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A New High-Throughput Screening Method for Phages: Enabling Crude Isolation and Fast Identification of Diverse Phages with Therapeutic Potential

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Abstract

Bacteriophage therapy and application of phages for biocontrol necessitate acquisition of suitable phages. The exclusivity of phage–host relations and the risk of phage resistance instigate a need to rapidly isolate and characterize novel phages and continually build sizeable phage libraries. Current methods for phage isolation are both laborious and time consuming, suitable for the isolation of a limited number of phages. The *high-throughput screening* method for phages upscales and organizes enrichment of phages for fast isolation and identification of potentially hundreds of distinct phages against single hosts. This enables screening of hundreds of samples, in multiple simultaneous setups with varying parameters, increasing the likelihood of isolating multiple distinct phages specific for the given conditions. The efficiency of the method is emphasized by our screening of 200 environmental samples, resulting in the identification of an abundance of unique phage species virulent to *Escherichia coli*, *Salmonella enterica*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*.

Keywords: bacteriophage, phage isolation, phage therapy, high-throughput, wastewater

Introduction

The upsurge of antibiotic resistant bacteria is one of the main health concerns of our time.[1] Pathogenic bacterial infections are becoming ever more difficult to treat, and even last resort antibiotics such as the glycopeptide antibiotics vancomycin and teicoplanin are falling short as efficient antimicrobial agents.[2] Bacteria are consecutively acquiring antibiotic resistance and develop multidrug resistance,[3] which necessitates the development of alternative antimicrobials or means to increase the efficiency of existing antibiotics.

Phage therapy (PT) is the therapeutic use of the viral antagonists of bacteria, the bacteriophages (phages), to treat bacterial infections in humans or animals. Most bacteriophages have narrow host ranges, limiting their infectivity to specific species or even strains. Consequently, PT does not instigate drastic perturbations of natural microbiota such as traditional antibiotic treatments.[4] Although studies have been limited, PT has not been shown to have any adverse side effects.[5] Moreover, PT has shown potential as a last resort treatment of multiresistant bacterial infections, when traditional antibiotics fall short.[6–8] Hence, PT is, especially when applied as a combination therapy together with conventional antimicrobials, foreseen to play an essential role in the multifaceted strategy required to combat the lurking antibiotic crisis.[1,9] Furthermore, the use of phages for biocontrol in plant production and food processing has displayed a promising potential,[10] and could be a sustainable alternative to traditional chemicals facing restrictions due to concerns for public health and the spread of resistance.[11]

Yet, a successful biocontrol or PT venture requires phages with different modes of action, and lots of them. Infection-specific phages and prepared phage cocktails are rarely generalizable.[12] Clinical infections can be unparalleled and call for *de novo* isolation or genetic engineering, as was the recent case with a 15-year-old patient with cystic fibrosis caused by *Mycobacterium abscessus*.[6] More than 10,000 phages infecting *Mycobacterium smegmatis* were screened in addition to 100 environmental samples, resulting in only 3 suitable phages, 2 of them requiring genetic engineering.[6] Indeed, one of the greater hurdles for effective PT is the availability of suitable phages.[12]

Methodologies for isolation of phages have not changed much since phages were discovered >100 years ago. The procedures are laborious and time consuming. In general, phages are isolated either by direct plating or by enrichment and then subsequent purification. Enrichment entails an introduction of a host to a phage-containing sample, which

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is afterward removed by centrifugation and filtration when phages have been amplified. Direct plating and purification are typically performed with the soft-agar overlay technique, first described by Gratia.[13]

Improvements to increase throughputs have been proposed, such as tube-free agar overlays,[14] and phage activity can now be measured by means more suitable for automation, such as colorimetric methods.[15] However, no truly high-throughput isolation method has, to our knowledge, been offered. A citizen science approach, like the great effort performed by The Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science, has resulted in the isolation of thousands of phages against *M. smegmatis*.[16] But this type of approach requires both substantial funding and facilities.

To establish and expand libraries of phages relevant for PT and biocontrol, affordable, fast, and efficient screening methods are needed to enable rapid isolation and identification of candidate phages. Large libraries of phages infecting the same single host also enable important phage–host interaction studies, expanding our understanding of phage taxonomy and ecology.

In this study, we present the *high-throughput screening* (HiTS) method for phages, an organization and upscaling of traditional enrichment and soft-agar overlay methodologies that enable a single person to go from a high number of samples to a plethora of identified phages within weeks. The focus is on easily identified hits (phage lysis plagues) from a larger number of samples as opposed to retrieving diverse phages from single samples. The simplicity of this approach enables >500 samples to be handled simultaneously. The HiTS method selects for predominantly lytic and easily culturable phages. The resolution is a single or a few phages from each sample processed. The integrated sequencing of the identified phages allows for an early assessment of genomes enabling the selection of candidates that do not possess any unwanted genetic traits and are thus suitable for further characterization and potential application as PT or biocontrol agents.

Materials and Methods

The method presented is host system independent and can thus be applied for screening of environmental samples for phages virulent to any culturable fast-growing aerobic or facultative anaerobic bacteria by adjusting host medium, incubation temperature, and time. The protocol enables a simple and fast (4 consecutive days), yet crude, purification of single or a low number of distinctive phages from a small sample volume (SV) (0.5–1.5 mL). The method allows for a high number of samples to be handled, with simultaneous investigation of diverse sample matrices or parallel screenings of the same sample set with varying parameters, for example, host, pH, medium, amendments, and incubation conditions (Fig. 1). This increases the likelihood of sequestering multiple distinctive phages from each sample. The method is suitable for both direct plaque sequencing (DPS)[17] and standard phage DNA extraction from lysate. The screening procedure entails four steps: (1) *phage amplification*, (2) *liquid purification*, (3) *spot test*, and (4) *phage collection and DPS or optional: plating of dilution series*.

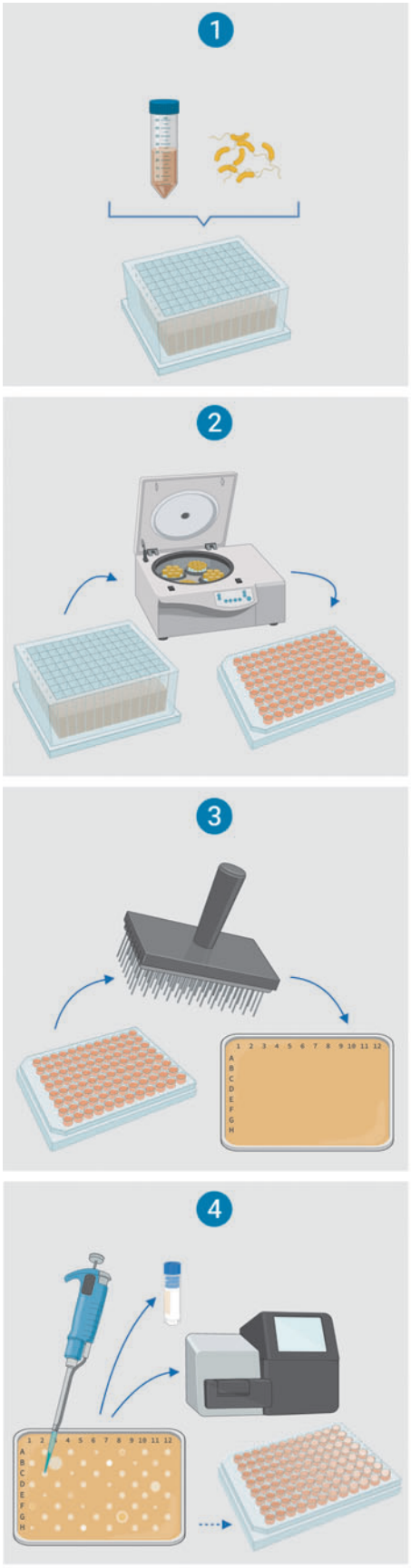

FIG. 1. The four steps in the HiTS method. Illustration created with BioRender. HiTS, *high-throughput screening*.

Protocol

HiTS method for phages. The method, which is scalable for robotics, requires a centrifuge suitable for 96-well plates and a 96-pin replicator or a 1 μL multichannel pipette. Multichannel pipettes or a pipetting robot may ease many of the steps involved. Dry samples should be suspended before processing and preferably centrifuged and filtered. The maximum number of samples per plate is 94. The sample volume can be adjusted as desired and as applicable to available well plates. By increasing the volume of raw sample input, the number of phages per incubation is also increased and thus the chance of isolating these. Initial sample volume only affects step 1: *phage amplification*. Volumes and concentrations suggested in step 0 and step 1 are suitable for screening 1.5 mL raw sample in deep-well plates with a working volume (wV) of 2.2 mL (e.g., 732-0612; VWR, Radnor, PA). All incubations should be performed under optimal host conditions (medium and temperature) and hence adjusted as required. In step 4, there is the option to either collect the phages and sequence them by DPS or to do titers and aim for single plaques, and then do DPS or sequence phage amplifications.

0. Preparation

   a. Prepare all media, solutions, and agar plates.
   b. Inoculate host cells in 2×10 mL liquid medium and incubate overnight (ON).

1. Phage amplification (day 1)

   a. Distribute a maximum of 94 samples ($\leq$1.5 mL) in a deep-well plate (no. 1) with pierceable sealing tape (e.g., Z722529-50EA; Excel Scientific, Victorville, CA). Sterilized water ($\leq$1.5 mL) is added as negative amplification controls to wells D6 and E6. If a sample volume <1.5 mL is used, then adjust volume and concentration of host medium accordingly. To each of the 96 wells add: 90 μL $CaCl_2$ (0.25 M) and $MgCl_2$ (0.25 M), final concentration 10 mM. 110 μL ON host culture, final concentration 5% v/v. 500 μL host medium (concentration $\times$ 4.4), final concentration $\times$ 1. During addition of medium, carefully pipette up and down a few times to mix. Close the well plate and incubate ON on a shaker (200 rpm).
   b. Inoculate ON host culture in 10 mL liquid medium for next day.

2. Liquid purification (day 2)

   a. Filter to remove host bacteria by transferring 200 μL (punch through pierceable tape) from each well to a 96-well filter plate (0.45 μm) (e.g., MSHAS4510; Merck Millipore, Burlington, MA), pipette up and down a few times before extracting. Centrifuge filter plate on top of a new well plate (no. 2, wV 200 μL, e.g., 269787; Nunc, Roskilde, DK) at $900\times g$ for 2 min. Then add pierceable sealing tape to well plate no. 2. Discard the filter plate.
   b. Prepare a third well plate (no. 3, wV 200 μL) with pierceable sealing tape and add: 180 μL host medium (concentration $\times$ 1). 10 μL of host culture, final concentration 5% v/v. 10 μL 0.2 M $CaCl_2$ and 0.2 M $MgCl_2$, final concentration 10 mM.

   Use the 96-pin replicator to transfer $\sim$1 μL of each lysate (punch through pierceable tape) in well plate no. 2 to each well in well plate no. 3. Close well plate no. 3 and incubate ON on a shaker (200 rpm). If processing more than one set of samples, clean the 96-pin replicator using ethanol and flame three times in between and make sure to cool it down before reuse.
   c. Inoculate ON host culture in 10 mL liquid medium for next day.

3. Spot test (day 3)

   a. Filter to remove host bacteria as described in step 2a.
   b. Prepare two large (Ø14 cm, e.g., 82.1184.500; Sarstedt, Nürnbrecht DE or at least 12×8 cm, e.g., 242811; Nunc) soft-agar overlay plates (A and B) of 0.5% agarose amended with: $CaCl_2$ and $MgCl_2$ (final concentration 10 mM). Host culture (final concentration 2.5–5%).
   c. While the plates solidify, remove every second row of pipette tips in a box of 200 μL pipette tips to facilitate the transfer of lysate from every second well in a chequered pattern into two new microtiter plates with pierceable sealing tape, number no. 4 (A) and no. 5 (B) (Supplementary Fig. S1).
   d. Use the 96-pin replicator to carefully transfer $\sim$1 μL of lysates from well plate A (no. 5) to the soft-agar overlay plate A. Make sure to specify direction on the plate. The chequered pattern ensures a safe distance between spotted samples, a negative amplification control (D6 or E6) on each plate and sterilization controls (every second tip) (Fig. 1). Clean the 96-pin replicator by ethanol and flame and repeat the procedure with well plate B (no. 5) and soft-agar overlay plate B. Incubate soft-agar overlay plates upside down ON. Seal well plates A (no. 4) and B (no. 5) and store at 4°C.
   e. Optional: Inoculate ON host culture in 10 mL liquid medium for titer estimation next day.

4. Phage collection and DPS, or optional: plate dilution series (day 4)

   a. The center of clearing zones (agar-plates A and B) is collected with a pipette tip for DPS. Additional clearing zone is dissolved in 100 μL SM-buffer,[18] filtered (0.22–0.45 μm), and stored for future purification and characterization. If clearing zones are too small for double collection, make amplifications of the phage-SM solutions (inoculate host bacteria in 10 mL medium, after $\sim$1 h add lysate, next day centrifuge and filtrate) and extract DNA for sequencing from these.
   b. Optional: titer the lysates by transferring phage solutions from positive (plaque-forming) wells to new wells in column 1 of as many new well plates (no. 6+, wV 200 μL) as required. Eightfold dilutions series are made within the well plates by adding 180 μL SM-buffer to all wells in columns 2–9 and then transferring 20 μL of the solution in column 1 to column 2, pipetting up and down to mix and repeating the procedure for the remaining columns. Spot ($\sim$1 μL) the dilution series on soft-agar overlay plates with a 96-pin replicator or multichannel pipette and incubate ON. Next day, count plaques or clearing zones for approximate titer. Do DPS of single plaques if present

and also collect clearing zone for phage storage. If single plaques are not present plate lysate dilution giving rise to 10–50 plaques on a full plate by the soft-agar overlay method (add the lysate to 4 mL of 0.5% agarose with 10 mM $CaCl_2$ and $MgCl_2$ and 2.5–5% (v/v) ON host culture, pour on standard petri dish with agar). Next day, pick diverting plaque morphologies for DPS or phage amplification, lysate hereof can be used for DNA extraction and phage storage.

Phage screenings

Five screenings were performed as described in *2.1 Protocol*, using *Escherichia coli*, *Salmonella enterica*, *Enterococcus faecalis*, or *Pseudomonas aeruginosa* as hosts (Table 1). For the *E. coli*, *E. faecalis*, and *S. enterica* screenings, 188 distinct wastewater samples divided into 2 sets of 94 samples were used, for the *P. aeruginosa* screening, 82 wastewater samples were used together with 8 soil samples and 4 organic waste samples (Supplementary Table S1). The *S. enterica* screenings were performed with both a SV of 0.5 mL and a large sample volume (LV) of 1.5 mL, whereas the *E. coli* and *E. faecalis* screenings were only performed with 0.5 mL (SV) and the *P. aeruginosa* screening only with 1.5 mL (LV). The SV screenings (*E. coli*, *E. faecalis*, and *S. enterica*) followed the protocol, with the exception that instead of DPS, lysates from step 3 corresponding to positive wells (those instigating clearing zones) were used for DNA extraction and sequencing, whereas phages were stored by collecting top agar of clearing zones or plaques. In step 4 of the LV screenings (*S. enterica* and *P. aeruginosa*), lysates from positive wells were titrated in the 96-well format and the most diluted lysates instigating single plaques or clearing zones were used for making 10 mL amplification lysates for DNA extraction, library preparation, sequencing, and phage storage. All incubations were performed at 37°C.

Bacteria and growth medium. The host bacteria used for phage screenings were *E. coli* (K-12, MG1655), *S. enterica* subsp. *enterica* serovar Enteritidis PT1, the vancomycin-resistant *E. faecalis* (strain ATCC 700802/V583), and the chloramphenicol-resistant *P. aeruginosa* (PAO1). The medium applied was LB (Alpha Biosciences, Baltimore, MD).

Samples. The 188 inlet wastewater samples (40–50 mL) were collected in time series of 2–4 days during July and August 2017, from 48 Danish wastewater treatment facilities geographically distributed in both rural and urban areas in Zealand, Funen and in Jutland. Upon receipt, the samples were centrifuged ($9000 \times g$, 4°C, 10 min), the supernatant filtered (0.45 μm), and then stored in aliquots at −20°C. The organic waste samples were collected from four different Danish facilities in February, May, and November 2017. The 12 soil samples ($\sim$5 g) were collected in Roskilde municipality, Denmark, in March 2019. Before screening, the soil was suspended in 5 mL LB and slowly and continuously inverted for 1 h at room temperature, then the samples were centrifuged ($9000 \times g$, 5 min) and the supernatant was filtrated (0.45 μm). Refer to Supplementary Table S1 for a list of all samples and facilities.

DNA extraction, library preparation, and sequencing. Phage DNA extractions were performed by an initial DNase treatment, 1 U of DNase 1 (New England Biolabs, Ipswich, MA) per $\sim$100 μL lysate (37°C, 30 min, inactivated by 10 μL of 50 mM ethylenediamine tetra-acetic acid), followed by addition of 3 U Proteinase K (A&A Biotechnology, Gdynia, Poland) and 10% (v/v) sodium dodecyl sulfate solution (55°C, 30 min, inactivated by 70°C, 10 min). The extracted DNA was then purified in the well plate format using the ZR-96 Clean and Concentrator Kit (Zymo Research, Irvine, CA), following manufacturer's instructions and eluting in 6 μL of the supplied elution buffer. Sequencing libraries were built according to manufacturer's instructions with minor modifications as described in Kot et al.[17] using the Nextera® XT DNA Kit (Illumina, San Diego, CA), the libraries were sequenced as paired-end reads on Illumina NextSeq platform using the Mid Output Kit v2 (300 cycles).

Assembly, annotation, identification, and phylogenomic analysis. The obtained reads were trimmed and assembled in CLC Genomics Workbench 10.1.1. (CLC BIO, Denmark); overlapping reads were merged with the following settings: mismatch cost, 2; minimum score, 15; gap cost, 3; and maximum unaligned end mismatches, 0; and then assembled *de novo*. Additional assemblies were constructed using SPAdes 3.12.0 (Ref.[19]). Gene prediction and annotation were

Table 1. Results from the Five High-Throughput Screenings with *Salmonella enterica*, *Escherichia coli*, *Enterococcus faecalis*, or *Pseudomonas aeruginosa* as Host

Host	*Sample (mL)*	*Samples* (n)	*Clearing zones* (n)	*Sequenced lysates*[a] (n)	*Phages* (n)	*Unique species*[b] (n)	*Novel species*[c] (n)
S. enteric	0.5 SV	188	51	42	47	33	28
S. enterica	1.5 LV	188	74	60	76	45 (26[d])	38 (24[d])
E. coli	0.5 SV	188	153	94	136	104	91
E. faecalis	0.5 SV	188	5	4	4	4	3
P. aeruginosa	1.5 LV	94[e]	48	38	46	22	8

Escherichia phage data from Olsen et al.[39]

[a]Includes all lysates for which sequencing yielded reads assembling to contigs with a coverage more than $\times$20.

[b]Phages with $\leq$95% nucleotide similarity to the other phages in this data set.

[c]Phages with $\leq$95% similarity to the other phages in this data set and those deposited in the National Center for Biotechnology Information database.

[d]Excluding the phages with >95% nucleotide similarity to phages in the *S. enterica* SV screening.

[e]Eighty-two wastewater samples, eight soil samples, and four organic waste samples.

LV, large sample volume; SV, small sample volume.

performed using a customized RASTtk version 2.0 (Ref.[20]) workflow with GeneMark,[21] with manual curation and verification using BLASTP,[22] HHpred,[23] and Pfam version 32.0 (Ref.[24]), or *de novo* annotated using VIGA version 0.11.0 (Ref.[25]) based on DIAMOND searches (RefSeq Viral protein database) and HMMer searches (pVOG HMM database). nucleotide (NT) similarity was determined as percentage query cover multiplied by percentage NT identity. Novel phages were categorized according to International Committee Taxonomy of Viruses (ICTV) taxonomy. The criterion of 95% DNA sequence similarity for demarcation of species was applied to identify novel species representatives and to determine species uniqueness within the data set. All unique phage genomes were deposited in GenBank (Table 1).

All genomes were assessed for antibiotic resistance genes (ARGs) and bacterial virulence genes using ResFinder 3.1 (Refs.[26,27]) and VirulenceFinder 2.0 (Ref.[28]). NT and amino acid (AA) similarities were calculated using tools recommended by the ICTV,[29] that is, BLAST[22] for identification of closest relatives (BLASTn when possible, discontinuous megaBLAST [word size 16] for larger genomes) and Gegenees version 2.2.1 (Ref.[30]) for assessing phylogenetic distances of multiple genomes, for both NTs (BLASTn algorithm) and AAs (tBLASTx algorithm), a fragment size of 200 bp and step size 100 bp were applied.

Evolutionary analyses for phylogenetic trees were conducted in MEGA7 version 2.1 (default settings).[31] These were based on the large terminase subunit *terL*, a gene commonly applied for phylogenetic analysis[32,33] and on the DNA encapsidation gene *gpA* for the <13 kb alleged *Podoviridae*. The NT sequences were aligned using MUSCLE[34] and the evolutionary history inferred by the maximum likelihood method based on the Tamura–Nei model.[35] The tree with the highest log likelihood is shown manually curated by adding color codes and identifiers in Inkscape version 0.92.2. The R package iNEXT[36,37] in R studio version 1.1.456 (Ref.[38]) was used for rarefaction analyses, species diversity ($q=0$, data type: incidence_raw), extraplation hereof made with estimateD, and estimation of sample coverage. Additional graphs were prepared in Excel version 16.31.

Results

Screening efficiency and resolution

Across all five screening between 3% ($n=5$ of 188) and 81% ($n=153$ of 188) of samples yielded clearing zones plausibly due to lysis by phages, the majority of these also gave rise to the identification of phages (Table 1). However, in some cases the DNA extraction was unsatisfactory or the sequencing failed. Between 61% (*E. coli* screening $n=94$ of 153) and 82% (*S. enterica* SV screening $n=42$ of 51) of clearing zones were successfully sequenced, that is, yielded reads assembling to phage contigs with an average coverage more than ×20 (Table 1). Regardless of host, a single phage was identified from the vast majority of sequenced samples (64–100% per screening), although in some instances 2 (0–

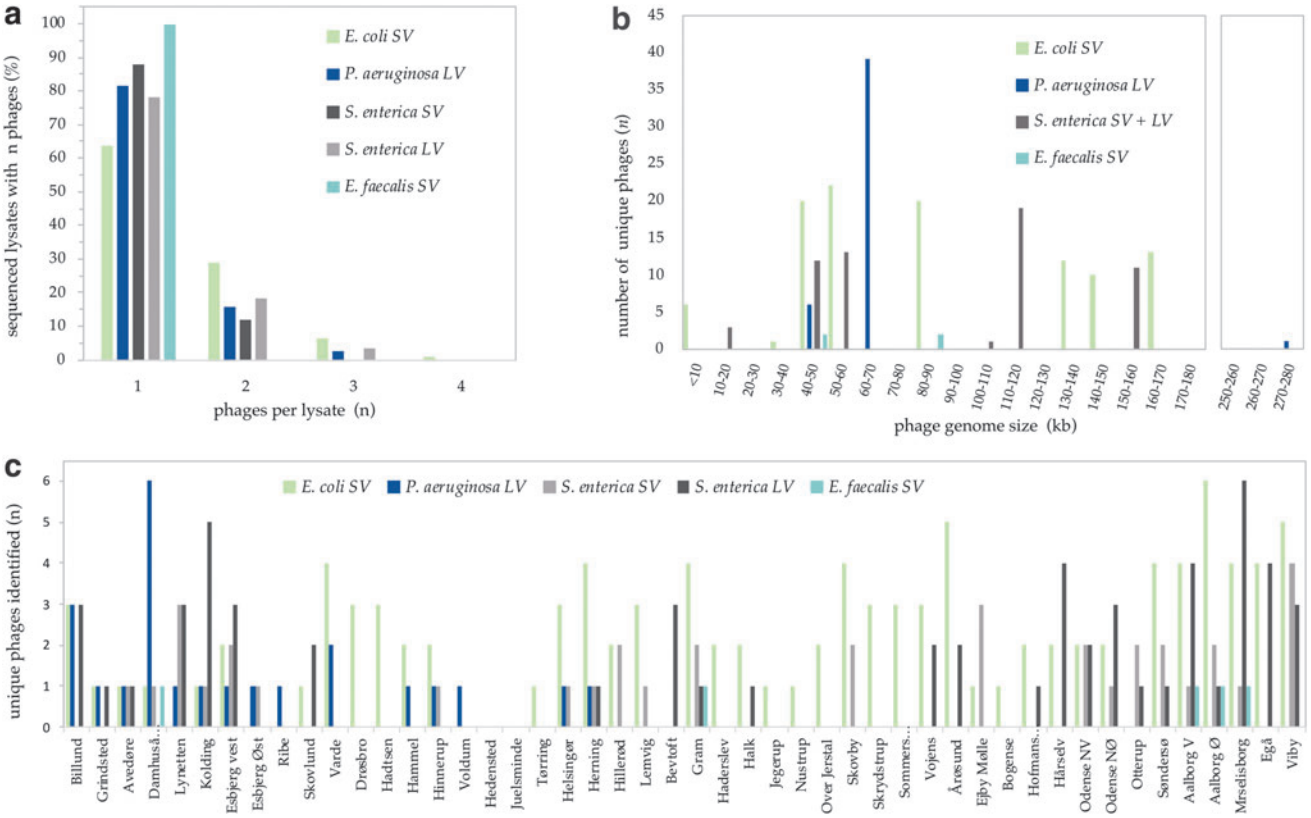

FIG. 2. **(a)** Number of phages per lysate (n) (x-axis) as occurring in percentage of all sequenced lysates (y-axis), presented according to individual screenings. **(b)** Average genome size (kb) of Gegenees-based clusters, of unique phage species (<95% nucleotide similarity to other phages in the data set) organized by host, from all screenings. **(c)** Distribution of all 309 phages identified organized per facility, only the first 21 facilities were included in the *Pseudomonas aeruginosa* screening. LV, large sample volume; SV, small sample volume.

29% per screening), 3 (0–6% per screening), or 4 (0–1% per screening) phages were identified from a single sample (Fig. 2a). The *Escherichia* phages were the most numerous (136 phages from 94 wells), they were more frequently (34 samples) isolated as >1 phage per sample and the only ones to be four phages in a sample[39] (Fig. 2a; Table 1). The number of phages per sample did not differ considerably between phages of *S. enterica* (123 phages from 102 wells) and *P. aeruginosa* (43 phages from 38 wastewater wells), whereas only 4 phages lytic to *E. faecalis* were identified in 4 separate samples (Fig. 2a). *Escherichia* phages were identified in samples from 43 different facilities out of the 48, *Enterococcus* phages in samples from 4 facilities, and *Salmonella* phages in samples from 22 of the 48 facilities included in these screenings. In the *P. aeruginosa* phage screening, phages were identified in wastewater samples from 95% of the 21 facilities included (20 out of 21). Furthermore, *P. aeruginosa* phages were identified in three of the four organic waste samples, but in none of the eight soil samples (Fig. 2c).

Of the 136 *Escherichia* phages, the majority (76%) represent unique species.[39] The many *Salmonella* and *P. aeruginosa* phages are more homogeneous. The two *S. enterica* phage screenings identified 123 phages. Out of 47 SV phages, 14 were shown to have >95% NT similarity with other SV phages, whereas 31 of the 76 LV phages were shown to have >95% NT similarity with other LV phages and an additional 19 of the LV phages had >95% NT similarity with SV phages. Hence, a total of 59 (48%) *Salmonella* phages of distinct species are identified. Similarly, of the 46 *P. aeruginosa* phages, 22 (48%) are unique, whereas all 4 *E. faecalis* phages represent distinctive species (Table 1).

Novelty and diversity of HiTS phages identified compared with National Center for Biotechnology Information database

The phages identified cover an impressive wide range of genome sizes (Figs. 2b and 4), guanine-cytosine (GC) contents, and predicted morphologies, representing nine different families: *Ackermannviridae*, *Autographviridae*, *Chaseviridae*, *Demerecviridae*, *Drexlerviridae*, *Myoviridae*, *Podoviridae*, and *Siphoviridae* of the order *Caudovirales* and also the *Microviridae* of the order *Petitvirales* (Table 2).[39] The numerous and diverse *Escherichia* phage genomes, of which only two are 100% identical, are described separately in

Table 2. Predicted Taxonomy of All Phages Identified in Screenings for *Escherichia*, *Salmonella*, *Pseudomonas aeruginosa*, and *Enterococcus* Phages, Based on Taxonomy of Closest Relative

Phage taxonomy	*Isolation hosts:*	E. coli	S. enterica	P. aeruginosa	E. faecalis
Caudovirales; *Ackermannviridae*; *Cvivirinae*; *Kuttervirus*		—	21	—	—
Caudovirales; *Autographviridae*; *Bonnellvirus*		10	—	—	—
Caudovirales; *Chaseviridae*		1	—	—	—
Caudovirales; *Demerecviridae*; *Markadamsvirinae*; *Eseptimavirus*		—	27	—	—
Caudovirales; *Demerecviridae*; *Markadamsvirinae*; *Tequintavirus*		—	1	—	—
Caudovirales; *Drexlerviridae*; *Tempevirinae*		1	—	—	—
Caudovirales; *Drexlerviridae*; *Tempevirinae*; *Hanrivervirus*		10	1	—	—
Caudovirales; *Drexlerviridae*; *Tempevirinae*; *Warvickvirus*		15	—	—	—
Caudovirales; *Myoviridae*		5	—	—	—
Caudovirales; *Myoviridae*; *Pbunavirus*		—	—	38	—
Caudovirales; *Myoviridae*; *Phapecoctavirus*		9	—	—	—
Caudovirales; *Myoviridae*; *Phikzvirus*		—	—	1	—
Caudovirales; *Myoviridae*; *Rosemountvirus*		—	18	—	—
Caudovirales; *Myoviridae*; *Ounavirinae*; *Felixounavirus*		33	—	—	—
Caudovirales; *Myoviridae*; *Ounavirinae*; *Suspvirus*		1	—	—	—
Caudovirales; *Myoviridae*; *Tevenvirinae*; *Dhakavirus*		3	—	—	—
Caudovirales; *Myoviridae*; *Tevenvirinae*; *Krischvirus*		2	—	—	—
Caudovirales; *Myoviridae*; *Tevenvirinae*; *Mosigvirus*		4	—	—	—
Caudovirales; *Myoviridae*; *Tevenvirinae*; *Tequatrovirus*		6	—	—	—
Caudovirales; *Myoviridae*; *Vequintavirnae*; *Vequintavirus*		15	—	—	—
Caudovirales; *Podoviridae*; *Bruynoghevirus*		—	—	6	—
Caudovirales; *Podoviridae*; *Murrayvirus*[a]		1	5	—	—
Caudovirales; *Podoviridae*; *Skarprettervirus*		1	—	—	—
Caudovirales; *Podoviridae*; *Sortsnevirus*		2	—	—	—
Caudovirales; *Podoviridae*; *Picovirinae*		—	3	—	—
Caudovirales; *Siphoviridae*		1	1	1	2
Caudovirales; *Siphoviridae*; *Efquatrovirus*		—	—	—	2
Caudovirales; *Siphoviridae*; *Dhillonvirus*		6	—	—	—
Caudovirales; *Siphoviridae*; *Seuratvirus*		1	—	—	—
Caudovirales; *Siphoviridae*; *Guernseyvirinae*; *Jerseyvirus*		1	46	—	—
Petitvirales; *Microviridae*; *Bullavirinae*; *Alphatrevirus*		1	—	—	—
Petitvirales; *Microviridae*; *Bullavirinae*; *Gequatrovirus*		7	—	—	—

Escherichia phage data from Olsen et al.[39]

[a]The new genus *Murrayvirus* is currently by mistake classified as of the *Siphoviridae* family, but a proposal has been made to move this genus to the *Podoviridae* family.

Olsen et al.[39] In summary, disregarding the jumbo phage Pseudomonas phage fnug (278.9 kb), the *Escherichia* phages cover the largest size range (5.3–170.8 kb) and have an impressive GC content span (35.3–60.0%).[39] Members of the families *Ackermannviridae* and *Demerecviridae* were only detected among the *Salmonella* phages, just as members of the *Microviridae*, a family of small single-stranded DNA phages, were only observed among the *Escherichia* phages.[39] The *Salmonella* and *P. aeruginosa* phages covered similar GC content spans of 36.9–56.5% and 39.0–59.5%, respectively. The *Salmonella* phage genomes vary in sizes from 11.6 kb (Salmonella phage astrithr) to 159.1 kb (Salmonella phage maane), whereas the nonjumbo *P. aeruginosa* phages are more uniform having genome sizes of 44.9 kb (Pseudomonas phage clash) to 66.5 kb (Pseudomonas phage shane). The *Enterococcus* phages have genomes of 39.7–85.7 kb, Enterococcus phage heks and nattely, respectively (Fig. 2b and Supplementary Tables S2–S4).

An impressive number ($n = 154$) of novel phage species have so far been identified with the HiTS method. Around 68% ($n = 92$) of the *Escherichia* phages,[39] 42% ($n = 52$) of the *Salmonella* phages, 22% ($n = 8$) of the *P. aeruginosa* phages, and 3 out of 4 *Enterococcus* phages represent novel phage species (Table 1; Fig. 3). Whereas most of the *Escherichia* (69%) and all of the *P. aeruginosa* phage species representatives have a high NT similarity (>89%) with their closest relatives, a larger proportion of the *Salmonella* phages differ more from their closest relatives as only 54% ($n = 42$) of the unique *Salmonella* phages species have >89% NT similarity to their closest relative (Fig. 3). Two of the *Escherichia* phages and three *Salmonella* phages share <50% NT similarity with published phages. Likewise, two of the *Enterococcus* phages (Supplementary Fig. S3 and Supplementary Table S3) and Salmonella phage Akira (63% NT similarity) are only distantly related to any published phage (62–65% NT similarity) (Fig. 3). The 189 unique phages (<95% NT similarity with other phages in the data set) and their GenBank accession numbers are listed in Supplementary Tables S2–S4 and Olsen et al.[39]

No virulence factors or ARGs were detected in any of the 189 unique phages. Furthermore, no integrases were identified and though putative recombinases do occur, it is uncertain whether they are involved in DNA repair or integration.

P. aeruginosa *and* Enterococcus *phages from Danish wastewater*

The *P. aeruginosa* phages group into two distinct clusters and two singletons (Supplementary Fig. S2). The vast majority ($n = 38$) of the *P. aeruginosa* phages are closely related (90.9–98.5% NT similarity) to phages of the genus *Pbunavirus* of the family *Myoviridae*, with genome sizes of 60.7–66.8 kb (89–95 coding sequences [CDSs], 54.8–55.7% GC, no transfer RNA [tRNAs]). Many of the *Pbunavirus* genomes are ≥99% identical including the 11 isolates of phage Debbie of which 9 are 100% identical and the 3 phage good old isolates also have 100% identical genomes. A smaller group of six *P. aeruginosa* phages (44.9–45.3 kb, 68–69 CDSs, 52.1–52.5% GC, 3–4 tRNAs) with two new species representatives are closely related (94.1–96.3% NT similarity) to phages of the genus *Bruynoghevirus* of the family *Podoviridae* (Supplementary Table S2). The jumbophage fnug is closely related (93% NT similarity) to phages of the genus *Phikvirus*, family *Myoviridae*. Although the last Pseudomonas phage Iggy (60.7 kb, 90 CDSs, 56.5% GC, no tRNAs) is closely related (94.6%) to the unclassified *Siphoviridae* Pseudomonas phage PBPA162 (MK816297), none of these share >8% NT similarity with any other published phages.

The *Enterococcus* phages are all predicted to have *Siphoviridae* morphology, but divide into two distinct clusters with NT inter-Gegenees scores of 0 (Supplementary Fig. S3). Phages heks and Nonaheksakonda (39.7–41.9 kb, 64–74 CDSs, 34.6–35.0% GC, no tRNAs) are related to efquatroviruses, but with only 59% NT similarity. The other two (85.3–85.7 kb, 131–134 CDSs, 30.2–30.3% GC, 1 tRNA) are more closely related (87–96% NT similarity) to unclassified *Siphoviridae* (Fig. 2b and Supplementary Tables S2–S4).

Salmonella *phages from Danish wastewater*

Based on NT similarity with closest relatives, 59 distinctive species of *Salmonella* phages were identified, of which 52 represent novel species. Estimations based on both the SV and LV screenings predict species richness of easily culturable phages lytic to *S. enterica* subsp. *enterica* serovar Enteritidis PT1 in Danish wastewater to be nearby 80 (Supplementary Fig. S4), whereas Shannon diversity estimates 68 and 61 and Simpson diversity 51 and 38 for the SV and LV screenings, respectively (Supplementary Fig. S4). The

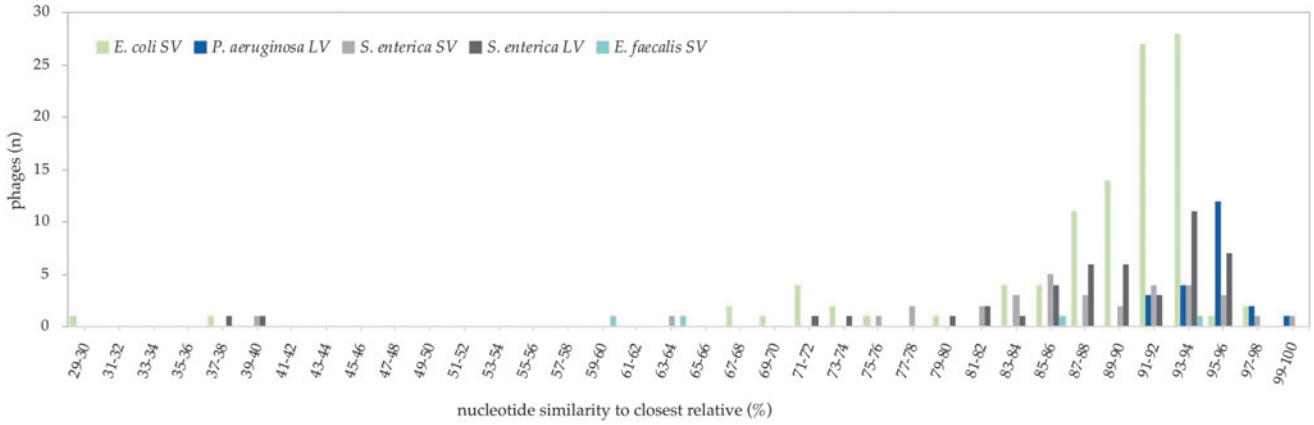

FIG. 3. Distribution of nucleotide similarity (%) to closest relative (Blast) of all phages from all five screenings.

estimates are, however, expected to be subject to large prediction bias due to the relatively small reference sample size, and a 95% confidence interval suggests a range for all diversity indices of 26–173. Sample completeness is estimated to be achieved at ~1300 samples for an SV screening and at ~800 samples for an LV screening (Supplementary Fig. S4). The HiTS *Salmonella* phages belong to at least six different families, *Ackermannviridae*, *Demerecviridae*, *Drexlerviridae*, *Myoviridae*, *Podoviridae*, and *Siphoviridae*, covering a wide range of genome sizes and GC contents (Supplementary Table S4). They group into five clusters and three singletons (inter-Gegenees scores = 0), corresponding to their proposed taxonomy, excluding phage Akira (Fig. 4). Despite an NT similarity of 63% and a Gegenees score of 38–39 with its closest relative, the unclassified *roufvirus* Salmonella virus KFS_SE2 (MK112901) Akira does not group with neither KFS_SE2 nor the type species of *roufvirus* Aeromonas phage pIS4-A (NC_042037) in the phylogenetic tree, resulting in a peculiar pattern in the Gegenees analysis (Fig. 4). Furthermore, Akira shares limited NT similarity (39%) with pIS4-A and a Gegenees score of only three to four (Fig. 4; Table 2).

All nine novel phages (143–159 kb, 187–209 CDSs, 44–45% GC, 3–4 tRNAs) of the *Ackermannviridae* are related (78–99% NT similarity) to phages of the genus *Kuttervirus*, subfamily *Cvivirinae*, defined by an average genome size of 158.1 kb, with an average GC content of 44.5% and averagely coding for 201 proteins and 4.3 tRNAs. These nine phages have an average NT intra-Gegenees score of just 60, but are more similar to one another and also to the type species of *Kuttervirus*, Escherichia virus CBA120 (JN593240) when comparing AAs (Gegenees score = 88–97).

The largest group of *Salmonella* phages is predicted to belong to *Siphoviridae* ($n = 47$, 11 species, 10 novel species). Apart from Akira, the *Siphoviridae* phages (41–44 kb, 56–70 CDSs, 50% GC, 0–1 tRNA) were found to be closely related to Jerseyviruses, subfamily *Guernseyvirinae*. This genus is defined by genome sizes of 40–44 kb, comparable morphology, and a shared DNA identity of $\geq 60\%$ and >68% protein content.[40] The wastewater Jerseyviruses-like phages are a heterogeneous group with varying NT intra-Gegenees scores of 38–91, yet the AA intra-Gegenees scores are all >69. However, the Gegenees NT scores between the novel phages and the type species Salmonella phage Jersey (NC_021777) are all <33, though the AA scores are 65–71. The novel Jerseyviruses-like phages are relatively abundant in the Danish wastewater samples and most of them were identified in several samples from different treatment facilities. Phages with >95% NT similarity with phage were found eight times (three with 100% NT similarity) in samples from five distinct facilities and phages with >95% NT similarity with phage demigod were found as many as 14 times (six with 100% NT similarity), in samples from seven different facilities (Supplementary Table S4).

The novel phage slyngel is related (92% NT similarity) to Escherichia phage vB_EcoS_G29-2 (MK373798) an unclassified *Hanrivervirus*, subfamily *Tempevirinae* of the order *Drexlerviridae*. Phage slyngel has 88% NT similarity with the type species Shigella phage pSf-1 (NC_021331), with which a more distant relationship is also suggested by the phylogenetic analysis (Fig. 4 and Supplementary Fig. S5).

The 18 unique *Demerecviridae* phages (105–115 kb, 151–171 CDSs, 39.3–40.1 GC, 23–31 tRNAs) are of the subfamily *Markadamsvirinae* and 17 of them are of the genus *Eseptimatrevirus*, whereas phage oldekolle that has low NT Gegenees scores with the rest ($n = 27$–32) is closely related to T5 (93% NT similarity) and is of the *Tequintavirus* genus. Even though *the Demerecviridae* phages cluster together, they have relatively low NT inter-Gegenees scores ($n = 59$–90) and they also differ from published phage genomes by 6–29% NT similarity (Fig. 4, Supplementary Fig. S5, and Supplementary Table S4).

Twelve of the unique *Salmonella* phages are based on NT similarity of the *Myoviridae*, they constitute the most homogeneous group and are all of the *Rosemountvirus* genus. They have comparable genomes (52–53 kb, 67–73 CDSs, 45.7–46% GC, no tRNAs) and cluster together in both the Gegenees analyses and in the phylogenetic tree. Although phage brorfarstad has slightly lower NT Gegenees scores ($n = 72$–74), than the rest, only a minor difference can be observed in the AA Gegenees analysis (Fig. 4, Supplementary Fig. S5).

The eight Podoviridae divide into a cluster of three phage species representatives (astrid, assan, and astrithr) with comparable (83–84 NT Gegenees score) small low GC genomes (11.6–11.7 kb, 15 CDSs, 39.7–39.8% GC, no tRNASs) and the singleton lumpael. Phages with >95% NT similarity with phage Lumpael were observed in five samples from five distinct facilities. Lumpael (41.1 kb, 58 CDSs, 59.9% GC, no tRNAs) is of the Murrayvirus genus, it has the highest GC content observed, and shares only 76% NT similarity with the type species Enterobacteria phage IME_EC2 (KF591601). The new *Murrayvirus* genus is erroneously classified as *Siphoviridae*, but as confirmed by transmission electron microscopy,[41] these phages belong to the *Podoviridae* family and a move of the genus will be included in the next ICTV ratification process (Personal communication with Evelien Adriaenssens). Astrid, assan, and astrithr all share <40% NT similarity with their closest relative Pectobacterium phage DU_PP_III (MF979562), and though they share NT Gegenees scores of only 0–1 and AA of 45, they do form a monophyletic clade (Fig. 4 and Supplementary Fig. S5).

Discussion

When it comes to phage isolation, three aspects are key: titer, sterility, and purity. These features are a prerequisite for any phage work, regardless of aim. However, purity and sterility are not the main focus in a screening such as the HiTS method. This screening method does not intend to provide a final PT or biocontrol product, but instead offers crude isolation of highly diverse phages in sufficient titers. This is to provide a fast and crude acquisition of numerous and diverse phages and thereby a basis for further phage isolation and establishment or expansion of phage libraries. If a phage of interest is in a mixed lysate, sequencing enables primer design for PCR verification when isolating the individually plaquing phages.

The numerous simultaneous enrichments enable the identification of an abundance of assorted phages while also facilitating crude isolation in a very short time span. This makes investigations of phage diversity easy and possible. The capacity of the HiTS method to uncover diverse phages is clearly illustrated by the impressive findings when screening for phages of PT relevance in wastewater, especially those infecting *E. coli* and *S. enterica*, as presented in

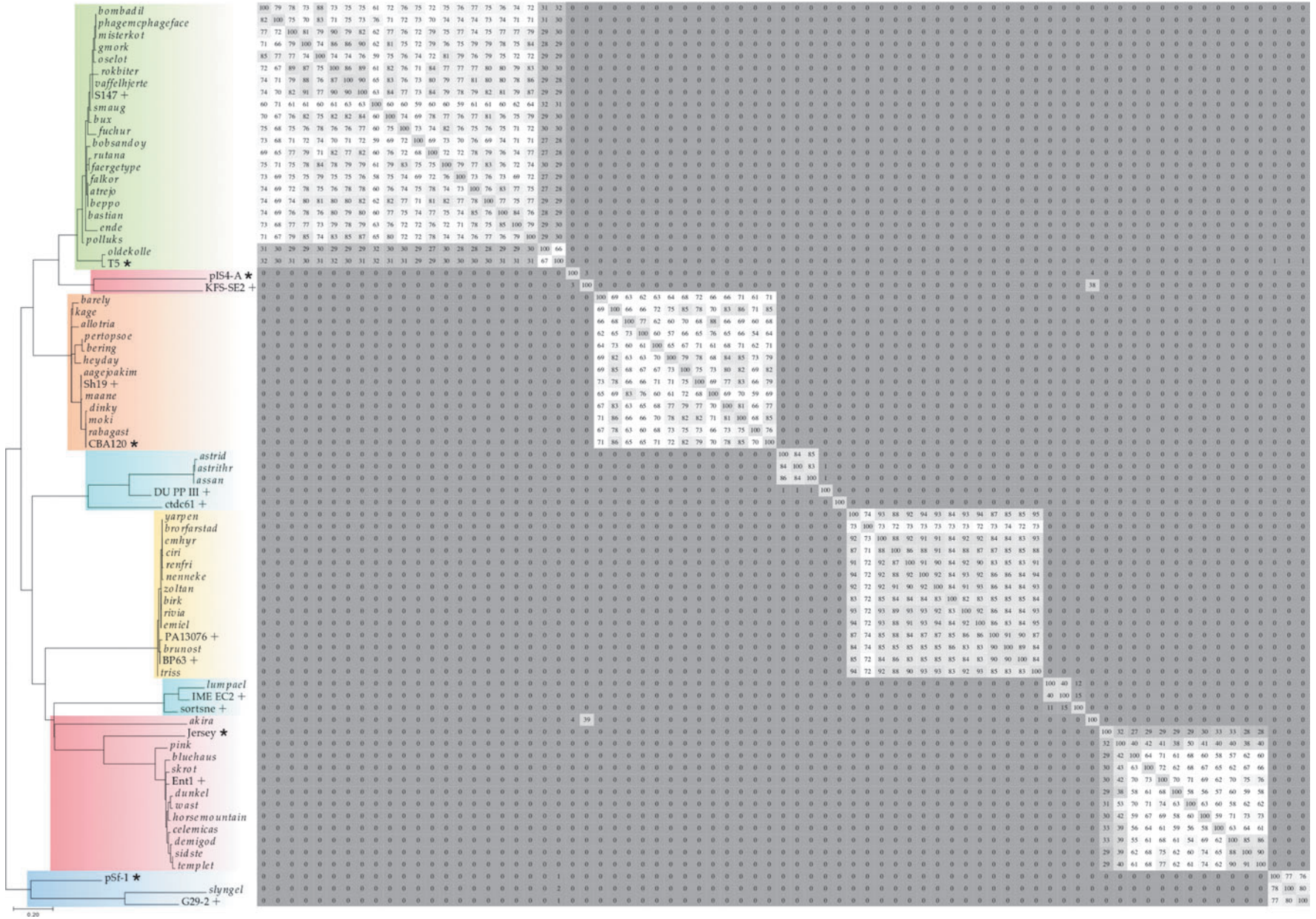

FIG. 4. Phylogenetic tree (maximum log likelihood: −4176.67, based on large terminase subunit or for the <13 kb *Podoviridae* the DNA encapsidation protein, scalebar: substitutions per site) and phylogenomic nucleotide distances (Gegenees, BLASTn: fragment size: 200, step size: 100, threshold: 0%). Predicted morphology is indicated by color bars, light orange *Ackermannviridae*, light green *Demerecviridae*, light blue *Drexlerviridae*, light yellow *Myoviridae*, light skyblue *Podoviridae*, light pink *Siphoviridae*, novel phages from this study are in italics, whereas close relatives are denoted by + and type species by *.

Olsen et al.[39] and in this study, respectively. Even for less abundant phages, diversity and novelty were uncovered, the *P. aeruginosa* phages represent three distinct families, and eight are novel phage species representatives, whereas all the *Enterococcus* phages are of the *Siphoviridae*, two of them have limited NT similarity (<60%) with published phages. The reported five screenings yielded 331 potential hits in the form of clearing zones, resulting in the identification of 154 novel phage species. Furthermore, none of these phages code for known virulence factors or ARGs and none appear to be lysogenic, making them all potential candidates for PT and biocontrol applications.

Unlike metagenomic sequencing approaches, this method provides actual phages with a direct link to the pathogenic host or any host in interest. The HiTS method does not reveal the diversity of individual samples; hence many phages remain undetected, especially those that are not easily grown under laboratory conditions. As the HiTS method is a semi-automated upscaling of traditional enrichment and soft-agar overlay methodologies, it is competition based and clearly selects for lytic phages with traits preferable in PT and biocontrol applications, that is, a high burst size and a short latency period. Consequently, the HiTS method enables the capture of the most prevalent phage(s) of the day in any sample. Thus, when screening numerous distinct samples, it provides an estimate of species richness of this type of phages in the given sample matrix. Accordingly, the species richness of easily culturable phages, presumably with high burst sizes and short latency times, lytic to the specific strains of *Escherichia* and *Salmonella* phages in Danish wastewater, was estimated to be at least in the range of 160–420 and 49–173, respectively (Supplementary Fig. S4).[39] This is likely an underestimation considering the relatively small sample sizes and the inherent bias in the method to only isolate a single or a few phages per sample combined with the many plaque-forming lysates for which DNA extraction or sequencing was unsuccessful.

If the aim of screening is to isolate phages with PT or biocontrol potential or phages that are easy to study under laboratory conditions, the targeting of lytic and highly reproductive phages is indeed an advantage. However, if the aim is to disclose true diversity or detect more difficult-to-culture specimen, other methods such as plaquing without amplification may be superior. Metagenomic sequencing approaches are constantly refined and now offer high detection levels of phageomes,[42] but phages of interest detected may be near impossible to isolate *in vitro*. The key advantage of the HiTS method is indeed that it offers both identification through sequencing and also provides physical isolates of all phages targeting the specific host species used as bait. Consequently, interesting discoveries such as rare and novel phages or the presence of remarkable genes with unexpected or desired functions can be investigated following a final isolation. In step 4.a of the reported SV screenings, the authors resorted to sequencing the lysates while storing phages collected from clearing zones. This approach is not recommended as it may result in sequencing of phages in lysate not present in the harvested soft-agar.

A 96-well setup carries a risk of cross-contamination; however, the use of pierceable sealing tape as recommended in the HiTS method reduces this risk. The presence of a negative amplification control in each spot test (agar-plates A and B) provides an indication of potential cross-contamination. No plaquing was observed in negative amplification controls in any of the screenings. Furthermore, the chequered pattern with empty wells between all lysates used when performing the spot tests ensures that in the case of improper sterilization, still no phages will be transferred to other wells in use during spotting with a 96-pin replicator, as opposite patterns are present in well plates A and B. Finally, if the sterilization of the 96-pin replicator is insufficient, any contaminating phages will plaque in between purposely spotted phages. This was not observed in any of the screenings.

A high number of distinct samples, as required by the HiTS method, may be cumbersome to collect and prepare, but once they are collected, they can be aliquoted, stored (–20°C), and used for numerous screenings of different target bacteria, as only very small sample volumes (0.5–1.5 mL) are required. The small sample volumes also permit for samples to be collected by sending out collection kits and having them returned by mail or carrier, provided that the applicable law allows it. Any sample matrix with high quantities of the target host is applicable. Furthermore, the suitability of time series of wastewater samples eases the sample collection and makes the screening method more feasible as it limits the number of distinct sampling sites. In this study, no *Enterococcus*, only two *P. aeruginosa* (9%), five *Salmonella* (8.5%), and nine *Escherichia* phage species (8.6%)[39] were detected more than once in samples from the same facility in any distinct screening. Wastewater treatment plants receive inlet wastewater in a constant yet changing flow, thus the presence of diverse phages can be expected to fluctuate and be interchangeable. Although some of the identified phages had 100% identical genomes also when originating from separate screenings, none of these were isolated from the same treatment plant.

Sequencing is continuously getting cheaper,[43] and even though this is the major expenditure of the HiTS method, it is economically feasible. Spending weeks and months on thoroughly isolating hundreds of phages is also a costly affair with regard to time and work hours. And still, also by individual isolation, resulting phages may end up being a similar specimen.

With the HiTS method, phage libraries can be built and sequenced after just 4 consecutive days of sample processing. The fast turnaround is of particular importance when screening for phages for PT, but not all phages are suitable for PT. Lysogenic phages should be avoided as they do not necessarily lyse their hosts and may also increase virulence of their hosts by lysogenic conversion.[44] Some phages, also those with a lytic lifestyle on the host in question, code for genes with unwanted genetic traits such as toxins, superantigens, intracellular survival/host cell attachments proteins, or ARGs that can be spread to bacterial communities through transduction.[45,46] This is especially relevant to consider when isolating phages from wastewater, as treatment plants can be considered hotspots for ARGs.[46] Fortunately, ARGs and other unwanted genetic traits can, for a large part, be deduced by genetic analyses and thus phages coding for them can with decent confidence be excluded. Hence, the HiTS method allows selection of new candidate phages after a few weeks of screening, sequencing, and analyzing. The ability of the candidate phages to infect the target host is already verified and the absence of undesired genetic traits confirmed, consequently the phages are now ready for experimental validation and final isolation, if required.

Conclusions

The upscaling and organization of enrichments as in the HiTS method presented here have the potential to efficiently detect the diversity of and crudely isolate phages relevant for PT and biocontrol that are abundant in the sample matrix explored. The HiTS method is simple, fast, and cost-efficient. It can prove to be a valuable scalable method in the case of urgent needs for PT suitable phages targeting specific clinical infections. With the HiTS method, establishment of sizeable discovery phage banks becomes fast and efficient. Such phage discovery banks could be lifesavers eliminating the need to spend time on isolating new PT phages and would also facilitate important phage taxonomy and ecology studies and can be explored for industrially relevant biotechnological applications.

Authors' Contributions

Conceptualization of this study was carried out by N.S.O., W.K., and L.H.H.; methodology was carried out by N.S.O., W.K., and L.H.H.; validation was by N.S.O., W.K., N.B.H., and L.H.H.; formal analysis was by N.S.O.; investigation was by N.S.O.; resources were by N.S.O. and L.H.H.; data curation was by N.S.O.; writing—original draft preparation was by N.S.O.; writing—review and editing were by N.S.O., W.K., N.B.H., and L.H.H.; visualization was by N.S.O.; supervision was by N.S.O., W.K., and L.H.H.; project administration was by N.S.O., W.K., and L.H.H.; and funding acquisition was by W.K. and L.H.H. All coauthors have reviewed and approved the article before submission. The article has solely been submitted to this journal and is not published, in press, or submitted elsewhere. A preprint is available at BioRxiv.

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Author Disclosure Statement

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
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Supplementary Figure S5
Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4

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