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Thesis

Set up of a Bacteriophage enrichment and titration workflow using the *Staphylococcus aureus* lytic phage P15S6

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Abstract

Antimicrobial resistance has renewed interest in bacteriophages as antibacterial agents, including against the bacteria that cause dental caries and periodontitis. Isolating phages from oral samples first requires a reliable laboratory workflow. The aim of this work was to set up and test an enrichment and titration workflow using a reference phage, P15S6 with *Staphylococcus aureus* as a model host and to test the same phage against the oral commensal *Streptococcus salivarius*. Exponential-phase cultures were incubated with the phage in the presence of $CaCl_2$, and the filtered lysate was titrated by spotting tenfold serial dilutions onto double-agar-layer plates. The phage titre assay on *S. aureus* showed clear countable plaques at the 10^{-5} dilution, with no lysis in the negative control, giving a titre of 2.3×10^8 PFU/mL. The phage titre assay on *S. salivarius* showed plaques on square 1 (10^{-1}) and square 2 (10^{-2}) but they were too many to count. The workflow works with *S. aureus* and, once plaque purification and phage characterisation are added, can be applied to oral bacterial hosts and oral samples.

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Statement on the use of AI tools

During the preparation of this thesis, the generative AI assistant Claude (Anthropic) was used to review the draft for the internal coherence (title, objectives, methods, results and conclusions), figure numbering and captions, and consistency of the references, and to draft a revised version of the introduction based on the objectives, methods and results of this work. Also Google Gemini was used to help research the articles used for the citations of this thesis. The author reviewed and edited all AI-assisted text, verified every cited reference against the original source and takes full responsibility for the content of this thesis. No AI tool was used to generate, modify or analyse the experimental data or the images.

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List of abbreviations

AMR - antimicrobial resistance

CRISPR-Cas - Clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins

dsDNA - double-stranded DNA

EOP - efficiency of plating

ICTV - International Committee on Taxonomy of Viruses

LPS - lipopolysaccharide

MOI - multiplicity of infection

MRSA - Methicillin-resistant *Staphylococcus aureus*

OD_{600} - optical density at 600 nm

OMV - Outer Membrane Vesicle

PFU - plaque-forming units

SM buffer - saline-magnesium buffer (phage dilution buffer)

ssDNA - single-stranded DNA

TSA - tryptic soy agar

TSB - tryptic soy broth

1. Introduction

Antimicrobial resistance (AMR) is one of the main threats to global health: In 2019, bacterial AMR was directly responsible for an estimated 1.27 million deaths and was directly associated with 4.95 million deaths (Murray et al., 2022), and attributable deaths are forecast to rise to 1.91 million per year by 2050 (Naghavi et al., 2024). This has renewed interest in alternatives to antibiotics, including bacteriophages (Dicks and Vermeulen, 2024). One setting in which phages are being explored is the oral cavity, where the two most common diseases, dental caries and periodontitis, are driven by bacterial biofilms (Szafrański et al., 2017). Before phages against oral bacteria can be studied, they have to be isolated, and this requires a reliable laboratory workflow to enrich, detect and count them. This thesis describes the set-up of such a workflow using a well-characterised model system.

1.1 Pathogens of the oral microbiome

The oral microbiome is a complex ecosystem of bacteria, archaea, fungi and viruses. When its ecological balance is disrupted, it can give rise to dental caries, which is one of the most prevalent and persistent diseases in the human population, despite the many preventive options available (Dalmasso et al., 2015), or to periodontal disease, whose two major forms are gingivitis and periodontitis. The dental plaque biofilm, together with other risk factors such as lifestyle and genetic factors, is the main etiological factor in the development and progression of periodontitis (Abdulkareem et al., 2023).

Streptococci are among the first bacteria to colonise the tooth surface: mitis-group species such as *Streptococcus sanguinis*, *Streptococcus gordonii* and *Streptococcus oralis* are the main early colonisers of the salivary pellicle (Kolenbrander et al., 2010). Among the mutans streptococci, *Streptococcus mutans* and *Streptococcus sobrinus* have long been recognised as cariogenic (Bloch et al., 2024). *S. mutans* is a Gram-positive, coccus-shaped, non-motile, facultatively anaerobic bacterium that resides in the human mouth. It is an opportunistic pathogen and the main cause of dental decay in humans: it attaches to the tooth surface within biofilm communities and contributes to the development of dental disease (Dalmasso et al., 2015). S. sobrinus also contributes to caries, but it has been studied much less than *S. mutans* (Damle, 2018). Lactobacilli have been associated with dental caries for over a century (Caufield et al., 2015). *Bifidobacterium*, *Scardovia* and *Parascardovia have been* detected in active caries lesions, although not consistently (Nair et al., 2017).

Periodontitis is associated with a shift from a symbiotic to a dysbiotic subgingival community dominated by Gram-negative anaerobes (Abdulkareem et al., 2023).

Porphyromonas gingivalis is regarded as a keystone pathogen: even at low abundance, it can shift the microbial community and the host response towards disease (Hajishengallis et al., 2012). *Fusobacterium nucleatum* aggregates with many other oral species, bridging early and late colonisers (Kolenbrander et al., 2010; Wang et al., 2022). *Aggregatibacter actinomycetemcomitans* is strongly associated with a rapidly progressing form of periodontitis that affects young people, formerly called localised aggressive periodontitis (Fine et al., 2019; Papapanou et al., 2018).

Not all oral streptococci are harmful. *Streptococcus salivarius* is one of the first colonisers of the oral mucosa and a mainly commensal species of the healthy oral cavity; some strains are used as oral probiotics (Bloch et al., 2024). As a Gram-positive oral streptococcus that is easy to grow in the laboratory, *S. salivarius* is a convenient first oral host on which to test laboratory methods before moving on to cariogenic species such as *S. mutans*.

1.2 Bacteriophages

Bacteriophages (phages) are viruses that infect bacteria (Dicks and Vermeulen, 2024). To infect a cell, a phage first adsorbs to specific receptors on the bacterial surface and then injects its genetic material into the cell (Chae, 2023). On the basis of their structure alone, phages can be grouped into four types: tailed, polyhedral, filamentous and pleomorphic phages (Chang et al., 2022). The vast majority are tailed phages with double-stranded DNA (dsDNA) genomes; only a few are single stranded DNA (ssDNA) or RNA viruses. Tailed phages were traditionally divided into three families according to tail morphology: *Siphoviridae*, with long, flexible, non-contractile tails (about 55%); *Myoviridae*, with contractile tails (about 25%); and *Podoviridae*, with short, stubby tails (about 20%) (Hatfull and Hendrix, 2011). In 2022 the International Committee on Taxonomy of Viruses (ICTV) abolished these morphology-based families: all tailed phages are now placed in the class *Caudoviricetes*, and siphovirus, myovirus and podovirus are used only as descriptions of morphotype (Turner et al., 2023).

Phages have two main life cycles. In the lytic cycle, the phage takes over the bacterial cell to replicate its genome and produce new virions; phage-encoded lysis proteins (holins and endolysins) then rupture the cell and release the progeny. In the lysogenic cycle, used by

temperate phages, the phage genome integrates into the host chromosome as a prophage, which remains latent and is replicated along with the host DNA until it is induced, typically when DNA damage triggers the SOS response, and enters the lytic cycle (Chae, 2023). Only strictly lytic (virulent) phages are suitable for therapy, because they kill their host and do not establish lysogeny. Temperate phages are avoided because lysogeny can protect bacteria from further phage infection and can transfer genes, including virulence and resistance genes, between bacteria (Caflisch et al., 2019).

1.3 Phage Therapy and oral biofilms

Phage therapy differs from antibiotic treatment in that most phages are host-specific, although some can infect several bacteria species or strains (Dicks and Vermeulen, 2024). Phages can also penetrate and disrupt biofilms, something that antibiotics do poorly, and for this reason they have been investigated against major cariogenic bacteria such as *S. mutans* (Wolfoviz-Zilberman et al., 2021). Biofilm infections are persistent and very hard to prevent and treat, because the biofilm matrix reduces the penetration of antimicrobials. Oral biofilms underlie the most prevalent polymicrobial infections in humans, caries and periodontitis, and phages have been shown to be active against bacteria organised in biofilms and to reduce their numbers (Szafrański et al., 2017). Isolated phages have been shown to infect and kill both planktonic and biofilm cultures of *S. mutans* in vitro, and to reduce caries in vivo (Wolfoviz-Zilberman et al., 2021). In addition, mucin supplementation has been reported to increase the susceptibility of *S. mutans* to phage infection (Sundberg et al., 2022).

Phages have also been found for periodontal pathogens. Phages infecting *Fusobacterium nucleatum* have been isolated and characterised and are considered to have good prospects for application (Wang et al., 2022). Prophages have been identified in the genomes of *Porphyromonas gingivalis* (Matrishin et al., 2023) and *Aggregatibacter actinomycetemcomitans* (Tang-Siegel et al., 2023). Temperate phages such as these are not candidates for therapy, but they may influence the ecology and virulence of their hosts. Overall the number of characterised phages active against oral bacteria is still small (Szafrański et al., 2017), so the isolation of new phages remains a necessary first step.

1.4 Challenges with Phage Therapy

Although phages are a promising way to fight bacteria, phage therapy faces several challenges. The first is bacterial resistance to phages, the product of the long-standing co-evolutionary arms race between phages and bacteria. Bacteria can resist phages at every stage of infection (Labrie et al., 2010). They can prevent adsorption by altering or masking phage receptors, such as lipopolysaccharide (LPS) in Gram-negative bacteria or wall teichoic acids and surface proteins in Gram-positive bacteria, through point mutations or phase variation. They can also block DNA injection (superinfection exclusion), cut incoming phage DNA with restriction-modification and CRISPR-Cas systems, or use abortive-infection systems, in which the infected cell dies before new phages are released (Labrie et al., 2010). Gram-negative bacteria also release outer membrane vesicles (OMVs), non-replicating structures that are part of a functionally diverse intercellular transport system. Because OMVs carry the same surface receptors as the cell, they can act as decoys that bind phages and reduce infection of the bacteria (Caflisch et al., 2019).

Phage selection is another challenge: therapeutic phages must be strictly lytic and highly virulent against the target bacterium. Because phages are very specific about which bacteria they infect, finding the right phage for a given pathogen can be difficult. In addition, the immune system can clear phages and produce neutralising anti-phage antibodies. Finally, phages are unfamiliar to many people: because they are viruses, they may be mistakenly perceived as potential pathogens (Zalewska-Piątek, 2023).

1.5 Detecting and counting phages: enrichment, spot assay and titration

In natural samples, phages are often too few to be detected directly. Their number is therefore increased by enrichment: the sample is incubated with an actively growing culture of the host in broth, so that any phage able to infect that host replicates; bacteria and debris are then removed by centrifugation and filtration, and the filtered lysate is tested for phage activity (Van Twest and Kropinski, 2009). Host cultures are used in the exponential phase, when phages replicate most efficiently, and divalent cations such as Ca^{2+} and Mg^{2+} are commonly added because many phages need them for efficient adsorption (Adams, 1959).

Detection and counting are usually based on the double agar overlay technique, in which host bacteria are mixed with molten soft agar and poured onto a hard agar base to form a uniform bacterial lawn. Each infective phage particle that reaches the lawn starts repeated

cycles of infection and lysis that produce a visible clear area, a plaque (Kropinski et al., 2009). In a spot assay, a small volume of lysate is placed on the lawn, and a clear zone after overnight incubation shows that the lysate contains something that lyses the host. A clearing, however, does not prove that the phages are replicating. When many phages adsorb to the same cell, the cell envelope can be damaged enough for the cell to lyse without any phage replication, a phenomenon called lysis from without (Abedon, 2011). Bacteriocins and other lytic agents carried over in the lysate, or abortive infection systems that kill the infected cell before progeny are released (Labrie et al., 2010), can also produce clear or turbid zones. For this reason, spot tests tend to overestimate the number of susceptible hosts compared with plaque-based methods (Khan Mirzaei and Nilsson, 2015). Productive infection is demonstrated when individual plaques appear at high dilutions, where each plaque originates from a single phage particle.

Titration measures the concentration of infective phages in plaque-forming units per millilitre (PFU/mL). Tenfold serial dilutions of the lysate are plated with the host, either mixed into the soft agar or spotted onto a pre-formed lawn, and plaques are counted at a dilution where they are well separated. The titre is the number of plaques divided by the volume plated (in mL) and multiplied by the dilution factor; whole-plate counts of about 30–300 plaques give the most reliable estimates (Kropinski et al., 2009). Spotting small volumes of each dilution onto a single gridded plate saves material and time, but gives lower plaque counts and therefore a less precise titre.

1.6 Phage host range

The host range of a phage is the set of bacteria it can infect. It is determined first by adsorption, because the phage must recognise a specific receptor on the cell surface, and then by intracellular factors such as restriction-modification, CRISPR-Cas and abortive infection systems (Hyman and Abedon, 2010; Labrie et al., 2010). Host range is usually described at the level of strains or species, although phages with broader host ranges may be more common than is generally assumed, depending on how they are isolated (Ross et al., 2016). Because a clearing in a spot test does not prove productive infection, host range is better confirmed by the efficiency of plating (EOP), the ratio between the titre of the phage on the test host and its titre on the reference host (Kutter, 2009; Khan Mirzaei and Nilsson, 2015).

1.7 The model system *Staphylococcus aureus* and P15S6

Staphylococcus aureus is a major human pathogen and a leading cause of bacteraemia, infective endocarditis and skin, soft-tissue, bone and joint infections, and Methicillin-resistant strains (MRSA) are a prominent example of antimicrobial resistance (Tong et al., 2015). Staphylococci are not considered members of the normal oral microbiota, but *S. aureus* can be recovered from the oral cavity, particularly in older and medically compromised people and in some oral lesions (Smith et al., 2001). All known *S. aureus* phages are tailed dsDNA phages. Most have siphovirus morphology and are temperate, whereas the strictly lytic phages include podovirus and the large myoviruses of the Twort-like group (Xia and Wolz, 2014). Twort-like phages such as phage K are obligately lytic and polyvalent, infecting most clinical *Staphylococcus* strains, which has made them leading candidates for phage therapy (O'Flaherty et al., 2005; Łobocka et al., 2012). Staphylococcal siphoviruses and myoviruses adsorb to wall teichoic acid, a cell wall polymer of *S. aureus* (Xia et al., 2011); because adsorption depends on such staphylococcal surface structures, productive infection of bacteria outside the genus *Staphylococcus* would be unusual (Hyman and Abedon, 2010).

The phage used in this work, P15S6, is a reference lytic *S. aureus* phage with a known titre on its *S. aureus* host strain. Because both the phage and its host are known, this system works as a positive control in which each step of the workflow can be validated before it is applied to unknown samples. Testing the same phage on *S. salivarius* provides a first contact with an oral streptococcal host and, given the expected specificity of staphylococcal phages, also shows how the workflow distinguishes productive infection (individual plaques at high dilutions) from lysis without phage replication.

2. Objectives

This project aimed to set up a reproducible laboratory workflow for phage enrichment, detection and titration that can serve as a starting point for phage hunting against oral bacteria. The specific aims were:

1- To set up and test an enrichment and titration workflow using P15S6 as reference phage and *S. aureus* as a model host;

2- to test the activity of the same phage against the oral commensal *S. salivarius*;

3- to define the steps needed to apply the workflow to oral samples and oral bacterial hosts (Chapter 5).

3. Materials and methods

3.1 Bacterial strains, growth conditions and phage

Staphylococcus aureus ATCC 890, and *Streptococcus salivarius* were streaked from glycerol stocks onto tryptic soy agar (TSA) plates and incubated overnight at 37 °C in a static incubator. The next day, a single colony from each plate was inoculated with a 10 µL loop into 2 mL of tryptic soy broth (TSB) in labelled tubes and incubated overnight at 37 °C with shaking at 180 rpm. The Phage used was P15S6, a reference phage which had a titre of 2.2×10^9 PFU/mL.

3.2 Enrichment

The next day, the OD_{600} of each culture was measured, and the cultures were diluted to an OD_{600} of 0.1 in 5 mL of TSB and incubated again with shaking until an OD_{600} above 0.4 was reached (exponential phase). At that point, 1 mL of each culture was mixed with 1 mL of phage suspension in separate labelled tubes and 2 mL of TSB were added to each tube along with $CaCl_2$ to a final concentration of 5 mM from a 1 M $CaCl_2$ stock. The MOI for the phages and the bacteria was not calculated in order to simulate an enrichment. The tubes were mixed and incubated for 4-6 h at 37 °C with shaking.

The samples were then centrifuged at 5000 × g for 10 min, and the supernatant was transferred to a new tube and passed through a 0.2 µm syringe filter, using a 1 mL syringe, into a sterile 13 mL tube to remove any residual bacteria. The filtered lysate was stored at 4 °C until use.

3.3 Phage titration

S. aureus and *S. salivarius* were each grown in 5 mL of TSB from an OD_{600} of 0.1 with shaking, and growth was monitored with a spectrophotometer until OD_{600} was above 0.4.

In the meantime, a gridded plate was prepared for each strain with 15 mL of TSA and left to dry. When each culture reached an OD_{600} above 0.4, 100 µL of it was mixed with 10 mL of melted soft agar, poured onto the corresponding gridded plate and left to solidify.

A tenfold serial dilution series (10^{-1} to 10^{-7}) of the phage lysate was prepared in seven 1.5 mL tubes, each containing 900 µL of SM buffer: 100 µL of phage suspension was added to the first tube and mixed by pipetting, then 100 µL of this 1:10 dilution was transferred to the second tube, and so on, using a fresh tip at each step. The squares of the gridded plate

were numbered from 1 to 9 and 10 µL of each dilution was spotted onto the corresponding square (tube 1 onto square 1, tube 2 onto square 2, and so on). Square 8 was used as a positive control with undiluted phage and square 9 as a negative control with SM buffer only. The spots were left to dry and the plate was incubated upside down overnight at 37 °C in a static incubator.

3.4 Titre calculation

The titre was calculated from the highest dilution giving countable (visible), individual plaques, as: titre (PFU/mL) = (number of plaques / volume spotted in mL) x dilution factor, where the dilution factor is the reciprocal of the dilution.

4. Results

The workflow was first evaluated with the reference phage P15S6 on its model host, *S. aureus* ATCC 890, and the same enriched lysate was then assayed against the oral commensal *S. salivarius*. For both hosts, 10 µL of each tenfold serial dilution of the filtered lysate (10^{-1} to 10^{-7}) was spotted onto a gridded double-agar-layer plate, with undiluted lysate (square 8) and SM buffer (square 9) serving as positive and negative controls, respectively. The outcome of each spot on both hosts is summarised in Table 1.

4.1 Titration of the phage lysate on *S. aureus*

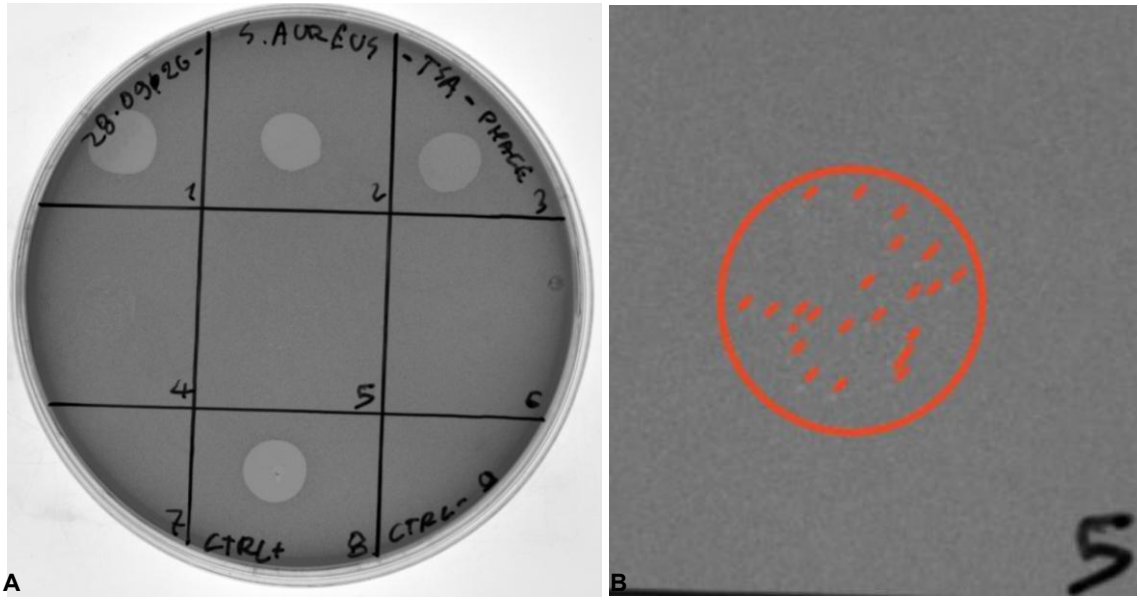

Figure 1: Titration of the enriched P15S6 lysate on *S. aureus* ATCC 890. (**A**) Gridded double-agar-layer plate on which 10 µL of each tenfold serial dilution of the lysate was spotted: squares 1–7, 10^{-1} to 10^{-7}; square 8, undiluted lysate (positive control); square 9, SM buffer (negative control). (**B**) Magnified view of square 5 (10^{-5} dilution); the 23 individual plaques counted are marked in red.

Titration on *S. aureus* produced a dilution-dependent pattern of lysis (Figure 1A). The 10^{-1} to 10^{-3} dilutions (squares 1–3) caused confluent clearing of the bacterial lawn, whereas the 10^{-4} dilution (square 4) produced a turbid lysis zone containing plaques too numerous to count (TNTC). At the 10^{-5} dilution (square 5), lysis resolved into 23 discrete plaques (Figure 1B), and no plaques were detected at the 10^{-6} and 10^{-7} dilutions (squares 6 and 7). The undiluted lysate produced a clear lysis zone, whereas SM buffer alone left the lawn intact, indicating that clearing was attributable to the lysate rather than to the diluent or the plating procedure.

As the 10^{-5} dilution was the highest dilution yielding individual, countable plaques, it was used to calculate the titre as described in Section 3.4 (23 plaques; spotted volume, 10 µL; dilution factor, 10^5):

$$\text{Titre} = (23 / 0.01\ \text{mL}) \times 10^5 = 2.3 \times 10^8\ \text{PFU/mL}$$

The enriched lysate therefore had a titre of 2.3×10^8 PFU/mL on *S. aureus*. This value is derived from a single spot in a single titration and was not replicated.

4.2 Titration of the phage lysate on *S. salivarius*

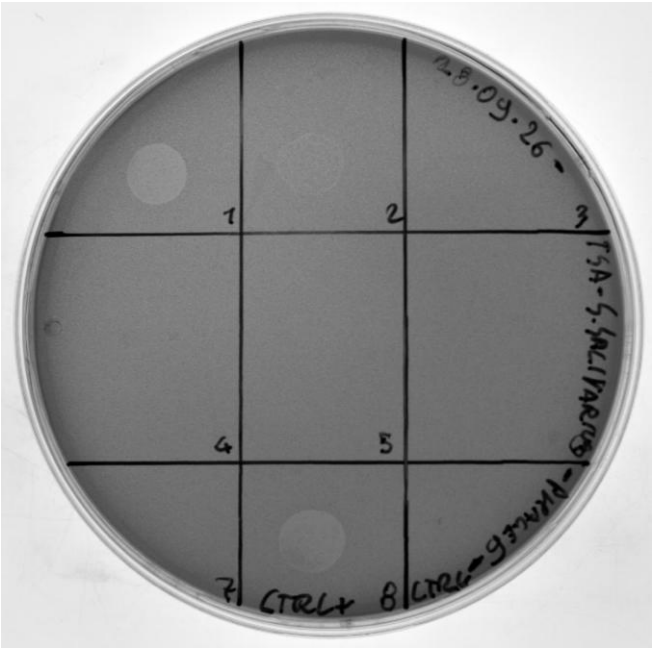

Figure 2: Titration of the enriched P15S6 lysate on *S. salivarius*. 10 µL of each tenfold serial dilution was spotted onto squares 1–7 (10^{-1} to 10^{-7}); square 8, undiluted lysate (positive control); square 9, SM buffer (negative control).

When the same lysate was titrated on *S. salivarius*, lysis was confined to the two lowest dilutions (Figure 2). The 10^{-1} dilution (square 1) caused confluent clearing, and the 10^{-2} dilution (square 2) produced a turbid lysis zone in which plaques were too numerous to count. No plaques were detected at the 10^{-3} to 10^{-7} dilutions (squares 3–7). As on *S. aureus*, the undiluted lysate produced a clear lysis zone and SM buffer caused no lysis.

The lytic endpoint on *S. salivarius* was therefore reached three tenfold dilutions earlier than on *S. aureus* (highest dilution showing lysis, 10^{-2} versus 10^{-5}). Because no dilution yielded discrete, countable plaques, the titre of the lysate on *S. salivarius* could not be determined (Table 1).

Table 1: Outcome of the spot titration of the enriched P15S6 lysate on *S. aureus* and *S. salivarius*. Each spot corresponds to 10 µL of the indicated sample. TNTC, too numerous to count.

Square	Sample spotted	*S. aureus*	*S. salivarius*
1	10^{-1}	Confluent lysis	Confluent lysis
2	10^{-2}	Confluent lysis	Turbid lysis, TNTC
3	10^{-3}	Confluent lysis	No plaques
4	10^{-4}	Turbid lysis, TNTC	No plaques
5	10^{-5}	23 plaques	No plaques
6	10^{-6}	No plaques	No plaques
7	10^{-7}	No plaques	No plaques
8	Undiluted lysate (positive control)	Clear lysis	Clear lysis
9	SM buffer (negative control)	No lysis	No lysis
Titre (PFU/mL)		**2.3×10^{8}**	**Not determined**

5. Discussion

The aim of this work was to set up a reproducible workflow for phage enrichment, detection and titration. With *S. aureus* as the host, each step worked as expected: in the titration of the enriched and filtered lysate, lysis decreased with dilution, giving individual plaques at 10^{-5}, no plaques at higher dilutions and no lysis in negative control. The individual plaques confirm that the lysis was caused by a replicating phage and not by lysis from without or by other lytic agents in the lysate.

On *S. salivarius, lysis was seen up to the* 10^{-2} *dilution, but the plaques were too many to count, so no titre could be determined.* Without individual plaques at higher dilutions, lysis from without or other lytic agents in the lysate cannot be excluded. This makes the results for *S. salivarius* inconclusive.

This work has several limitations. First, a known reference phage was used, so the workflow was tested on amplification rather than on the isolation of a new phage from an environmental sample. Second, 23 plaques on a single spot are a small number for an accurate titration, at this count, Poisson counting error alone is ±20%. Titrating in triplicate, or plating whole dilutions in soft agar to obtain 30-300 plaques per plate, would give a more precise estimate. Third, further tests have to be made to assess that *S. salivarius* was not contaminated with *S. aureus*.

The next step is to apply the workflow to oral samples such as saliva, dental plaque or dental wastewater, which has recently been described as a reservoir of oral phage diversity (Roush and Whiteley, 2026), using oral bacteria such as *S. mutans* or *S. salivarius* as hosts. To obtain new phages, the workflow will also need plaque purification (at least three rounds of single-plaque isolation), amplification to high-titre stocks and characterisation, including host range, efficacy of plating, transmission electron microscopy and genome sequencing to confirm that the phages are strictly lytic and carry no virulence or resistance genes.

6. Conclusions

An enrichment and titration workflow was set up and tested with a reference phage on *S. aureus* and *S. salivarius*. A clear lytic response, individual plaques and a titre of 2.3×10^8 PFU/mL were obtained on *S. aureus*, with the controls behaving as expected (clear lysis on square 8: Positive control, and no lysis on square 9: negative control). On *S. salivarius*, lysis

was observed only at low dilutions and no plaques were resolved; whether this reflects productive infection remains to be determined.

Although the results on *S. salivarius* were inconclusive, the steps taken can be applied to the oral microbiome for enrichment and titre assay.

Once replicate titrations, plaque purification and phage characterisation are added, the workflow provides a starting point for isolating phages against oral bacteria.

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