Glycerol Monolaurate Inhibits the Production of β-Lactamase, Toxic Shock Syndrome Toxin-1, and Other Staphylococcal Exoproteins by Interfering with Signal Transduction

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Glycerol monolaurate (GML) is a naturally occurring surfactant that is used widely as an emulsifier in the food and cosmetics industries and is generally regarded as lacking in important biological activities. The recent observation that it inhibits the production of staphylococcal toxic shock toxin-1 (P. M. Schlievert, J. R. Deringer, M. H. Kim, S. J. Projan, and R. P. Novick, Antimicrob. Agents Chemother. 36:626–631, 1992) is therefore rather surprising and raises the interesting question of how such a compound might interact with cells. In this report, we show that GML inhibits the synthesis of most staphylococcal toxins and other exoproteins and that it does so at the level of transcription. We find that GML blocks the induction but not the constitutive synthesis of β-lactamase, suggesting that it acts by interfering with signal transduction.

We have recently observed that the surfactant glycerol monolaurate (GML) inhibits the production of toxic shock syndrome toxin-1 (TSST-1), the toxin responsible for toxic shock syndrome (4, 5), by Staphylococcus aureus and also of the streptococcal erythrogenic toxin SpeA (3, 26). These findings are remarkable inasmuch as GML, which is nontoxic and used as a mild surfactant and emulsifier, had been assumed to lack important biological activities. Therefore, we have undertaken an investigation of the mechanism by which this inhibition might occur. The hydrophobicity of GML makes it likely that any biological effect would stem from an interaction between GML and the cell membrane rather than between GML and any intracellular process. Therefore, it seemed likely that GML inhibition of TSST-1 production would affect a regulatory step involving the cell membrane. We consider the regulation of exoprotein production to involve two membrane-associated steps, namely, activation of the exoprotein genes by external metabolites via signal transduction and processing and secretion of the exoprotein precursors.

TSST-1 production in S. aureus occurs during postexponential growth and is responsive to a number of environmental factors such as the composition of the growth medium, the presence of oxygen, and a neutral pH (24). Expression of TSST-1 may also be subject to catabolite repression (22, 23). Control of TSST-1 production is therefore similar to that of a large number of other staphylococcal exoproteins, e.g., alpha-hemolysin (7, 10, 17).

These factors may act through one or more global regulatory systems of which one, the agr system (accessory gene regulator), has been characterized in some detail (9, 10, 14, 17). The agr system up-regulates most exoproteins during the postexponential phase of growth (10, 21) and down-regulates surface proteins such as protein A and coagulase (10, 14, 19, 21). The agr locus encodes two putative proteins with sequence motifs characteristic of the signal receptor and response regulator of the classical two-component bacterial signaling systems, and it is likely that this system functions by sensing external metabolites. agr regulates the level but not the timing of exoprotein production; timing is regulated by an independent but as-yet-uncharacterized system (28). Either of these systems is a potential target of GML inhibition. A third potential target, the secretion process, has not been characterized in S. aureus but is likely to resemble that of other bacteria.

In this report, we confirm that GML inhibits the production of TSST-1 and show that it also affects many other secreted exoproteins, including alpha-hemolysin. We also demonstrate that the observed inhibition is at the level of transcription. We find, moreover, that GML blocks the induction but not the constitutive synthesis of β-lactamase, suggesting that transmembrane signal transduction is the target of GML action.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and plasmids are listed in Table 1. MN8 is a TSST-1 staphylococcal enterotoxin A-producing strain (25). RN11 is a derivative of S. aureus NCTC8325 carrying pII258 (a naturally occurring β-lactamase plasmid [15]). β-Lactamase production is inducible in this strain (16). RN24, derived from RN11, is a constitutive, high-level producer of β-lactamase because of a mutation in the β-lactamase repressor (blaI) gene (8, 16). RN6390B is a wild-type (agr+) strain, RN6911 is an agr null strain (10, 17), and RN6734 is a derivative of RN6390B that does not produce beta-hemolysin because of lysogenization with phage d13, which inserts within the beta-hemolysin structural gene (6). RN6734 also contains pRN554. RN6734 was used for alpha-hemolysin assays (17). pRN66832 is a derivative of the promoter probe vector pWPN2019 (29) in which the alpha-hemolysin (hla) promoter is transcriptionally fused to the β-lactamase coding sequence. The fusion point corresponds to the +1 position of the hla...
| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|-------------------|
| MN8               | TSST-1* SEA*; naturally occurring* | 26 |
| RN11              | pRN6832(p258) | 18 |
| RN24              | pRN6832(pRN3038) | 8, 18 |
| RN6390B          | pRN6832 cured of 3 prophages; agr* | 19 |
| RN6734           | Beta-hemolysin-negative derivative of RN6390B, owing to lysogenization with Phi13 prophage; also contains Tn554 | 6; This work |
| RN6911           | Derivative of RN6390B with tetM replacing agr | 19 |
| RN7220           | RN6911(pRN6735) | 19 |
| RN8052           | RN6390B(pRN6832) | This work |
| RN8053           | RN6911(pRN6852) | This work |
| p258             | Naturally occurring β-lactamase plasmid | 18 |
| pRN3038          | p258bla443 (constitutive mutant) | 18 |
| pWN2019          | Promoter-probe shuttle vector containing pC194 and CoIEI replicons and a promoterless derivative of the p258 blaZ gene preceded by the pUC19 poly linker | 29 |
| pRN6832          | Derivative of pWN2019 containing the bla promoter transcriptionally fused to blaZ | 28 |
| pRN6725          | pC194 containing the p258 bla promoter and two-thirds of the blaZ gene, followed by several cloning sites derived from the pUC19 poly linker | 19 |
| pRN6735          | pRN6725:agr-a27 (agr-a27 is an agr fragment consisting of the malIII determinant lacking its promoter) | 19 |

*SEA, staphylococcal enterotoxin A.

| Strains          | Description                                                                 |
|------------------|-----------------------------------------------------------------------------|
| MN8              | TSST-1* SEA*; naturally occurring*                                          |
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| RN6911           | Derivative of RN6390B with tetM replacing agr                               |
| RN7220           | RN6911(pRN6735)                                                            |
| RN8052           | RN6390B(pRN6832)                                                           |
| RN8053           | RN6911(pRN6852)                                                            |
| p258             | Naturally occurring β-lactamase plasmid                                     |
| pRN3038          | p258bla443 (constitutive mutant)                                            |
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| pRN6735          | pRN6725:agr-a27 (agr-a27 is an agr fragment consisting of the malIII determinant lacking its promoter) |

*SEA, staphylococcal enterotoxin A.

Laemmli (13) with a Bio-Rad Mini Protean apparatus. Proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred electrophoretically to nitrocellulose filters (Schleicher & Schuell BA85) and treated with anti-TSST-1 (26) or with anti-S. aureus enterotoxin A serum (Toxin Technology, Sarasota, Fla.). Filters were subsequently treated with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), and the blots were developed as described by Blake et al. (2).

**RNA analysis.** Northern (RNA) blots of whole-cell lysates were prepared by the method of Kornblum et al. (11) and probed with the cloned TSST-1 gene (25) or a PvuII fragment encompassing nucleotides 330 to 4,150 of the agr sequence (10, 14).

**Assay of enzymatic activities.** β-Lactamase activity was determined as described previously (18) on samples taken at the indicated time points and normalized to total cell mass. Assay of alpha-hemolysin activity was done as described previously (1). Note that delta-hemolysin (which is encoded by agr-malIII [9]) is essentially inactive toward rabbit erythrocytes and therefore does not contribute to alpha-hemolysin titer.

### RESULTS

**Inhibition of exoprotein synthesis by GML.** To test for the generality of the GML effect, we analyzed four specific exoproteins in addition to TSST-1, namely enterotoxin A, alpha-hemolysin, staphylococcal protein A, and β-lactamase, and also studied the overall exoprotein patterns in supernatants of GML-treated and nontreated cultures, using subinhibitory concentrations of the ester. In Fig. 1a and b are shown Western blots (immunoblots) confirming the inhibition of TSST-1 synthesis by GML and demonstrating inhibition also of enterotoxin A production. Inhibition of alpha-hemolysin synthesis was demonstrated by hemolytic titration, as shown in Fig. 1c. The generality of this effect is demonstrated further in Fig. 1d, which depicts an SDS-PAGE analysis of post-exponential-phase supernatants of a culture of our standard laboratory strain, RN6390B, and of an agr mutant, RN6911, grown in the presence or in the absence of GML and stained with Coomassie brilliant blue. Note that most of the clearly defined exoprotein bands seen with the wild-type strain RN6390B are absent from the GML-treated culture and those that are still visible are present at much lower levels. The identity of most of these bands, however, is unknown. Similar results have been obtained with other strains (not shown), although in some cases, exoprotein bands that are not especially sensitive to GML are observed. With RN6911, in which protein A is overproduced because of the mutation in agr, it can be seen that protein A production is also inhibited by GML. A one-dimensional electropherogram of intracellular proteins (not shown) showed no obvious effect of GML, which is expected since GML cannot be a general inhibitor of protein synthesis at the level used, since the bacteria grow normally. In Fig. 1e are shown the comparative growth curves for strain RN6390B in the presence and absence of GML, demonstrating the lack of growth inhibition by the ester at concentrations sufficient to cause the inhibition of exoprotein synthesis shown in Fig. 1a to d. The fate of GML in the culture flask is somewhat uncertain. We infer that GML is degraded by one or more staphylococcal esterases. The major evidence for this comes from the observation that cells incubated with inhibitory concentrations of GML (>30 mg/ml) grow after a lag that is inversely proportional to the inoculum size and that the culture medium loses its inhibitory activity after the organisms have grown to saturation (20). Cultures containing a starting GML
FIG. 1. Effect of GML on exoprotein synthesis. (a) TSST-1. Supernatants from a culture of the TSST-1-producing strain MN8 were separated by SDS-PAGE, blotted onto nitrocellulose, treated with rabbit anti-TSST-1 serum and then with goat anti-rabbit alkaline phosphate conjugate, and then developed as described in the text. Panels A, B, and C correspond to points A, B, and C in Fig. 1e. –, without GML; +, with GML. (b) Enterotoxin A. Supernatants from a culture of MN8 (which also produces enterotoxin A) were separated by SDS-PAGE, blotted onto nitrocellulose, treated with rabbit anti-enterotoxin A antiserum and then with goat anti-rabbit alkaline phosphate conjugate, and then developed as described in the text. Panels A, B, and C correspond to points A, B, and C in Fig. 1e. –, without GML; +, with GML. (c) Alpha-hemolysin. Culture supernatants of strains RN6734 and RN7220 taken at points corresponding to A, B, and C in Fig. 1e plus one earlier point were analyzed for alpha-hemolysin production by hemolytic titration using rabbit erythrocytes. Hemolysin titers are expressed as the reciprocal of the dilution giving 50% lysis. Symbols: ○, RN6734; □, RN6734 plus GML; △, RN7220; ○, RN7220 plus GML. (d) Overall exoproteins. Culture supernatants for two different strains, RN6390B and RN6911, taken at the time corresponding to point B in Fig. 1e were analyzed by SDS-PAGE by the method of Laemmli and stained with Coomassie brilliant blue. RN6390B is a wild-type agr strain, and RN6911 is an agr strain in which protein A (arrow) is overproduced. RL, rainbow ladder (molecular weight standards; Bethesda Research Laboratories). –, without GML; +, with GML. (e) Growth of S. aureus in the presence (□) and absence (○) of GML. Inocula were prepared and cultures were grown as described in Materials and Methods. The asterisks indicate the times at which additional GML (10 μg/ml) was added to the GML culture. A, B, and C indicate sampling time points for Western and Northern blot analyses.
concentration of 20 mg/ml are initially inhibited for TSST-1 and alpha-hemolysin production but later regain the ability to produce toxin unless periodically supplemented with GML (unpublished data), and this is true for agr as well as for agr\(^+\) strains even though agr regulates the synthesis of at least one lipase (21a). These results suggest that there is at least one GML esterase whose production is neither inhibited by GML nor regulated by agr. For these reasons, we have adopted the supplementation scheme shown in Fig. 1e. This regimen maintains the inhibition of TSST-1 production but does not affect growth.

**GML acts before the secretion step.** Exotoxins such as TSST-1 are accessory proteins that are produced postexponentially by a facultative pathway with a membrane-dependent step at either end. As noted, GML is most likely to act by blocking one of the two membrane functions in this pathway, namely, signal transduction or processing-secretion. If processing-secretion were blocked, one would expect the accumulation of precursors in the cytoplasm. It was tested by preparing whole-cell lysates of post-exponential-phase cultures of GML-inhibited organisms and testing these for TSST-1 antigen by SDS-PAGE and Western blotting. These tests revealed no TSST-1-specific antigen, whereas lysates of untreated cells showed the expected low level of the TSST-1 precursor (not shown). We conclude, therefore, that GML inhibition is at a step earlier in the exotoxin biosynthetic pathway than the secretion step.

**GML blocks \(tst\) and \(hla\) transcription in \(S. aureus\).** If we are correct in assuming that GML acts at the cell membrane, then signal transduction would be the most likely target. If exotoxin genes are activated by a signal transduction mechanism, then one would expect that blockage of signal transduction would block exotoxin synthesis at the level of transcription. Accordingly, we determined the effect of GML on transcription of the \(tst\) and alpha-hemolysin (\(hla\)) genes. In Fig. 2a is a Northern blot hybridization analysis of RNA samples prepared from the MN8 culture samples used to analyze TSST-1 production (as shown in Fig. 1a) and probed with a \(tst\)-specific probe. As can be seen, there is no detectable signal in samples from the GML-treated culture, whereas there is a clear signal from the untreated samples, strongest at the latest time point. The sensitivity of these blot hybridization patterns indicates at least 98% inhibition of \(tst\) transcription by GML. At earlier time points, there was no detectable signal in either culture (not shown), in keeping with the known post-exponential-phase expression of TSST-1. Figure 2b shows measurements of the activity of \(\beta\)-lactamase produced by two strains carrying a plasmid containing the alpha-hemolysin promoter transcriptionally fused to the \(\beta\)-lactamase structural gene, in the presence and absence of GML. One strain, RN6390B, is an \(agr\)^- strain, and the other is the \(agr\) null strain. The transcriptional activity of the \(hla\) promoter seen with RN8053 (the \(agr\) null strain) represents the basal activity of the promoter in the absence of \(agr\). This activity is higher than that seen with the normal chromosomally located copy of the \(hla\) promoter, probably because of the gene dosage of the fusion vector (~15 copies per cell). \(agr\) inhibited the \(hla\) promoter in both strains to about the same extent. Here, the slight residual activity observed indicates approximately 80-fold inhibition. Since, for alpha-hemolysin, the function of a transcriptional gene fusion is inhibited, it may be concluded that GML directly affects transcription rather than translation or mRNA stability.

**GML inhibits \(\beta\)-lactamase induction.** To test directly for the inhibition of signal transduction by GML, we utilized the \(\beta\)-lactamase induction system, which is the best-known signal transduction system in \(S. aureus\). In this system, a transmembrane receptor, BlaR1, binds a \(\beta\)-lactam compound and transmits a signal to the cytoplasm that relieves repression by BlaI of the \(\beta\)-lactamase promoter. Mutations affecting \(blaI\) cause high-level constitutive \(\beta\)-lactamase synthesis (8, 16, 30).

As shown in Fig. 3, GML completely blocked induction of
β-lactamase, but only when it was added 75 min before the inducer, carboxyphenyl-benzoyl aminopenicillanic acid (CBAP). When GML was added at the same time as the inducer (CBAP), only partial inhibition was seen. Subsequent experiments have shown that it is necessary to treat cultures for 30 min with GML to observe full inhibition of induction.

In an attempt to localize the GML-sensitive step in the β-lactamase induction pathway, we tested the effect of the inhibitor on β-lactamase production by a strain containing a mutation in blaI and consequently producing the enzyme at a high constitutive level (16). Remarkably, high-level β-lactamase synthesis by this mutant was indifferent to the presence of GML, indicating that the inhibition is at a prior regulatory step. Additionally, the β-lactamase produced by this mutant was secreted to the same extent in the absence or presence of GML, indicating that GML does not block either processing or export of the enzyme. These results support the conclusion that the signal transduction step in β-lactamase induction is the site of GML inhibition.

**GML does not affect agr transcription.** It has been shown previously that transcription of the tsf gene is under the control of the staphylococcal *agr* operon (10, 21). The *agr* locus controls the production of a large number of extracellular and cell wall-bound proteins. For example, *agr* strains of *S. aureus* do not produce TSST-1 or alpha-hemolysin but overproduce protein A and coagulase. *Agr* activation is thought to involve signal transduction (10), and so it was considered likely that GML inhibition of exoprotein synthesis would involve inhibition of *agr* activity. In the *agr* system, it has been shown that the 500-nucleotide transcript, RNAII, is the positive effector for *agr*-regulated genes including *tsf*, *hla*, and *hlb* (9, 17). However, Northern blot analysis of RNAII and RNAIII transcription (Fig. 4) revealed that production of the *agr* transcripts is not inhibited by GML. This result rules out the possibility that GML inhibition of TSST-1 and alpha-hemolysin production involves inhibition of the *agr* activation pathway. This conclusion is supported by the observation that GML inhibits protein A production since protein A is normally overproduced in *agr* mutant strains (10). Since *agr* is not affected by GML, we suppose that there must be other signal transduction pathways involved in TSST-1, alpha-hemolysin, and protein A expression and that one or more of these must be blocked by GML. The existence of one such pathway has been suggested by recent experiments showing that a post-exponential-phase activation signal, distinct from *agr*, is required for the expression of up-regulated exoproteins (28). It is possible that GML acts by interfering with the transduction of this temporal signal.

**DISCUSSION**

This report has demonstrated that the commonly used surfactant GML, at concentrations that do not affect cell growth, inhibits the production of a large number of exoproteins by *S. aureus*. We have shown, for TSST-1 and alpha-hemolysin, that the observed inhibition is at the level of transcription. We have also shown that GML blocks the induction but not the constitutive synthesis of β-lactamase.

Although these results implicate transmembrane signal transduction as the target of GML inhibition, they do not reveal the particular target for exotoxins, whose synthesis is activated jointly by the global regulator *agr* and by an independent post-exponential-phase signal of unknown nature (28). Although sequence analysis suggests that *agr* activation involves a two-component signaling pathway (9, 14, 17), transcription of the *agr* locus was not inhibited by GML. Figure 1c dramatically illustrates the existence of a second pathway up-regulating toxin production and sensitive to GML. Total inhibition of alpha-hemolysin production was seen in the *agr* strain RN6734, despite the lack of inhibition by GML of *agr*...
transcription, and the same inhibition was seen when the agr effector, RNAIII, was produced constitutively under control of the bla promoter in an agr null strain. In Fig. 2b, it can be seen that the residual transcription, directed by the alpha-hemolysin promoter in an agr mutant, was inhibited as fully as that seen with the agr wild type. These results are consistent with the implication that the GML-sensitive step in exotoxin synthesis is not agr, and they indicate either that the above-mentioned post-translational-phase signal is the target or that there is some other unknown signal transduction system(s) regulating exotoxin synthesis.

Our current working hypothesis is that GML may intercalate into the cytoplasmic membrane and subtly modify membrane structure to interfere with the conformational shifts in the structure of transmembrane proteins by which signals are projected through membranes. Alternatively, GML may interfere with the normal placement of signaling proteins in the membrane. It is also possible that GML inhibits ligand binding. Effects on cytoplasmic elements of the signal transduction pathway are less likely because of the nature of the inhibitor. It is possible that GML is not a universal inhibitor of signal transduction; indeed, we have obtained preliminary evidence suggesting that GML is mitogenic for rabbit splenocytes (unpublished data). This would suggest that GML can activate as well as inhibit signal transduction. These results have a number of obvious implications for cellular regulation in eukaryotes as well as prokaryotes.

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