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Glycerol Monolaurate Inhibits Virulence Factor Production in *Bacillus anthracis*

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Anthrax, caused by *Bacillus anthracis*, has been brought to the public's attention because of the 2001 bioterrorism attacks. However, anthrax is a disease that poses agricultural threats in the United States as well as human populations in Europe, China, Africa, and Australia. Glycerol monolaurate (GML) is a compound that has been shown to inhibit exotoxin production by *Staphylococcus aureus* and other gram-positive bacteria. Here, we study the effects of GML on growth and toxin production in *B. anthracis*. The Sterne strain of *B. anthracis* was grown to post-exponential phase with 0-, 10-, 15-, or 20-μg/ml concentrations of GML and then assayed quantitatively for protective antigen (PA) and lethal factor (LF). After 8 h, GML at concentrations greater than 20 μg/ml was bacteriostatic to growth of the organism. However, a 10-μg/ml concentration of GML was not growth inhibitory, but amounts of PA and LF made were greatly reduced. This effect was not global for all proteins when total secreted protein from culture fluids was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Through quantitative reverse transcription-PCR assays, this toxin-inhibitory effect was shown to occur at the transcriptional level, since amounts of mRNA for *pagA* (PA), *lef* (LF), and *cya* (edema factor) were reduced. Surprisingly, mRNA levels of *atxA*, a regulator of exotoxin gene expression, rose in the presence of GML. These data will be useful in developing therapeutic tools to treat anthrax disease, whether in animals or humans. These results also suggest that mechanisms of virulence regulation exist independent of *atxA*.

Glycerol monolaurate (GML) is a mild surfactant commonly used in the food and cosmetics industry as an emulsifier. Although not identified with having significant human toxicity, GML inhibits the growth of some gram-positive bacteria at a concentration of 20 μg/ml, and at more dilute concentrations it inhibits virulence factor production. Previous studies have shown inhibition of toxic shock syndrome toxin 1, alpha-hemolysin, and protein A from *Staphylococcus aureus*, as well as inhibition of superantigens from *Streptococcus pyogenes* (9, 12). GML also inhibits the induction of vancomycin resistance in *Enterococcus faecalis* (11). Because of the success of GML with these organisms, GML is being investigated as a possible therapeutic agent to be used in wound dressings or in tampons to prevent the toxin production by these organisms.

The mechanism of GML action on these organisms is unclear. GML contains the fatty acid lauric acid, attached to a glycerol molecule. GML has 12 carbon molecules as its fatty acid backbone chain, which makes it span exactly one-half the width of a lipid bilayer. Because of its length and lipophilic nature, GML is thought to act at the membrane of organisms by interfering with signal transduction, most likely through two-component mechanisms. Such inhibition was suggested in at least one case through studies showing that GML inhibits the induction of vancomycin resistance in *E. faecalis*, which is a system that is controlled by a well-known two-component system, VanS and VanR (11). However, GML does not affect RNA III, which acts through the well-characterized *agr* two-component system in *S. aureus* that controls expression of secreted virulence factors (9). This leaves the possibility that there are yet-uncharacterized two-component systems through which GML may be acting.

Because of GML effects on other gram-positive organisms, we undertook experiments to determine the effect GML on *Bacillus anthracis. B. anthracis*, the causative agent of anthrax, is a spore-forming organism whose natural environment is the soil. Although an important agricultural risk, anthrax is currently in the limelight, being ranked as a top risk as an agent of biological terrorism. Therefore, it is important to understand mechanisms that control the expression of virulence factors as well to develop potential treatments in order to be well prepared for sudden outbreaks of the disease.

Anthrax has three proteins that make up two anthrax toxins, lethal factor (LF) and edema factor (EF). The toxin components consist of protective antigen (PA), LF, and EF. Their genes, *pagA* encoding PA, *lef* encoding LF, and *cya* encoding EF, are located on pXO1, one of two plasmids in *B. anthracis* that are essential for virulence. These toxin proteins are unique in that they are not active by themselves. Both LF and EF must be coupled to PA to form the active toxins lethal toxin and edema toxin. The other plasmid, pXO2, carries the genes *capABC*, coding for an antiphagocytic capsule.

Environmental factors are extremely important for the expression of toxin genes, most notably temperature and a CO_2 environment (1, 13). The toxin genes are controlled by a regulator, *atxA*, also located on the pXO1 plasmid, as well as *abrB*, a chromosomal gene, inhibiting toxin production until post-exponential phase (2, 3, 6). Yet, there is much unknown about toxin regulation and under what roles certain genes play in regulation. For example, although the presence of *atxA* is essential for anthrax toxin expression, modified expression levels

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of *atxA* have yet to be matched with a changing CO_2 environment (2).

In the present studies, GML was added to broth cultures, and growth and toxin production were analyzed. It was found that GML concentrations greater than 10 μg/ml had growth-inhibitory effects on *B. anthracis*. However, a 10-μg/ml concentration of GML inhibited the virulence factors PA and LF. Through quantitative reverse transcription-PCR (qRT-PCR), it was determined that the inhibition was at the transcriptional level, supporting other evidence that GML acts via signal transduction. *atxA* was found to be up-regulated in the presence of GML. These studies provide insights into toxin gene expression as well as treatments for anthrax that may be useful as adjunct therapies with antibiotics.

MATERIALS AND METHODS

Bacteria and growth conditions. The Sterne strain (342F; Colorado Serum Company, Denver, Colo.) was used in all studies. For growth and toxin experiments, one colony from a plate was inoculated into Luria broth and grown to stationary phase over 18 h. Approximately 750 μl of the overnight culture was added to fresh medium to give an absorbance at 600-nm wavelength of 0.1 (7, 10) in 25 ml of R medium supplemented with 0.8% sodium bicarbonate; the culture was grown at 37°C with 5.0% CO_2. CFU per milliliter were determined at 2, 6, 8, 12, 16, and 24 h after inoculation through a standard plate count technique. GML (Henkel, Gulph Mills, Pa.) was dissolved in absolute ethanol at a concentration of 10 mg/ml. GML was added to the cultures at 0, 5, 10, 15, or 20 μg/ml at time zero.

Toxin measurements. Cell-free culture medium was harvested after 8 h, filtered through 0.22-μm filters, and concentrated approximately 10-fold. Toxin was harvested at 8 h, because our laboratory experiments have shown that the postexponential time point is optimal to harvest exotoxins. Concentration of the medium was accomplished by dialyzing the medium in 6,000-to-8,000 molecular weight cutoff tubing (Spectrum Laboratories, Rancho Dominguez, Calif.) against water for 18 h and then air drying the tubing until the volume was about 1/10 of the starting volume. The culture medium concentrates were then used in Western immunoblotting and enzyme-linked immunosorbent assay (ELISA) analyses. Primary antibodies were purchased from Chemicon International, Temecula, Calif. LF antibody (MAB8086) was used at 1/10,000 dilution for ELISAs and 1/3,000 for Western immunoblot assays. PA antibody (MAB8081) was used at a 1/1,000 dilution for ELISAs and Western blotting. Secondary antibody, an anti-mouse immunoglobulin G-alkaline phosphate conjugate (Sigma-Aldrich, St. Louis, Mo.) was used at a 1/30,000 concentration. Standard curves for the ELISAs were constructed from purified PA or LF purchased from List Biochemicals, Campbell, Calif. To develop the ELISA, 200 μl of a 1-mg/ml solution of *p*-nitrophenyl phosphate dissolved in 1 M diethanolamine was added to each well, and the 96-well plate was read in a spectrophotometer at 405 nm after developing and stopping the reactions with H_2SO_4. The ELISAs were done in triplicate and were repeated from three independent experiments. The Western immunoblots were developed colorimetrically by addition of 50 μg of nitroblue tetrazolium/ml and 1 μg of 5-bromo-4-chloro-3-indoyl phosphate/ml.

Protein identification. Bands excised from silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were sequenced at the Protein Sequence Facility, Mayo Clinic, Rochester, Minn. The bands excised from the gels were digested with trypsin. The resulting fragments were then analyzed by liquid chromatography-tandem mass spectrometry. The identified peptides were aligned with the proteins from the *B. anthracis* genome. The proteins were definitively identified as PA and LF.

qRT-PCR. Cultures were grown under the conditions described above in R medium supplemented with 0.8% sodium bicarbonate. Three milliliters of the culture was harvested at 4 h (0.7 absorbance at 600-nm wavelength), pelleted (1,000 × *g*, 15 min), and resuspended in TE buffer (0.01 M Tris [pH 8.0], 0.005 M EDTA). The bacteria were digested with 20 μg of lysozyme (Sigma-Aldrich)/ml followed by total RNA isolation with an RNAqueous kit (Ambion, Austin, Tex.) according to the manufacturer's instructions. The total RNA was then treated with DNA*free* (Ambion) and LiCl precipitation to rid the RNA sample of contaminating DNA. Approximately 1 μg of total RNA was then reverse transcribed with Superscript II (Invitrogen, Carlsbad, Calif.) using random hexamer primers. One microliter of the 20-μl RT reaction mixture was used as template for real-time PCR with SYBR-Green PCR reagents from QIAGEN (Valencia, Calif.), and the reactions were performed in an icylcer machine (Bio-Rad, Hercules, Calif.). Reactions were performed at 95°C for 15 min for a hot start and then 95°C for 20 s, 54°C for 20 s, and 72°C for 20 s, for a total of 40 cycles. Primers for the mRNAs quantified, *gyrB*, *pagA*, *lef*, *cya*, and *atxA*, are listed in Table 1. The *gyrB* gene was used as a housekeeping gene, because growth of the organism in 10 μg of GML/ml was not severely affected and this gene has been used successfully in other studies measuring mRNA in *B. anthracis* (4). Data were analyzed by the ΔΔ*Ct* method described by Livak and Schmittgen (8). Briefly, the *Ct* value of the housekeeping gene (*gyrB*) was subtracted from the value of each experimental gene. Then, the differences between the samples grown with and without GML were found and taken to the power of 2 to find the fold difference in the amounts of starting mRNA in the sample. The data reported represent four independent experiments.

TABLE 1. Primers used in study

Gene	Forward primer (+) and antisense primer (−)
gryB	(+)5′-GGTGTTGGGGCATCTGTACGT-3′
	(−)5′-TAAATCCGCAACCGGAATAC-3′
lef	(+)5′-CTTAAGGAACATCCCACAGAC-3′
	(−)5′-CTGTGGCTGCATATAATATCG-3′
pagA	(+)5′-AAGTGCATGCGTCGTTCTTT-3′
	(−)5′-TTTCAGCCCAAGTTCTTTCC-3′
cya	(+)5′-TGCGCTTTTCTTTAGCGTTTTC-3′
	(−)5′-TTTCTCAAATCCCCCTTTTTC-3′
atxA	(+)5′-GTGTTGTTCGCTATAACAATCTCC-3′
	(−)5′-GCTCTGAGGTTATAGACGCTACTG-3′

RESULTS

GML effect on *B. anthracis* growth. To access the effect of GML on the growth of *B. anthracis*, various amounts of GML were added to liquid cultures at time zero and the growth was monitored over 24 h (Fig. 1). GML did not have a significant effect on *B. anthracis* growth in the presence of either 5.0- or 10.0-μg/ml concentrations. However, GML concentrations of 15 and 20 μg/ml inhibited growth for 12 and 16 h, respectively. All cultures, independent of the GML concentration, were ultimately able to recover and grow to stationary phase by 24 h. These results correlate with other experiments done to assess the effects of GML on growth of *S. aureus* (12).

GML does not have a global effect on secreted proteins. Once the GML effect on growth was determined, we evaluated the effect GML would have on total protein secretion at GML concentrations insufficient to inhibit growth. Cultures incubated with and without 10 μg of GML/ml were compared. Cell-free culture media from both 8-h cultures grown to the same approximate cell density were analyzed on a silver-stained SDS-PAGE gel (Fig. 2). The majority of the bands were the same on each lane of the gel and were of similar intensities. However, there were three bands at molecular masses of approximately 160, 150, and 120 kDa that were consistently present in cultures grown without GML and were not present in cultures incubated with 10 μg of GML/ml. These bands were excised from the gel and submitted for sequencing to the Protein Sequencing Facility at the Mayo Clinic. The 150-kDa band was identified as a mix of PA and LF. The other two bands were both identified as only LF. The 160-kDa band was most likely a dimer of LF, whereas the 120-kDa band was most likely a partially degraded combination of LF proteins. These results were then confirmed through Western immunoblotting for both PA and LF (Fig. 3). The immunoblot assays showed significant decreases in the amounts of PA and LF in cultures grown in the presence of GML. The quantities of toxin in each culture were measured with ELISAs. PA was found at

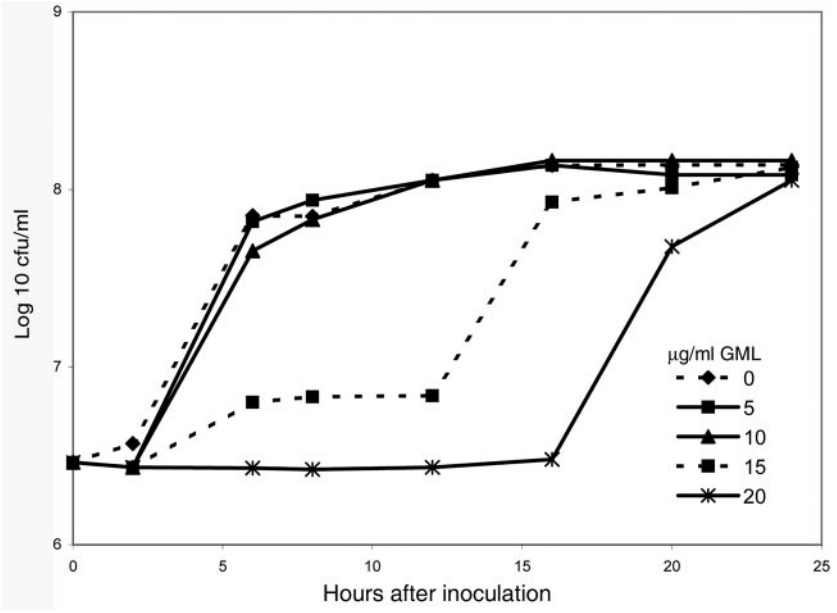

FIG. 1. Effect of GML on growth of *B. anthracis.* Samples of a stationary-phase culture (750 μl) were inoculated into 25 ml of R medium supplemented with 0.8% sodium bicarbonate. Cultures were grown with GML concentrations of 0, 5, 10, 15, and 20 μg/ml, and growth was monitored at 2, 6, 8, 12, 16, 20, and 24 h after inoculation.

9.3 and 0.6 μg/ml with 0- and 10-μg/ml amounts of GML, respectively. LF levels were found to be at 1.5 and 0.2 μg/ml at 0 and 10 μg/ml, respectively. These results showed that GML had a specific effect on *B. anthracis* virulence factors and not on total exoprotein secretion.

GML is acting at the level of transcription to inhibit virulence factors of *B. anthracis*. Since previous studies with GML showed that GML was acting via signal transduction to affect protein targets at the transcriptional level, it was hypothesized that GML was acting in a similar manner to interfere with PA and LF. Our hypothesis was addressed by performing qRT-PCR assays. Cultures with and without 10 μg of GML/ml were grown as before, and RNAs were harvested while the cells were in mid-exponential phase. The RNAs were reversed transcribed to produce cDNA before assay in real-time PCRs. In accordance with the ELISAs, LF mRNA was decreased in the presence of GML by approximately 18-fold (Table 2). EF (*cya*) mRNA was measured and was also found to be present at a 50-fold-lower level when the cells were grown in the presence GML. Although PA (*pagA*) mRNA levels were reduced, the

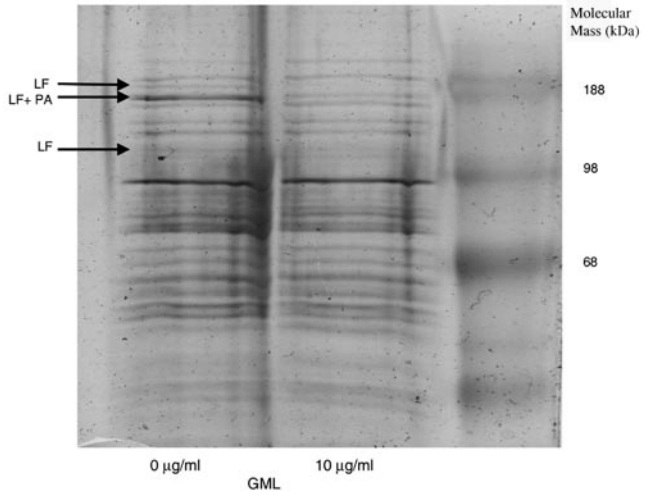

FIG. 2. Effect of GML on global protein secretion by *B. anthracis*. A GML concentration of 10 μg/ml was used because of its minimal effect on growth. Cultures were grown, and media were harvested at 8 h. The media were concentrated to 10× solutions, 10 μl of media samples was electrophoresed on an SDS–13% PAGE gel, and proteins were stained with a silver stain.

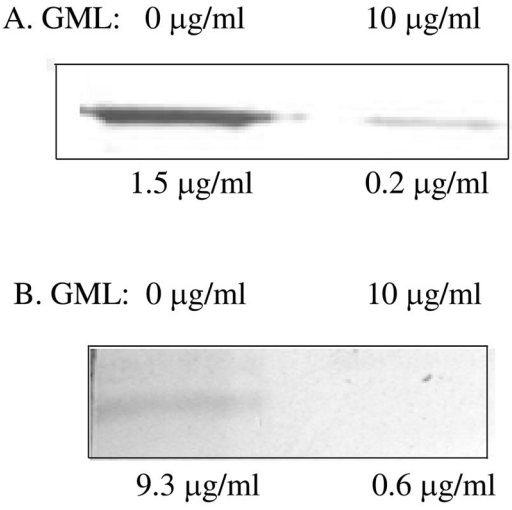

FIG. 3. Effect of GML on PA and LF production. Cultures were grown with and with out 10 μg of GML/ml for 8 h. The culture media were collected through filtration and concentrated 10 times. Samples were then assayed by Western immunoblotting and ELISA for the amount of LF (A) or PA (B) in the culture medium. The concentrations shown are from ELISA calculations.

TABLE 2. Differences in mRNA levels for virulence factor genes and regulator

Gene	Fold increase (+) or decrease (−) in mRNA with 10 μg of GML/ml
lef	−18.66 (±4.62)
pagA	−2.69 (±1.52)
cya	−50.57 (±16.6)
atxA	+4.74 (±2.32)

amount was not as striking as for the other two toxin genes. Levels of the *pagA* regulator, *pagR*, were not measured, but it is possible that GML regulates *pagA* by a different mechanism than the other two toxin genes. mRNA levels of *atxA* were surprisingly increased, although modestly, in response to GML. This suggests that the mechanism of GML action is not down-regulation of transcription of the entire pX01 plasmid, and its effect still appears to be toxin specific. Also, since *atxA* is thought to be an activator of toxin expression, it was surprising to find an increase in its expression in the presence of GML. This could indicate a separate level of regulation that is independent of the *atxA* regulation.

DISCUSSION

In this study, GML was found to be growth inhibitory to the Sterne strain of *B. anthracis* at GML concentrations greater than 10 μg/ml, an amount that is nearly 500-fold lower than the concentrations added to food and cosmetic products. A GML concentration of 10 μg/ml was not growth inhibitory but did have a direct and specific effect on toxin regulation. GML acted at the transcriptional level to reduce the amounts of all three anthrax toxin components. In contrast, mRNA levels of the anthrax toxin regulator *atxA* were actually increased in the presence of GML. This indicates that there may be another, undefined mechanism of toxin regulation that is sensitive to the effects of GML. It is also possible that up-regulation of *atxA* in the presence of GML represents a response by the organism to counteract the inhibition, which would indicate the organism has the ability to sense the amount of toxins made.

There has not yet been a system characterized that senses the essential CO_2 environment and then regulates toxin expression. A system of this type is likely to be a two-component system in which one component is embedded in the membrane, the sensing the environment, while the second component, a response regulator, is activated by the sensor and then regulates target genes. GML has previously been found to interfere with two-component systems in *E. faecalis* (11), and it is possible that the same mechanism is occurring in *B. anthracis. Bacillus subtilis* has over 23 two-component systems, and it is predicted that *B. anthracis* has a similar number (5). It is unknown whether or not GML acts on all two-component systems or on a subset of systems. It is also unknown how many two-component systems may be involved in *B. anthracis* toxin regulation. However, the data here suggest a specific effect. To examine other genes in our future studies, including the two-component system genes *abrB* and *pagR* that GML may affect in *B. anthracis*, microarray experiments are being done and will be used to examine differing expression profiles. This information will further our understanding of the mechanism of action of GML, and it should identify additional genes that may be involved in anthrax toxin gene regulation.

It is not yet known how GML will affect a fully virulent strain of *B. anthracis*, nor is it known if GML will affect the pXO2 plasmid and the capsule genes it contains. We believe that all strains of *B. anthracis* will be affected, since our prior studies suggested that all strains of *S. aureus* and group A streptococci tested were affected similarly. Since the capsule genes appear to be regulated by a similar mechanism as the toxin genes, it is expected that GML will reduce capsule formation as well.

Lastly, because GML has been shown to be an effective inhibitor of anthrax toxin production, our studies may lead to promising new adjunct therapies for the disease. GML is unlikely to be used in treatment without antibiotics. However, if the host is unable to inhibit the growth of the organism early enough to prevent serious disease, GML may be useful to at least block toxin production, and this may give antibiotics and the host's immune system chances to eliminate the organism. Future studies will examine the effects of GML in in vivo systems, including cutaneous, gastrointestinal, and inhalation models. Since GML is considered to be generally recognized as safe by the Food and Drug Administration for oral use and this compound is already used in food and other products, it is not expected that GML will be toxic in vivo.

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