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Identifying a *B.thuringiensis* var. *kurstaki* Receptor Binding Protein for Bacteriophage Riley

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Integrated Science and Engineering
James Madison University

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ABSTRACT

With the rapid emergence of antibiotic resistant bacteria affecting people around the world, research into new therapies using bacteriophages (phages) is increasing in the United States. Phages are viruses that can only infect bacteria and are able to co-evolve alongside the bacteria they infect. A researchers' ability to pinpoint which phage to use in the therapy is important to combat an infection effectively. To do so, the genes that control the interaction between phages and the bacteria they infect, such as receptor binding proteins on the surface of a bacterial cell, need to be identified. Transposon mutagenesis was used in our study to find the receptor binding protein of *Bacillus thuringiensis kurstaki* (Btk). Btk was chosen as the bacterial host because it is a naturally occurring soil bacteria that is commonly used as an insecticide in agriculture, but is nonpathogenic to humans. The bacterium is also a close relative to *Bacillus anthracis*, the causative agent of anthrax, and may share some phages. Using the EZ-Tn5™ <R6Kyori/KAN-2> Tnp transposon kit, 134 individual mutant colonies were isolated on kanamycin plates. Virulent bacteriophage Riley, a well-characterized phage infecting BtK, was used to find phage-resistant bacteria in the mutant population. Three mutants, 1041, 1043, and 1221, were found to be resistant to bacteriophage Riley and will be further studied to determine the interrupted gene.

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INTRODUCTION

On September 3, 1928, the major discovery of penicillin was made by Alexander Fleming that would soon be deemed the miracle drug (Aminov, 2010). For decades after that discovery, the western world has depended solely on antibiotics to treat bacterial infections. What many did not realize was that every time a person was prescribed antibiotics, it increased the risk of bacteria becoming resistant and, consequently, deadlier to the human population. Due to the Cold War, many of the advances in antibiotics did not reach past the iron curtain into Eastern Europe. Consequently, research into bacteriophage (phage) therapy increased, instead of being pushed to the side like it was in the West (Nobrega, Costa, Kluskens, & Azeredo, 2015). With the increase of the number of antibiotic resistant bacterial strains, phages are once again being looked at as a treatment for bacterial diseases.

Unlike antibiotics, that have the tendency to kill more than just bacteria causing the infection, bacteriophages have a high specificity to the bacteria they infect (Nobrega et al., 2015). Their high specificity is also a limitation in phage therapy because they can only infect one type of bacteria while an infection may include multiple types of pathogenic bacteria. To combat this problem many scientists are creating phage cocktails that have multiple different types of phages that can infect and destroy different types of bacteria (Chan, Abedon, & Loc-Carrillo, 2013). Another advantage to bacteriophages is that they only replicate at the site of infection. Phages are self-limiting and self-dosing, preventing them from persisting when their specific bacterial pathogen becomes absent (Nobrega et al., 2015). From a developmental point

of view, phages have the advantage of rapid isolation, versatility of formulation and application, and lower developmental costs than antibiotics (Nobrega et al., 2015).

Phage therapy has the advantage of being able affect multi-drug resistant bacteria and biofilms because phages have specific receptor binding sites that allow them to infect a bacterium even if it is resistant to antibiotics. Phages have a general lower tendency to induce resistance and cross-resistance to antibiotics (Nobrega et al., 2015). Phage-resistance can, however, occur in bacterial cells if the bacterium uses the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system that is able to cause the degradation of the inserted phage DNA or RNA, or phage absorption is blocked due to the mutation or loss of the bacterial receptor on the surface of the bacterial cell (Nobrega et al., 2015). To combat bacteria's natural way of immunizing themselves against phages, a proposed solution is to genetically engineer the phages using the CRISPR system.

Before genetic engineering can be done, research on the genes that control phage and interaction with the bacterial cell must be completed. An important protein for the attachment of the bacteriophage to the bacteria's cell wall is the Receptor Binding Protein (RBP) located on the surface of the bacterial wall. The first step in the infection by a bacteriophage is the adsorption of the phage to the host cell (Bielmann et al., 2015). RBP is recognized by phage receptor and allows the phage to attach to the cell wall (Bielmann et al., 2015). The RBP recognition and binding is extremely specific, and high affinity is required for rapid and efficient virus attachment (Bielmann et al., 2015). According to Bielmann, the precise mechanism by

which a phage particle recognizes, adsorbs to, and infects a bacterial cell is only poorly understood, especially for phages infecting Gram-positive pathogens (Bielmann *et al.*, 2015).

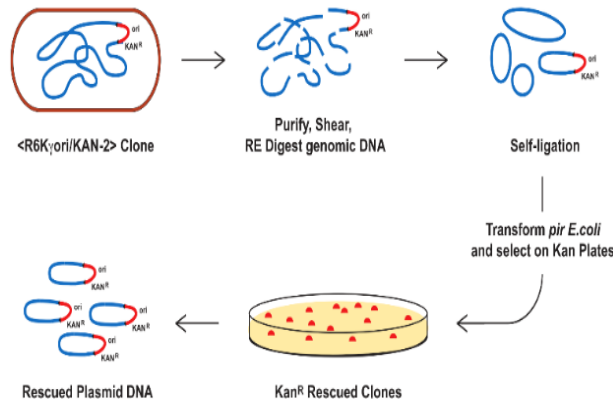


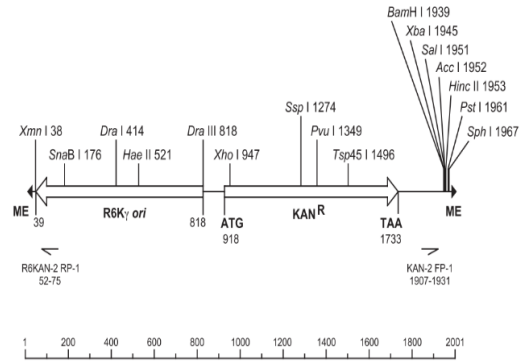
Figure 1: Rescue Cloning of Interrupted Gene and Transposon (TNP & KIT, 2017)

Though they are difficult to identify using standard procedures, RBPs can be used as diagnostic tools and therapeutics in the biotech industry (Sacher, 2016). For these therapeutics to be successful, the structures of the host cell that will allow phage interaction must be analyzed. (Sacher, 2016).

Transposon Mutagenesis

To analyze RBPs, the technique of transposon mutagenesis can be used to find and isolate the gene. Transposons are genetic elements that go through the recombination process of transposition in which they can relocate from one genomic location to another (Reznikoff, 1993)(Hayes, 2003). Transposons are found in both prokaryotic and eukaryotic cells and can be used as *in vivo* or *in vitro* interrupters (Hayes, 2003). Tn5 is a type of transposon that moves from its resident position to another location by a cut-and-paste mechanism (Hayes, 2003). The Tn5 transposon from the EZ-Tn5 <R6k_{ori}/KAN-2> transposon kit contains the kanamycin resistant gene KAN^R that allows antibiotics resistance to be used as a selection phenotype (Tnp & Kit, 2017). The R6k_{ori} gene allows the transposon to self-ligate into a plasmid after the DNA from the mutant is extracted and sheared so that it can be transformed into *E. coli* to be

rescued, as seen in Figure 1 (Tnp & Kit, 2017). The transposon map (Figure 2) indicates that the KAN^R gene and the R6k γ ori gene fall in the middle of the transposon's genomic sequence. The additional genes allow the researcher to ensure that the transposon has interrupted a gene. The Tn5 transposon can be inserted into the bacteria's chromosome using electroporation (Figure 3). Electroporation is the process of using electrical



Note: Not all restriction enzymes that cut only once are indicated above.
See the following pages for further information.
Primers are not drawn to scale.

R6KAN-2 RP-1 Reverse Primer 5' CTACCCTGTGGAACACCTACATCT 3'
KAN-2 FP-1 Forward Primer 5' ACCTACAACAAAGCTCTCATCAACC 3'
ME = Mosaic End 5' AGATGTGATATAAGAGACAG 3'

Figure 2: Ez-Tn5 <R6k γ ori/Kan-2> Transposon (Tnp & Kit, 2017)

pulses to create pores that allow genetic material to permeate the bacterial membrane of

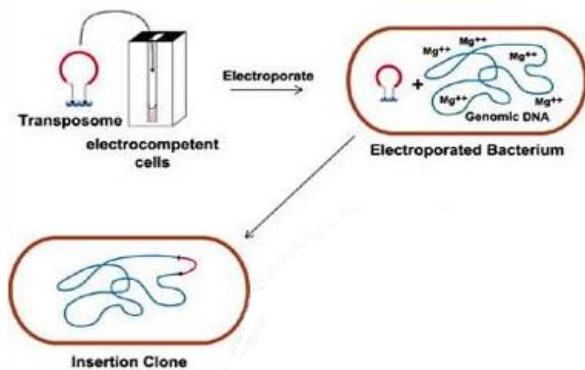


Figure 3: Transposon mutagenesis using electroporation (Tnp & Kit, 2017)

electrocompetent cells ("Electrocompetent Cells," n.d.). The EZ-Tn5 transposase is then activated by the Mg²⁺ that is located in the cell's environment and randomly inserts into the host's DNA (Tnp & Kit, 2017). The cells that took up the transposon can be grown on kanamycin (KAN) plates so that the genes that were

interrupted can be found and analyzed.

Bacillus thurgiensis kurstaki and Bacteriophage Riley

Bacillus thurgiensis kurstaki (BtK) (Figure 4) is a Gram-positive bacterium that is found abundantly in soil. It is used in industry as a natural insecticide to certain types of caterpillars (Olkowski, Daar, & Olkowski, 2009). It is a non-pathogenic bacterium that can be safely handled in a Biosafety Level 1 lab. Since it is in the lowest Biosafety Risk Group, BtK is a safe candidate to create mutants with transposon mutagenesis.

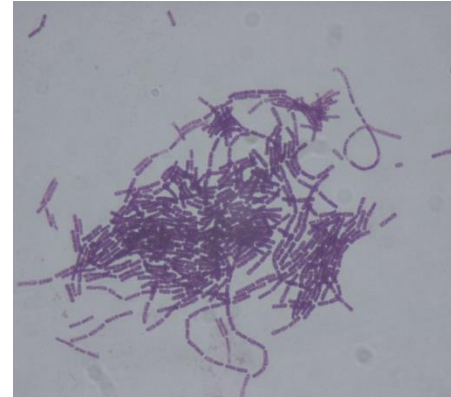


Figure 4: *Bacillus thurgiensis* Kurstaki (Sahay, 2013)

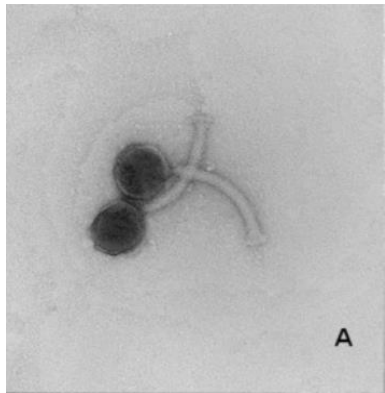


Figure 5: Bacteriophage Riley (Sauder *Et Al.*, 2016)

Riley (Figure 5) is a myovirus bacteriophage that was discovered and characterized by students at Mary Washington University. It is a virulent phage that only goes through the lytic cycle. This attribute allows Riley to be a candidate for phage therapy because it would infect and destroy the bacteria without going into the lysogenic cycle, which could leave some bacteria alive.

OBJECTIVE

The main purpose of this project was to further our knowledge about the genes that allow the interactions between bacteriophages and the bacteria they infect. The overall goal was to find the receptor binding protein using transposon mutagenesis to construct mutants that are resistant to bacteriophage Riley and then to sequence the interrupted gene.

METHODS:

Multiplicity of Infection:

The Multiplicity of infection (MOI) for *Bacillus thurgiensis* kurstaki (BtK) was calculated to find the amount of BtK needed to infect LB plates. To find the MOI, 3.0 mL of enriched BtK and SM buffer (100mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris-Cl (1 M, pH 7.5), 0.01% (w/v) Gelatin (2%, w/v), 1 liter H₂O) was placed in a tube labeled 10⁰. A 10-fold dilution out to 10⁻⁴ was then made with SM buffer and the bacterial culture. A Bausch and Lomb Spectronic 20 spectrometer was used to measure the optical density (OD) at 600nm for each dilution. Then 100 μL of each dilution was spread on separate LB plates. The plates were then placed into an incubator at 37°C for 24 hours. The titer was then calculated for the 10⁻⁴ dilution using the following formula: $\left(\frac{\text{number of cells}}{\text{amount placed on plate}}\right) \times \left(\frac{1000 \mu\text{L}}{1\text{mL}}\right) \times \text{the dilution factor}$

Testing Bacteriophages:

The bacteria culture was diluted to 10⁻² and 100 μL of the dilutions 10⁰ to 10⁻² were pipetted into 5 mL top agar and poured onto separate plates. Five microliters of high titer lysate of bacteriophages Riley, Troll, Megaron, and CAM003 were placed onto designated spaces on each plate. The plates were then placed into the incubator at 37°C for 24 hours. The test was used to see which phage would infect the bacteria with the clearest spot with the given concentration of phages.

Transposon Mutagenesis:

The purpose of this protocol was to prepare the cells for electroporation and then deliver the transposon. The BtK cells were prepared for electroporation by inoculating a colony of BtK in 10 mL of LB in a 125-mL flask and then incubating the culture overnight at 37°C in a shaking incubator at 250 RPM. Five hundred milliliters of BHI were incubated overnight as well. After 24 hours, 2 mL of the BtK culture were pipetted into the heated BHI flask and incubated at 28°C in the shaking incubator at 300 RPM. The cultures were monitored until the OD₆₀₀ reached 0.3 OD, which is approximately 1.0×10^7 cells/mL as calculated by colony forming units. At this point, the culture was chilled on ice for 10 minutes. The cells were then transferred into two chilled 250 mL centrifuge bottles and centrifuged at 4°C for 10 minutes at 10,000 x g to form a pellet at the bottom of the tube. After discarding the supernatant, the pellet was resuspended with 50 mL of sterile ice-cold EP buffer (0.5 mM K₂HPO₄–KH₂PO₄, 0.5 mM MgCl₂, 272 mM Sucrose). The cells were pelleted as before with the chilled centrifuge. After pelleting the cells, the supernatant was discarded and the pellet was resuspended in 15 mL of sterile, ice cold EP buffer. The cells were then transferred into 40 mL Oak Ridge tubes and centrifuged for 10 minutes at 10,000 x g. The supernatant was discarded and the pellet was resuspended in 0.5 mL of ice cold EP buffer and kept on ice.

To electroporate the cells, 1 µL of transposon DNA was pipetted into 100 µL of the electrocompetent BtK cells. The solution was mixed and incubated on ice for 5 to 10 minutes. After incubating the cells, 100 µL of the transposon-cell suspension was transferred to a chilled 0.2 cm cuvette on ice. A microcentrifuge tube for each sample was prepared with 2.0 mL of LB

broth and incubated at room temperature. The cuvette was tapped to get the solution to the bottom of the cuvette. The Bio-RAD Gene Pulser X cell (Bio-Rad, [Hercules, CA]) was used with a *Bacillus*-specific protocol from the instrument manufacturer to electroporate the cells. The cuvette was then placed in the ShockPod and the chamber lid was closed. The pulse parameter was checked to see if the time constant was approximately 8.6 milliseconds and the voltage was approximately 1.0 KV. After pressing the pulse button, the cuvette was removed from the chamber and 200 μ L of the room temperature LB broth was immediately added to the cuvette. The cells from the cuvette were then transferred to the tube containing the LB broth. The process was then repeated for the negative control (no DNA added to the bacterial cells). The two tubes were then incubated for 1.5 hours at 37°C in the shaking incubator at 250 rpm. The cell cultures were then plated on kanamycin-LB (50 μ g/mL) plates at 100 μ L per plate and incubated at 37°C for 48 hours. The colonies that grew were cultured eight at a time on KAN plates.

Bacteriophage Resistance testing:

The mutants were named by taking the number of their plate and their position on the plate. An example of this would be mutant 1043 where the mutant was on plate 10 as the 43rd mutant cultured. The next step was to use full plates infections (FPI) to find mutants that are resistant to bacteriophage Riley. The FPI started by labeling five microcentrifuge tubes 10^0 to 10^{-4} . For the 10^0 microtube, 100 μ L of LB was pipetted into the tube. Ninety microliters of LB was pipetted into the rest of the labeled tubes. Eight mutants were then picked and put into one 10^0 tubes to make a pool. Ten microliters from the 10^0 tube was pipetted into the 10^{-1}

tube. This was then repeated for the rest of the dilution series. One hundred microliters of phage HTL was pipetted into each tube. After the tubes were mixed by vortexing, 190 μL of each dilution was pipetted onto separate KAN plates and incubated at 37°C for 24 hours. This technique was then repeated for the rest of the mutants, but only using dilutions 10^{-3} and 10^{-4} since their lawns had the most coverage on the plate.

The colonies were then tested against phage Riley using spot tests to identify mutants that were resistant to the bacteriophage. To do this, the mutant colonies were individually picked and then placed in 5 μL of LB broth and incubated in the shaker at 37°C for 48 hours. The cultures were then plated on KAN plates by pipetting 50 μL of the culture onto the plate and spread by using an L-shaped spreader. Five microliters of the high titer lysate (HTL) of Riley was then spotted on the plate and left to dry. The plates were placed in the incubator at 37°C for 48 hours. The spot test protocol was repeated twice more using 100 μL of the culture on the plates until there were 8 mutants that showed consistent and stable phage resistance. After repeated testing, 3 mutants - 1041, 1043, and 1221 - were retained due to highly consistent resistance results.

RESULTS

In this study, a suitable phage was chosen and optimized for infectivity against BtK. Lack of infection by this phage was used to screen a transposon mutant library of BtK. The process yielded three resistant mutants, which are currently being analyzed.

Multiplicity of Infection and testing bacteriophages:

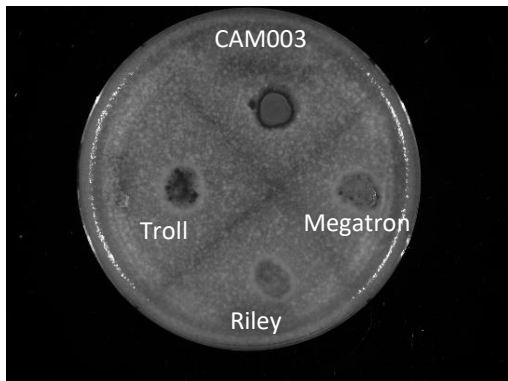


Figure 6: Dilution 10^0 with phage spots

BtK was diluted to 10^{-4} and was determined that the 10^{-4} dilution yielded a confluent lawn of approximately 7.6×10^6 cfu/mL. Bacteriophage Riley was chosen as the phage to test against the mutants (Figure 6). The MOI chosen was 10^0 since it had the most consistent lawn coverage, as seen in Figure 6.

Transposon Mutagenesis:

The time constant for the control BtK for electroporation was 9.6 msec and the voltage was 989 V. The time constant for the bacteria-plasmid solution was 8.1 msec and the voltage was 986 V. A total of 134 mutants (Figure 7) were produced in two experiments. A few cells were found on the control, but were not consistent enough throughout the plates to warrant a new test.

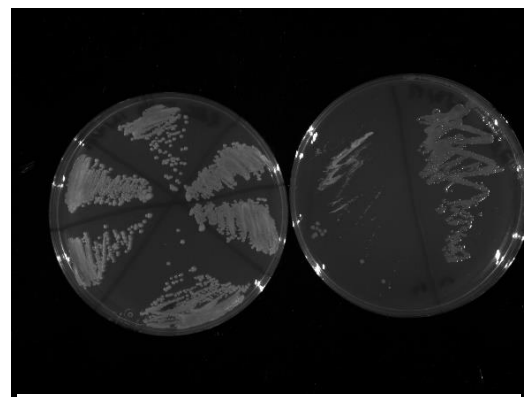


Figure 7: 7 out of 134 Btk mutant cultures created by transposon

Bacteriophage Resistance

Mutants from plates 10, 11, 12, and 13 were able to grow on the plates after being tested against bacteriophage Riley. The rest of the plates were fully lysed. Table 1 shows that mutants 1041, 1043, and 1221 were resistant to bacteriophage Riley. The other mutants either did not grow in culture well enough to plate or they were infected by Riley. The results of the spot tests are illustrated in Figure 8 where there is only a clearing in D, as indicated by the red circle, while A, B, and C did not have clearings. Mutants 1041, 1043, and 1221 are resistant to bacteriophage Riley since the phage could not make a clearing in their lawns. There were some mutants, such as 1335, that did show some promise of being resistant. However the resistance results were inconsistent between each spot test, so they were disregarded.

Table 1: Mutants Tested Against Bacteriophage Riley

Mutant	Resistant (Yes/No)
1034	No growth
1041	Yes
1043	Yes
1048	No
1221	Yes
1335	No
1336	Inconsistent resistance between spot tests
1339	No growth

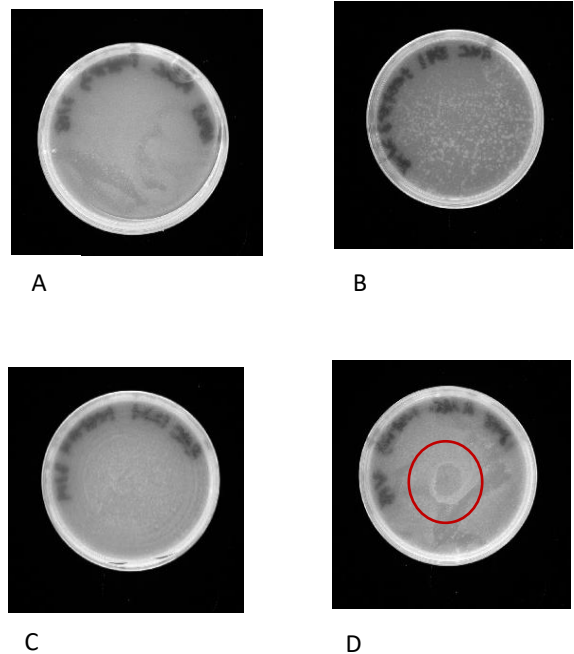


Figure 8: Spot Tests: **A)** 1043 against Riley **B)** 1041 against Riley **C)** 1221 against Riley **D)** 1048 against Riley

DISCUSSION AND CONCLUSIONS

From the data we obtained, we can conclude that transposon mutagenesis can be used to create phage resistant mutants of Gram-positive bacteria. From these mutants, three were found to be resistant to bacteriophage Riley. The BtK gene that encodes the receptor for Riley can be said to have been interrupted by the transposon by these results.

Currently, there is a lack of information on *Bacillus* species surface receptors for phages. Analyzing the genes that allow for phages to interact with BtK will allow researchers to find specific phages that can be used for phage therapy. One reason why there is a lack of information for *Bacillus* RBPs is that the bacteria are Gram-positive, thus making it harder to complete mutagenesis and DNA extraction.

The Ez-Tn5 <R6kyori/Kan-2> transposon kit was chosen because it has been proven to be effective for multiple different strains of gram-positive bacteria including *Bacillus subtilis*. A problem that kept arising was that the yield of mutants was always small. The number of mutants created should have been in the hundreds, but only a total of 134 mutants were isolated on the KAN plates. We expected that the number of mutants needed to find a few that were resistant to Riley would have to be in the hundreds. Instead, we were able to isolate three that were consistently resistant. Having a large percentage of the mutants being resistant might mean that there are multiple receptors for this phage instead of just one.

Once the information about the genes that control the interaction between phages and the bacteria they infect has been found, it can be used to enhance phage therapy. Researchers

may be able to genetically engineer phages that bind more tightly to the receptor to guarantee high rate of infection. This can only be done if the researcher knows what protein controls the interactions and what makes up its macromolecular structure. Researchers could also use this information to genetically engineer other phages that are highly virulent so that they can infect the same bacterial strain.

FUTURE WORK

Future work will be to extract DNA from the mutant cells, ligate the DNA with the transposon into a plasmid, and then complete plasmid transformation into *E. coli*. The transformation step will allow for the plasmid to be separated from the mutant's chromosomal DNA. The DNA can then be extracted and purified from the *E. coli* cells to allow for it to be sequenced. The sequenced DNA can then be compared to the BtK library to find which protein was interrupted.

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