excess aeration [15, 24] either maintained culture oxygen levels well above those for cultures in our study or lacked sufficient carbon dioxide concentrations in the cultures.) These observations suggest sensory mechanisms that act in the regulation of virulence factors in *S. aureus*.

Wagner et al. (23) showed that carbon dioxide levels on the vaginal surface recovered from near atmospheric levels (<1% [vol/vol]) to normal in vivo levels (5 to 7% [vol/vol]) within a half hour after insertion of a tampon, whereas oxygen levels remained well above normal in vivo levels for the entire 8-h observation period. This suggests that vaginal *S. aureus* will encounter both elevated carbon dioxide and oxygen levels concurrently, conditions which this study demonstrates are optimal for TSST-1 production.

It is not readily apparent why the thin-film cultures responded differently than the batch cultures to various oxygen levels when carbon dioxide was not present (Fig. 1B and 5). At least two factors might be responsible for the observed differences in the response to oxygen levels. First, the physical environments of the batch and thin-film cultures are quite different. Unlike the constantly stirred cells in the batch culture, which are evenly exposed to atmospheric gases, the relatively stationary cells of the thin films are possibly exposed to concentration gradients of oxygen and carbon dioxide. Cells near the surface of the bacterial film are exposed to atmospheric levels of these gases, while cells closer to the polystyrene base may experience reduced oxygen and elevated carbon dioxide levels due to the aerobic metabolism of cells nearer the surface. Second, the batch cultures were continuously flushed with gas mixtures, whereas the thin-film cultures were placed into chambers initially flushed with the gas mixture and then sealed for the duration of the experiment. Atmosphere replacement may have prevented the accumulation of some metabolic by-products in the batch cultures, while lack of atmosphere replacement allowed accumulation of by-products in the thin-film cultures. This accumulation may have significantly altered the response of *S. aureus* in the thin-film cultures by inducing toxin production in the presence of high oxygen levels.

Oxygen-scavenging agents might make effective antitoxicogenic compounds, as anaerobic conditions inhibit TSST-1 production by *S. aureus*. It would be necessary, however, to find one that is both nontoxic to humans and a particularly effective scavenging agent, as even low levels of oxygen permit toxin production. A more effective approach to combating staphyloccocal infection might be to target the oxygen-sensing mechanism that staphylococci use to regulate virulence factor production. It is not difficult to envision a two-component histidine kinase (HK)-response regulator (RR) pair that is sensitive to oxygen levels and that interacts with *S. aureus* global regulators of virulence factors, including toxins. A search of the *S. aureus* genome databases at TIGR and at the University of Oklahoma with a TBlastN search program (1) revealed the presence of putative homologs to the ResDE HK-RR system that has been implicated in global regulation of aerobic and anaerobic respiration in *Bacillus subtilis* (14, 20). The putative *S. aureus* RR (PorA) is 68% identical to ResD, while the putative *S. aureus* HK (PorB) is 34% identical to ResE. The C terminus of PorB, which contains the conserved regions integral to HK activity, is 45% identical to the ResE C-terminal region. The N terminus has less identity (26%) with the corresponding region in ResE, but this region contains transmembrane helices that are less highly conserved among members of the HK family. We have confirmed the presence of these ResDE homolog genes in MN8 using PCR methods. Our laboratory is investigating the role of this putative two-component system in the control of virulence factors in *S. aureus*.

Indeed, the targeting of two-component systems has been shown to be effective against gram-positive pathogens, includ-
ing methicillin-resistant \textit{S. aureus} and vancomycin-resistant \textit{Enterococcus faecium} (2, 10). It is conceivable that such a targeted drug may inhibit oxygen sensing by staphylococci through two-component systems and thus prevent toxin production. Although these specialized drugs remain toxic to humans, future efforts are likely to produce safe and effective compounds for use in antistaphylococcal chemotherapy. Glycerol monolaurate has also been shown to have antitoxicogenic effects, while it has only weak antimicrobial action (18). A possible mechanism of glycerol monolaurate, which acts at the lipid-water interface, is to interfere with the activity of membrane-bound sensor proteins that are used by \textit{S. aureus} and that sense environmental conditions and regulate toxin production. Inhibition of toxin production would reduce systemic disorders in patients, while they would help to prevent further spread of the organism. Treatment with combinations of drugs that interfere with staphylococcal environmental sensing mechanisms and standard antibiotics may provide an effective two-pronged approach by quickly eliminating toxin production and clearing the organism from the patient.

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