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REVIEW

Phage lytic enzymes: a history

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There are many recent studies regarding the efficacy of bacteriophage-related lytic enzymes: the enzymes of 'bacteria-eaters' or viruses that infect bacteria. By degrading the cell wall of the targeted bacteria, these lytic enzymes have been shown to efficiently lyse Gram-positive bacteria without affecting normal flora and non-related bacteria. Recent studies have suggested approaches for lysing Gram-negative bacteria as well (Briersa Y, et al., 2014). These enzymes include: phage-lysozyme, endolysin, lysozyme, lysin, phage lysin, phage lytic enzymes, phage-associated enzymes, enzybiotics, muralysin, muramidase, virolysin and designations such as Ply, PAE and others. Bacteriophages are viruses that kill bacteria, do not contribute to antimicrobial resistance, are easy to develop, inexpensive to manufacture and safe for humans, animals and the environment. The current focus on lytic enzymes has been on their use as anti-infectives in humans and more recently in agricultural research models. The initial translational application of lytic enzymes, however, was not associated with treating or preventing a specific disease but rather as an extraction method to be incorporated in a rapid bacterial detection assay (Bernstein D, 1997).The current review traces the translational history of phage lytic enzymes–from their initial discovery in 1986 for the rapid detection of group A *streptococcus* in clinical specimens to evolving applications in the detection and prevention of disease in humans and in agriculture.

KEYWORDS **bacteriophage; phage lytic enzymes; translational application; lysin**

INTRODUCTION

The first reported discovery of antibacterial-like activity linked to bacteriophages was made by British bacteriologist Ernest Hanbury Hankin in 1896. Studying malaria and cholera in India, he suggested that the presence of antimicrobial activity in the waters of the Ganga and Jamuna rivers might have restricted outbreaks of cholera (Hankin, 1896). However, most researchers credit Fredrick Twort, an English bacteriologist, who in 1915 actually isolated filterable entities capable of destroying bacterial cultures, there by producing small cleared areas on bacterial lawns (Twort, 1915). A few years later, Felix d'Herelle, a French Canadian microbiologist working at the Pasteur Institute in Paris, reported the same phenomenon. It was d'Herelle who recognized the nature of the discovery as a result of viral parasitic action on bacteria and has therefore been given much of the credit for the discovery (d'Herelle, 1917 and 1931). He named the virus "bacteriophage" or bacteria-eater (Summers, 2001). A further review of the early history of the phage is provided by Abedon (2011).

In 1910, George Eliava of the Soviet Union observed the bactericidal action of the waters of the Mtkvari River in Tbilisi. The first reported application of phages to treat infectious diseases occurred in France in 1921 when Bruynoghe and Maisin used bacteriophages to treat staphylococcal skin disease (Bruynoghe and Maisin, 1921). Eliava and d'Herelle subsequently founded the Bacteriophage Institute of Tbilisi in 1923 (Summers, 2001). This facility later became known as the George Eliava Institute of Bacteriophage, Microbiology and Virology and it has remained in continuous use since that time.

Phages were used for prophylaxis and therapy in the

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USA in the early 1930s and for over 80 years in eastern Europe and the Soviet Union. In fact, there have been several hundred reports on phage therapy over the years. For example: phages have been reported to be effective in treating skin infections caused by Pseudomonas, Staphylococcus, Klebsiella, Proteus, *Escherichia coli* (*E. coli)*, staphylococcal lung and pleural infections and *Pseudomonas Aeruginosa* (*P. aeruginosa)* infections in cystic fibrosis patients, neonatal sepsis and surgical wound infections. Phages were administered orally, applied directly to wounds, as suppositories and in eye drops. Depending on the pathogen, success rates ranged from 75 to 100% (Abedon et al., 2011). d'Herelle provided the first studies in animal health (Sulakvelidze and Barrow, 2005). He suggested treatments for Salmonella infections and methods for controlling diarrhea in (bovine) calves simply by spraying the litter, in the rooms that were used to house calves, with an aqueous phage suspension. Calves could then be kept in rooms that had not been cleaned but that had previously been occupied by calves whose *E. coli* infections had been treated with phage.

Although early reports of bacteriophage therapy in the western world were somewhat favorable, continued clinical use showed that therapy was at times inconsistent and limited in its effects (Ho, 2001). These failures were due primarily to inappropriate phage choice, poor phage preparation and phage that had become degraded prior to application (Harper and Kutter, 2009). Reports of unsuccessful treatment ultimately led to a decline in attempts to develop clinical applications of phage therapy. That decline accelerated once antibiotics were introduced in the 1940s. Disappointment with phage therapy increased because the significant potential of these viruses to kill bacteria *in vitro* was not translated *in vivo* (Abedon et al., 2011).

Despite the general failure of phage therapy, isolated groups of physicians saw its potential and continued their attempts to use these agents to treat infectious diseases. Many of these efforts were concentrated in former Soviet Union countries and Eastern Europe, where high costs and a lack of availability of antibiotics continued to stimulate a search for alternative therapies. In Russia, d'Herelle of the Eliava Institute in Tbilisi generated a database of more than 300 phage types (Summers, 2001). As of 2014, almost 4,000 different phages have been identified by the Institute, which has probably the world's most extensive print library of phage studies and reports.

PHAGE LYTIC ENZYMES

The emergence of a bacteriophage technology revolution is not just limited to whole phages. There has been an increased focus on phage-lytic or phage-associated enzymes (PAE), also referred to as lysins. Lysins are specific molecules produced by bacteriophages; they are expressed as recombinant proteins designed to "punch holes" in the cell wall of a bacterium (usually Gram-positive). PAEs cause rapid lysing and cell death, seconds after contact with a targeted bacterium. For example: 10 ng of enzyme can lyse 10^7 bacteria in < 30 seconds (Schuch et al., 2002; Fischetti, 2005). The enzymes work by creating holes in the bacterial cell wall, resulting in osmotic lysis and bacterial death (Nelson et al., 2001). Other than chemical agents, these are among the most active and safest substances able to kill bacteria. Lysins have evolved over time, based on the need for the bacteriophages to escape effectively from the infected bacterium.

In the West, this resurgence of interest included studies on various aspects of *Streptococcus pyogenes*, the causative bacteria of group A *streptococcus* (GAS) infections. Specifically, Maxted (1957), Krause (1958), and Fischetti (1971) studied the characteristics of an enzyme produced by group C *streptococcus* after being infected by bacteriophage C1. The enzyme, designated as lysin, was found to be effective against the cell wall of GAS and other streptococci. The researchers reported on the effectiveness of this enzyme to lyse GAS and expose the cell-wall carbohydrate. However, they did not address in depth any potential applications nor identify any specific uses.

The 1980's saw the advent of rapid antigen detection methods destined for the physician's toolkit. These methods were developed to provide the doctor with culture similar results of a sample collected from the patient during the outpatient visit without waiting for the bacteria to "grow" in a laboratory. The *streptococcus* cultures took 24-72 hours; however, when patients were administered antibiotics, cultures were inconclusive and erroneous results were reported in GAS-positive patients. Enzyme linked immunosorbent assay (ELISA) and agglutination assays were developed but were not as sensitive and easy to use. Therefore, a new technology using colloidal gold for the rapid identification of bacteria was developed. Initial use of this gold-based "dip-stick" format was the rapid detection of GAS by antibody/antigen detection. Unfortunately, the antibody that was used targeted a cell-wall carbohydrate antigen, which required an extraction step to fragment the antigen for efficient binding. This additional step limited the acceptance of this new method. The group developing this new colloidal gold method (Figure 1), the New Horizons Diagnostics Corp (NHD, Columbia, MD, USA), collaborated with Bernstein and Fischetti to refine the production methods and formulate the lysin for incorporation into a detection assay (Bernstein et al., 1997). It should be noted that these technologies were new; it took more than 10 years

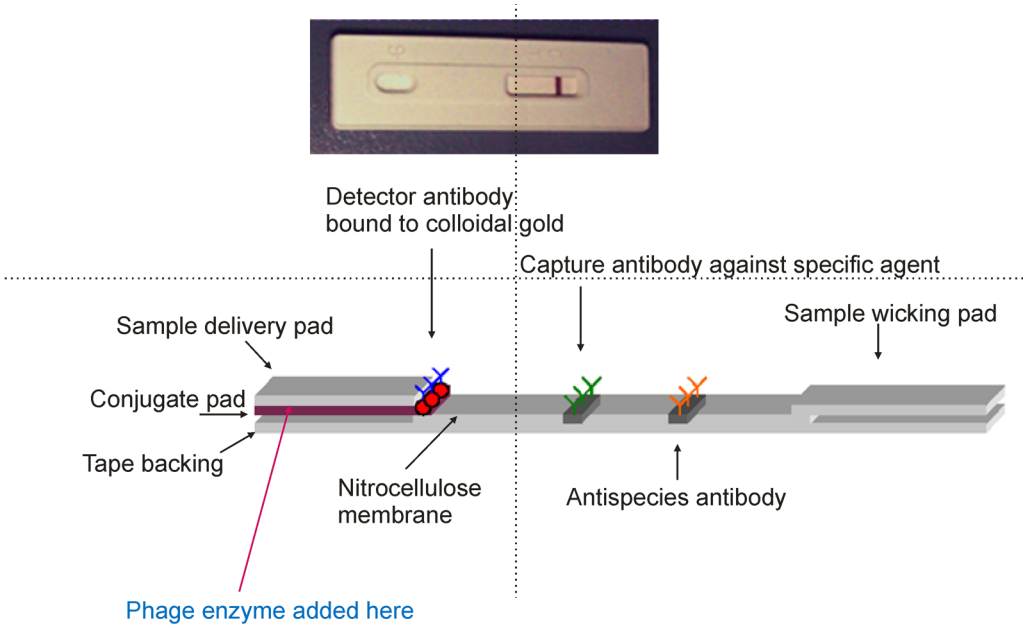

Figure 1. Lateral flow dip stick assay.

for the US Patent and Trademark Office to issue a patent for this method.

It was in the mid 1980's that history was made. The idea of attempting to control a GAS infection in patients' throats rather than just detecting it began to materialize. Experiments were designed and initiated. These efforts led to the first of several patents for the use of lytic enzymes for therapeutic, preventative and biocontrol applications assigned to the NHD (Fischetti and Loomis, 1999).

It is worth mentioning another twist in the history of phage lytic enzymes. The initial work on the diagnostics for GAS began in the mid-1980s. This new "gold-based" simple detection method also caught the eye of the US Department of Defense (DOD). The sensitive membrane antigen rapid test (SMART™ II) developmental team changed direction and began to develop rapid methods for the detection of selected bio-warfare agents, such as anthrax, botulism toxin and others. The assays were subsequently used during the First Gulf War (1990-1991). The hiatus in the work on therapeutic phage lytic enzymes was only temporary, however. It was in the 1990s, as a result of relationships gained from supporting the US National Biological Defense Program (http://en.wikipedia.org/wiki/United_States_biological_defense_program) under the DOD, that new potential uses for phage were proposed and subsequently funded. These included a major effort to develop an application for the use of phage to treat anthrax exposure. The project was funded by the Defense Advanced Research Projects Agency (http://www.darpa.mil/default.aspx), a long-range research arm of the US DOD. As a result, phage lytic enzyme research was up and running.

Compared to antibiotics, the use of PAEs offers distinct advantages for the prevention and treatment of diseases. The actions of the lysins are rapid. They do not depend on bacterial growth and can be directed to the mucosal lining where, owing to their specificity, they will kill colonizing bacteria without disturbing the normal flora. They appear to have a low probability of inducing resistance and new mutations, and they have fewer toxicity issues than antibiotics (Nelson et al., 2001). It also appears that lysins can be used as an effective preventative and decontamination reagent, for example against anthrax and other agents (Fischetti, 2005). Work has continued on the use of lytic enzymes directed against *B. anthracis* (BA), *Y. pestis* and others. Specifically, the BA-associated lytic enzyme system has been shown to be both effective and rapid. When lytic enzyme was introduced into a low-heat fogger, effective destruction in a closed environment was observed.

Although current lytic enzymes appear to have acceptable stability, it is necessary to ensure their stability under extreme conditions. Consequently, the NHD working with the US Department of Energy's Global Initiatives in Proliferation Prevention (http://www.darpa.mil/default.aspx), a project using a reverse-cell micelle matrix was initiated. It was discovered in 1977 that reverse micelles could entrap proteins (enzymes) and at the same time retain the catalytic activity of the enzyme within these micelles. When the enzyme is incorporated into the reverse micelle, the protein acquires a shell made of hydrated surfactant molecules. This micellar matrix protects the protein from the environment, as demonstrated in the sta-

bilization of PlyC (Yu et al., 2008; Klyachko et al., 2008).

Lysins can have many uses, including prophylactic and therapeutic treatments of a variety of illnesses caused by *S. pneumoniae*, *S. faesciae, H. influenza* and others. Also, since some of the lysins are heat-stable up to 60 °C and others can be stabilized, they can be incorporated into syrup, enemas and animal feed to kill bacterial infections of the digestive system (*Salmonella*, *E.coli,* etc.) and replace antibiotics currently used as growth promoters. Other uses include inclusion in bandages (*Staphylococcus* and *Pseudomonas*), tampons for vaginal infections (Group B *streptococcus*), eye drops (*Haemophilus*, *Pseudomonas*, etc.) and in inhalers.

In addition to their rapid and specific lytic activities, lysins are attractive compounds from a chemical viewpoint. Their recognition ability can be more specific than that of monoclonal antibodies. They have a high avidity that results in greater binding and improved activity and sensitivity. The modular design of lysins with their independent functional domains makes them ideal for domain-swapping studies in which bacterial specificities and catalytic activities are improved or adapted for use with other pathogens (Schmelcher et al., 2012; Yang et al., 2014).

DECONTAMINATION

Due to the threat posed by various antibiotic-resistant strains of common bacteria, there is an ever-increasing need to find new ways to address this important issue. Looking beyond current therapies, it is apparent that there is a need to address environmental threats affecting both humans and animals. These include methicillin-resistant *Staphylococcus* aureus (MRSA) in hospitals, nursing homes and other areas. Florin reported in 2006 on the growing concern of antibiotic-resistant strains in a field-hospital setting in Iraq (Florin, 2006). Combining this threat with the complex issues of decontaminating a facility contaminated with a biological threat agent, an ultrasonic aerosol-generator fogger was developed by the US Department of Energy's Pacific Northwest National Laboratory (PNNL, http://www.pnl.gov/). This proprietary vortex aerosol-generator is based on the application of an electro-activated aqueous solution in aerosol (fog) form at very low heat (Trudil and Rainina, 2006).

The PNNL ultrasonic aerosol fogger generates ~ 2 µm particles of fog via a remote fogging technology that enters all spaces and is deposited on all surfaces without a human presence (Figure 2). Non-fixed assets can be decontaminated in a collective protection unit. The system uses ambient temperature and an ultrasonic aerosol generator in combination with various enzymes capable of deactivating/decontaminating a variety of biological contaminants. In the current application, the biocatalytically active fog behaves like a gas, filling the space in an enclosed environment. The fog reaches difficult-to-access areas and is deposited on all surfaces, including objects with complex geometries. There are no detectable effects upon computers or other electronic devices.

The system has two key elements: the equipment and the enzyme. Results have shown that this system killed all nosocomial pathogens tested and ≥ 99% of microbial spores. The generators have only one expendable component, an ultrasound transducer that is inexpensive and easily replaced. The system was developed in a 20 lb. portable version (Trudil and Rainina, 2006), however, current systems offer reduced weight. There are currently several organizations developing lower-cost foggers using ultrasonic aerosol generation. They plan to perform further trials in 2015, with phage as well as lytic

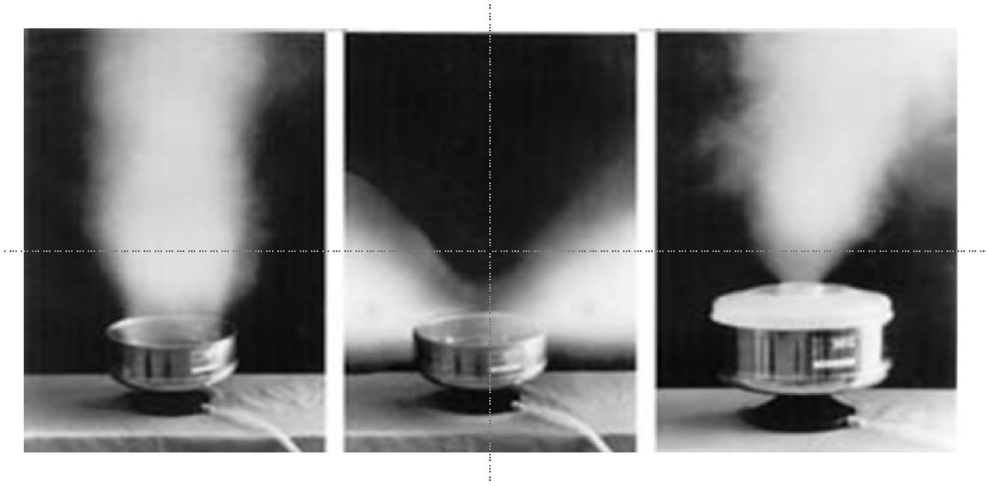

Figure 2. Ultrasonic aerosol fogger. Electro-activated aqueous solutions in aerosol (fog) form by "Vortex" Aerosol Generator producing 1-3 µm particles.(Courtesy of the Pacific Northwest National Laboratory)

enzymes, and in humans as well as in animal bacterial pathogen controls. Additional studies on the effectiveness of lytic enzymes for the control of strangles, a highly infectious bacterium in horses, have demonstrated significant advantages over normal chemical disinfectants (Hoopes et al., 2009)

DETECTION

In a unique blend of mature and old technologies, the lytic enzymes of isolated phage can be used in bioluminescence. Previously, measurement of microbial adenosine triphosphate (ATP) by bioluminescence measurement was used to determine bacterial populations in water, wastewater, marine environments, urine, milk, various foods and jet fuel, to name just a few. However, two main difficulties were encountered in the application of this method: 1) interference by non-microbial sources of ATP, and 2) chemicals and metals in the sample. These factors have resulted in limited sensitivity, as well as false positives and negatives. Advances in instrumentation, reagents and the inclusion of membrane filtration methods have resolved many of these problems (Siragusa et al., 1995; Cutter et al., 1996; Hasan et al., 1997).

Studies performed in the USA, Canada and Poland have shown the rapid ATP test (within 5 min) to have a 85–95% correlation with the standard 48-haerobic plate count (APC) (Siragusa et al., 1995; Cutter et al., 1996; Stopa et al., 1999) for total bacteria. This technology can also be used for the specific identification of selected organisms by using selected phage lytic-enzyme lysing reagents (*Listeria spp., Bacillus anthracis, Enterococcus*, etc.) instead of a total extracting reagent as used in the total APC method, which provides for generic lysing of all bacteria. The specific assays were first developed for GAS and then modified for other bacteria. They can be accomplished within 20 minutes, with a sensitivity of less than 10^3 CFU/mL for non-spores and as low as 100 cells for spore-formers (anthrax) when phage lytic enzymes are used (Trudil, 1999 and 2002).

Researchers at Rockefeller University further demonstrated that a phage lytic enzyme (PlyG) specific for anthrax, when used with the filtration bioluminescence method, could detect as few as 100 spores in 60 minutes. No signal was detected in the presence of other germinating spores, indicating the specificity of the assay (Schuch, 2002). In this method, the assay was performed using NHD's PROFILE®-1 reagent kit and model 3550i bioluminometer with a field spore protocol (Figure 3), as detailed by researchers from the US DOD and the Polish Military Institute of Hygiene and Epidemiology (MIHE) (Trudil, 1999 and 2002). Researchers suggest that this method could have useful applications in monitoring domestic and battlefield environments for *B. anthracis*, as well as in other field conditions to determine water or food safety (Stopa et al., 1999).

Additional studies are underway examining the utilization of this method with newly isolated anthrax phage, and also using other phage lytic enzymes, at the MIHE and at Eliava Institute. Additionally, other detection targets such as Group D enterococcus are being explored by Eliava with the NHD and other institutes. Measurement of Group D enterococcus is a regulatory requirement for safe recreational water. Previous technologies required a minimum of 18-24 hours for results. A rapid field-based assay would be of significant benefit to swimming facilities, as well as providing commercial opportunities for the technology. Currently, this method appears to be most suited for Gram-positive bacteria, but studies are underway to expand the effectiveness in Gram-negatives that have a more complex outer cell structure.

Additionally, just as the initial work with lytic enzymes focused on GAS detection, the same issues of extraction surround group B *streptococci*., Currently studies are underway to evaluate inclusion of a lytic enzyme in a 'dipstick' assay and as a specific extractant in a luminescence-based method. Studies have also been expanded to include *Streptococcus* and *Staphylococcus*

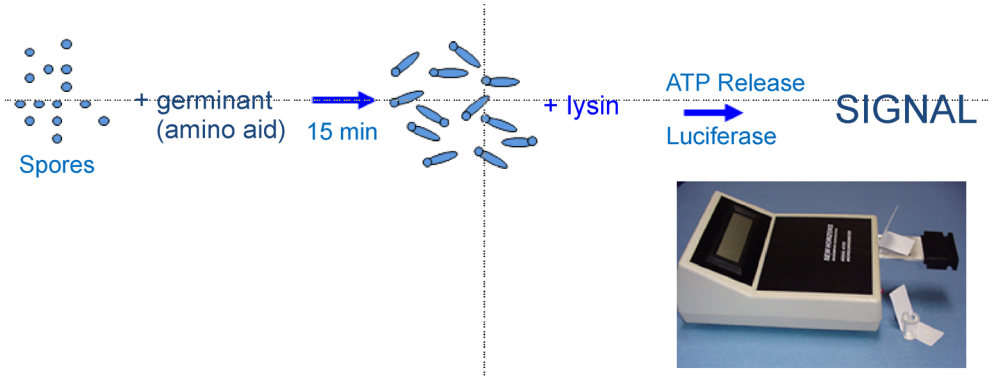

Figure 3. Use of gamma phage lytic enzyme for rapid identification of *B. anthracis* via Bioluminescence.

lytic enzymes in a mastitis assay, using the luminescence method (NHD personal communication).

DISCUSSION

The concept of using Mother Nature's antibiotics (bacteriophages) as a tool in the war against microbes is most appealing. The fact that this approach has been used and adapted for many thousands of years and remains effective is remarkable. In the grand scheme of things, the antibiotics developed by man have only been available for a small period of time and yet microbes continue to quickly adapt.

Bacteriophages have been in use for almost 90 years in human, animal and environmental applications. Their initial use was remarkable to early 20th century scientists. The panacea was short-lived, however, since antibiotics took the spotlight as a more defined and dependable alternative. Today, due to a rise in emerging pathogens, including antibiotic-resistant forms, there is a need for a new alternative or, in this case, a more defined and refined old alternative.

The early lack of success of phage therapy is thought to have been the result of a lack of information regarding basic phage biology. With increased experience regarding the study of phage, lytic enzymes and molecular biology, researchers have now progressed to the point where sufficient knowledge and molecular tools are available to quantify and improve on these technologies scientifically, so as to develop specific next-generation applications.

Fischetti and colleagues reported on various isolated phage lytic enzymes that are effective in selectively and rapidly lysing specific bacteria. The list of scientists working with this technology continues to expand into other fields: from Donovan (USDA) isolating *Staphylococcus aureus* for human and large animal diseases (Schmelcher et al., 2012) to Siragusa (formerly of USDA) for poultry disease, and the USDA efforts on fermentation control (Roach et al., 2013), to name but a few.

It is apparent, if not only from the increased numbers of phage-related meetings and sessions, that interest in phage and lytic enzymes continues to increase. With studies in the areas of antibiotic resistance, food safety, improved food production, inexpensive treatments, environmental control, improved detection and more, the use of phage-related technologies is bearing fruit today.

It appears that due to necessity, the scientific community is turning back to Mother Nature (phage) to address the growing issues that are sometimes man-induced (antibiotic resistance). Researchers can leverage current and future tools such as bioinformatics and proteomics to discover new opportunities for the development of specifically engineered designer lysins. What is needed now is to understand fully the capabilities and limitations of these methods and technologies and to determine how they can best be translated to effective use, especially given the seriousness of the threat of emerging pathogens, both natural and man-made.

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COMPLIANCE WITH ETHICS GUIDELINES

All the authors declare that they have no competing interests. This article does not contain any studies with human or animals subjects performed by any of the authors.

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