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Next generation sequencing technologies and the changing landscape of phage genomics

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The dawn of next generation sequencing technologies has opened up exciting possibilities for whole genome sequencing of a plethora of organisms. The 2nd and 3rd generation sequencing technologies, based on cloning-free, massively parallel sequencing, have enabled the generation of a deluge of genomic sequences of both prokaryotic and eukaryotic origin in the last seven years. However, whole genome sequencing of bacterial viruses has not kept pace with this revolution, despite the fact that their genomes are orders of magnitude smaller in size compared with bacteria and other organisms. Sequencing phage genomes poses several challenges; (1) obtaining pure phage genomic material, (2) PCR amplification biases and (3) complex nature of their genetic material due to features such as methylated bases and repeats that are inherently difficult to sequence and assemble. Here we describe conclusions drawn from our efforts in sequencing hundreds of bacteriophage genomes from a variety of Gram-positive and Gram-negative bacteria using Sanger, 454, Illumina and PacBio technologies. Based on our experience we propose several general considerations regarding sample quality, the choice of technology and a "blended approach" for generating reliable whole genome sequences of phages.

Introduction

Bacteriophages (phages) are natural viral predators of bacteria. They are in a constant evolutionary arms race with host bacteria; the survival of phages over millions of years is a testament to their ability to overcome bacterial resistance mechanisms by constantly evolving in parallel with their hosts. Isolation of new phages is rapid, facile and inexpensive, and there is an abundant supply of phages in nature, making them ideal weapons to combat bacterial infections. Despite the fact that they are non-toxic to animals and plants,[1] phages are not as widely used for biocontrol and therapeutics as one would imagine. Since the introduction of antibiotics in the 1940s to treat bacterial infections in humans and livestock, the widespread use, and in many instances misuse, has resulted in the current crisis with multidrug resistant bacteria. This activity, combined with a decline in the discovery of new classes of antibiotics that are effective against these resistant bacteria in the past several decades, has brought about a renewed interest in alternatives to antibiotics, such as phages or phage-encoded lytic enzymes.[2–5]

Since their discovery around 1915–1917, phages have served as excellent research tools,[6] although the promise of their antibacterial potential has not been fully realized.[7] Despite the apparent attractiveness of phages as antimicrobials, history is replete with false starts that have suppressed the field for decades at a time.[1–3,7]

Besides human therapy approaches, whole-phage preparations have also been widely evaluated as biocontrol agents for food production. Numerous studies attest to the efficacy of selected phages or phage cocktails against foodborne pathogens, such as *Listeria*, *Salmonella* or *E. coli*.[8–13] First phage preparations, such as Listex™ (Micreos) or ListShield™ (Intralytix), have received approval from regulatory agencies and are being used in food production. Phage lytic enzyme application in food production has received intensive research interest, as they present highly effective and practical means of decontamination (reviewed in refs. 14 and 15). Phage particles or their components have also been used successfully as detection agents for pathogens. Phage-based detection methods confer a faster and more sensitive detection. Newer developments include phage-amplification assays coupled with MALDI-MS,[16,17] detection by lysis products (reviewed in ref. 18), reporter bacteriophages (reviewed in ref. 19) or detection by receptor binding, to list just a few.

Today, technologies exist that allow cost-effective sequencing of hundreds of viral or bacterial genomes per year, and we can anticipate in the not-too-distant future further advances that might allow routine whole-genome screening of every pathogen encountered in a clinic[20] or on contaminated foodstuff. The majority of the sequenced bacterial genomes reveal the presence of one or more partial or complete prophage genomes. Even closely related genomes appear to possess different sets of prophages.[21] Thus, the phage gene pool is larger and more diverse than the rest of the chromosome. In our experience, some prophage regions are recalcitrant to cloning, most likely due to toxicity of the gene products to the bacterium. Genes revealed by whole genome sequencing and screening of phage collections will potentially yield new generations of antimicrobials. Whole-genome sequencing has become mandatory for regulatory approval of any healthcare or food-industry application of phage or phage products,[22–24] but today's researchers are faced with an array of sequencing platforms and assembly options and the massive amounts of data they produce.[25]

The current wave of high throughput sequencing efforts began in 2005 with the introduction of the Roche/454 sequencer followed by other platforms such as SOLiD, Solexa (Illumina), Helicos, Ion Torrent and PacBio and another wave of platforms yet to be released such as a nanopore-based platform (MinIon and GridIon of Oxford Nanopore).[26] Furthermore, the relatively small footprint, both in terms of laboratory space and personnel, required by these technologies brought about the democratization of genome sequencing in the sense that whole-genome sequencing can be done in any laboratory with limited resources and is therefore no longer just a prerogative of large Genome Centers. Each of the 2nd and 3rd generation sequencing platforms has its own unique features and distinct advantages over other platforms. However, all these platforms produce very high sequence outputs compared with the throughput of conventional Sanger sequencing platforms. Conservative estimates by the Genomic Standards Consortium in 2009 placed the prokaryotic and eukaryotic genomes completed by 2012 at over 10,000 and ~2000 respectively.[27] According to the GOLD genomes database, currently there are a total of approximately 15,000 prokaryotic and 3,000 eukaryotic genomes listed, of which only about 20% are finished genomes.

Although phage genomes are orders of magnitude smaller in size, whole-genome sequencing of phage has not kept pace with the current trend in high throughput sequencing of bacteria and other organisms. There has only been a slow increase in the number of complete bacteriophage genomes published.[28] The NCBI genome database contains around 600 Caudovirales genomes to date as well as some unclassified phage genomes.

The lack of parity in phage genome sequencing can be attributed to several problems unique to sequencing and assembly of phage genomes. (1) Phages are not self-replicating and rely on their host macromolecular machinery for their replication and growth and hence isolation of phage genomic material completely devoid of host genetic material involves extensive purification steps. Although one can, in many cases, separate the reads pertaining to the host bioinformatically post-sequencing, the presence of prophage sequences in the host chromosomes may pose a problem for such filtration. (2) Sometimes phage preparations are associated with host debris and cellular membrane fractions that contaminate the genomic material and interfere with subsequent steps of DNA sequencing. (3) Phages, especially the exclusively lytic phages, have notoriously highly methylated genomes because bacteria possess restriction-modification systems to safeguard the integrity of their genomes from invading DNAs. In order to overcome such restriction systems, phages have evolved mechanisms such as genome methylation so that they are able to infect and grow in their host bacteria. From a practical standpoint, such highly methylated sequences are recalcitrant to many of the routine genetic manipulations including shearing, cloning and DNA sequencing. In conventional cloning-based shotgun Sanger sequencing, many of the phage fragments are underrepresented and/or unclonable due to toxicity of the genes for the cloning host, usually an *E. coli* strain. This problem is avoided in the next generation sequencing platforms, by virtue of cloning free PCR amplification of fragments in oil and water microreactors, or emulsion PCR. However, in many instances, highly methylated DNA is a poor template for PCR and sometimes even for fragmentation of the DNA by usual procedures, such as nebulization by compressed Nitrogen gas. (4) Some phage genomes are notoriously rich in extreme GC content that is different from that of their host. Such extremes may pose a problem for PCR and sequencing. (5) Phage genomes are also known to contain complex genomic structures such as extremely long direct or inverted repeats and terminal redundancies that are problematic for assembly of the whole-genome sequence from the reads. Many assembly algorithms break the contigs at these repeats, requiring further evaluation by the human eye and confirmatory sequencing by other methods, such as PCR, restriction analysis or Sanger sequencing.[29,30] (6) Regions of uneven sequence depth along the length of the genome, when amplifying or generating libraries using random-priming methods, may cause problems for many of the common assembly algorithms because the programs assume that this uneven coverage is due to repeats or contamination, resulting in artificially poor assemblies. (7) Almost 80% of the genome sequences in the genome online database (GOLD) are unfinished draft sequences. For bacteria and other organisms, complete genome finishing may not be a requisite for many applications, but for small genomes such as bacteriophages, finishing the genome sequencing is essential to obtain a more complete understanding of their biology, i.e., obtain confirmation of their lifestyle by identification/exclusion of genes encoding lysogeny control functions; or to identify their potential for generalized transduction of host DNA by assessing the physical genome structure.[29] Hence, phage researchers are faced with an increased demand in resources in order to finish and polish phage genomes before publication is possible. (8) Whereas in bacterial and human genomics, mapping of reads to a finished reference genome can be a powerful analytical tool not just for genome assembly, but for discovery of genetic variations such as insertions/deletions (indels) and single nucleotide polymorphisms (SNPs), in phage genomics this is very seldom feasible due to the absence of a reference genome for any given phage. Phage genomes are extremely mosaic in nature[31] and even closely related phages are highly divergent, rendering reference mapping a futile effort. (9) In general, a lack of resources for phage genome sequencing within the reach of individual phage researchers coupled with a general lack of interest and support for phage genomics by the journals and the funding agencies have resulted in too few complete phage reference genomes.

Despite all the challenges outlined above, 2nd and 3rd generation sequencing platforms offer the best opportunity for whole-genome sequencing of phages. In this report, we describe our efforts to sequence a large number of bacteriophages using both conventional and 2nd/3rd generation sequencing approaches. We also present general guidelines for obtaining a complete genome sequence of phages using a blended approach.

DNA Preparation and Quality

A common prerequisite to all DNA sequencing technologies is the necessity for high-quality nucleic acid preparations, free from contaminating RNA, proteins or solvents. Traditionally, the organic extraction method for DNA purification originally developed for bacteriophage Lambda[32] is used to produce highly pure DNA samples, which are sufficient even for the rigorous sample quality demands of the newest sequencing technologies. Superior results have been obtained from high-titer phage stocks purified by CsCl, sucrose or Optiprep® stepped density gradient ultracentrifugation and subsequently dialyzed against buffer of choice. Genomic DNA is then extracted by cracking the phage capsid with heat and proteinase K and purified using organic extraction as described elsewhere.[32] The DNA usually features A280/260 values of ~1.8 and forms a clear, sharp band on agarose gel electrophoresis runs. Co-purification of host RNA or proteins is virtually impossible using these ultracentrifugation techniques.

Alternatively to ultracentrifugation, pure, phage plate lysates or lysates from liquid cultures can be filtered through membranes with pore diameters of 200 nm and concentrated by high-speed centrifugation for several hours. Phage particles collected in the centrifugation pellet are resuspended in buffer and the DNA prepared by organic solvent extraction as described elsewhere.[32] A DNase and RNase-treatment before centrifugation and the inclusion of a second phenol-extraction step is recommended to increase DNA purity. DEAE anion exchange chromatography can also be used to remove contaminating free-floating nucleic acids.[33]

In contrast, we found that DNA purified with commercial phage DNA preparation kits was often not completely free of contaminants, and additional purification steps were necessary. The overall yield is quite low (which may be attributable to smaller starting sample sizes) compared with the methods outlined above, although the kits are far less labor-intensive and do not require a preparative ultracentrifuge.

If it is difficult to obtain sufficient quantity of DNA for library construction, a variety of random-priming DNA amplification methods exist for generating large quantities of pure DNA from a few nanograms of starting material, including, multiple displacement amplification (MDA) using the phi-29 DNA polymerase[34] or sequence-independent single primer amplification (SISPA).[35,36]

Sanger Chain-Termination Sequencing

Most bacteriophage DNA sequences have been obtained using a shotgun library approach followed by Sanger sequencing on capillary sequencers (e.g., ABI 3730XL). In this procedure, a phage genome is fragmented enzymatically, by sonication or by other methods, to a convenient fragment size and these fragments are randomly cloned into a high copy-number plasmid, such as pBluescript (Stratagene) or pHOS2.[37] Using primers located on the vector sequence, the unknown insert can be sequenced. Sanger reads usually exceed 1500 bp in length on a capillary sequencer and yield a Phred20 quality-corrected[38–40] length of approximately 950–1100 basepairs of high-quality sequence with high accuracy. Although labor-intensive, the Shotgun-sequencing approach features a considerable advantage: Dependent on the choice of fragment size, reads from both flanking regions result in a mate-pair of sequences, which is either overlapping (fragment size below 2 kb) or has a known distance (fragment size above 2 kb) between the reads from both sides of the insert. This additional information on the physical distance between two reads can be computed into the sequence assembly in state-of-the-art software suites, such as products from CLC Bio, Geneious, DNAstar and many more, and aids scaffolding (called linkage) and correct placement of individual sequence reads within the contigs.

Usually, a shotgun sequencing approach leaves the bacteriophage researcher with a handful of high-quality contigs. Gap-closure between contigs can be done by Sanger sequencing on PCR amplified regions between contigs, generated using combinations of primers facing outward of the contigs, or by primer walking directly on phage DNA. Such direct genome primer walking, if done by an expert technician on a well-calibrated capillary sequencer, has advantages over PCR-based gap closure; it is equally fast and omits the error-prone amplification step. Primer walking can also be used to check regions of low coverage or low confidence contig-join regions in the sequence assembly.

Additionally, restriction maps of the phage genome should be generated and if possible pulsed-field gel electrophoresis performed, in order to verify computed genome size and contig alignment with experimentally obtained data. This step is especially critical in the discovery of multiple phage variants, such as in the case of Gamma/Cherry phages of *Bacillus anthracis*.[41]

Some potential drawbacks of shotgun sequencing have been mentioned. One concern is cloning bias, which can occur when the target DNA exhibits extensive secondary structures or stretches of non-clonable DNA (e.g., due to encoded proteins/enzymes toxic to the cloning host, usually an *E. coli* strain). A second shotgun library with smaller insert sizes (i.e., < 500 bp) might be able to help circumvent the cloning problem, because the toxic ORF might be incomplete in the clone, and therefore no functional protein would be made in *E. coli*.

Small, temperate siphoviruses, such as A500 of *Listeria*[42] or TP21-L of *Bacillus*[43] usually feature a genome size of approximately 40 kb and are easy targets for sequencing. A shotgun library of roughly 250 clones carrying distinct inserts, sequenced from both sides is sufficient to assemble a high-quality draft genome, which needs generally no more than 10–15 runs of primer walking to finish and polish. Most small siphoviruses can be sequenced efficiently in a timeframe of 4–5 weeks, using the approach outlined above. Average genome coverage of 4–7-fold is obtained, which is sufficient for a reliable assembly. If necessary, primer walking can be used to ensure that each part of both DNA strands was sequenced completely at least once. Higher throughput Sanger-sequencing pipelines, such as the JCVI pipeline, sequence in a minimal unit of 384-well plates or blocks. By sequencing the ~40kb phage phiEf11 using 384 clones of 2–3 kb insert size in both directions, we were able to assemble the complete genome with no further finishing needed.[44] **Table 1** summarizes the sequencing data on a selected number of bacteriophages and the approximate time for sequencing and assembly in working days. Sequencing of larger viruses or complex myoviruses might require significantly more time and resources than that outlined for sequencing of small temperate siphoviruses.

However, some viruses exhibit an exceptional cloning bias, which makes them unsuitable for shotgun cloning. **Figure 1** depicts a read pile-up in one contig of *Listeria* phage P70 genome assembly from 589 Sanger reads using CLC Genomics Workbench 5.1. Although the reads equal an average overall coverage of 8.5-fold, no complete genome could be assembled due to the biased representation of clones from certain regions of the phage genome. No apparent difference between these regions and the rest of the genome could be found, besides the presence of putative promoter sequences and a slightly elevated GC content.

Roche / 454 Sequencing

The 454 pyrosequencing technology, marketed by Roche, was the first of the second generation technologies available to researchers since 2005. Several thousand genomes and genomic fragments have been sequenced using the 454 technology and it has become somewhat the standard in genome sequencing. The newest FLX+ system together with Titanium XL+ reagents promises a read length of up to 1000 bp (mode read length 700 bp) and an average sequence output of 700 Mbp per sequencing plate in 23 h of runtime. Roche claims 99.997% accuracy at 15-fold coverage and provides the possibility to multiplex up to 132 samples on one plate or 16 samples when using gaskets. At a theoretical maximum output of 700 Mbp, an unbelievable number of 582 phage genomes of 40 kb could be sequenced in one run with 30-fold coverage, provided the plate would allow for more than 132 samples per run (http://my454.com/products/gs-flx-system) and each DNA species would be equally distributed on the sequencing plate. This can be done using the SISPA method (sequence-independent single-primer amplification) since up to 1,500 error-correcting barcodes can be designed.[36,45]

We have used the current 454 FLX Titanium sequencing for a number of *Listeria* and *Bacillus* phages with mixed outcomes. Generally, the large amount of sequence data poses problems with regard to IT requirements. Also, the choice of sequence assembly algorithm has to be made based on the phage genome in question and no universal solution is available. Due to the lack of reference genomes for most phages, de novo assembly is often the only option. For some phages, the *GSAssembler* software[46] that comes with the instrument is sufficient to quickly produce a single contig or only a handful of contigs, but in many cases, due to repeat issues discussed above, the software produces a hundred or more contigs even for a small phage genome. If mate-pair information is available, we have found the Celera Assembler[47] version 7.0 (http://wgs-assembler.sourceforge.net) to give very good results.

An advantage of the Roche/454 technology is the option for sample multiplexing combined with average to long read

Table 1. Summary of bacteriophage shotgun genome sequencing projects

Phage name (host bacteria)	Virus family	Genome size	Number of reads	Average read length	Complete sequencing in	Reference
P40 (*Listeria*)	*Siphoviridae*	35.64 kb	164	942	55 d	44
ΦS63 (*Clostridium*)	*Siphoviridae*	33.61 kb	263	915	27 d	45
B653 (*Listeria*)	*Siphoviridae*	31.17 kb	235	923	31 d	unpublished
NF5 (*Brochothrix*)	*Siphoviridae*	36.95 kb	287	850	25 d	46
BL3 (*Brochothrix*)	*Siphoviridae*	41.52 kb	242	871	28 d	46

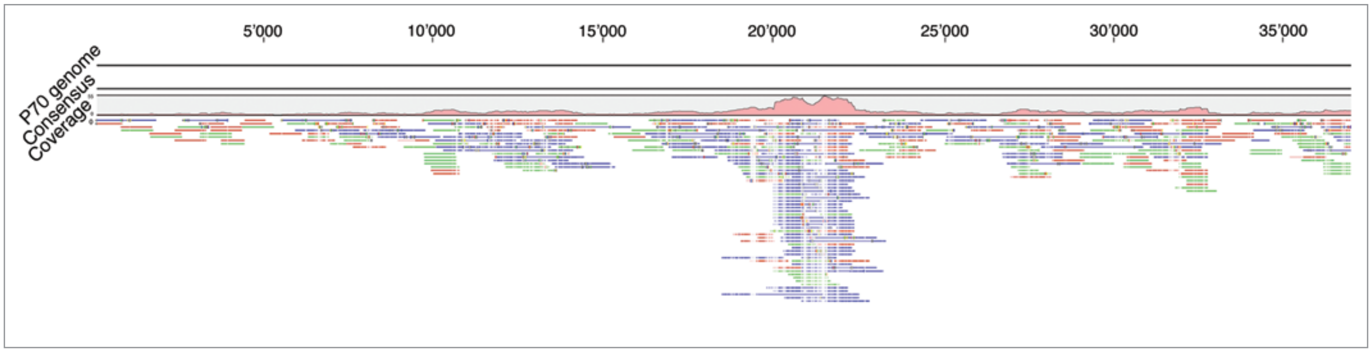


Figure 1. Sanger read pile-up in the assembly of a shotgun library sequencing approach of *Listeria* phage P70. Image captured from CLC Genomics Workbench 5.1. Upper scale shows sequence length in bp. Green are forward reads, red are reverse reads. Blue are mate-pair reads. Light green and light read color indicates trimmed sequence parts. The coverage plot shows the region of sequence and cloning bias, which features a significant higher coverage (up to 55-fold) than the rest of the contig sequence (2–21 fold).

lengths. We have run up to 16 standard phage DNA libraries on a single 454 plate, each individually barcoded for later read separation. The results of one such run on a FLX machine using XLR70 chemistry are depicted in **Table 2**. The total read number of 85461924 bases is quite equally distributed between most of the phages, with exception of *Bacillus* CP-51 phage, (sample 7, **Table 2**) which did not sequence well in all 454 approaches, most likely due to problems with library preparation or unequal pooling of multiple MID libraries (**Table 2A**). In addition, we have run up to 25 SISPA individually barcoded libraries on a half 454 plate with mixed results (e.g., only one phage assembling completely) (**Table 2B**).

Illumina

The Illumina HiSeq2000 sequencer uses the sequencing-by-synthesis technology and delivers an unrivalled number of short sequencing reads (i.e., up to 6 billion paired-end reads, equaling 600 Gb of sequence information in one ten day run using 2 flow cells). Generally speaking, the Illumina technology delivers an incredible amount of data, which is too much for the typical laboratory workstation computer to assemble; thus, necessitating the use of high throughput computing grids or cloud services. Also, because of the sheer volume of data, several (incorrect) variants of the phage genome can be constructed with good confidence from the bulk of read data. Such an example is best illustrated in the case of sequencing of *Cronobacter* phage A19 in which 599,997,144 100 bp paired-end reads were generated. Results generated by de novo assembly are depicted in **Figure 2**. A total of 219 large contigs could be produced, each with reliable coverage of > 15-fold. However, the 178 kb contig stands out, because the coverage is by far higher than for any of the other contigs. This is the actual A19 genome, as confirmed by restriction profiling and partial re-sequencing. The large number smaller chaff contigs likely represent reads with sequencing errors, variants within the population and contaminants that only show up due to the large number of reads. Assemblers such as Newbler[46] have trouble with this large volume of read data and hence artificially fragment a genome that is completely represented (e.g., mapping assembly generated in one piece yet the de novo assembly is fragmented). Various labs have been working on methods to reduce redundant reads, reduce sequence error and flatten pile-up regions to try to address these issues. This is particularly a problem with using random-primed amplification to generate libraries. For example, SISPA can generate pile-up regions due to partial matches to the bar-code sequence within the genome.

Illumina sequencing generates a large amount of data but with very short read lengths and is therefore problematic when phage genomes contain repetitive sequence stretches. The maximum distance that can be bridged, using the HiSeq 2500/1500 instruments announced for 2012 and paired-end library prep is 2 × 150 bp (2 × 100 bp on current model instruments) plus a variable 200–500 bp pair distance, which equals 800 bp on the HiSeq 2500/1500 and 700 bp on the current models HiSeq2000/1000 (www.illumina.com/systems/hiseq_systems.ilmn). Also, two or more genes with similar sequence in a single phage genome can lead to assembly mistakes. Such short read data are also insufficient to resolve genome end repeats, which are present in a large fraction of bacteriophages. In our opinion, Illumina sequencing has only limited usefulness for de novo assembly of phage genomes, but is rather the method of choice for re-sequencing of existing genomes, for increasing depth of coverage and/or in combination with a certain amount of sequences with longer read lengths (i.e., Sanger or PacBio). Error-correction of PacBio reads by using Illumina reads and the Celera Assembler is a particularly attractive possibility.[48]

Pacific Biosciences RS

PacBio is currently one of the newest and most discussed of the next-generation sequencing technologies. The RS device is capable of single-molecule sequencing omitting amplification steps. Also, the expected read length is by far greater than that of any of the other sequencing technologies. Individual read lengths of up to 23 kb have been generated,[49] which makes the RS results the ultimate resource for genome scaffolding and supposedly speeding up assembly by several orders of magnitude. However, due to the high error-rate of the polymerase-based sequencing, some

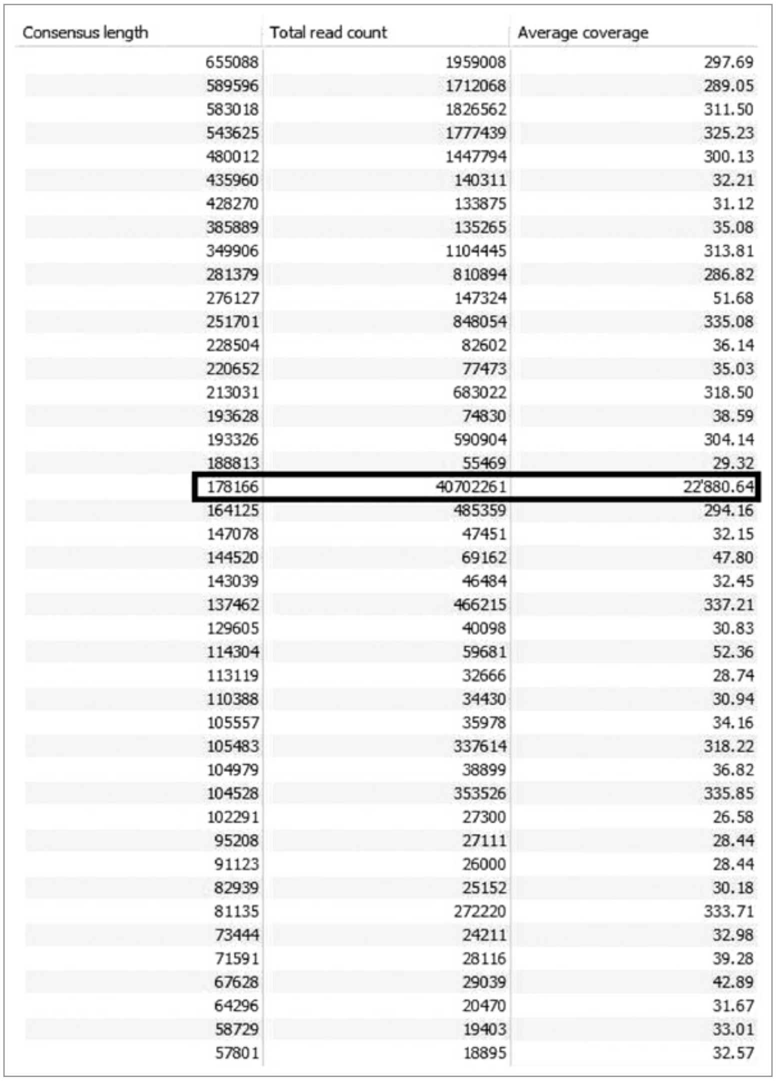
Table 2. Results of 454 sequencing of bacteriophage genomes

(A) 16 bacteriophage genomes on one sequencing plate

Sample (host)	Number of sequences	Number of bases	Average read length	Average coverage	# Contigs > 500 bp
1 (*Listeria*)	11344	2161998	191	48x	4
2 (*Listeria*)	30271	6025214	199	46x	2
3 (*Salmonella*)	26871	4771285	178	58x	7
4 (*Listeria*)	39479	7344858	186	198x	16
5 (*Bacillus*)	27507	5082812	185	34x	14
6 (*Listeria*)	30877	6066035	196	46x	3
7 (*Bacillus*)	668	116563	174	0.83x	9
8 (*Salmonella*)	34842	6112171	175	76x	3
9 (*Listeria*)	16325	3237309	198	27x	6
10 (*Listeria*)	25081	4662743	186	34x	7
11 (*Listeria*)	25805	5450722	211	39x	3
12 (*Listeria*)	45510	8086412	194	71x	3
13 (*Erwinia*)	37291	6585482	177	78x	8
14 (*Listeria*)	35671	7045886	198	201x	11
15 (*Staphylococcus*)	37561	7474743	199	59x	19
16 (*Bacillus*)	23148	4517691	195	30x	9
TOTAL	**448251**	**85461924**	**190.13**		

(B) 25 bacteriophage genomes on a half sequencing plate using SISPA

Sample (host)	Number of sequences	Number of bases	Average read length	Average coverage	# Contigs > 500 bp
1 (*Actinomyces*)	362	70907	196	4x	7
2 (*Pseudomonas*)	3701	1075632	291	9x	11
3 (*Pseudomonas*)	4055	1074532	265	10x	6
4 (*Pseudomonas*)	6063	1886107	311	26x	1
5 (*Pseudomonas*)	5709	1810952	317	11x	5
6 (*Pseudomonas*)	5520	1662547	301	14x	6
7 (*Pseudomonas*)	7427	2277470	307	11x	3
8 (*Pseudomonas*)	14793	4810715	325	81x	3
9 (*Pseudomonas*)	28320	8545926	302	61x	3
10 (*Pseudomonas*)	22228	6431342	289	107x	8
11 (*Pseudomonas*)	9433	2679172	284	20x	15
12 (*Pseudomonas*)	1793	533638	298	7x	4
13 (*Pseudomonas*)	8050	2397310	298	34x	1
14 (*Pseudomonas*)	3318	993033	299	11x	8
15 (*Pseudomonas*)	9989	2828957	283	93x	1
16 (*Pseudomonas*)	2150	674149	314	5x	18
17 (*Escherichia*)	544	146829	270	3x	10
18 (*Escherichia*)	722	206078	285	4x	14
19 (*Escherichia*)	1976	493767	250	5x	30
20 (*Escherichia*)	29373	8630949	294	21x	40
21 (*Escherichia*)	16190	4299180	266	9x	66
22 (*Escherichia*)	15366	4633923	302	10x	47
23 (*Actinomyces*)	132966	45175179	340	33x	41
24 (*Streptococcus*)	45747	14678094	321	37x	27
25 (*Actinomyces*)	40314	12187399	302	11x	183
TOTAL	**416109**	**130203787**	**292**		



Consensus length	Total read count	Average coverage
655088	1959008	297.69
589596	1712068	289.05
583018	1826562	311.50
543625	1777439	325.23
480012	1447794	300.13
435960	140311	32.21
428270	133875	31.12
385889	135265	35.08
349906	1104445	313.81
281379	810894	286.82
276127	147324	51.68
251701	848054	335.08
228504	82602	36.14
220652	77473	35.03
213031	683022	318.50
193628	74830	38.59
193326	590904	304.14
188813	55469	29.32
178166	40702261	22'880.64
164125	485359	294.16
147078	47451	32.15
144520	69162	47.80
143039	46484	32.45
137462	466215	337.21
129605	40098	30.83
114304	59681	52.36
113119	32666	28.74
110388	34430	30.94
105557	35978	34.16
105483	337614	318.22
104979	38899	36.82
104528	353526	335.85
102291	27300	26.58
95208	27111	28.44
91123	26000	28.44
82939	25152	30.18
81135	272220	333.71
73444	24211	32.98
71591	28116	39.28
67628	29039	42.89
64296	20470	31.67
58729	19403	33.01
57801	18895	32.57

Figure 2. De novo assembly of approximately 60 million Illumina reads generated for a 178 kb *Cronobacter* phage. Two hundred and nineteen large contigs were produced and at least 20 of them are of similar size or larger than the actual phage genome, which sticks out because of the unusual high sequence coverage of 22,880-fold. Several other assemblies also feature reliable coverage when viewed separately from the rest.

researchers have so far refrained from using this technology at all. In making such a decision it is vitally important to understand the technical limitations vs. the advantages of a platform such as PacBio. The PacBio technology utilizes so-called SMRT-cells, which produce about 40,000–50,000 reads each. Each such SMRT cell is patterned with 150000 zero mode waveguides, basically small cavities containing an immobilized DNA polymerase which sequences DNA by synthesis.[50] Briefly, SMRT-bell hairpin adapters[51] are ligated to fragmented DNA and this is sequenced on one strand, and in ideal cases, the polymerase passing the template multiple times, generating plus- and minus-strand reads. A good approach for de novo genome sequencing would be to combine a short insert library (250 bp) that is passed multiple times [circular consensus sequencing (CCS)] and which can be sequenced with 100% accuracy, with a larger insert library (2 kb or 10 kb) which is sequenced with 85–87% accuracy (post-filter). Alternatively, PacBio data can be error-corrected with other data from short-but-high-coverage sequencing technologies, i.e., Illumina.[48] The drawback is that such a combination of technologies requires at least two cost-intensive sequencing runs.

Pacific Biosciences has recently released the C2 upgrade, consisting of changed chemistry, SMRT-cells and software, which is aimed at improving accuracy and read length. From our experience, the new SMRTAnalysis software trades number of valid reads for accuracy with some assembly settings. The number of reads (and post-filter bases) in our data sets has nearly dropped to half the number from version 1.2.2 to 1.3 using protocol RS_Assembly.1, whereas the read accuracy went up 2.5%. In extreme cases, less than 10% of the actual sequencing reads pass the quality-filtering step (**Table 3**). For bacteriophage genomes, we believe that PacBio is a very valuable technology to generate a sequence assembly scaffold and provide an orientation for contig alignment. The bulk of sequence data should

Table 3. Results from PacBio RS sequencing of bacteriophage CP-51 (*Bacillus*) and P70 (*Listeria*) DNA using SMRTanalysis version 1.3

Phage name	# of SMRT-cells (# of 45 min movies)	Pre-filter # of bases	Post-filter # of bases	# of post-filter reads	Post-filter mean read length (library insert size) in nt	Post-filter mean read quality
P70	3 (6)	354960481	70378003	33848	1881 (1800–2000)	0.871
CP-51	6 (12)	676462435	212266847	107146	1770 (2800)	0.875

be generated with a small-insert library and CCS sequencing or a complementary technology, e.g., 454 or Illumina, where 10 or more phage genomes can be run on one sequencing plate.

We have so far sequenced two bacterial genomes (approximately 4.5 Mbp each) and five bacteriophage genomes by this method (including 67 kb *Listeria* phage P70 and 140 kb *Bacillus* phage CP-51)[55] (**Table 3**). The overall single-pass sequence accuracy (2 kb insert libraries) did not exceed 87.5%, meaning 12.5% of the base calls were incorrect. Assemblies of reads with this magnitude of error can be computed by an assembly algorithm with tolerant settings (i.e., low insertion and gap penalties, low overlap identity) against a good reference genome, but requires a large amount of redundant sequence information for de novo sequencing projects, either from a small-insert PacBio library or from a second sequencing technology. However, the very long PacBio reads (on average, a good proportion of a large insert library yields 2–3 kb long individual reads) make a good starting point for genome scaffolding (i.e., in cases where other, amplification-based sequencing technologies fail to sequence a certain genomic region because of amplification biases). Also, for small viral genomes, a single SMRT-cell output provides enough data for multiple-fold genome coverage and therefore massively reduced error rate. The average PacBio read length is far greater than on any other current technology, which considerably speeds up genome assembly.

Summary and Conclusion

Although next-generation sequencing technology moves at an incredible pace, most researchers, especially in the phage biology field, have barely adopted the first generation of the new sequencing technologies due to technical difficulties or lack of funding. Furthermore, researchers are faced with large amounts of raw data with limited bioinformatics support. Commercial solutions to phage sequencing are virtually nonexistent, as most sequencing companies focus on genomes of bacteria or eukaryotes and have little expertise for the specific requirements of virus genome sequencing. Commercial sequencing is also expensive and the wait-time for results is rather long. University-based sequencing centers have filled this niche and many research groups have also acquired their own sequencing infrastructure.

Here we report our experiences with different commercial and university/institute-based sequencing facilities and describe some of the rather extreme hurdles which a modern-day phage biologist might face. It must be stated that most bacteriophage genomes from Gram-positive and Gram-negative organisms are fairly easy to sequence and require no specific bioinformatics expertise. Usually, 454 sequencing is used and the vendor software does a satisfying job of genome assembly. Only a few error-corrections, usually done by PCR-amplification and Sanger sequencing, are needed to obtain a complete sequence of the virus genome with good depth of coverage in all parts of the sequence.

Large phage genomes, the presence of modified bases, extensive DNA secondary structures, DNA-attached proteins or segmented genomes present a different story and require the application of a combination of sequencing technologies. A scaffold for read placement and contig orientation and alignment are the critical features in these cases. Currently, only three technologies, Sanger, PacBio and 454 (only with long insert mate-pair libraries), deliver the required read length for scaffolding phage genomes. Gap-filling, increasing depth of coverage and/or error correction (i.e., in case of PacBio data) can be done with a second technology that generates accurate, but rather short reads, such as Illumina or IonTorrent.

The sheer variety of commercial and freely-available software for processing sequencing data is overwhelming. Commercial software solutions are available for a variety of operating systems, usually integrated with a central database with a graphical user interface and intuitive handling (point-and-click). However, the software licenses are normally rather expensive and often coupled to maintenance contracts which add follow-up costs. Most open-source tools and most vendor software have to be installed under Linux (sometimes requiring a specific Linux distribution to run) and are mostly command-line operated. This may be routine for experienced bioinformaticians, but may be a problem for biologists.

Generally speaking, the best software for homogenous data sets from one technological source is the vendor software, i.e., gsAssembler (a.k.a. Newbler) from Roche or SMRT-Portal from PacBio. Other than that, large commercial software suites, such as DNAStar or CLC Bio products, as well as open-source products such as Mira,[52] Velvet[53] or wgs-Assember (Celera Assembler),[47] offer the possibility to integrate data from various sources and assemble them together or as separate data sets. However, manufacturer software assembling only one source of data might lead to hard-to-resolve ambiguities and mis-assemblies in the final genome draft. Thus, assembly should be attempted using different tools or iterative steps in two or more software tools. It is not the aim of this article to provide an overview of software options. The reader is kindly referred to reviews specifically dealing with this topic.[54]

Another problem for individual labs performing next-generation sequencing is data storage. Results of NGS sequencing runs can easily add up to several hundred gigabases of raw sequence and large assemblies files. A reliable, redundant data storage option is mandatory to ensure data safety and consistency. Also, powerful computer workstations and computing grids are needed for data processing and result visualization.

In conclusion, next generation sequencing technologies offer a thrilling variety of methods to obtain bacteriophage genome sequences quickly, reliably and rather inexpensively. While traditional costs of shotgun library preparation and Sanger sequencing roughly amounts to several thousand USD per genome, modern technologies can sequence an individual phage genome for $800 USD or less if several samples are combined into one run. However, not all bacteriophage genomes sequence effortlessly and potential obstacles to sequencing, such as DNA structure, sequence repeats and problems due to DNA methylation must be taken into account. We propose a blended approach of a long-read technology for scaffolding purposes combined with a large number of short reads from a second technology for efficient DNA sequencing of bacteriophage genomes.

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