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Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and *E. coli* virulence genes in bacteriophage and prophage nucleotide sequences

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Extensive research is currently being conducted on the use of bacteriophages for applications in human medicine, agriculture and food manufacturing. However, phages are important vehicles of horisontal gene transfer and play a significant role in bacterial evolution. As a result, concern has been raised that this increased use and dissemination of phages could result in spread of deleterious genes, e.g., antibiotic resistance and virulence genes.

Meanwhile, in the wake of the genomic era, several tools have been developed for characterization of bacterial genomes. Here we describe how two of these tools, ResFinder and VirulenceFinder, can be used to identify acquired antibiotic resistance and virulence genes in phage genomes of interest. The general applicability of the tools is demonstrated on data sets of 1,642 phage genomes and 1,442 predicted prophages.

Introduction

The current dramatic increase in antibiotic resistance among pathogenic bacteria has led to renewed interest in the West in the use of bacteriophages as a treatment alternative.[1,2] In the former USSR, this interest has never faded. The Eliava Institute in Georgia in particular has played a leading role in the application of phages to treat a wide range of bacterial infections.[3,4] In the West, several studies in both animal models and human infections have demonstrated the effectiveness of phages as antibacterial treatment.[5-7] Furthermore, in the UK, the first clinical phase II trial on the use of phages to treat antibiotic resistant *Pseudonomas aeruginosa* has been performed with success[8] and more trials are to follow.

Besides their use in treatment of human infections, phages have been suggested for use in the agriculture and food industries,[9] e.g., to reduce *Campylobacter jejuni* colonisation of broiler chickens[10] and to reduce the growth of *Escherichia coli* in milk.[11] Genetically modified phages have furthermore been suggested as a detection tool for *Bacillus anthracis* in deliberately contaminated food.[12]

One concern that has been raised in regards to extensive application and dissemination of phages is related to the fact that they are important vehicles of horisontal gene transfer (HGT) between bacteria within the same[13,14] or different species.[15] Phages accordingly play a major role in the HGT that transforms benign bacteria into pathogens by introducing genes encoding virulence factors. This process is known as phage-lysogenic conversion.[16] Examples include the transfer of Cholera toxin[17] and Shiga toxin.[18,19] The outbreak of *E. coli*, which started in Germany in May 2011 and spread across Europe in the following months, was indeed caused by an *E. coli* serotype O104:H4 that had acquired a lambdoid prophage carrying the Shiga toxin gene.[20]

Phages are also involved in the transfer of antibiotic resistance genes,[21,22] although this is usually by the process of generalized transduction, and only few examples exist of these genes being an integrate part of the phage genome.[23] A few studies report of prophages containing resistance genes: A prophage in *Streptococcus pyogenes* carries the resistance genes *mef(A)* and *tet(O)*,[24] while a prophage in a *Streptococcus suis* isolate has been found to contain a *tet(W)* gene along with other complete or fragmented genes for antibiotic and heavy metal resistance.[25]

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Considering the impact phages have on bacterial evolution, they should be handled with care to avoid accelerating the spread of undesirable genes. As a first step, thorough characterization of any phage under consideration for therapeutic or industrial use is necessary. Furthermore, only few large-scale functional genomics studies of bacteriophages have been conducted.[22] This is despite the fact that increased research in this area would help us to gain a better understanding of the antibiotics resistance phenomenon and mechanisms, and potentially aid in limiting the antibiotics resistance and its consequences.[26]

The recent advances in nucleotide sequencing techniques and the resulting easy availability of whole genome sequences (WGS) of bacteria has led to the development of a multitude of tools aimed at analysis of this type of data.[27] One such tool is ResFinder,[28] which is aimed at the identification of acquired antibiotic resistance genes in WGS bacterial data. A study that compared traditional phenotypic methods for antimicrobial susceptibility testing to the results obtained by ResFinder, showed high concordance (99.74%) between phenotypic and predicted antimicrobial susceptibility.[29] ResFinder has also been used to search for antibiotic resistance genes in *Acinetobacter baumannii*,[30,31] *Escherichia coli*,[32] *Salmonella enterica*,[33,34] and metagenomic samples from permafrost.[35]

The database of acquired antibiotic resistance genes, which is the foundation of ResFinder, is compiled from existing databases, e.g., the ARDB (http://ardb.cbcb.umd.edu/) and a thorough literature search. Therefore, the database is considered to be reasonably complete, and new genes are continuously being added as they are described in the literature. On the contrary, no comprehensive database exists of virulence genes, and the scientific community is far from comprehending all the factors that influence bacterial pathogenicity. A number of web-services are available for identification of known or predicted bacterial toxins, e.g, BTXpred (http://www.imtech.res.in/raghava/btxpred/), DBETH (http://www.hpppi.iicb.res.in/btox/), and VICMpred (http://imtech.res.in/raghava/vicmpred/), but they all require amino acid sequences as input, and are accordingly not optimal for easy identification of virulence genes in nucleotide sequences. The VFDB database (http://www.mgc.ac.cn/VFs/) enables the search of genes encoding virulence factors from 26 different pathogenic bacterial genera in nucleotide sequences. However, the aim of VFDB is to enable comparisons between different pathogenic bacterial strains and it strives at being the most comprehensive database of virulence factors, containing intrinsic as well as acquired virulence factors. It even contains hypothetical proteins. On the contrary, the VirulenceFinder tool (Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM, Evaluation of Real-Time WGS for Routine Typing, Surveillance and Outbreak Detection of Verotoxigenic *Escherichia coli*. J Clin Microbiol; Under review) aims at the identification of known virulence genes in nucleotide sequences. It contains no housekeeping or hypothetical proteins. It is hence optimized for the examination of phage nucleotide sequences for the discovery of unwanted genes. So far, only virulence genes related to *E. coli* have been included, but work is currently being conducted to extend the databases, e.g., for *Enterococcus* and *Staphyloccus aureus*.

In the present study, we describe how the freely available web-services ResFinder and VirulenceFinder can be used to examine genome sequences of phages and prophages of interest to detect antibiotic resistance and virulence genes. The general applicability of the tools is demonstrated on data sets of 1,642 phage genomes and 1,442 predicted prophages.

Materials and Methods

Data sets

Whole phage genome sequences were obtained from the three public databases RefSeq,[36] INSDC (www.insdc.org), and PhageSEED.[37] Archaeal viruses and bacterial genomes wrongly annotated as bacteriophages were removed. Additionally, duplicate genomes were removed on the basis of their accession numbers. The final data set contained 1,642 phage genomes and in the remainder of the text this will be referred to as the $phage_{db}$ set. An overview of the data set is available in **Supplemental Material**.

Furthermore, the PhiSpy prophage prediction method[38] was used to predict prophages in 1,571 complete bacterial genomes collected from NCBI (http://www.ncbi.nlm.nih.gov/genome) in August 2011. Each analysis by PhiSpy was repeated 24 times as the number and locations of predicted prophages in the same bacterial genome can differ between runs. The final data set of prophages was established as follows: Each predicted prophage had to be detected in all 24 iterations of PhiSpy. A prophage was considered identical between two iterations, if the lengths did not differ by more than 1%, and the start and end coordinates did not differ by more than 0.5% of the length. The final data set contained 1,442 predicted prophage genomes and will be referred to as the prophage set. They are available for download at http://cge.cbs.dtu.dk/services/data.php.

Procedure

Identification of acquired resistance genes

A local, command-line version of the ResFinder tool[28] was used to identify antibiotic resistance genes in the $phage_{db}$ and prophage sets. ResFinder is based on a database of more than 2,000 resistance genes covering 12 types of antimicrobial resistance agents (aminoglycoside, betalactamase, fluoroquinolone, fosfomycin, fusidic acid, glycopeptide, macrolide-lincosamide-streptograminB, phenicol, rifampicin, sulphoamide, tetracycline, and trimethophorim), which is searched using BLAST.[39] The threshold for reporting a match between a gene in the ResFinder database and the input phage genome was set to be 50% identity over at least 3/5 of the length of the resistance gene.

ResFinder is freely available at http://cge.cbs.dtu.dk/services/ResFinder/. **Figure 1** shows the front page of the tool. Only four steps are necessary to perform a prediction: 1) The file containing the sequence of the genome to be analyzed is selected using the "Browse" button. 2) The types of antimicrobial agents toward which the user wishes to search for resistance genes are selected under "Select Antimicrobial configuration." By default, ResFinder searches for resistance genes for all 12 types of antimicrobial agents. 3) The threshold for the percent identity between genes in the ResFinder database and genes in the input genome sequence is selected using the dropdown menu marked "Select

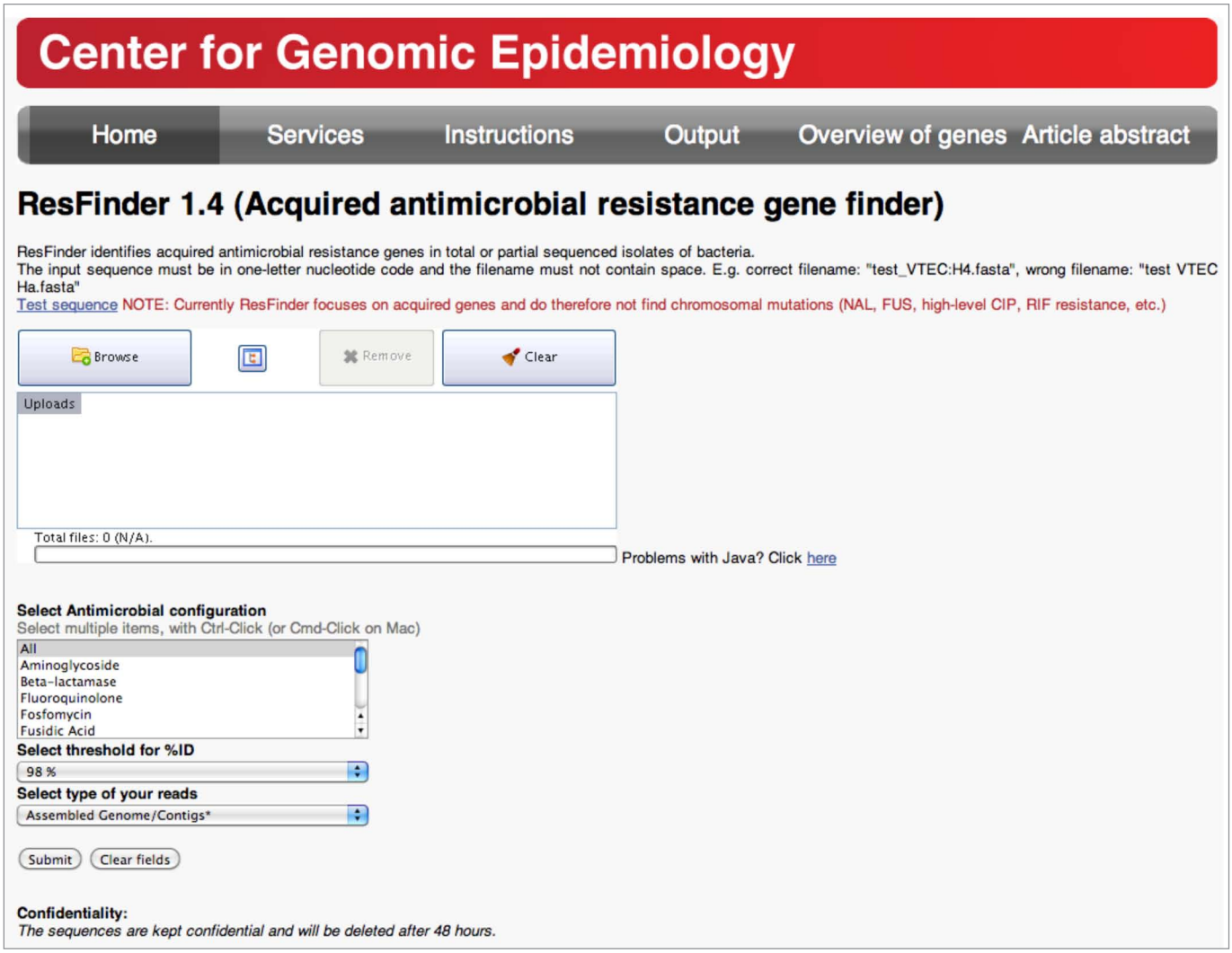

Figure 1. Front page of the ResFinder web-service that searches for acquired antibiotic resistance genes in WGS data. ResFinder is freely available at http://cge.cbs.dtu.dk/services/ResFinder/.

threshold for %ID." By default, only genes in the input genome that are 98% identical to genes in the ResFinder database are reported. 4) The format of the input genome must be specified using the dropdown menu marked "Select type of your reads." By default, the input sequence is expected to be a draft or complete genome in FASTA format, and it is not advisable to change this default setting when analyzing phage genomes.

The time it takes to analyze one genome depends on a number of factors including the network bandwidth capacity of the client computer and the number of jobs queued on the server. Typically, it is below 10 min.

Identification of E. coli *virulence genes*

A local, commandline version of the VirulenceFinder tool (Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM, Evaluation of Real-Time WGS for Routine Typing, Surveillance and Outbreak Detection of Verotoxigenic *Escherichia coli*. J Clin Microbiol; Under review) was used to identify virulence genes associated to *E. coli* in the phage$_{db}$ and prophage sets. The *E. coli* database of virulence genes contains 874 genes and was searched using BLAST.[39] The threshold for reporting a match between a virulence gene and a gene in the input phage genome was set to be 50% identity across at least 3/5 of the length of the virulence gene.

VirulenceFinder is freely available at http://cge.cbs.dtu.dk/services/VirulenceFinder/. It is used in the same manner as ResFinder. However, instead of selecting the types of antimicrobial agents toward which the user wishes to search for resistance genes, he/she should select for which taxonomic group of bacteria he/she wishes to search for virulence genes.

Results

The phage$_{db}$ data set, which contains phage genomes collected from public databases, and the prophage data set, which contains the nucleotide sequence of predicted prophages, were analyzed using ResFinder[28] and VirulenceFinder (Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM, Evaluation of Real-Time WGS for Routine Typing, Surveillance

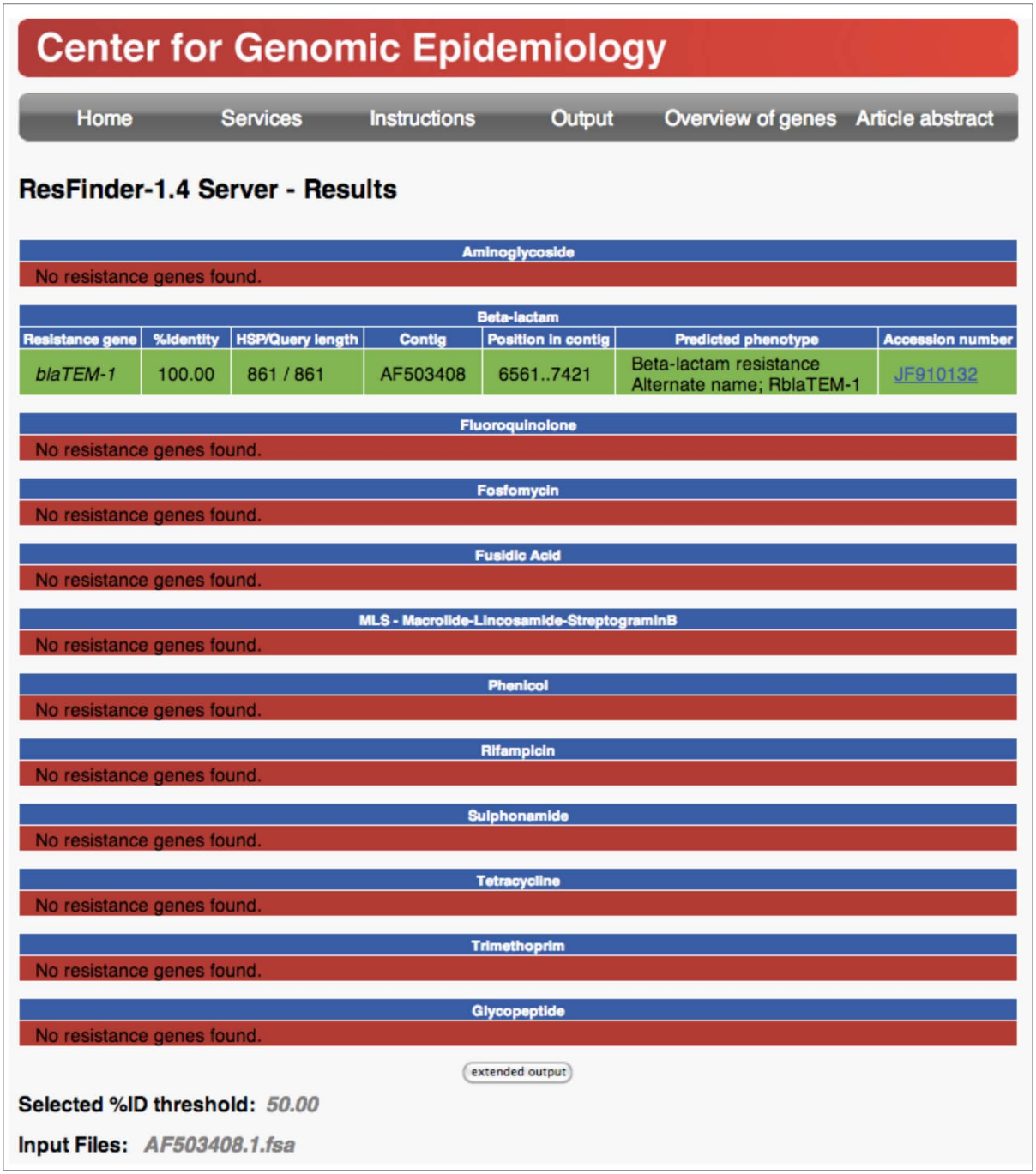

Figure 2. Example of output from the ResFinder web-service, when used to analyze the genome of the Enterobacteria phage P7 (GenBank accession number AF503408). %Identity: Percent identity between the best matching antibiotic resistance gene in the ResFinder database and the corresponding sequence in the input genome. A perfect match is 100%, but must also cover the entire length of the resistance gene (see below). HSP/Query length: HSP length is the length of the High-Scoring Segment Pair (HSP), which is the alignment between the antibiotic resistance gene in the ResFinder database and the corresponding sequence in the input genome. Query length is the length of the antibiotic resistance gene in the ResFinder database. For a perfect match, these two lengths are identical. Contig: The name of the input sequence. Position in contig: Start position and end position of the gene in the input sequence.

and Outbreak Detection of Verotoxigenic *Escherichia coli*. J Clin Microbiol; Under review). ResFinder is aimed at the identification of acquired antibiotic resistance genes and **Figure 2** shows the output page after it has been used to analyze the genome of the Enterobacteria phage P7 (GenBank accession number AF503408). One resistance gene, *blaTEM-1*, which provides Beta-lactam resistance, was identified.

Table 1 lists all the complete and partial antibiotic resistance genes that were identified in the two data sets. In the $phage_{db}$ set, only three genes were identified in three different phage

Table 1. Acquired antibiotic resistance genes in the phage$_{db}$ and prophage data sets

Gene	Acc. no. of gene	Type of resistance	%ID[a]	HSP/Query length[b]	Phage/pro-phage ID	Position in phage/ prophage genome[c]	Host	Data set
blaTEM-1	JF910132	Beta-lactam	100.00	861/861	AF503408	6561..7421	*Enterobacteria*	Phage$_{db}$
aph(3′)-Ia	V00359	Aminoglycoside	100.00	816/816	AY598820	322..1137	NA[d]	Phage$_{db}$
catA1	V00622	Phenicol	99.85	660/660	HM208303	22125..22784	*Escherichia coli*	Phage$_{db}$
mef(A)	AF227521	Macrolide	100.00	1218/1218	uid12469_0.1	47285..48502	*Streptococcus pyogenes* MGAS10394	Prophage
msr(D)	AF227520	Macrolide, Lincosamide and Streptogramin B	100.00	1464/1464	uid12469_0.1	45702..47165	*Streptococcus pyogenes* MGAS10394	Prophage
blaOXA-48	HM755942	Beta-lactam	79.17	600/691	uid13386_0.2	15133..15732	*Shewanella baltica* OS155	Prophage
catB9	AF462019	Phenicol	77.06	497/630	uid13389_0.1	39274..39770	*Shewanella baltica* OS195	Prophage
aac(6′)-aph(2″)	M13771	Aminoglycoside	100.00	1440/1440	uid15757_0.4	21394..22833	*Staphylococcus aureus* subsp. aureus JH9	Prophage
blaZ	AP003139	Beta-lactam	99.76	846/846	uid15757_0.4	9927..10772	*Staphylococcus aureus* subsp. aureus JH9	Prophage
aac(6′)-aph(2″)	M13771	Aminoglycoside	100.00	1440/1440	uid15758_0.4	1378..2817	*Staphylococcus aureus* subsp. aureus JH1	Prophage
blaZ	AP003139	Beta-lactam	99.76	846/846	uid15758_0.4	20339..21184	*Staphylococcus aureus* subsp. aureus JH1	Prophage
erm(A)	AF002716	Macrolide	100.00	732/732	uid16366_0.1	31169..31900	*Streptococcus pyogenes* MGAS10750	Prophage
mef(A)	AF227521	Macrolide	94.16	1215/1218	uid19065_0.0	22045..23259	*Clostridium kluyveri* DSM 555	Prophage
msr(D)	AF274302	Macrolide, Lincosamide and Streptogramin B	92.90	14515/1464	uid19065_0.0	20469..21919	*Clostridium kluyveri* DSM 555	Prophage
aph(3′)-III	M26832	Aminoglycoside	100.00	795/795	uid29179_0.0	111911..112705	*Streptococcus pneumoniae* CGSP14	Prophage
erm(B)	AF368302	Macrolide	99.86	711/711	uid29179_0.0	110153..110863	*Streptococcus pneumoniae* CGSP14	Prophage
cat(pC194)	NC_002013	Phenicol	100.00	651/651	uid29179_0.0	79438..80088	*Streptococcus pneumoniae* CGSP14	Prophage
aac(6′)-aph(2″)	M13771	Aminoglycoside	100.00	1440/1440	uid29567_0.1	33796..35235	*Staphylococcus aureus* subsp. aureus str. JKD6008	Prophage
dfrC	GU565967	Trimethoprim	100.00	486/486	uid29567_0.1	15356..15841	*Staphylococcus aureus* subsp. aureus str. JKD6008	Prophage
qacA	FR821778.1	Multidrug efflux pump toward monovalent and divalent antimicrobial cations	100.00	1545/1545	uid29567_0.1	36751..38295	*Staphylococcus aureus* subsp. aureus str. JKD6008	Prophage
qacE	NC_008253.1	Toward quarternary ammonium compounds and dyes like ethidium bromide	100.00	330/330	uid33411_0.8	27254..27583	*Escherichia coli* IAI39	Prophage
aadA5	AF137361	Aminoglycoside	100.00	789/789	uid33415_0.1	10621..11409	*Escherichia coli* UMN026	Prophage
mph(A)	D16251	Macrolide	100.00	906/906	uid33415_0.1	18792..19697	*Escherichia coli* UMN026	Prophage
sul1	AY224185	Sulphonemide	100.00	840/840	uid33415_0.1	14171..15010	*Escherichia coli* UMN026	Prophage
dfrA17	FJ460238	Trimethoprim	100.00	474/474	uid33415_0.1	10017..10490	*Escherichia coli* UMN026	Prophage
blaTEM-1	JF910132	Beta-lactam	100.00	861/861	uid33415_0.1	21108..21968	*Escherichia coli* UMN026	Prophage
catA1	V00622	Phenicol	99.85	660/660	uid33415_0.1	2216..2875	*Escherichia coli* UMN026	Prophage
qacEdelta1	AB733642.1	Toward quarternary ammonium compounds and dyes like ethidium bromide	100.00	348/348	uid33415_0.1	15004..15351	*Escherichia coli* UMN026	Prophage
aph(3′)-Ia	V00359	Aminoglycoside	100.00	816/816	uid33775_0.1	3096..3911	*Escherichia coli* BW2952	Prophage
tet(L)	M29725	Tetracycline	100.00	1377/1377	uid34729_0.0	32749..34125	*Streptococcus gallolyticus* UCN34	Prophage
tet(M)	EU182585	Tetracycline	96.46	1920/1920	uid34729_0.0	34319..36238	*Streptococcus gallolyticus* UCN34	Prophage
oqxB	EU370913	Olaquindix	79.09	2449/2450	uid50601_0.6	1241..3687	*Rahnella sp.* Y9602	Prophage
VanZ-F	AF155139	VanF vancomycin operon, (VanR-F, VanS-F, VanY-F and VanZ-F)	84.49	445/621	uid60447_0.0	23599..24043	*Bacillus thuringiensis* serovar finitimus YBT-020	Prophage
VanZF-Pp	AF155139	VanPp vancomycin operon, (VanAE-Pp, VanHE-Pp, VanXE-Pp, VanYF-Pp and VanZF-Pp)	84.49	445/621	uid60447_0.0	23599..24043	*Bacillus thuringiensis* serovar finitimus YBT-020	Prophage

[a]Percent identity between the best matching antibiotic resistance gene in the ResFinder database and the corresponding sequence in the input genome. [b]HSP length: The length of the High-Scoring Segment Pair (HSP), which is the alignment between the antibiotic resistance gene in the ResFinder database and the corresponding sequence in the input genome. Query length: The length of the antibiotic resistance gene in the ResFinder database. [c]Start position..end position. [d]This phage is a helper phage for phage display.

genomes. In the prophage set, 14 predicted prophages were found to contain a total of 31 resistance genes. Some of the identified resistance genes diverged from the reference genes in the ResFinder database. As an example, the *blaOXA-48* gene, which is contained in a predicted prophage in *Shewanella baltica* OS155, covered only 600 of 691 nucleotides of the reference gene and was only 79.17% identical. Further analysis would be necessary to examine whether the gene is still functional.

Table S1 lists all virulence genes known to be associated with *E. coli* that were identified in the two data sets. In the $phage_{db}$ set 54 complete or partial genes were identified in 24 phage genomes. In the prophage set, 70 complete or partial genes were identified in 51 predicted prophages.

Discussion

In the present study, we have analyzed the genomes of phages collected from public databases and of prophages predicted from bacterial genomes with regards to the presence of acquired antibiotic resistance genes and virulence genes associated with *E. coli*.

Among the phage genomes, only three phages contained antibiotic resistance genes. Small as this number is, two of the phages were even engineered to carry the resistance genes (HM208303[40,41] and AY598820[42]). Only P7, with accession number AF503408, is one of the rare examples of phages carrying antibiotic resistance determinants without human interventions. In the late 1970s, P7 was found to carry the gene for ampicillin resistance.[23]

Fourteen predicted prophages contained a total of 31 complete or partial resistance genes. We are aware that some of the predicted prophages might be wrongly identified by the PhiSpy prediction tool, and hence may not be *actual* prophages. However, we have not performed further analysis to confirm the validity of these prophages, since the main purpose of this study was to demonstrate the usefulness of freely available web-services for the identification of unwanted genes. Even so, the result may have biological relevance, as even cryptic prophages as well as genetic elements with close homology to parts of phages, have the potential to be involved in the dissemination of antibiotic resistance or virulence genes, as has been shown in a study of defective prophages in *E. coli*[43] and described in a review by Casjens.[44]

The current version of VirulenceFinder only enables the identification of virulence genes related to *E. coli* and as such it is far less complete than the database of antibiotic resistance genes. However, work is being performed to extend the tool to include virulence genes related to other taxonomic groups, e.g., *Enterococcus* and *Staphylococcus aureus*.

Conclusion

The concern that has been raised about the application of phages in relation to their ability to disseminate antibiotic resistance and virulence genes can be addressed by analyzing the phage genomes using freely available web-services. Two such web-services, ResFinder and VirulenceFinder, were tested in this study, and found to be suitable for identifying both types of genes in phage and prophage genomes. Similar in silico screenings are likely to become increasingly important to enable more specific and efficient investigations in the laboratory. Additionally, they can be used for large-scale functional genomic studies and characterisations of phages.

Note

ResFinder and VirulenceFinder are freely available at http://cge.cbs.dtu.dk/services/ResFinder/ and http://cge.cbs.dtu.dk/services/VirulenceFinder/. Analyzing one phage genome typically takes less than 10 min.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/bacteriophage/article/27943

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