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Note

Cell-free synthesis of the *Salmonella* specific broad host range bacteriophage, felixO1

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ABSTRACT

Phage-based biocontrol of foodborne *Salmonella* is limited by the requisite use of *Salmonella* to propagate the phages. This limitation can be circumvented by producing *Salmonella* phages using a cell-free gene expression system (CFE) with a non-pathogenic chassis. Here, we produce the *Salmonella* phage felixO1 using an *E. coli*-based CFE system.

Bacteriophages (phages) are an effective control strategy for foodborne pathogens including *Salmonella*, a bacterium responsible for over one million illnesses annually in the U.S. (Scallan et al., 2011). Phages can reduce *Salmonella* loads on foods by 1 to 2 logs alone or by up to 6 logs as part of a hurdle strategy (Moye et al., 2018; Wessels et al., 2021). However, the use of phages as biocontrol agents has a significant limitation that is the requisite use of pathogens to propagate them. The direct handling of pathogens provides a risk to workers, requires strict regulatory compliance, and demands safe disposal of contaminated materials. Additional challenges come from optimizing phage yields given numerous phage-host variables which must be controlled including growth rates, nutritional requirements, adsorption kinetics, burst sizes, and spontaneous development of host resistance (João et al., 2021). Attempting to manage these variables for multiple phages in a cocktail amplifies production difficulties.

A method that circumvents many of these biological variables is cell-free expression (CFE) which is gaining popularity in biomanufacturing (Chiba et al., 2021). In CFE, a cell lysate, containing transcription and translation machinery, is mixed with template nucleic acids to produce a desired product in a one-pot reaction (Garenne et al., 2021). Expressing phage genomes directly via CFE eliminates many biological variables and uses a non-pathogenic *E. coli* cell lysate, improving safety. Note that for employing CFE in phage biomanufacturing, challenges of scalability and cost must be addressed (Vilkhovoy et al., 2020). Recent developments demonstrate that an *E. coli*-based CFE can produce phages of *E. coli*, *Yersinia pestis*, and *Klebsiella pneumoniae* (Emslander et al., 2022; Garenne et al., 2021; Rustad et al., 2018). Additionally, the *Salmonella* broad host range phage felixO1 (Felix and Callow, 1943), which lyses 98.2% of *Salmonella* strains and is used commercially in the food industry (Kocot et al., 2023; Whichard et al., 2010), has been rebooted by electroporating its genome into *E. coli* (Jia et al., 2023). However, *E. coli*-based CFE of *Salmonella* phages remains undemonstrated.

To test *Salmonella* phage production in CFE, we isolated genomic DNA (gDNA) from felixO1 and an *E. coli*-infecting relative, VpaE1 (Šimoliūnas et al., 2015). Phage lysates of felixO1 and VpaE1 at $>10^{10}$ and $>10^{11}$ PFU/ml, respectively, were produced in Lysogeny Broth (LB) with hosts *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain LT2 (Zinder and Lederberg, 1952) and *E. coli* strain B^E (Šimoliūnas et al., 2015), respectively. These phage lysates were treated with DNase I [New England Biolabs (NEB) 2000 units/ml] and RNase A (NEB 20 mg/ml) using 0.5 μl enzyme per ml phage lysate at 37 °C for 1 h to remove host-associated nucleic acids before centrifugation at 20,000 xg and 4 °C for 1 h to generate visible phage pellets (Fig. 1A). Pellets were resuspended in SM buffer [100 mM NaCl, 8 mM $MgSO_4 \bullet 7H_2O$, 25 mM Tris-HCl (pH 7.5)]. Several methods were tested to free gDNA from the phage capsid including proteinase K digestion, heat denaturation at 75 °C, and purification with the Promega Wizard Genomic DNA Purification Kit (Fig. 1B). These tests revealed that heat denaturation and the commercial kit released gDNA. Proteinase K digestion did not release gDNA but reduced the molecular mass of the produced band, possibly via partial digestion of phage tails but not the capsid. Digestion of the kit extract by DNase I, but not by RNase A or proteinase K, further indicated gDNA was purified (Fig. 1B). Kit-extracted gDNA, at concentrations of ~5.2 nM and ~ 8.9 nM for felixO1 and VpaE1, respectively, was used for

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subsequent CFE reactions.

CFE reactions were run as previously described (Garamella et al., 2016). Reactions with and without supplemental energy mix were run for felixO1 while VpaE1 was tested in a reaction without the energy solution. Samples of completed reactions were serially diluted and spot plated onto LB soft agars (Adams, 1959) containing appropriate bacterial host mentioned above. Spot plates were incubated overnight at 37 °C, and PFUs were counted to determine reaction titer. These plates revealed that ~5.4×10^7 PFU/ml were generated from the base felixO1 reaction. Supplementing the felixO1 reaction with an energy mix which includes dNTPs resulted in a titer of ~4.0×10^8 PFU/ml (Fig. 2A). These titers demonstrate that felixO1 can be produced using an *E. coli*-based CFE system and that the phage's gDNA may have been replicated during the reaction. When compared to spot plates of felixO1 propagated in vivo, plaque size and turbidity of CFE-produced felixO1 appeared the same (Fig. 2A). The CFE reaction using VpaE1 gDNA generated a titer of 7.5×10^{10} PFU/ml which was higher than felixO1, potentially because the CFE system is derived from *E. coli* and remains better adapted to phages that infect this host bacterium. Negative controls were performed by spot plating samples of gDNA extracts and CFE reactions without added gDNA, neither of which generated detectable PFUs (data not shown).

While viable felixO1 was produced via CFE, it was unclear if the virion structure would be consistent with felixO1 propagated in vivo. Scanning a sample of the felixO1 CFE reaction with transmission electron microscopy revealed very few phage parts (e.g., capsids, tails), possibly due to the low titer and limited volume of the reaction. Most of the components observed were either capsids or tails unassembled with other parts of the phage. However, one particle was observed which appeared to have a connected capsid and tail, though it could not be determined whether tail fibers were also assembled with the phage tail (Fig. 2B). The tail of the virion measured 18 × 117 nm in length while the capsid had a diameter of 78 nm, consistent with the previously reported dimensions of felixO1 (Whichard et al., 2010).

Overall, this work has established an effective and convenient method for isolating felixO1 and VpaE1 gDNA and demonstrated that a *Salmonella* phage can be produced using an *E. coli*-based CFE system. CFE of felixO1 may advance the use of phages as biocontrol agents for foodborne *Salmonella* by removing the requirements to use pathogenic bacteria in their production.

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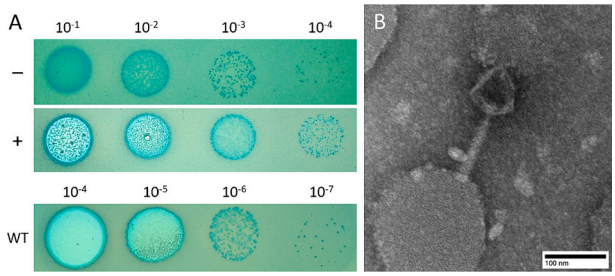

Fig. 2. Viable felixO1 phages were produced in an *E. coli*-based cell-free expression system. (A) Spots of felixO1 produced in a CFE reaction without (−) and with (+) supplemental energy solution added, along with felixO1 propagated normally on *S. enterica* serovar Typhimurium LT2 (WT). Exponents indicate relative serial dilutions from an undiluted stock (10^0). Soft agars were formulated with 5% *v*/v glycerol and 0.01% *w/v* Coomassie Brilliant Blue to improve plaque visibility. (B) Transmission electron microscopy image of an assembled felixO1 virion from a CFE reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Declaration of generative AI in Scientific writing

No artificial intelligence software was used in the preparation of this manuscript.

Ethics statement

Use of all biological entities in this study was approved by the Institutional Biosafety Committee of the University of Minnesota, Minneapolis, Minnesota, USA. All biological entities were handled according to good laboratory practices and disinfection protocols.

CRediT authorship contribution statement

John A. McFarlane: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **David Garenne:** Investigation, Methodology, Writing – review & editing. **Vincent Noireaux:** Formal analysis, Funding acquisition, Resources, Supervision, Writing – review & editing. **Steven D. Bowden:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

All authors declare no competing interests.

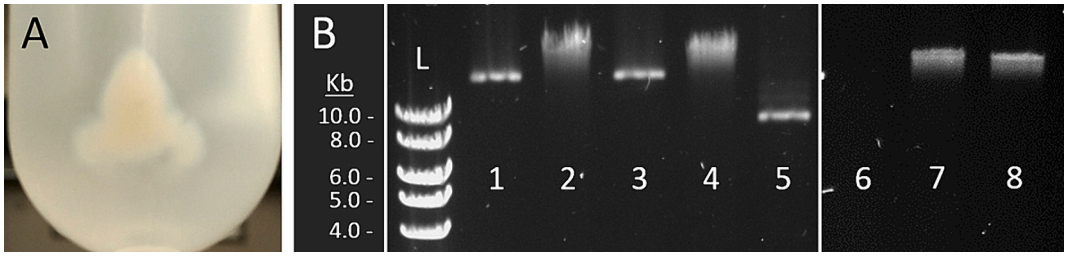

Fig. 1. Troubleshooting gDNA isolation from bacteriophage felixO1. (A) An image showing a pellet of felixO1 produced from a phage lysate of $>10^{10}$ PFU/ml following nuclease pre-treatment and high-speed centrifugation at 20,000 xg. (B) Samples of a felixO1 phage lysate (1–5) and elution following Promega Wizard Genomic DNA Purification Kit processing (6–8) were migrated through an agarose gel following various treatments. L, 1 kb ladder (NEB); 1, untreated; 2, incubated at 75 °C for 20 min; 3, treated with DNase I and RNase A at 37 °C for 2 h; 4, treated as in 3 and then treated as in 2; 5, treated with proteinase K at 55 °C for 2 h; 6, treated with DNase I overnight at 37 °C; 7, treated with RNase A overnight at 37 °C; 8, treated with proteinase K overnight at 37 °C. Both gels were formulated with 0.7% *w/v* agarose in TBE buffer and 1× SYBR Safe DNA stain before migration at 110 V for 90 min.

Data availability

Data will be made available on request.

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