

Microarray Bactericidal Testing of Natural Products
Against *Yersinia intermedia* and *Bacillus anthracis*

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ABSTRACT

To address the Defense Department's need for safe, effective protection against biological warfare agents (BWA's), natural products with a historical record of bactericidal efficacy such as bacteriocins, biosurfactants, lytic enzymes and essential oils or their active components were tested against non-pathogenic *Bacillus anthracis* and the plague pathogen surrogate *Yersinia intermedia*. Unlike the traditional well diffusion or spot lawn tests, the microplate/microarray test method used in these studies greatly simplified screening and interpretations of dose-response testing of the natural compounds as it relates to decontamination applications. Growth of treated cultures was measured turbidometrically with a microplate reader. Dose-response plots were made from the resulting turbidometric data to assess bactericidal efficacy of the test compounds. Several natural compounds were found effective against *Yersinia* and *B. anthracis* cells and spores using this method. These results show the promise of natural compounds in the development of environmentally responsible BWA decontamination preparations.

INTRODUCTION

The US Department of Defense recognizes the threat of biological warfare agents (BWA's) as a weapon of mass destruction from foreign military powers and terrorists. Although the chemical warfare agent (CWA) decontamination solution 2 (DS2) is also an effective bactericide, it is corrosive and its use produces hazardous waste. As the DoD is concerned with the environmental impact of decontaminants for fixed site and large area applications, decontamination solutions with an adverse effect on the environment are less desirable so alternatives were sought.

Microbial chemical agent decontaminating enzymes such as Organophosphorus Acid Anhydrolase (OPAA) and Organophosphorus Hydrolase (OPH) are good alternatives to DS2 for nerve agent decontamination, but they lack bactericidal activity. Inclusion of a bactericide to these enzymes for an anti-CWA/BWA preparation would require chemicals with little effect on enzyme activity or the environment.

Several natural products are effective bactericides and have low environmental impact, making them good candidates as biological decontaminants. Among these are the bacteriocins, essential oils and biosurfactants.

Bacteriocins are anti-microbial polypeptides that are bactericidal to non-host bacteria. Some bacteriocins have characteristics that make them desirable biological decontaminants, such as resistance to proteolytic degradation, heat stability and a wide target range. Commercially available Nisin, from *Lactococcus lactis*, has these characteristics and in addition is a FDA GRAS food additive.

Plant essential oils (e.g. oil of cloves, eucalyptus, etc) are attractive as BWA bactericides because of their historical use in the preservation of food, embalming and medicine. These oils and their active components (AC's) are also commercially available. As the bacteriocins from the lactic acid bacteria were more noted for their effectiveness with gram positive bacteria than gram negative bacteria, we also included the active components of plant essential oils in our decontamination studies for the *Yersinia* in addition to *Bacillus anthracis*.

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Biosurfactants are detergent compounds produced from biological sources. Microbial surfactants are harvested as secondary metabolites from bacteria such as *Pseudomonas*, *Acinetobacter*, *Arthrobacter*. Although the role of these compounds is usually associated with mobilization of hydrophobic nutritional sources, some of them are bactericidal to non-producer bacteria. Because the rhamnolipid microbial surfactant from *Pseudomonas aeruginosa* is a bactericide for *Bacillus subtilis* with low human toxicity, we also included it in our screening procedures.

Catalytic biodegradation agents such as the enzymes lysozyme and lysostaphin effect the structural integrity of many bacterial cells. Because of this property, these potential bactericides were also included in our studies.

Although procedures to quantitate the effects of bactericides were in place (e.g. spot lawn, well diffusion, sensidisks, tube and plate culture etc.), these methods were cumbersome for our BWA decontamination studies. We needed to know what concentration of bactericide (e.g. wt/vol or M) in aqueous media was needed to kill a given population of BWA at a designated growth stage. We also had to compare our results with natural product bactericides against results obtained with the harsh chemical agent decontaminants slated for replacement with more innocuous materials. A microarray/microplate testing method was devised to test the susceptibility of non-pathogenic *Bacillus anthracis* cells and spores and the plague pathogen surrogate, *Yersinia intermedia* to Natural products.

MATERIALS AND METHODS

Strains and Culturing Methods: Bacterial test strains are listed in Table 1. Isolation medium for the Lactococcus and Lactobacilli derived from cheese and buttermilk was TYM at either pH 5.5 or 7 incubated at either 37°C or 30°C. Growth medium for bacteriocin isolation was Lactobacillus MRS Broth. Nutrient broth was used as the test medium at 37° for *B. anthracis* and 30°C for *Y. intermedia* unless otherwise indicated (Brain Heart Infusion Broth and Turnbull' sporulation medium). Overnight cultures of *B. anthracis* were grown for 20 h prior to inoculation in the microplates. The ON or spore inocula were used routinely as they provided the most rigorous antimicrobial test matrices. Vegetative cultures of *B. anthracis* or *Y. intermedia* were obtained from an early-log culture (A600= 0.2). *B. anthracis* spores originated from frozen spore stocks, and diluted to 10⁷ spores/ml in the test wells with growth medium.

TABLE 1
Test Strains of *Bacillus anthracis*, *Yersinia intermedia*
and Lactic Acid Bacterial Food Isolates

Strain	Strain
<i>Yersinia intermedia</i> ATCC 33647	<i>Bacillus anthracis</i> NNR1-Δ 1
<i>Bacillus anthracis</i> ΔAmes	<i>Bacillus anthracis</i> VNR1-Δ 1
<i>Bacillus anthracis</i> ΔSterne	LJ (LAB cheese isolate)
<i>Bacillus anthracis</i> ΔNH-1	UB (LAB cheese isolate)

Isolation of the LJ and UB LAB isolate Bacteriocins: Bacteriocins were obtained from a one liter culture of each organism using the cell elution method of Yang, et al.¹.

Microarray testing: All microplates were read with a Bio-Tek 340i microplate reader. All assays were performed in duplicate with replicate negative and positive controls. All the outside wells on the plates were filled with uninoculated medium to provide an evaporation barrier for the test wells. Plant essential oils active components (AC's) were diluted with ethanol from a 0.1 M stock to make final concentrations of

10 mM to 10 μ M in the wells. The ethanol was evaporated from the plates in a Biosafety cabinet before medium or inoculum addition. Biosurfactants were either diluted in water and filter sterilized before use, or diluted in isopropanol and dispensed. The isopropanol was evaporated from the plates in a biosafety cabinet overnight before inoculation. AC's tested and their sources are shown in Table 2.

Table 2
Active Components of Essential Oils Tested against
B. anthracis and *Y. intermedia* in Microarray Format

AC	Plant Source	AC	Plant Source
Cineole	<i>Eucalyptus globulus</i>	Carvacrol	Oregano (<i>Origanum vulgare</i>)
Estragole	Tarragon(<i>Artemisia Dranunculus</i>)	Thymol	Thyme (<i>Thymus vulgaris</i>)
Linalool	Lavender(<i>Lavendula officianalus</i>)	Eugenol	Cloves (<i>Eugenia carophyllata</i>)
Linalyl acetate	Lavender(<i>Lavendula officianalus</i>)	Terpenin-4-ol	Tea Tree (<i>Melaleuca alternifolia</i>)

Stock solutions of Lysozyme and Lysostaphin (Sigma Chemical) were filter sterilized and prior to dilution (1 mg/ml to 0.5 μ g/ml final) with culture medium in the microplate wells. Nisin (Sigma Chemical), 1 mg/ml (Nisin), was sterile filtered and diluted in growth medium to a final concentration (in the well) ranging from 1 mg/ml to 1 pg/ml. Bacterial inocula were added last to initiate the test (zero time). Untreated wells were also included on all microplates, and the turbidity in these wells (after the blank correction) used to calculate % growth treated/untreated data that is presented in the dose-response plots. Turbidity readings were taken at 630 nm periodically (15 min to hour intervals) and after overnight incubation. For compounds producing aqueous emulsions (e.g. biosurfactants), a set of uninoculated controls was run concurrently and these turbidity values subtracted from the inoculated+test compound turbidity readings. Real time data for single microplates used microplate lids treated with sterile diluted and dried antifog to prevent fogging. The incubation temperature was 37°C. The reader was programmed to shake at low speed for 10 seconds prior to each read for real time monitoring.

Micrographs: The effects of Nisin at varying concentrations on the Bacilli after ON incubation was captured with an Olympus BX-50 Phase Contrast Microscope at 1000X on Kodak 400 ASA Gold Film. Photos were scanned and exported to MS Power Point.

RESULTS

Testing candidate BWA decontaminants in microarrays proved a convenient and rapid method to gather viability data on treated bacteria. Because the wells hold less liquid than the culture tubes (0.2 ml vs. 5 ml), the amounts of biological waste and treatment candidates were greatly reduced. Several hundred assays could be run simultaneously using this method. However, many technical refinements were needed to obtain consistent results.

Technical issues:

Lid fogging: Solved with sterile antifog to plate lids or incubating plates in a plate incubator, then heating the lids prior to reading. Other solutions are a plate reader that heats evenly or a warm room for all operations.

The one plate/reader limit: Incubate plates in a plate incubator; remove plates periodically to read them.

Uniform culture distribution: Critical for non-motile *B. anthracis*-solved with a rotating platform in the plate incubator during the incubation period. *Y. intermedia* was not as problematic, as it is motile.

Evaporation: Solved by adding uninoculated and untreated media to “barrier wells” in all the outside wells.

AC vapor migration: Minimized with barrier wells between rows of different AC’s.

Addition of Matrix components: Solved with 12 well reservoirs.

Insoluble (turbid) anti-BWA components: Not suited to turbidometric analysis in microplates or tubes: plate counts are more reliable. Reasonable compensation for the emulsion’s contribution to the total optical density can be obtained by running a set of treated, but uninoculated wells concurrently with the treated and inoculated wells. The data obtained from the uninoculated set is subtracted from the total optical density reading to obtain turbidity produced by the growing cells.

Data reduction: Export data to a communications program (e.g. MS hyperterminal) then to Spreadsheet program (Excel, Quattro Pro, etc.). Better solution: acquire the appropriate reader software.

Findings

Several natural compounds gave excellent bactericidal activity against the test BWA’s. The Minimum Inhibitory Concentrations (MIC) was the concentration of treatment agent in aqueous medium that prevents culture growth. Nisin and preparations of LJ and UB lactic acid bacterial isolates all showed good activity against *B. anthracis* at low concentrations (Figure 1, Table 3). Only the AC’s thymol, eugenol and carvacrol were effective against both *B. anthracis* and *Y. intermedia* (Figures 2, 3, 7, Table 4). None of the biosurfactants tested were effective against *Y. intermedia*, but the Petrogen, Battelle and Janeil biosurfactants were effective against *B. anthracis* (Table 5). The highly purified Janeil biosurfactant (99% rhamnolipid) was the most effective (Figure 9), and also formed transparent solutions (no emulsion) at all but the highest concentration tested. Lack of an emulsion greatly simplified data reduction and results interpretation. The enzymes lysozyme and lysostaphin were not effective against *B. anthracis* or *Y. intermedia* cells at any concentration.

Table 3
Minimum Inhibitory Concentration of Bacteriocin Needed
for the Several *Bacillus anthracis* Strains

Strain	MIC, µg/ml, for ON zero growth
ΔAmes	600*
ΔSterne	1000*
ΔNH-1	850*, 50**, 850***
NNR1-Δ 1	950*
VNR1-Δ 1	1000*

*Nisin, using overnight cultures as the inoculum.

**LJ preparation (ref. 1), using spores as inoculum.

***UB preparation (ref. 1), using spores as inoculum.

Table 4

Minimum Inhibitory Concentration of the Active Component of Essential Oils needed for an Overnight Inocula of *B. anthracis* VNR1-Δ1 and *Y. intermedia*

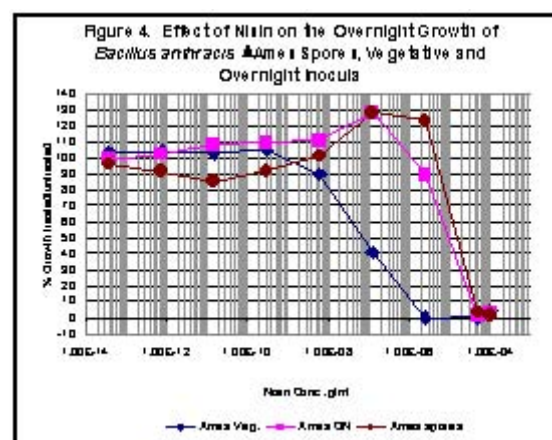
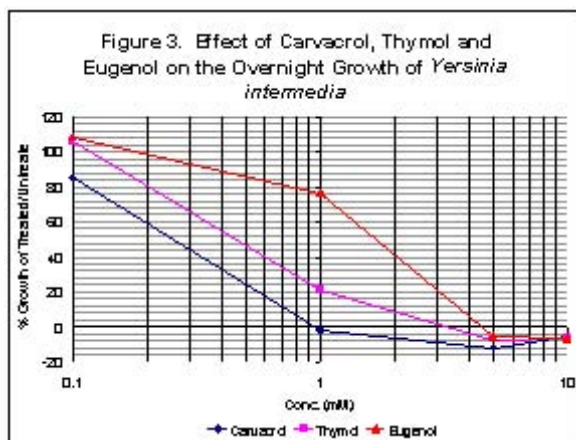
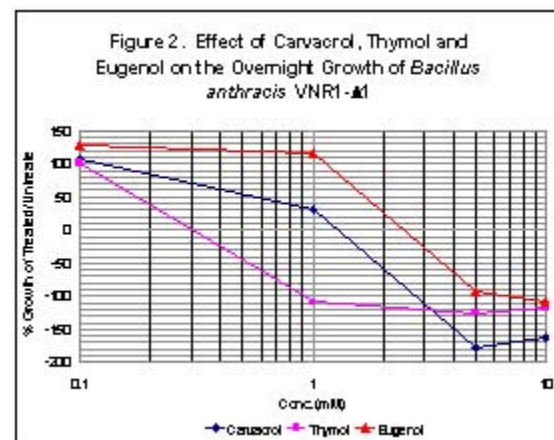
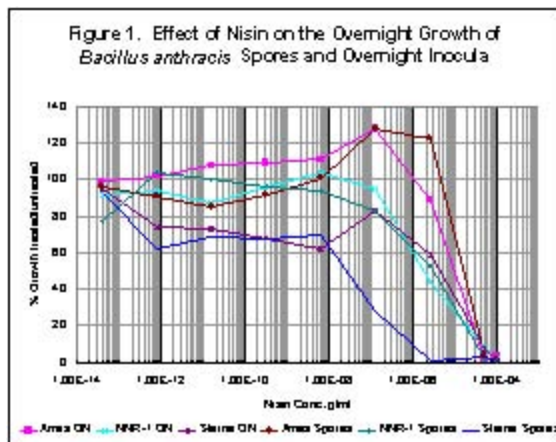
Active component	MIC (mM) B.A.	MIC (mM) Y.I.
Carvacrol	1.2	1.0
Thymol	0.3	3.5
Eugenol	2.5	4.5

Table 5

Bactericidal Testing of Biosurfactants with *Bacillus anthracis* ΔNH-1

Biosurfactant*	MIC, μg/ml, for ON zero growth
Janeil (<i>P. aeruginosa</i>)	100 (spores)
Petrogen (<i>P. aeruginosa</i>)	100 (spores), 10 (cells)
Battelle (unknown)	500 (cells)
ML2 or ML3 (<i>P. aureofasciens</i>)	Not effective (600 to 0.6 μg/ml)

* The rhamnolipid purity of the Janeil biosurfactant was 99%. The surfactant content for the Petrogen or Battelle surfactants was not provided.



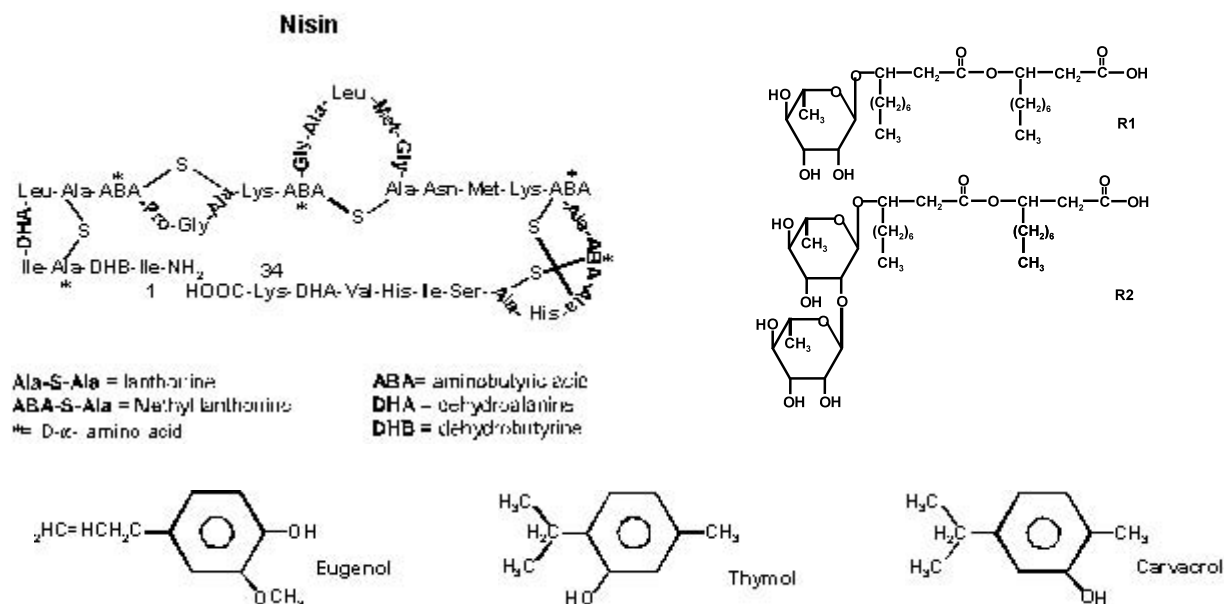
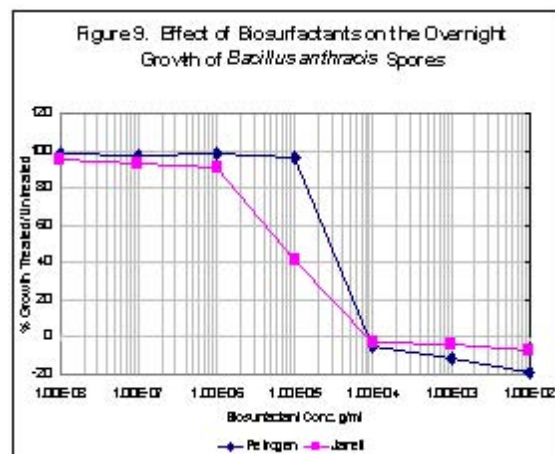
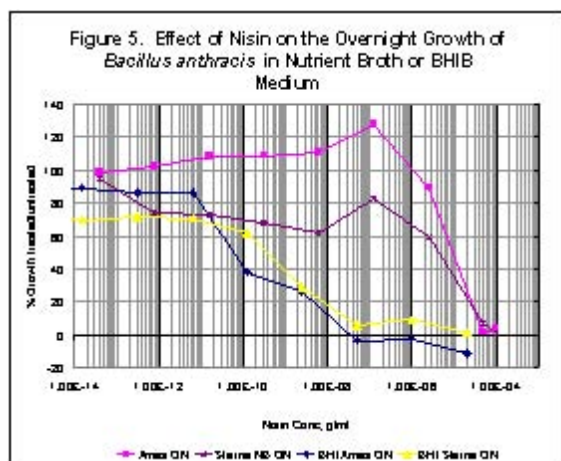
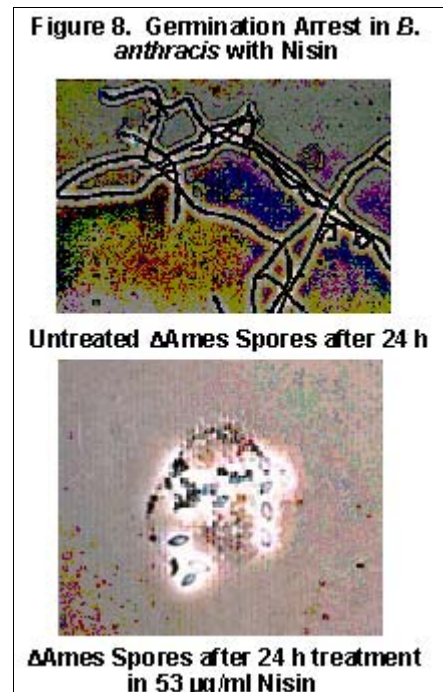
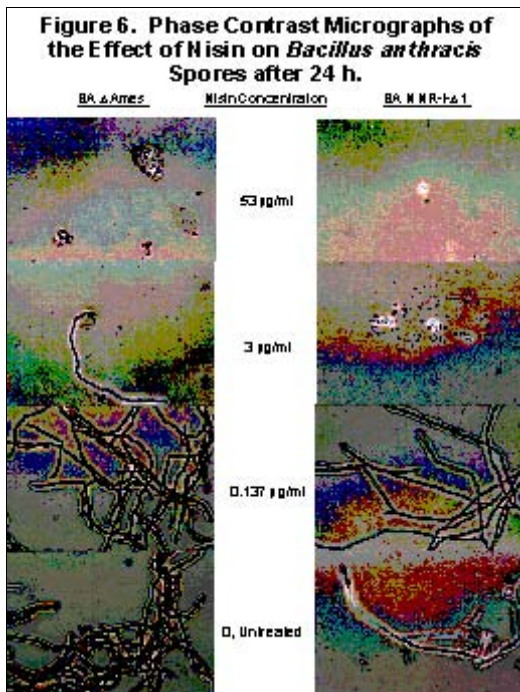


Figure 7. Natural Product Biological Decontaminants. R1 & R2 are the *Pseudomonas aeruginosa* biosurfactants. Nisin is the bacteriocin from *Lactococcus lactis*³. Eugenol, Thymol and Carvacrol are essential oils active components (ACs).

Comparative testing between different growth stages of *Bacillus anthracis* showed that exponentially growing cells are the most vulnerable to Nisin, followed by either overnight cultures or spores (Figure 4). Many investigators use BHI as a growth medium for anti-microbial testing of the Bacilli. We learned from our early studies that sporulation of *B. anthracis* is inhibited by BHI, and therefore more susceptible to growth inhibition and lysis by Nisin than those tested on Nutrient Broth (Figure 5). The best results were obtained with Turnbull's sporulation medium, which gave excellent growth and also supported good sporulation.

There was considerable strain to strain variation in the susceptibility of the Bacilli to Nisin over the concentration range tested, but the growth of all strains (including the spores) was prevented at 1 mg/ml Nisin (Figure 1). It was also observed that cell lysis of the inoculum occurred at these higher concentrations (negative zero% treated/untreated values). Microscopic observations also



confirmed cellular lysis and indicated that the spores met the same end (Figure 6). At lower Nisin concentrations, the spores were “arrested” during germination (refractile football shapes in the Figure 8 micrographs). Some of the dose/response plots show an increased level of growth at lower Nisin concentrations for spores or ON cultures (Fig. 1, 4). We speculate that lower Nisin concentrations may in fact speed the spore germination process, as pretreatment of the spores with hot water is done by some investigators to get the same effect.

CONCLUSIONS

Microarray viability testing is an expedient method of testing a large number of samples with careful handling. Data obtained from these studies was comparable to that obtained with the spot lawn assay.

1. Data obtained from these testing methods indicates that the bacteriocins from some Lactic Acid bacteria are lethal to spores, vegetative and sporulating *B. anthracis* at low concentrations (1 mg/ml or less). These bacteriocins were not effective against *Y. intermedia*, *Y. ruckeri* or *Y. rhodei* (spot lawn).
2. The active components of some essential oils (Thymol, Eugenol and Carvacrol) were also very good bactericides on both *B. anthracis* (MIC=0.3-2.5 mM) and *Y. intermedia* (1-4.5 mM). The observed MIC for carvacrol and *B. anthracis* (1.5 mM) is very close to that determined for *B. cereus*² (3 mM).
3. Commercial preparations of the Microbial surfactants of *Pseudomonas aeruginosa* were effective sporicides (MIC 100 μ g/ml) and bactericides (MIC 10 μ g/ml) for *B. anthracis*, but ineffective against *Y. intermedia* at the concentrations tested (10 mg/ml to 10 μ g/ml).

4. This method has was used to test the anti-BWA activity of current chemical agent decontamination solutions by the DoD. MIC values for these chemicals ranged from 62 µg/ml to 125 mg/ml ⁴.

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