

Original Research

Searching for a Bacteriophage Lysin to Treat *Corynebacterium bovis* in Immunocompromised Mice

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Corynebacterium bovis is the causative agent of *Corynebacterium*-associated hyperkeratosis in immunocompromised mice. The resulting skin pathology can be profound and can be associated with severe wasting, making the animals unsuitable for research. Although the administration of antibiotics is effective in resolving clinical symptoms, antibiotics do not eradicate the offending bacterium. Furthermore, antibiotic use may be contraindicated as it can affect tumor growth and is associated with *Clostridioides difficile* enterotoxemia in highly immunocompromised murine strains. Lysins, which are lytic enzymes obtained from bacteriophages, are novel antimicrobial agents for treating bacterial diseases. The advantage of lysins are its target specificity, with minimal off-target complications that could affect the host or the biology of the engrafted tumor. The aim of this study was to identify lysins active against *C. bovis*. Chemical activation of latent prophages by using mitomycin C in 3 *C. bovis* isolates did not cause bacteriophage induction as determined through plaque assays and transmission electron microscopy. As an alternative approach, 8 lysins associated with other bacterial species, including those from the closely related species *C. falsenii*, were tested for their lytic action against *C. bovis* but were unsuccessful. These findings were congruent with the previously reported genomic analysis of 21 *C. bovis* isolates, which failed to reveal bacteriophage sequences by using the PFAST and PFASTER web server tools. From these results, we suggest *C. bovis* is among those rare bacterial species devoid of lysogenic bacteriophages, thus making the identification of *C. bovis*-specific lysins more challenging. However, *C. bovis* may be a useful model organism for studying the effects of antiphage systems.

Abbreviation: TEM, transmission electron microscopy

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In 1998, the causative agent of *Corynebacterium*-associated hyperkeratosis, commonly known as ‘scaly skin disease,’ was identified as *Corynebacterium bovis* via 16S rRNA gene sequence analysis.^{12,34} *C. bovis* is a small gram-positive rod belonging to the family Corynebacteriaceae that colonizes the superficial layers of the epidermis of immunocompromised mice (for example, athymic nude, NOD.CB17-Prkdc^{scid}/J [NOD SCID], and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ [NSG] mice) and is highly contagious.^{5,9,11,12,31,41,52,63} The use of immunocompromised mouse models has increased exponentially over the past decade, given that they serve as valuable tools in oncology, immunology and other scientific disciplines because they support the growth of xenografts and allografts.^{29,50} Due to the prevalent use of these mouse models, infection with *C. bovis* has become increasingly problematic. The resulting skin pathology can be profound and,

on occasion, can be associated with severe wasting, making the animals unsuitable for research.¹⁶ A recent study demonstrated the effect of *C. bovis* in a patient-derived chronic myelomonocytic leukemia model, where *C. bovis* infection decreased tumor engraftment in the immunocompromised murine host (NSGS mice).⁶⁰ Recently, oral administration of amoxicillin (the therapy of choice for *Corynebacterium*-associated hyperkeratosis) to SCID and NSG mouse strains presumptively led to microbial dysbiosis, *Clostridioides difficile* colonization, enterotoxemia, diarrhea, and death.⁴⁰ Furthermore, although the administration of antibiotics is effective in resolving disease, it does not eradicate the causative *C. bovis* organism.⁶

Currently, alternative antimicrobial therapies are being developed in response to both an increase in antibiotic-resistant bacteria and the decrease in antibiotic development.²³ One such therapy involves the use of bacteriophage (that is, phage) lysins. At the end of phage replication and assembly inside a host bacterium, the progeny phage must escape.^{18,23,48} To this end, phage-encoded peptidoglycan hydrolases, termed lysins, degrade the bacterial cell wall, resulting in hypotonic lysis of the bacterium and release of phage progeny. When applied exogenously, purified forms of these enzymes are likewise able to access the peptidoglycan layer of the gram-positive cell envelope to produce the same lytic effect. Most lysins demonstrate high specificity, with lethal activity directed against the species

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that the lysin-encoding phage infects.²³ However, some lysins exhibit broad-acting lytic activity.²⁰ For example, PlySs2 displays strong lytic activity toward numerous *Staphylococcus* and *Streptococcus* species.²³ Unlike broad-spectrum antibiotics, lysins do not disrupt the normal microbiota of the host.⁴⁹ These enzymes have been shown to be highly effective in preclinical models of sepsis, endocarditis, pharyngitis, pneumonia, meningitis, and mucosal and skin decolonization, and a lysin against *S. aureus* has recently successfully completed phase 2 clinical trials for use in human medicine.^{18,48,49}

With regard to using lysins as antimicrobial agents, several features distinguish lysins from traditional antibiotics for use in the animal research setting: (1) rapid bactericidal activity against both stationary and exponentially growing bacteria; (2) lack of observed resistance; (3) no loss of efficacy or adverse effects after repeated administration; and (4) minimal disruption of the resident microbiota—unlike antibiotics, which can be toxic, influence physiologic responses of the host (for example, immune function, gastrointestinal dysbiosis), and affect the use of animals as models. Lysins, due to their targeted mode of action, would not be expected to have the same negative effects.^{2,18,49} Using whole-genome sequencing and comparative genomic analyses, we recently reported on the genetic diversity of 21 *C. bovis* isolates obtained from various hosts, geographical locations, and time points.^{3,4,8,21,36,37,57,61} Genotypic differences between isolates, including the number of associated virulence factors, reflected the host from which they were obtained.^{8,19} Analyses using the PHAST (Phage Search Tool) and PHASTER (Phage Search Tool Enhanced Release) algorithms failed to identify the presence of intact or true (completeness score greater than 90) or questionable (completeness score, 70 to 90) phage in any of the isolates.^{1,64} Both PHAST and PHASTER are used to identify, annotate, and graphically display prophage sequences within bacterial genomes or plasmids. However, these prophage prediction methods, which are based on databases of previously identified prophage sequences, are limited with regard to determine the probabilities of putative phage sequences in submitted genomes.¹ Notably, the prophage sequence databases used by the aforementioned algorithms are largely incomplete due to the ubiquity of phages. This understanding, along with their genetic diversity, allows PHAST and PHASTER to miss prophage. When phages are labeled as incomplete by these programs, excessive distance between possible prophage genes would prevent their combination into a single predicted region, and the completeness score would decrease. This limitation allows a prophage sequence to be classified as questionable or incomplete even though it represents an intact or true prophage. Finally, previous work demonstrated a sensitivity (no. of true positives / [no. of true positives + no. of false negatives]) of 85% for PHASTER (79% for PHAST), implying that about 15% of prophages would be missed.¹ Furthermore, although no specific lysin sequences were found in our previous study, these methods did identify several incomplete phages in each isolate (completeness score of less than 70).⁸ For these reasons, additional in vitro studies are necessary to definitively conclude that *C. bovis* lacks phages.

The primary aim of the current study was to identify a phage lysin to use as a potential therapeutic for *Corynebacterium*-associated hyperkeratosis. Given both the ubiquity and abundance of phages and the known analytical limitations of PHAST and PHASTER, we hypothesized that *C. bovis* phage and their corresponding lysins potentially could be identified by using traditional phage induction methods.^{1,13} To this end, multiple *C. bovis* isolates were treated with mitomycin C to stimulate potential

prophage to enter into a lytic cycle. The supernatant of the induced bacterial cultures was then visually inspected for phage by using transmission electron microscopy (TEM) and subsequently assayed for its ability to generate plaques when applied to multiple *C. bovis* strains. In addition, with the understanding that some lysins exhibit broad lytic activity, we analyzed the susceptibility of *C. bovis* to a diverse library of lysins.^{22,49,54} In total, 8 lysins were screened against *C. bovis*, including PlyCf, which is a lysin derived from a prophage from the closely related *Corynebacterium falsenii*.⁸ We hypothesized that lysins originating from non-*C. bovis* phage might exhibit nonspecific lytic activity against *C. bovis*. For example, the lysin from corynephage BFK20 obtained from *Brevibacterium flavum* has shown effective lytic activity against other bacterial species, including *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*.²⁰

Materials and Methods

Bacterial strains and culture conditions. Strain information relating to *C. bovis* clinical isolates 7894, CUAMC1, 4826, 4828, MI 82-1021, F6900, and WCM1 was previously described.⁸ Each strain, as well as the *C. falsenii* strain ATCC BAA-596, was initially plated on Columbia colistin and nalidixic acid agar with 5% (v/v) sheep blood (Becton Dickinson, Franklin Lakes, NJ) and then routinely grown in brain–heart infusion medium (Becton Dickinson) supplemented with 0.1% (v/v) Tween 80 (Fisher Scientific, Hampton, NH). *Streptococcus pyogenes* strain D471 and *S. pneumoniae* strain DCC1490 were grown in Todd–Hewitt broth (Becton Dickinson) with 1% (w/v) yeast extract (Fisher Scientific). *Clostridium difficile* strain ATCC 43255 was grown anaerobically in brain–heart infusion medium supplemented with 0.5% (w/v) yeast extract and 0.1% (w/v) L-cysteine. *Bacillus anthracis* strain Sterne and *Staphylococcus aureus* strain RN4220 were grown in tryptic soy broth (Becton Dickinson).

Determining *C. bovis* growth kinetics. A growth curve for *C. bovis* was generated by using previously described methods.^{14,28,43} Briefly, a turbid culture of *C. bovis* strain 7894 was diluted 1:100 in fresh growth medium and subsequently incubated at 37 °C with aeration for a total of 48 h. Every 6 h, an aliquot of bacteria was quantitated by either (1) measuring the optical density at 600 nm or (2) serially diluting and plating the bacteria to calculate the number of cfu per milliliter. The resulting growth curves were fit with a Boltzmann sigmoidal curve by using GraphPad Prism (GraphPad, San Diego, CA). All error bars correspond to the SEM of duplicate experiments.

Bacteriophage induction and TEM. We attempted to chemically induce phage from *C. bovis* isolates WCM1, 7894, and CUAMC1 by using mitomycin C according to previously described methods, with modifications.^{15,39} In the initial set of experiments, each strain was grown to an OD_{600nm} of 0.5. The culture was then divided, with one half being treated with 1 µg/mL mitomycin C and the other half serving as an untreated control. After 30 min, the bacteria were pelleted, resuspended in fresh growth medium, and grown for an additional 6 h. Growth curves for each strain were monitored by measuring the OD_{600nm} at 1-h increments. In the second set of experiments, *C. bovis* strain 7894 was grown to an OD_{600nm} of 0.5 and then divided into 5 flasks. Each flask was treated with either 0, 0.01, 0.1, 0.5 or 1 µg/mL mitomycin C. The subsequent steps were the same as described for the first set of experiments.

At the culmination of each induction experiment, the cells were pelleted by centrifugation. The supernatant was filtered and incubated with polyethylene glycol to precipitate any induced phage. Each sample was then centrifuged at 16,000 × g for 15 min at 4 °C. The supernatant was removed, and the pellet

containing phage, if present, was resuspended in SM buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 8 mM MgSO₄). The samples were negatively stained with 3% (w/v) uranyl acetate and imaged by using TEM at the Core Laboratories Center Imaging Core Facility of Weill Cornell Medicine. The images were visually inspected for structures resembling phage components according to previous published TEM images of phages associated with other *Corynebacterium* spp.⁵⁸

Plaque assay. *C. bovis* strains 7894, CUAMC1, 4826, 4828, MI 82-1021, F6900, and WCM1 were each embedded at an initial concentration of approximately 10⁶ cfu/mL in growth medium supplemented with 0.75% (w/v) agarose. Next, the filtered supernatant from *C. bovis* strain 7894 treated with 0.1 or 0.5 µg/mL mitomycin C was spotted onto the bacteria-containing plates. The filtered supernatant from bacteria absent mitomycin C treatment was spotted as a negative control. The plates were incubated at 37 °C for a maximum of 72 h to determine whether plaques formed.

Molecular cloning. The *plyCf* gene encoding the translated *C. falsenii* lysin PlyCf (GenBank AHI04498) was synthesized and codon-optimized for protein expression in *E. coli* (GeneWiz, South Plainfield, NJ). The *plyCf* gene was PCR-amplified and then cloned into the NcoI and BamHI sites of the *E. coli* expression vector pET28a by using the NEBuilder HiFi DNA Assembly method (New England Biolabs, Ipswich, MA). After sequence confirmation, the pET28a::*plyCf* construct was transformed into *E. coli* BL21(DE3) for protein expression.

Lysin expression and purification. The lysins PlyC, PlyCD, Cpl-1, ClyS, PlySs2, LysK, and PlyG were expressed and purified to homogeneity as previously described.^{10,22,38,45,46,53,62} The PlyCf lysin was expressed in Luria-Bertani medium (Fisher Scientific) supplemented with kanamycin (50 µg/mL; Fisher Scientific) for 16 h at 18 °C. Protein expression was induced at midlog phase by using 1 mM isopropyl β-D-1-thiogalactopyranoside (Biosynth Chemistry and Biology, Staad, Switzerland). Cells were washed and resuspended in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl supplemented with 1 mM phenylmethanesulfonyl fluoride (Fisher Scientific). The bacteria were lysed by using a homogenizer (Emulsiflex-C5, Avestin, Ottawa, Ontario). The lysate was cleared by centrifugation at 27,000 × *g* for 1 h at 4 °C. The soluble lysate fraction was dialyzed against 20 mM Tris-HCl (pH 8.0) and then applied to a 5-mL HiTrap Q FF column (GE Healthcare Life Sciences, Pittsburgh, PA) in 20 mM Tris-HCl (pH 8.0). PlyCf was eluted from the column using a linear salt gradient from 0 to 500 mM NaCl. After removal of salt via dialysis, the protein sample was applied to a 5-mL HiTrap DEAE FF column (GE Healthcare Life Sciences) in 20 mM Tris-HCl (pH 8.0). The flow-through was collected and dialyzed against PBS (pH 7.4). Finally, the PlyCf sample was concentrated by using an Amicon Ultra Ultracel-10K filter (EMD Millipore, Burlington, MA) and applied to a HiLoad 16/60 Superdex 200 Prep Grade column (GE Healthcare Life Sciences) in PBS. Highly pure PlyCf elution fractions were combined and stored at -80 °C until use. All chromatography experiments were performed by using an AKTA fast protein liquid chromatography system (GE Healthcare Life Sciences, Pittsburgh, PA).

Turbidity reduction and ATP release assays. Seven non-*Corynebacterium* lysins (PlyC, PlyCD, Cpl-1, ClyS, PlySs2, LysK, and PlyG) were initially assayed for bacteriolytic activity toward susceptible bacterial species by using the turbidity reduction assay to confirm that each lysin was enzymatically active. In brief, midlog *S. pyogenes* (PlyC), *C. difficile* (PlyCD), *S. pneumoniae* (Cpl-1), *S. aureus* (ClyS and PlySs2), and *B. anthracis* (PlyG) were incubated with their specific lysin(s) at 25 µg/mL (final

concentration) in 50 mM sodium phosphate (pH 7.0) in 96-well microtiter plates. By using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA), bacteriolytic activity was quantitated by measuring the OD₆₀₀ of each sample every 15 s for 1 h at 37 °C. After confirmation of enzymatic activity, each lysin was then incubated with midlog *C. bovis* strain 7894 by using the aforementioned turbidity reduction assay protocol. All resulting data points were fit by using a 1- or 2-phase exponential decay curve (Prism, GraphPad Software). Each sample set was analyzed in duplicate and included untreated negative controls.

To further determine *C. bovis* susceptibility to each of the previously mentioned non-*Corynebacterium* lysins, ATP release assays were performed (ENLITEN, Promega, Madison, WI). Briefly, at the conclusion of the previously mentioned *C. bovis* turbidity reduction assays, the contents from each sample well were transferred to polypropylene microcentrifuge tubes. Intact cells and insoluble cellular debris were removed by centrifugation at 14,500 × *g* for 10 min. By using new microcentrifuge tubes, the sample supernatant was added to an equal volume of 1 M Tris-HCl (pH 7.75). After gentle mixing, 100 µL from each sample was transferred to a white, opaque 96-well microtiter plate and mixed with an equal volume of rL/L reagent (ENLITEN, Promega). The luminescence of each sample well was measured at 560 nm in a microplate reader by using a 1.5-s integration time. *S. pyogenes* treated with PlyC was used as positive control for ATP release, and ATP-free water, buffer, and untreated bacteria were used as negative controls. All error bars correspond to the SEM of duplicate samples.

A modified version of the turbidity reduction assay was used for evaluating the lytic activity of the *C. falsenii* lysin PlyCf toward *C. bovis*. Briefly, midlog *C. bovis* strain 7894 was incubated with 125 µg/mL PlyCf (final concentration) in PBS (pH 7.4). OD₆₀₀ was measured every 30 s for 1 h at room temperature by using the microplate reader. As a positive control, PlyCf was incubated at 125 µg/mL with midlog *C. falsenii* strain ATCC BAA-596. Untreated bacteria were used as a negative control for lytic activity. The resulting data points were fit to a 1-phase exponential decay curve by using Prism (GraphPad Software). All samples were assayed in duplicate.

Results

***C. bovis* growth kinetics.** Generally, *C. bovis* are slow-growing bacteria, but a *C. bovis*-specific growth curve has yet to be published. In order to identify a lysin with lytic activity toward *C. bovis*, we must first understand when the bacterium has entered various stages of growth, given that logarithmic-phase bacteria are generally more susceptible than stationary phase cells to lysins.

To that end, *C. bovis* strain 7894 was grown in brain-heart infusion medium supplemented with Tween 80 at 37 °C for a total of 48 h, with the bacterial concentration calculated according to OD₆₀₀ (Figure 1 A) or number of cfu per milliliter (Figure 1 B) measured every 6 h. The bacteria were in lag phase during the initial 6 to 12 h and then transitioned into the exponential phase of growth from 12 to 30 h. Early-log phase occurred at around 12 h, midlog phase was observed at 18 h, and late-log phase developed between 24 and 30 h. *C. bovis* began to enter stationary phase at approximately 30 h after inoculation.

Bacteriophage induction, electron microscopy, and plaque assays. Three *C. bovis* clinical isolates (WCM1, 7894, CUAMC1) were treated with 1 µg/mL mitomycin C for 30 min. After removal of mitomycin C, the OD₆₀₀ of each sample was monitored for a total of 6 h at 37 °C. If lytic phage were induced successfully, a rapid decrease in the OD of the bacterial culture would

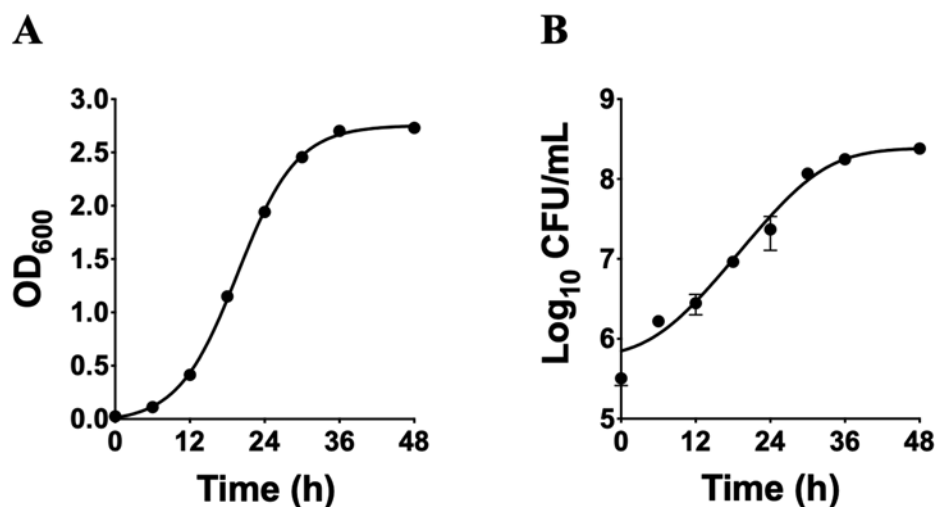


Figure 1. Growth curve of *C. bovis*. A turbid culture of *C. bovis* strain 7894 was diluted 1:100 in fresh BHI medium supplemented with Tween 80 and subsequently incubated at 37 °C with aeration for a total of 48 h. The bacterial concentration was quantitated every 6 h by either (A) measuring the OD₆₀₀ or (B) serially diluting and plating to determine the number of cfu per mL. Error bars, SEM of duplicate experiments.

be expected. Compared with untreated controls, the growth rate of each *C. bovis* strain treated with mitomycin C was reduced. However, the OD of each sample slowly increased during the 6-h incubation, suggesting either that lytic phage were not induced or only partially induced in the 3 *C. bovis* strains.

Given the potential that this finding could be an artifact resulting from the mitomycin C concentration used,¹⁵ additional induction experiments using various mitomycin C concentrations were performed. *C. bovis* strain 7894 was treated with mitomycin C concentrations of 0, 0.1, 0.5 and 1.0 µg/mL for a total of 30 min. After removal of mitomycin C, bacterial growth at 37 °C was determined for a total of 6 h (data not shown). The growth of the culture treated with 0.01 µg/mL mitomycin C was similar to that of the untreated control. Although the growth rates of *C. bovis* were significantly altered at mitomycin C concentrations of 0.1 µg/mL or greater, none of these concentrations rapidly decreased the OD of the culture over the duration of the experiment, indicating that phages likely were not induced. The absence of phage-like particles in the supernatant of mitomycin C-treated *C. bovis* was further confirmed through visual inspection by using TEM (data not shown). Furthermore, the same supernatants lacked the ability to generate plaques after spotting on soft agar overlays embedded with various strains of *C. bovis* (data not shown).

Evaluation of *C. bovis* susceptibility to various lysins. Several previously studied non-*Corynebacterium* lysins were assayed for lytic activity toward *C. bovis*. The lysins tested included PlyC, PlySs2, PlyG, Cpl-1, PlyCD, LysK, and ClyS. Because bacteria lyse on treatment with lysin, the turbidity reduction assay has become a standard method to determine the lytic activity of lysins.^{20,22,23} To confirm that each was enzymatically functional, all 7 lysins initially were incubated at a concentration of 25 µg/mL with a bacterial species known to be sensitive to the particular lysin^{10,22,38,45,46,53,62} (Figure 2 A). A rapid and significant reduction in turbidity was observed for PlyC (compared with *S. pyogenes*), PlySs2 (compared with *S. aureus*), PlyG (compared with *B. anthracis*), Cpl-1 (compared with *S. pneumoniae*), PlyCD (compared with *C. difficile*), LysK (compared with *S. aureus*), and ClyS (compared with *S. aureus*), thus confirming that each lysin was functional. Next, each lysin (concentration, 25 µg/mL) was incubated with *C. bovis* strain 7894 for a total of 1 h at 37 °C. Compared with the untreated control, a reduction in turbidity was observed when *C. bovis* was treated with PlyC, PlyG, Cpl-1,

and ClyS (Figure 2 B). However, according to visual inspection of these samples, the decline in turbidity did not appear to be due to bacteriolytic activity of the lysins. Instead, the decrease in OD resulted from the formation of bacterial aggregates and subsequent settling of the sample, seen as white clumps at the bottom of the wells.

Luminescence associated with ATP release is an assay used to confirm that the bacterial cell is disrupted as a result of lytic activity. To help verify the previous observation, ATP release was measured for each of the lysin-treated *C. bovis* samples (Figure 3). If the bacterial cells are indeed being lysed, then intracellular ATP would be released, with the amount of ATP observed in the supernatant directly correlating with the total number of cells lysed. As a positive control, *S. pyogenes* was treated with the streptococcal-specific lysin PlyC. The negative controls consisted of ATP-free water, buffer only, and untreated bacterial cells. The results show that all of the *C. bovis* samples treated with lysin had extracellular ATP concentrations comparable to that of the untreated control. This finding indicates that none of the tested lysins was capable of lysing *C. bovis*, thereby further confirming that the reduction in OD observed at the conclusion of the turbidity reduction assays was indeed a direct result of bacterial aggregation and settling and not due to lysis.

A lysin derived from a phage that infects a close relative of *C. bovis*, such as another *Corynebacterium* species, would be expected to have a greater probability of displaying nonspecific bacteriolytic activity toward the bacterium. Therefore, we tested the sensitivity of *C. bovis* against PlyCf, a lysin derived from a prophage encoded by *C. falsenii* strain DSM 44353. First, to establish that PlyCf was lytically active, it was incubated at 125 µg/mL with *C. falsenii* (its native target bacterial species) for 1 h (Figure 4 A). Results from the corresponding turbidity reduction assay confirmed that the lysin was enzymatically functional. PlyCf was subsequently incubated at 125 µg/mL with *C. bovis* for 1 h (Figure 4 B). Similar to the untreated negative control, PlyCf did not significantly reduce the turbidity of the bacterial sample, indicating that PlyCf lacks bacteriolytic activity toward *C. bovis*.

Discussion

The eradication of *C. bovis* from vivaria that house immunodeficient mouse strains is particularly challenging because

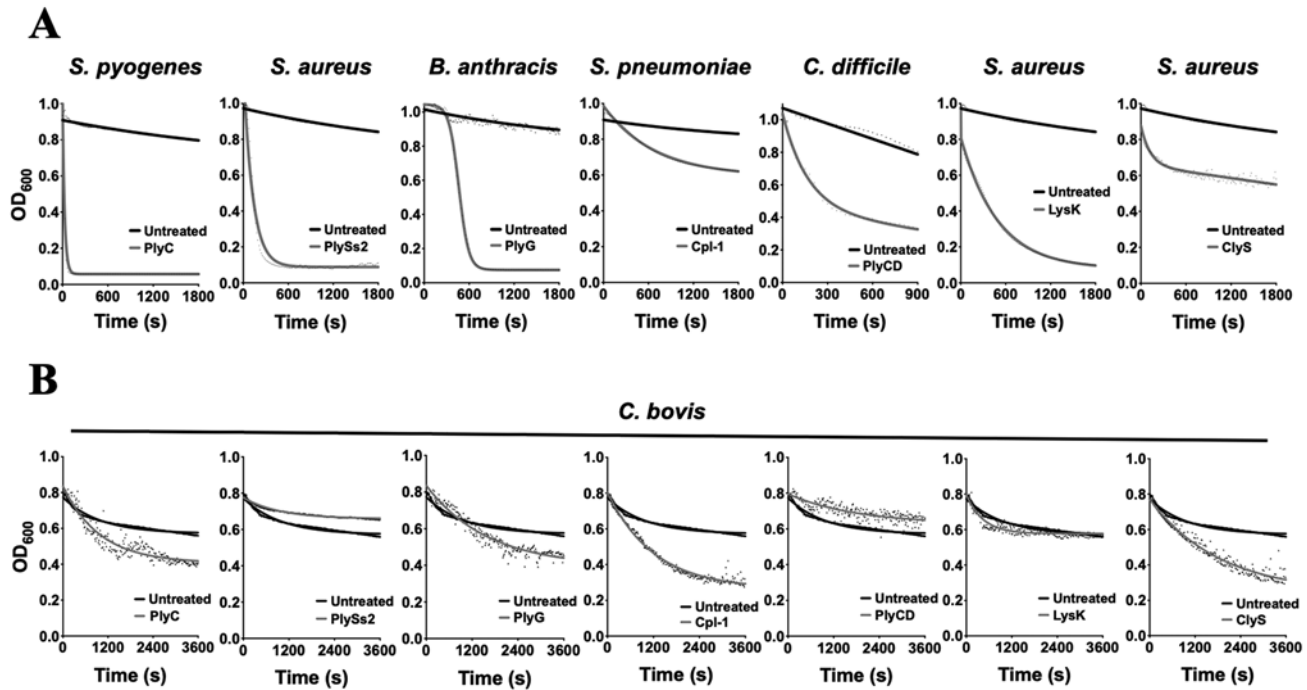


Figure 2. Analyzing the lytic activity of several non-*Corynebacterium* lysins against *C. bovis* by means of the turbidity reduction assay. (A) To confirm each lysin was enzymatically functional, PlyC (*S. pyogenes*), PlySs2 (*S. aureus*), PlyG (*B. anthracis*), Cpl-1 (*S. pneumoniae*), PlyCD (*C. difficile*), LysK (*S. aureus*) and ClyS (*S. aureus*) were incubated at 25 µg/mL with a susceptible bacterial species. (B) The bacteriolytic activity of each lysin at 25 µg/mL was determined against *C. bovis* strain 7894. For each turbidity reduction assay, the OD_{600nm} was measured every 15 s for 1 h at 37 °C. A decrease in turbidity is indicative of bacteriolytic activity. Bacteria absent lysin treatment were used as a negative control for lytic activity. Each sample was analyzed in duplicate.

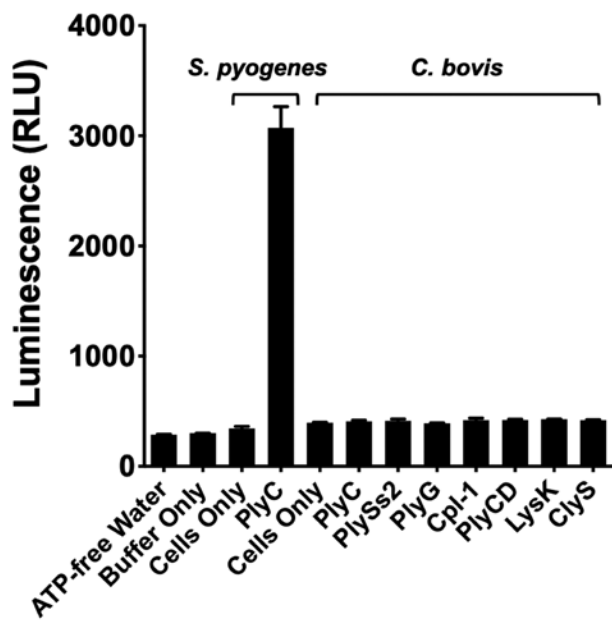


Figure 3. Measuring the concentration of ATP released by *C. bovis* when treated with various non-*Corynebacterium* lysins. ATP assays were used to measure the amount of intracellular ATP released, as a function of luminescence, by *C. bovis* strain 7894 after incubation with PlyC, PlySs2, PlyG, Cpl-1, PlyCD, LysK, and ClyS (final concentration, 25 µg/mL) for 1 h at 37 °C. *S. pyogenes* treated with PlyC was used as a positive control; ATP-free water, buffer only, and untreated bacteria were used as negative controls. Error bar, SEM of duplicate experiments. RLU, relative light units.

the organism can be found in drinking water, tumor and cell lines, within biosafety cabinets, and on fomites, including cages, bedding, and instruments.^{7,41} Although adherence to aseptic handling and cage-changing techniques can decrease the spread of *C. bovis*, studies have concluded that once *C. bovis* becomes enzootic, it is extremely difficult or even impossible to eradicate.^{6,41} Variable success in the management and eradication of *C. bovis* has been achieved through antibiotic therapy, rederivation, colony depopulation, and disinfection.⁶ However, control and elimination in academic programs is often unsuccessful because depopulation and restricted colony access usually are not options. Further evidence of the significance of the problem is demonstrated by the National Cancer Institute's *Requests for Applications for Patient-derived Xenograft (PDX) Development and Trial Centers (PDTC)*, which now requires that applicants indicate whether *C. bovis*-free animals can be maintained.⁴⁴

The principal aim of this study was to identify a phage lysin for use as a potential therapeutic against *Corynebacterium*-associated hyperkeratosis. We attempted to use mitomycin C to chemically activate potential prophages latent within several *C. bovis* isolates. According to plaque assays and transmission electron microscopy, no phages were induced. We then analyzed the susceptibility of *C. bovis* to 9 lysins, including PlyCf, a lysin derived from a prophage of the closely related *C. falsenii*. None of the lysins tested showed lytic activity against *C. bovis* (Figures 2 B, 3, and 4 B).

Although treatment with mitomycin C at concentrations of 0.1 µg/mL or greater markedly decreased the growth rate of *C. bovis*, a sharp decline in optical density was never observed during the 6-h incubation. If lytic phage were induced, bacterial lysis—indicated as a rapid decrease in turbidity—would be expected 2 to 3 h after exposure.^{26,56} Therefore, when compared with untreated controls, the reduced growth rate of the

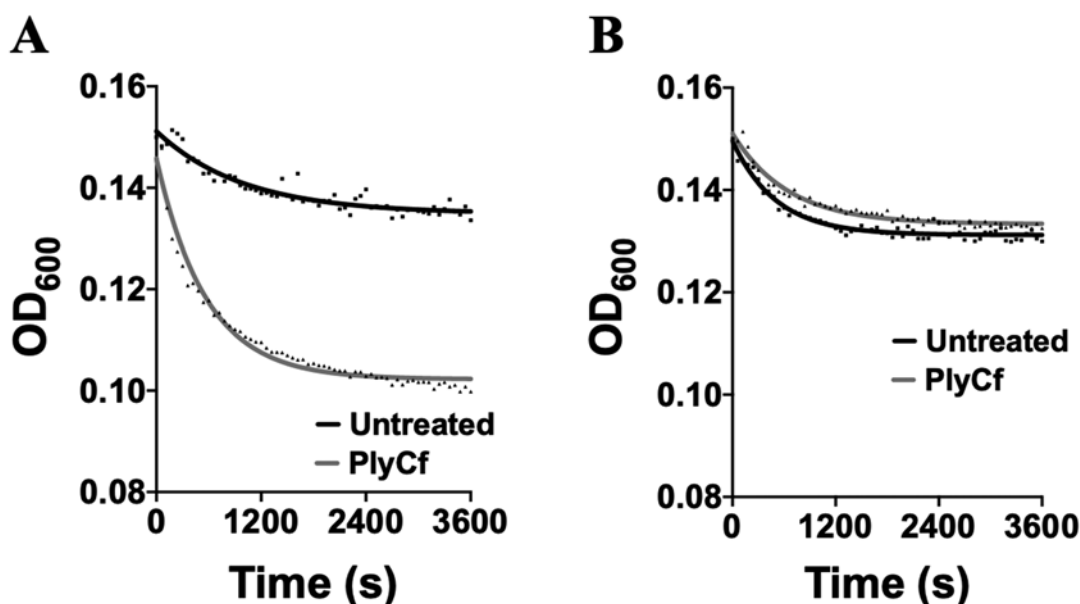


Figure 4. Evaluating the bacteriolytic activity of the *Corynebacterium* lysin PlyCf against *C. bovis*. In a turbidity reduction assay, the *C. falsenii* lysin PlyCf (final concentration, 125 $\mu\text{g}/\text{mL}$) with either (A) *C. falsenii* strain ATCC BAA-596 (positive control) or (B) *C. bovis* strain 7894. OD_{600} was measured every 30 s for 1 h at room temperature. A decrease in turbidity is indicative of bacteriolytic activity. Bacteria absent lysin treatment were used as a negative control for lytic activity. Each sample was analyzed in duplicate.

mitomycin C-treated bacteria was not due to phage induction but instead to the toxic effects of the antibiotic. Visual inspection using TEM coupled with plaque assays further confirmed the absence of phage in the supernatant of bacteria treated with mitomycin C. Our inability to detect phage in *C. bovis* is consistent with recently reported findings, in which no prophage DNA was found in any of the 21 *C. bovis* isolates sequenced and analyzed with PHAST and PHASTER.⁸

Although *C. bovis* does not appear to have any chromosomally-integrated prophage, phages can be present extrachromosomally as lysogenic linear and circular plasmidial elements in the cytoplasm of the bacteria. These phages may switch between extrachromosomal and chromosomally integrated states.⁵⁹ Although genomic sequencing might be biased toward bacterial chromosomes and thus ‘overlook’ the smaller, low-copy extrachromosomal elements unless they are isolated specifically, the methods used in the recently published *C. bovis* sequencing study would be expected to detect extrachromosomal elements.⁸ Therefore, the absence of phage in *C. bovis* is surprising, given the abundance and ubiquity of phages in bacterial populations.¹³ In addition, close relatives of *C. bovis* have associated phages. For example, *C. falsenii* contains the corynephage $\phi\text{CFAL8171I}$ in both circular (extrachromosomal) and linear (chromosomally integrated) states.²⁴

Evidence is available—albeit sparse—to show that not all bacteria can be infected by phages.³² Several antiphage systems found in bacteria have been acquired through evolution or horizontal transfer.^{51,55} These systems include surface alterations to block phage adsorption, inhibition of phage DNA penetration, DNA restriction or modification systems, phage-specific immunity (clustered regularly interspaced short palindromic repeats [CRISPR]–Cas systems), and abortive infection.^{13,17,26,27,30,51} Some of these bacterial defense mechanisms that have evolved against phage predation can help to explain the unusual lack of phages in *C. bovis*. Two likely phage defense mechanisms used by *C. bovis* were identified in our previous comparative

genomic analyses: (1) CRISPR–Cas systems, which function as a form of adaptive immunity that targets foreign nucleic acids, and (2) phage-abortive infection proteins (toxin–antitoxin systems) that are activated by phages and elicit cellular inhibition by interfering with essential metabolic processes.^{8,13,25,27,33,35,42,47} Both of these defense mechanisms were detected in most of the *C. bovis* isolates analyzed. Whether these or other methods are used by *C. bovis* to resist phage attack remains unknown. As such, *C. bovis* may serve as a useful model for studies of phage resistance.

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