Isolation and Characterization of $\phi$km18p, a Novel Lytic Phage with Therapeutic Potential against Extensively Drug Resistant Acinetobacter baumannii

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Abstract

**Aims:** To isolate phages against extensively drug resistant Acinetobacter baumannii (XDRAB) and characterize the highest lytic capability phage as a model to evaluate on phage therapy.

**Methods and Results:** Eight phages were isolated from hospital sewage and showed narrow host spectrum. Phage $\phi$km18p was able to effectively lyse the most XDRAB. It has a dsDNA genome of 45 kb in size and hexagonal head of about 59 nm in diameter and no tail. Bacterial population decreased quickly from $10^8$ CFU ml$^{-1}$ to $10^5$ CFU ml$^{-1}$ in 30 min by $\phi$km18p. The 185 kDa lysis protein encoded by $\phi$km18p genome was detected when the extracted protein did not boil before SDS-PAGE; it showed that the lysis protein is a complex rather than a monomer. Phage $\phi$km18p improved human lung epithelial cells survival rates when they were incubated with A. baumannii. Combination of phages ($\phi$km18p, $\phi$TZ1 and $\phi$S14) as a cocktail could lyse all genotype-varying XDRAB isolates.

**Conclusion:** Infections with XDRAB are extremely difficult to treat and development of a phage cocktails therapy could be a therapeutic alternative in the future. Phage $\phi$km18p is a good candidate for inclusion in phage cocktails.

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Introduction

Acinetobacter baumannii, a gram-negative, non-fermentative bacterium, is one of the most important nosocomial pathogens in hospitals, especially in intensive care units. A. baumannii is an opportunistic pathogen that causes serious nosocomial outbreaks. The mortality rate is elevated and the length of hospitalization is prolonged if drug resistance develops [1,2]. In recent years, multidrug resistant A. baumannii have been spread worldwide including Taiwan [3–5]. More recently, the term “extensively drug resistant” A. baumannii (XDRAB) has been used to characterize the bacterial isolates resistant to all authorized antibiotics except 2 category of antibiotic e.g. tigecycline and polymyxins [4,6,7]. Given the increasing prevalence of XDRAB infection with high mortality rate, new antibiotics and are needed to combat this challenge, and one possible solution is the therapeutic use of phages [9–13].

Phages have been used as pharmaceutical agents for more than 90 years. Because of the threat of serious multi-drug resistant bacterium infection, there has been renewed interest in the use of phages for the treatment of infectious diseases of humans and other animals [8–13]. Numerous animal studies have demonstrated the effectiveness of phages against multi-drug resistant bacteria, such as vancomycin-resistant Enterococcus faecium [14], Escherichia coli [15], Pseudomonas aeruginosa [16–17], Staphylococcus. aureus [18] and Vibrio vulnificus [19]. Some phages of common bacterial pathogens, such as Listeria monocytogenes, Salmonella spp. and Pseudomonas, have been approved for commercial use in food preservation [20–21].

The phages of Acinetobacter spp. have been studied previously, but most were used for transduction, phage typing or classification of phages [22–25]. Previous reports have described the phage BS46, which specifically infects A. baumannii AC54 and protects infected mice from death [26]. An ssRNA phage, AP205, that propagates in Acinetobacter genospecies 16 (A. radioresistens) was also isolated and analyzed [27]. Due to the limited efficacy and potential side effect of current antibiotic against XDR-AB, we want to isolate phages against XDR-AB and characterize highest
lytic capability phage as a model to evaluate the potential on phage therapy.

Materials and Methods

Bacterial strains

Thirty-four clinical strains of A. baumannii from five medical centers (Taichung Veterans General Hospital, National Taiwan University Hospital, Kaohsiung Medical University Chung-Ho Memorial Hospital, Hualien Tzu Chi Medical Center and Tri-Service General Hospital) were used in this study (Table 1). All isolates underwent MIC testing for various drugs, including carbapenems, anti-pseudomonal cephalosporins, anti-pseudomonal penicillins, monobactams, aminoglycosides, tetracyclines, fluoroquinolones, sulbactam and polymyxins, using CLSI (Clinical and Laboratory Standards Institute) guidelines (National Committee for Clinical Laboratory Standards, USA).

Phage isolation

Sewage samples were collected from various aquatic ecosystems in the Taichung Veterans General Hospital (Taichung, Taiwan) and E-Da Hospital (Kaoshiung, Taiwan) for phage isolation. The presence of phages was investigated using a phage enrichment technique [28]. A 50-ml supernatant form centrifugated sewage was mixed with 50 ml double-strength Luria-Bertani broth containing exponential-phase A. baumannii strains. After a 24-h incubation at 37°C, the bacterial cells were centrifuged and the supernatant was filtered through a 0.22-μm filter. Then, 10 μl of filtrate and the indicator strain were mixed with soft agar and poured onto an LB agar plate. Phage plaques would be visible after overnight incubation at 37°C. Single plaques were selected by a sterile tooth pick and suspended in 500 μl PBS. This condensed phage suspension was loaded onto a discontinuous CsCl gradient diluted in PBS buffer and centrifuged at 14,700 g for 9 h at 4°C. The banded phage particles were collected with a SW41Ti rotor. The banded phage particles were washed in TE buffer three times for 30 min each. For restriction enzyme digestion analysis, the plasmid was washed in sterile distilled water three times. The phage DNA was subjected to restriction digest analysis according to the manufacturer’s instructions. The plug was loaded onto 1% agarose gel prepared with 0.5× Tris-Borate-EDTA (TBE) running buffer.

Pulsed Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed according to a modification of the protocol described by Tseng et al. [30]. Purified phage suspension (ca. 1×10^10 PFU ml^-1) was mixed with an equal volume of molten 1% agarose (SeaKem Gold agarose, Cambrex), which was allowed to solidify in a mold (13-mm length×1-mm width×10-mm depth). The solid block was lysed at 55°C overnight by proteinase K (10 mg ml^-1) in cell lysis buffer (50 mmol l^-1 Tris, 50 mmol l^-1 EDTA at pH 8.0, 1% Sarkosine). After the proteinase K lysis step, the block of agarose was washed in TE buffer three times for 30 min each. For restriction enzyme digestion, a plug (13-mm×1-mm×2-mm) was cut from the agarose block containing the phage genomic DNA and washed in sterile distilled water three times. The phage DNA was subjected to restriction digest analysis according to the manufacturer’s instructions. The plugs were loaded onto 1% agarose prepared with 0.5× TBE (pH 8.0) running buffer. The restriction fragments were separated by PFGE (using the CHEF-DR III apparatus from Bio-Rad, Richmond, CA, USA) at 14°C with a ramping time of 2 s to 12 s for 8 h, at a field strength of 6 V/cm.

Electron microscopic examination of phage morphology

Samples for electron microscopic examination were prepared as follows: equal amounts of phage suspension (1×10^10 PFU ml^-1) and 0.1% bacitracin were mixed well, and one drop of this mixture was spotted onto a mesh grid for 3 min. The grid was then touched with a piece of Whatman filter paper to drain away any excess suspension and the grid was then stained with 2% uranyl acetate for 30 s. The prepared samples were examined under a JEM-2000 EX II electron microscope (JEOL, Japan) at an operating voltage of 80 kV.

SDS-PAGE under non-denaturing conditions

The method was according to the description by Jyothistri et al. [31]. To investigate the lysis protein of phage ϕkm18p, phage lysate was centrifuged at 14,700×g for 30 min at 4°C, and the supernatant was filtered through a 0.22-μm filter. Ammonium sulfate (30%) was added to precipitate the proteins in the filtered suspension, and then the suspension was dialyzed with PBS buffer (137 mmol l^-1 NaCl, 2.7 mmol l^-1 KCl, 10 mmol l^-1 Na_2HPO_4, 2 mmol l^-1 KH_2PO_4). The protein suspension was mixed with sample buffer without β-mercaptoethanol. The samples were then run on SDS-PAGE without boiling. The resolved gel containing the extracted proteins was plated onto L-agar and soft agar mixed with A. baumannii KM18 was poured onto the gel, and incubated at 37°C overnight. Clear zones on the overlay indicate lytic proteins.
A549 cell survival test

To ensure the safety of phage therapy, we investigated the toxicity of crude isolated phages to A549 human lung epithelial cells. A549 cells (10^5 cell well^{-1}) were plated in a 24-well ELISA plate and incubated for 12 h (37°C, 5% CO\textsubscript{2}) in 100 μl per well of Dulbecco’s modified Eagle medium (DMEM) (Sigma, D5523, USA) supplemented with 5% fetal calf serum. Then, 10^6 CFU A. baumannii KM18 were added to the cells, followed by the immediate addition of phage at different titers (MOI = 0, 1000, 1, 0.1, 0.01). The cells were cultured for 24 h at 37°C in a 5% CO\textsubscript{2} incubator. As a control, A549 cells were treated with 10^9 PFU phages without A. baumannii KM18. After incubation, the wells were washed twice with PBS and incubated with trypsin-EDTA solution (0.05% trypsin, 0.5 mM EDTA-tetra sodium) to allow surviving cells to separate from the well. The cell counts of

### Table 1. The antibiotic sensitivity results of reference strains and the spot test of eight phages against the reference strains of A. baumannii used in this study.

| Strain   | Phages | Antibiotics sensitivity¹ | Source² |
|----------|--------|---------------------------|---------|
|          | φkm18p | φTZ1 | φ2449N | φAb21 | φKMS | φ314 | φ134 | φ2449p |
| Ab016    | +++    | –   | +     | –     | –    | –    | –    | –     |
| Ab019    | +++    | –   | –     | –     | –    | –    | –    | –     |
| Ab14     | –*     | –   | +     | –     | –    | –    | –    | +++    |
| Ab15     | –      | –   | +++   | –     | –    | –    | –    | +++    |
| Ab16     | –      | –   | +++   | –     | –    | –    | –    | +++    |
| Ab19     | –      | –   | ++    | –     | –    | –    | –    | –     |
| Ab23     | +++    | –   | –     | –     | +    | –    | –    | +++    |
| Ab26     | –      | –   | +     | –     | –    | –    | –    | ++     |
| Ab29     | +++    | –   | –     | –     | –    | –    | –    | +++    |
| Ab35     | +++    | –   | –     | –     | –    | –    | –    | +++    |
| Ab39     | –      | –   | +     | –     | –    | –    | –    | +     |
| Ab40     | +++    | –   | –     | –     | –    | –    | –    | ++     |
| Ab54     | –      | –   | –     | –     | –    | –    | –    | +++    |
| TVG55    | –      | –   | +++   | –     | –    | –    | –    | –     |
| NTU2449  | –      | –   | +++   | –     | –    | –    | –    | +     |
| KM5      | –      | –   | –     | –     | –    | –    | –    | –     |
| Ab21     | +++    | –   | –     | –     | –    | –    | –    | +     |
| TVG68    | +++    | –   | –     | –     | –    | –    | –    | –     |
| Ab002    | +++    | –   | +     | –     | –    | –    | –    | –     |
| Ab010    | +++    | –   | –     | –     | –    | –    | –    | +     |
| Ab011    | +++    | –   | –     | –     | –    | –    | –    | –     |
| Ab015    | +++    | –   | –     | –     | –    | –    | –    | +     |
| Ab021    | +++    | –   | –     | –     | –    | +    | –    | –     |
| KM16     | –      | +++ | –     | –     | –    | –    | –    | –     |
| KM18     | +++    | –   | –     | –     | –    | –    | –    | +     |
| TSG2     | –      | –   | +     | –     | –    | –    | –    | –     |
| TSG4     | –      | –   | +++   | –     | –    | –    | –    | –     |
| TSG5     | –      | +++ | –     | –     | –    | –    | –    | +     |
| TSG6     | –      | +++ | –     | –     | –    | –    | –    | –     |
| TVG46    | +++    | –   | –     | –     | –    | –    | –    | –     |
| TVG52    | –      | +++ | –     | –     | –    | –    | +    | –     |
| TVG57    | –      | +++ | –     | –     | –    | –    | –    | –     |
| TVG58    | –      | +++ | –     | –     | –    | –    | –    | –     |
| TZ1      | –      | +++ | –     | –     | –    | –    | –    | –     |

**注**: no effect; ‘+’: turbid zone; ‘+++’: clear zone.

¹CS: carbapenem sensitive A. baumannii; CRAB: carbapenem resistant A. baumannii (including imipenem and meropenem resistant); MDRAB: multi-drug resistant (resistance to 3 or more of the following classes of antipseudomonal cephalosporin, antipseudomonal carbapenem, ampicillin-sulbactam, fluoroquinolone, aminoglycoside); XDR-AB: extensively drug resistant Acinetobacter baumannii (resistant to all antibiotics except colistin or tigecycline).

²TVG: Taichung Veterans General Hospital, Medical Center; NTU: National Taiwan University Hospital, Medical Center; KM: Kaohsiung Medical University, Chung-Ho Memorial Hospital; TZ: Hualien Tzu Chi Medical Center.; TSG: Tri-Service General Hospital, Medical Center.

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surviving A549 cells suspended in trypsin solution were determined by microscopic observation (Nikon, E2000, Japan).

Results

Antibiotic susceptibility and bacterial strain identification

Table 1 showed that 16 strains of A. baumannii were XDR-AB (resistant to all antibiotics except colistin or tigecycline). Three strains were carbapenem-resistant (including imipenem- and meropenem-resistant). Two strains resistant to fluoroquinolone were termed MDR-AB. The genomic DNA of these 34 strains was digested with Apol and separated by PFGE. Different DNA patterns were seen after PFGE and indicated that all 34 bacterium were different strains (data not shown).

Isolation of eight lytic phages

Thirty-four clinical A. baumannii strains were used as host indicators for the isolation of lytic phages. Finally, eight lytic phages were isolated and their host ranges were determined by spot tests on these host strains and presented in Table 1. Phages ϕkm18p and ϕ2449p were isolated from the sewage of Taichung Veterans General Hospital, and the other phages, ϕTZ1, ϕ2449N, ϕAb21, ϕKM5, ϕ314 and ϕ134, were isolated from the sewage of E-Da Hospital. Table 1 shows that 15 of 34 indicated strains were sensitive to ϕkm18p and distributed in the carbapenem-sensitive and XDRAB groups. Phage ϕ134 was found to have almost the same host range as ϕkm18p. Phage ϕ2449 was most virulent to carbapenem-sensitive strains, but was non-lytic to XDRAB strains. Only three bacterial strains were subject to lysogeny by ϕ2449p. The hosts of ϕTZ1 and ϕ314 did not overlap with those of phage ϕkm18p. Therefore, this combination of phages could be used as a cocktail to target all XDRAB. Phages ϕAb21 and ϕKM5 presented the most narrow host range as only one bacterium strain was sensitive to each of them. From the results shown in Table 1, phage ϕkm18p was found to be the most powerful phage. The spot tests of phage ϕkm18p on clinical strains of A. baumannii showed the widest range of hosts, including imipenem-susceptible and XDRAB strains. Compared with other phages, ϕkm18p produced more lysis of XDRAB strains than other phages did. We also found that the plaques of ϕkm18p were 2 mm larger in diameter than those of the other seven phages and that ϕkm18p lysed the bacteria more quickly in liquid medium (data not shown).

Electron micrograph of phage morphology

An electron micrograph of phage particles revealed that the phage has a hexagonal head 59 nm in diameter and lacks a tail (Fig. 2). The ϕkm18p phage particles were concentrated in a visible band at a density of 1.5 g ml⁻¹ in a CsCl gradient. Phage ϕkm18p was similar in morphology and size to phage PM2 [32] and should be assigned to the family Cystoviridae according to the taxonomic database of ICTVdB [33].

Phage genome size and protein profiles

Restriction fragments of phage DNA were separated by gel electrophoresis and PFGE to determine the genome size. HindII fragments of phage DNA were separated by gel electrophoresis, resulting in DNA patterns that included double bands for approximately 19 fragments (Fig. 3, A). The optimal second enzyme XhoI was also used to digest genomic DNA, resulting in six fragments visible on a PFGE gel (Fig. 3, B). From the HindII and XhoI digest patterns, the genome size of phage ϕkm18p was found to be approximately 45 kb. Phage virion structural proteins were at least 12 protein patterns and the most abundant protein was 39 kDa which was predicted to be a major coat protein (Fig. 4).
Phage endolysin activity was shown by bacterium overlay of a PAGE gel

Upon release, the phages burst the bacteria, causing endolysin to flow outside into the medium. Proteins of supernatant were precipitated by 30% ammonia sulfate and separated by SDS-PAGE, as described in the materials and methods. Bacterium overlay on SDS-PAGE showed a clear band at 185 kDa, but clear band disappeared when the sample was boiling treatment before electrophoresis (Fig. 5). From this result, we suggest that the endolysin functions in a complex rather than as a monomer.

Phage ensures A549 cell survival under bacterial infection

To ensure the safety of phage therapy, A549 human lung epithelial cells were used as an indicator for phage toxicity. The phage \( \phi \text{km}18p \) was found to provide the highest protection against \( A. \text{baumannii} \) KM18 infection of cells (105 cells) (Fig. 6). Phage at an MOI of 1, 0.1 or 0.01 enabled cells inoculated with \( A. \text{baumannii} \) KM18 (104 CFU) to survive as well as uninoculated controls. Cells treated with phages at an MOI of 1000 (109 PFU), but not inoculated with bacteria, survived as well as the control cells. Cells inoculated with the bacterial strain \( A. \text{baumannii} \) KM18 only without any phage treatment were completely killed. The results showed that \( \phi \text{km}18p \) eliminated bacteria and protected A549 cells from immediate killing by KM18 bacteria. They also suggested that a high dose of phages did not impact A549 survival.

Discussion

Phage \( \phi \text{km}18p \) has assigned to the family \textit{Corticoviridae} and was a new phage of \textit{Ac. baumannii}. Previously, several phage species have been isolated. They belong to the \textit{Myoviridae}, \textit{Siphoviridae} and \textit{Podoviridae} families of tailed phages [23]. An ssRNA bacteriophage of \textit{Acinetobacter} from the family \textit{Leviviridae} was also identified [27].

Our purpose was to investigate the possibility of phage therapy to eliminate XDRAB and found phage \( \phi \text{km}18p \) was a good candidate. From the report of Soothill [26], the phage BS46 provided protection to \( A. \text{baumannii} \)-infected mice, indicating that it is possible to control antibiotic-resistant \( A. \text{baumannii} \) by phage therapy. In our study, clinical \( A. \text{baumannii} \) isolates were used to survey lytic phages. We chose the most active phages for further analysis of their plaque morphology, effect on bacterial growth, genome size, endolysin activity and protection of cells. We examined the antibiotic sensitivity of these 34 strains of \( A. \text{baumannii} \), and classified them as CS, CRAB, MDRAB and XDRAB. All of the bacterial strains were sensitive to the phages
isolated in this study. The phage φkm18p was unique among the phage isolates; 15 bacterial strains were sensitive to it, including seven XDRAB strains. Although host specificity of phages is a major limitation for therapy, a phage cocktail could combat bacteria in an emergency. Table 1 shows that the host ranges of the studied phages are narrow. Phage φkm18p (44.1%) had the broadest range of hosts and triggered lysis in all hosts; the other phages showed decreased efficiency of infecting these strains. The major drawback of phages is their narrow host range; therefore, a large combination of phages is needed for therapy. The lytic and lysogeny phage, such as φ2449p was most virulent to carbapenem-sensitive strains. But non-lytic and lysogeny to XDRAB strains was not a suitable candidate for cocktail combination. The lytic or lysogeny pathway was determined when the phage DNA was injected into host, if the genome of phage integrate into host DNA and coexist in it, some antibiotic resistant gene or other host fragment would be assembly into the new synthesis phages. Using the lysogenic phage as the agent would dangerously transfer the multidrug resistant genes or toxic genes to other hosts. The candidates of phage for biocontrol or phage therapy should be obligate lytic to bacterium. These phages shouldn’t have integrases; would degrade the host genome DNA and lack the opportunities to coexist with their host. However, many genes of isolated phages still lack function information and database matches [34]. We tried to isolate phages to combat all of the bacterial strains shown in Table 1. None of the phages had efficient activity against most bacterial strains, but each bacterial strain could be lysed by at least one phage. We believe that every derived bacterial strain can be lysed by one type of phage in nature.

Phage φkm18 decreased the concentration of A. baumannii from 10^6 CFU ml^{-1} to 10^3 CFU ml^{-1} within 30 min and maintained this concentration for about 2 h. But the high titer of phage with MOI = 10 presented the same lytic activity as MOI = 1 (Fig. 1[A]). It was thought that the higher titer of phage inoculation would get higher efficiency on cleaning the bacterium, but the complex dynamic interactions between hosts and the phages always have to optimize the efficiency of phage as the agent for phage therapy. Each phage has its’ optimal concentration to interact to its’ host.

Higher titer of phage would raise the attaching rate on the host and decrease the bacterium concentration quickly. But sometimes phage would induce the phage-immune system and refuses other phage infection. There is also possible that the phage titer is saturate and receptors for phage are occupied and the bacterium reduction will not increase with increase MOI values. [35–36]. Thus it would be a property of phage that only interacts with a small portion of hosts in the purpose to their reproductivity. The burst size would be the factor to explain the phages could evolve to use a small proportion of host, probability of death of hosts and the time for phage production. But also interpret that the maturation of progeny is asynchronous with actual time of lysis and leaving the flexible control point [37–38]. As with any phage-host interaction, bacterial growth increases over time [29,39–40]. Another possible explanation for this finding is the production of mutant phage-resistant strains. Phage-resistant bacteria have been mentioned in many articles [41–43]. However, phage sensitivities also resulted in a change in Omp (outer membrane protein) and LPS (lipopolysaccharide) profiles of the bacteria [42]. Bacterial growth eventually rises after infection by one type of phage, but a cocktail of lytic phages completely eliminates the pathogen [29].

Human lung epithelial cells A549 (10^5 cells well^{-1}) were killed when inoculated with 10^8 CFU of A. baumannii KM18. In this study, a higher concentration (10^9 CFU) of bacteria was used to infect the cells and to investigate the protective efficacy of phages on the cells. The data presented in Fig. 6 showed that φkm18p increased the survival rate after 24-h incubation to the same rate as the control without added pathogen. Otherwise, the phage φkm18p did not affect the growth of A549 cells, indicating that the phage φkm18p is a potential candidate for phage therapy. Also, bacteria infected with phage φkm18p started to proliferate at two hour after infection, causing the growth curve to rise again. The bacterial density was also found to increase after a 24-h incubation of A549 cells, A. baumannii KM18 and φkm18p (Fig. 1[B]). But why did the bacterial cells survive under a high titer of A. baumannii KM18? We analyzed the surviving bacteria and found that phage-resistant bacterial strains had developed (data not shown). Further animal model experiments should be perform to confirm that phage φkm18p protect animal infected by XDRAB.
When the phage particles matured in the bacteria, the endolysin encoded by phage genes hydrolyzed the cell wall of the host and disrupted the bacterium, causing the phage particles to burst [18,44]. When the bacteria were destroyed, endolysin was also released into the lysate. The protein with lytic activity showed a clear pattern on the bacterial overlay, indicating a protein size of about 185 kDa (Fig. 5). From the results, it was suggested that the lytic protein is a multimer because the protein with lytic activity disappeared after denaturation by boiling. Purified phage-encoded peptidoglycan hydrolase (lysin) is also reported to be effective for the treatment of bacterial infections [43].

Current treatment of XDRAB included tigecycline and colistin [45–46]. Colistin is limited by its potential renal toxicity [45]. Tigecycline is limited by lack of efficacy bloodstream and urinary tract infection [46]. Besides, emerging resistance to colistin and tigecycline has been reported [3,4,7]. Phage therapy is a revitalized therapy against bacterial infectious diseases. Previous reports have shown that appropriate administration of living phages can be used to manage life-threatening infectious diseases caused by gram-negative bacteria, such as *E. coli*, *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* [26,47–48]. The Poles and the Soviets have administered phages orally, topically and systemically to treat different antibiotic-resistant pathogens, including *Staphylococcus*, *Streptococcus*, *Escherichia*, *Proteus*, *Pseudomonas*, *Shigella*, *Salmonella* and *Acinetobacter* spp. [8]. The phages specific for XDRAB described in this study will hopefully be prepared to cope with the resistance.

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**Author Contributions**

Conceived and designed the experiments: GHS CHH. Performed the experiments: FSW KMC CHH. Analyzed the data: JLW CHH. Contributed reagents/materials/analysis tools: CFK CHL HRL. Wrote the paper: GHS JLW CHH.

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