ანტიბიოტიკორეზისტენტობა შავი ზღვის მიკრობულ პოპულაციებში და ბაქტერიოფაგების როლი რეზისტენტობის გენების ჰორიზონტალურ გადატანაში

Antibiotic Resistance in Black Sea Microbial Populations and the Impact of Bacteriophages on Horizontal Transfer of Antibiotic Resistance Genes

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Declaration

For the presented dissertation „Antibiotic Resistance in Black Sea Microbial Populations and the Impact of Bacteriophages on Horizontal Transfer of Antibiotic Resistance Genes“, I declare that the dissertation is my own work, to the best of my knowledge and belief, it does not include plagiarism from other authors' previously published publications, I have fully cited and referenced all material and results that are not original to this work. All information in this thesis has been obtained and presented in „accordance with academic rules and ethical conduct.

Ekaterine Gabashvili

List of Published Papers

1. **Gabashvili E**, Kobakhidze S, Chkhikvishvili T, Tabatadze L, Tsiklauri R, Dadiani K, Koulouris S, Kotetishvili M (2022) Metagenomic and recombination analyses of antimicrobial resistance genes from recreational waters of Black Sea coastal areas and other marine environments unveil extensive evidence for their both intrageneric and intergeneric transmission across genetically very diverse microbial communities. Marine Genomics 61:100916. https://doi.org/10.1016/j.margen.2021.100916 PMID: 34922301

(Gabashvili et al. 2022)

2. **Gabashvili E**, Osepashvili M, Koulouris S, Ujmajuridze L, Tskhitishvili Z, Kotetishvili M. Phage Transduction is Involved in the Intergeneric Spread of Antibiotic Resistance-Associated *blaCTX-M*, *mel*, and *tetM* Loci in Natural Populations of Some Human and Animal Bacterial Pathogens. Curr Microbiol. 2020 Feb;77(2):185-193. doi: 10.1007/s00284-019-01817-2. Epub 2019 Nov 21. PMID: 31754824.

(Gabashvili et al. 2020)

3. **Gabashvili E**, Kobakhidze S, Chkhikvishvili T, Tabatadze L, Tsiklauri R, Dadiani K, Kotetishvili M. Bacteriophage-Mediated Risk Pathways Underlying the Emergence of Antimicrobial Resistance via Intrageneric and Intergeneric Recombination of Antibiotic Efflux Genes Across Natural populations of Human Pathogenic Bacteria. Microb Ecol. 2021 Sep 1. doi: 10.1007/s00248-021-01846-0. Epub ahead of print. PMID: 34467445.

(Gabashvili et al. 2021a)

4. **Gabashvili E**, Kobakhidze S, Koulouris S, Robinson T, Kotetishvili M. Bi- and Multi-directional Gene Transfer in the Natural Populations of Polyvalent Bacteriophages, and Their Host Species Spectrum Representing Foodborne Versus Other Human and/or Animal

Pathogens. Food Environ Virol. 2021 Jun;13(2):179-202. doi: 10.1007/s12560-021-09460-6. Epub 2021 Jan 23. PMID: 33484405.

(Gabashvili et al. 2021b)

Additional Activity:

Panosyan H, Margaryan A, Poghosyan L, Saghatelyan A, **Gabashvili E**, Jaiani E Birkeland N. K(2018) Microbial Diversity of Terrestrial Geothermal Springs in Lesser Caucasus. In: Egamberdieva D., Birkeland NK., Panosyan H., Li WJ. (eds) Extremophiles in Eurasian Ecosystems: Ecology, Diversity, and Applications. Microorganisms for Sustainability, vol 8. Springer, Singapore. https://doi.org/10.1007/978-981-13-0329-6_4

(Panosyan et al. 2018)

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სარჩევი

1.1 Introduction

1.1.1 Antimicrobial Resistance Genes in Microbial Populations of Recreational Waters of the Black Sea Coastal Areas of Georgia

Antimicrobial resistance is one of the major challenges of global public health (Mootapally et al. 2019) and is of particular concern for developing countries (Cuadrat et al. 2020). Clinical bacterial strains are increasingly becoming resistant to antibiotics, which poses a serious threat to human health (Beceiro et al. 2013). Each year, at least 700,000 people worldwide die from infections caused by bacterial pathogens resistant to multiple drugs (Rodríguez-Verdugo et al. 2020), and if preventive measures are not taken, the human mortality rate could reach 10 million by 2050 (O'Neill 2014; Jasovský et al. 2016). Antibiotic-resistant bacteria have been isolated from various environments, including marine ecosystems, especially those that are exposed to human activities (Alonso et al. 2001; Dang et al. 2007; Zhao and Dang 2012; Buschmann et al. 2012). The marine microbiome has therefore been recognized as a complex reservoir of antimicrobial resistance genes (ARGs) (Mootapally et al. 2019). Hense, the monitoring of ARGs in aquatic environments, including coastal waters, has being actively pursued worldwide. Recent studies on natural aquatic environments (rivers, lakes, and coastal areas) indicate that a pollution of these ecosystems by ARGs, exhibiting a fairly broad spectrum of diversity, is clinically significant. This has been primarily due to the contamination of these environments by various antibiotics, and to the selective pressures that these antibiotics exert on bacteria (Kolář et al. 2001; Zhao and Dang 2012; Du et al. 2019; Su et al. 2020). Moreover, in the natural aquatic environment, these ARG genes gradually become more widespread favored partially by climate warming (McGough et al. 2018; Rodríguez-Verdugo et al. 2020), diversifying further at fairly high rates (Hatosy and Martiny 2015; Su et al. 2020).

The variation in the environmental resistome (a global pool of ARGs) has been due to mutations and horizontal gene transfer (HGT) (Bengtsson-Palme et al. 2018) leading to the incorporation of new DNAs into the genomes of recombinant strains (Su et al. 2020; Cuadrat et al. 2020). HGT has contributed also to bacterial speciation (Lawrence and Retchless 2009). Genetic variations, introduced in living organisms, can lead to phenotypic heterogeneity in a population that is both heritable and adaptive (Payne and Wagner 2019). In this context, genetic recombination of ARGs has already been suggested to influence antibiotic tolerance, long-term persistence of antimicrobial resistance, and ecological fitness of different bacterial populations (McGough et al. 2018; Rodríguez-Verdugo et al. 2020). Therefore, studying the distribution, diversity and HGT of antibiotic resistance genes in aquatic environments, contributes significantly to our understanding of public health crisis caused by the continued emergence of antimicrobial resistance. Thus, more in-depth studies are needed to elucidate the molecular-genetic mechanisms underlying the emergence and persistence of antimicrobial resistance, its determinants, as well as the diversity of ARGs in marine microbial populations. It must be indicated that our knowledge of the genetic diversity of ARG-carriers and that of a spectrum of ARGS across various marine environment, especially across the recreational waters of the Georgian Black Sea coastal area, is very limited.

The chapter 3.1 describes our first pilot study, using metagenomic approaches, elucidating the genetic diversity of ARGs in recreational waters of the Black Sea coastal areas of Batumi, Georgia (Gabashvili et al. 2022). Here, we also describe HGT events and their trajectories involving these ARGs across microbial populations from the above area. In particular, our metagenomic study, examining the Green Cape and Batumi Boulevard coastal recreational waters, has revealed ARGs exhibiting predominantly those intrinsic antimicrobial resistance mechanisms that can be present in microbial populations even in the absence of selective antibiotic pressures.

Some of the ARGs from the Black Sea surface waters were found to encode both drug-specific and multidrug efflux transporters associated with certain human pathogens (e.g., some species from the genera of *Vibrio* and *Aeromonas*) from aquatic environments, and with non-pathogenic marine bacteria such as *Synechococcus, Rhodobacteraceae, Pseudoalteromonas, Altererythrobacter, Erythrobacter, Altererythrobacter, Loktanella,* and several other genera. Using in *silico* recombination analyses, our studies have also shown that the bacteria that are carriers of ARGs can be involved in the genetic recombination, transferring these ARGs across genetically fairly distinct aquatic microbial communities.

1.1.2 Bacteriophage Transduction-Mediated Genetic Recombination of Antimicrobial Resistance Genes

Microbes can interact better in an aquatic environment, facilitating dissemination of antibiotic resistance genes (Coutinho et al. 2014). Acquisition of antimicrobial resistance genes by bacteria can occur through bacteriophage transduction, although this phenomenon still needs to be more rigorously studied. Generally, bacterial DNA is transferred from a donor bacterium to a recipient bacterium by phages, which, along with some other environments, also commonly takes place in aqueous environments. For example, a quantitative model suggests that 1.3×10^{14} phage transduction events per year can be expected in Tampa Bay (Jiang and Paul 1998), while a global rate of the above phenomenon is estimated to equal approximately 2×10^{16} per second (Wang et al. 2016).

Phage-mediated transduction is expected to contribute significantly to the dissemination of ARGs (Colavecchio et al. 2017). Bacteriophages, also known as bacterial viruses, are the most widespread organisms, including 10^{31} phages with tails (Hendrix et al. 1999). They are widely distributed and are found in oceans, lakes, soils, sewage, drinking

water, and microbial plant populations (Wommack and Colwell 2000; Brüssow and Hendrix 2002; Hendrix 2002; Wilhelm et al. 2002; Hambly and Suttle 2005; Suttle 2005; Clokie et al. 2011). Phages, as agents of horizontal gene transfer, are thought to have a significant impact on the evolution of microbes. Phage transduction can occur even when the donor and recipient bacteria are not in the same location or at the same time, as the free phage particles can carry ARG(s) before infecting the recipient strain. In addition, free phages have been found to persist longer in the environment than their hosts (Muniesa et al. 2011; Marti et al. 2014). Moreover, HGT can be mediated by transducing phages regardless of their lifestyle (Chiang et al. 2019). Therefore, bacteriophages may play an important role in the dissemination of ARGs among bacteria. For example, monovalent phages, infecting only one species of bacterial host, have been found to transfer ARG(s) within *E. coli* natural populations (Colavecchio et al. 2017). In addition to monovalent phages, there exist also polyvalent bacteriophages that can infect a wide range of bacterial strains, not only from a single genus, but also from different bacterial genera (Jensen et al. 1998; Sullivan et al. 2003; Bielke et al. 2007; Grose and Casjens 2014). Some polyvalent phages with various host species are suggested to facilitate the spread of ARGs on both the inter-species and even intergeneric levels (Muniesa et al. 2013). ARGs dissmeniation by polyvalent phages on the interspecies levels within the genera *Enterococcus* (Mazaheri Nezhad Fard et al. 2011), *Staphylococcus*, (Zeman et al. 2017) and *Salmonella* (Zhang and LeJeune 2008) have been studied under laboratory conditions.

Our analyses (Gabashvili et al. 2020), using *in silico* recombination detection methods, showed that polyvalent phages can exchange ARGs not only at the intra- and inter-species levels, but also at intergeneric levels in natural populations of bacteria. We found that genetic recombination of *blaCTX-M* (broad-spectrum β-lactamase-encoding gene), *mel* (ABC-type efflux-permease-encoding gene) and *tet*M (ribosome-protective protein-encoding gene), can

be mediated by bacteriophages between the genetically very diverse strains collectively representing *Escherichia, Salmonella, Streptococcus, Bacillus* and *Erysipelothrix*.

Our study also strongly suggested that the *Salmonella* phage can serve as a vehicle for the transmission of the *bla*CTX-M gene between *E. coli* and *S. enterica* at the intergeneric levels. Moreover, the recombination tests suggested that in natural populations of *S. suis* and *E. faecium*, the *tet*M gene could be propagated by the *Erysipelothrix* phage. Furthermore, our study detected an exchange of the *mel* gene between phages from different genera, namely *Erysipelothrix* and *Streptococcus*.

Antimicrobial resistance in bacteria can be caused not only by acquired resistance genes, but also by a bacteria-specific intrinsic resistance mechanism associated with inherited structural and/or functional traits, such as efflux pumps (Lynch et al. 2013), which can be sometimes also acquired. The efflux pump transporters in gram-positive and gram-negative bacteria expel toxic compounds and antibiotics from their intracellular environment (Webber 2003). In bacteria, efflux pumps are commonly implicated in generating antimicrobial resistance and biofilm formation (Poole 2007; Nikaido and Pagès 2012; Soto 2013; Alcalde-Rico et al. 2016; Alav et al. 2018). These genes, encoding for membrane transport proteins, are not restricted to bacteria phyla. They are also present in some viruses including certain bacterial viruses, bacteriophages (Greiner et al. 2018). Various membrane transporters have been discovered in phages, including K^+ channel, Mg^{2+} transporter, sodium glucose transporter, sodium calcium transporter, and nucleotide transporters (Greiner et al. 2018).

Efflux pumps encoded by some genes found in certain genomes of both bacteria and bacteriophages could also serve as antibiotic transporters. Antibiotic efflux pump genes (MFS [Major Facilitator Superfamily], the ATP-binding cassette family, and RND family [resistance-nodulation-division]) were identified in *A. baumannii* prophages elements (Costa et al. 2018). The prophages represent integrated phage genomes in the bacterial chromosome.

Nevertheless, the presence of efflux antibiotic resistance genes in phages remains controversial, because such prophages genomes can be affected and modified by bacterial defense systems during integration into a bacterial chromosome as a prophage, and thus can sometime become defective (Canchaya et al. 2004; Pfeifer et al. 2021). We have yet to determine whether phages contribute to the dissemination of the genetic loci encoding for antibiotic efflux pumps, especially those that confer multi-drug resistance, in bacteria.

We have conducted meta-analysis screening for antimicrobial resistance-associated efflux pump genes across phage genomes deposited to the NCBI viral database, which includes the genomes of lytic and temperate phages, as well as induced prophage genomes.

Our study (Gabashvili et al. 2021a) demonstrated that bacteriophages can acquire genes encoding for efflux pumps leading to antimicrobial resistance, such as the Major Facilitator Superfamily (MFS) tranposters, ATP-binding cassette (ABC) transporters, resistance-nodulation-division (RND) family transporters. The phages containing efflux transporter genes are isolated from a wide range of pathogens, including *Salmonella enterica, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Burkholderia* pseudomallei, and they can spread these genes even further.

The array of recombination analysis, performed *in silico*, revealed that antimicrobial resistance efflux pump genes are transferred by bacteriophages at both intra- and inter- species levels in the natural bacterial population, represented by *Salmonella enterica, Mycobacterium smegmatis, Pseudomonas aeruginosa, Burkholderia pseudomallei, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus suis*, and *Streptococcus gallolyticus*.

1.1.3 The Life Cycle of Phages and Their Evolutionary Divergence

Understanding the coevolution of phage-host and phage-phage is essential for phage preparations designed for therapeutic, food safety or environmental safety applications. Recent

observations, as well as our own, collectively suggest that antimicrobial resistance or virulence genes are mainly associated with phages that exhibit a temperate life cycle (de la Cruz and Davies 2000; Boyd 2012; Beceiro et al. 2013; Brown-Jaque et al. 2015; Manaia 2017; Deng et al. 2019; Gabashvili et al. 2020; Gabashvili et al. 2021b), remaining latent for prolonged periods when infecting their bacterial hosts.

Lytic phages are presumed to have a wide range of therapeutic applications against pathogens given that they lyse bacteria immediately after replication of their virions. However, recently, the traditional methods, used for classification of phages, have been challenged by the discovery of phage plasmids, which disrupt the clear distinction between lytic and temperate phages and highlight the impact of phages evolution on their distinct lifestyles (Hockenberry and Wilke 2020; Pfeifer et al. 2021). The evolution of phages can be also driven by interactions between co-infecting phages (Joseph et al. 2009; Refardt 2011; Roux et al. 2015), which could be crucial for bacterial host cell survival. In this context, it is also common for phages to participate in horizontal gene transfer (Worobey and Holmes 1999; Dang et al. 2004; Casjens 2005; Čičin-Šain et al. 2005; Kupczok et al. 2018). Consequently, obtaining a better understanding of host coinfections caused by specific phages and phage-phage or phage-host interactions, which in turn affect the larger microbiome, becomes essential.

Our study describes that intra-species, interspecies, and even intergeneric recombination events occur not only among the virulent phages or their temperate conspecifics, but also between phages with these different lifestyles during coinfections (Gabashvili et al. 2021b). In addition, it provides evidence for both bi- and multi-directional intergeneric recombination of genes involved collectively in phage morphogenesis, host specificity, and replication.

1.2 ვრცელი აბსტრაქტი

1.2.1 ანტიმიკრობული რეზისტენტობის გენები შავი ზღვის მიკრობულ პოპულაციებში

ანტიმიკრობული რეზისტენტობა მსოფლიო ჯანდაცვის ერთ-ერთი ძირითადი გამოწვევაა (Mootapally et al. 2019) და განსაკუთრებით მწვავე პრობლემას წარმოადგენს განვითარებადი ქვეყნებისთვის (Cuadrat et al. 2020). ბაქტერიული შტამები საკმაოდ ხშირად ინვითარებენ რეზისტენტობას ანტიბიოტიკების მიმართ, რითიცმნიშვნელოვან საფრთხეს უქმნიან ადამიანის ჯანმრთელობას (Beceiro et al. 2013). ყოველწლიურად მსოფლიოში მინიმუმ 700 000 ადამიანი იღუპება მულტირეზისტენტული ბაქტერიული პათოგენებით გამოწვეული ინფექციების შედეგად (Rodríguez-Verdugo et al. 2020) და ნავარაუდებია, რომ სათანადო პრევენციული ზომების გატარების გარეშე, მათგან განპირობებულმა ადამიანთა გარდაცვალების მაჩვენებელმა შესაძლოა წლიურად 10 მილიონს მიაღწიოს 2050 წელს (O'Neill 2014; Jasovský et al. 2016). ანტიბიოტიკების მიმართ რეზისტენტული ბაქტერიები, შესაძლოა გამოყოფილ იქნას სხვადასხვა გარემოდან, მათ შორის ზღვის იმ ეკოსისტემებიდან, რომლებიც განიცდიან ანთროპოგენულ ზემოქმედებას (Alonso et al. 2001; Dang et al. 2007; Zhao and Dang 2012; Buschmann et al. 2012). შესაბამისად, ზღვის მიკრობიომები უკვე მიიჩნევა ანტიბიოტიკორეზისტენტობის გენების (ARGs) კომპლექსურ რეზერვუარებად (Mootapally et al. 2019). ამასთანავე, გარემოში გავრცელებული ბაქტერიები მნიშვნელოვან როლს ასრულებენ ანტიმიკრობული რეზისტენტობის გენების ფართოდ გადაცემაში სხვადასხვა ბაქტერიულ პოპულაციაში, მათ შორის ადამიანისა და ცხოველების მრავალ პათოგენურ ბაქტერიულ სახეობაში და მაშასადამე ხელს უწყობენ ანტიბიოტიკების მიმართ რეზისტენტულობის უნარის მქონე შტამების წარმოქმნას. შესაბამისად, დღესდღეობით ARG–ების შესწავლა და მონიტორინგი წყლის გარემოში,

განსაკუთრებით კი ზღვის სანაპირო რეკრეაციულ წყლებში, აქტიურად ხორციელდება მსოფლიოში (Baker-Austin et al. 2009; Port et al. 2012; Hatosy and Martiny 2015; Du et al. 2019). აღსანიშნავია, რომ სხვადასხვა ბუნებრივი წყლის გარემოს (მდინარეები, ტბები და ზღვის სანაპირო ადგილები) შესწავლისას გამოვლენილია კლინიკურად მნიშვნელოვანი ARG–ების შემცველობის მაღალი დონე ამ ეკოსისტემებში, რაც ძირითადად უკავშირდება სხვადასხვა ანტიბიოტიკებით ამ გარემოს დაბინძურებასა და ანტიბიოტიკების მიერ ბაქტერიებზე არსებულ შერჩევით წნეხს (Kolář et al. 2001; Zhao and Dang 2012; Du et al. 2019; Su et al. 2020). ამასთანავე, მოსალოდნელია, რომ გლობალური კლიმატური დათბობის (McGough et al. 2018; Rodríguez-Verdugo et al. 2020) ხელშეწყობით აღნიშნული AR გენები წყლის ბუნებრივ გარემოში შესაძლოა კიდევ უფრო სწრაფად გავრცელდნენ და შეიძინონ გენეტიკური მრავალფეროვნება (Hatosy and Martiny 2015; Su et al. 2020).

გარემოს რეზისტომების (ARG-ების გლობალური აუზი) ცვალებადობა შესაძლოა გამოიწვიონ როგორც მუტაციებმა, ისე გენთა ჰორიზონტალურმა გადაცემამ (HGT) (Bengtsson-Palme et al. 2018), რომლის დროსაც ახალი დნმ-ის ფრაგმენტების ჩართვა რეკომბინანტული შტამების გენომებში ხორციელდება გენეტიკური რეკომბინაციის გზით (Su et al. 2020; Cuadrat et al. 2020). აღსანიშნავია, რომ ზოგადად, HGT-ამ შესაძლოა გამოიწვიოს ახალი ბაქტერიული სახეობების ჩამოყალიბებაც (Lawrence and Retchless 2009). ცოცხალ ორგანიზმებში შეტანილმა გენეტიკურმა ვარიაციებმა შესაძლოა განაპირობონ ფენოტიპური ჰეტეროგენულობა პოპულაციებში, რაც მემკვიდრეობითია და ასევე ადაპტირებადი (Payne and Wagner 2019). შესაბამისად, ნავარაუდებია, რომ ARG-ების გენეტიკური რეკომბინაციამ, შესაძლოა, გავლენა მოახდინოს ანტიბიოტიკების მიმართ ბაქტერიის ტოლერანტობაზე, პერსისტენტობაზე და სხვადასხვა ბაქტერიული პოპულაციის ეკოლოგიურ ადაპტირებაზე (McGough et al. 2018; Rodríguez-Verdugo et al. 2020). ამრიგად,

ანტიმიკრობული რეზისტენტობის აღმოცენებისა და გავრცელების შესაძლო ახალი ტრენდებისგამოსავლენად, კრიტიკულია შესწავლილ იქნას ანტიმიკრობული რეზისტენტობის მაინდუცირებელი გენების შედგენილობა, მათი მრავალფეროვნებადა გავრცელების მოლეკულურ-გენეტიკური მექანიზმები წყლის გარემოში. შესაბამისად, საჭიროა სიღრმისეული კვლევები იმ მოლეკულურ-გენეტიკური მექანიზმების დასახასიათებლადაც, რომლებიც საფუძვლად უდევს ანტიმიკრობული რეზისტენტულობის და გარემოს მიმართ პერსისტენტობის სინერგიულ აღმოცენებას წყლისმიერ ბაქტერიებში, მათ შორის ზღვის მიკრობულ პოპულაციებში. ამ თვალსაზრისით, უნდა აღინიშნოს, რომ ჩვენი ცოდნა ძალიან მწირია იმ ARG-ების და მათი მტარებელი ბაქტერიების გენეტიკურ მრავალფეროვნებაზე, რომლებიც არსებობენ საქართველოს შავი ზღვის სანაპირო ზონის რეკრეაციულ წყლებში.

ჩვენ მიერ,მეტაგენომური დნმ-ის სიქვენირების და ანალიზის გამოყენებით, განხორციელებულია პირველი მნიშვნელოვანი საპილოტე კვლევა, რომელიც აღწერს საქართველოში, შავი ზღვის ბათუმის სანაპირო ზოლის რეკრეაციულ წყლებში არსებული ARG-ების გენეტიკურ მრავალფეროვნებას ამ გენების ჰორიზონტალური გადაცემის მოლეკულურ-გენეტიკურ მექანიზმებს და ამასთანავე, მათი გადაცემის ტრაექტორიებს მიკრობულ პოპულაციებში (Gabashvili et al. 2022).

კერძოდ, ჩვენს მიერ ჩატარებული მეტაგენომური კვლევებით გამოვლენილ იქნა, რომ მთელი რიგი ანტიბიოტიკების იფლაქსის ტუმბოების მაკოდირებელი გენების არსებობა მიკრობულ პოპულაციებში, შეიძლება განპირობებული იყოს ანტიბიოტიკების შერჩევითი წნეხის გარეშეც. ჩვენს მიერ აღმოჩენილი ზოგიერთი ARG-ები წარმოადგენენ ანტიმიკრობული რეზისტენტობის, მათ შორის მულტირეზისტენტობის, განმსაზღვრელ ანტიბიოტიკების იფლაქსის ტრანსპორტერების მაკოდირებელ გენებს, რომლებიც გამოვავლინეთ როგორც

ადამიანის პათოგენებში (მაგალითად, *Vibrio*-ს და *Aeromonas* გვარებიდან) და ისე არაპათოგენურ ბაქტერიებში *Synechococcus*-ის, *Rhodobacteraceae*-ის, *Pseudoalteromonas*-ს, *Altererythrobacter*-ის, *Erythrobacter*-ის, *Altererythrobacter*-ის, *Loktanella*-ს და ზოგიერთი სხვა გვარებიდან. მნიშვნელოვანია აღინიშნოს, რომ ჩვენ მიერ ჩატარებულ *In Silico* რეკომბინაციული ტესტების შედეგების თანახმად, რომლებიც ჩატარდა დნმ-ის ანალიზის პროგრამულ პაკეტებში, როგორებიცაა Splitree, RDP4, GARD და Simplot, იმპლემენტირებული არსებითად განსხვავებული რეკომბინაციის დეტექციის ალგორითმებით, გამოვლინდა ამ რეზისტენტობის გენების ჰორიზონტალური გადაცემა როგორც ახლომონათესავე, ისე გენეტიკურად მნიშვნელოვნად განსხვავებულ წყლის ბაქტერიულ მიკრობიოტაში. ამასთანავე, რეკომბინაციული ტესტებიდან ჩვენს მიერ მიღებულმა შედეგებმა ასევე გამოავლინეს ზემოაღნიშნული სამიზნე გენების ბი- და მულტი-ლატერული გადაცემის ტრაექტორიები დონორ და რეციპიენტ ბაქტერიებს შორის. რეკომბინაციულმა ანალიზებმა უჩვენეს ისიც, რომ ერთი და იგივე ბაქტერიული შტამი, ერთის მხრივ, შეიძლება წარმოადგენდეს ანტიმიკრობული რეზისტენტობის მაკოდირებელი გენეტიკური ლოკუსის რეციპიენტს (რეკომბინანტს), ხოლო მეორე მხრივ, თავად დონორს.

1.2.2 ბაქტერიოფაგების როლი ანტიმიკრობული რეზისტენტობის გენების ჰორიზონტალურ გადაცემაში

წყლის გარემო ოპტიმალური პიროებებს ქმნის ანტიმიკრობული რეზისტენტობის განმსაზღვრელი გენების გავრცელებისათვის , რადგან იგი ხელს უწყობს მიკრობთა შორის ადვილ ურთიერთქმედებას (Coutinho et al. 2014). ცნობილია,

რომ ანტიმიკრობული რეზისტეტობის გენების გადაცემა ბაქტერიებში შესაძლოა განხორციელდეს ბაქტერიოფაგების მიერ ტრანსდუქციის გზით, თუმცა აღნიშნული ფენომენი ჯერ კიდევ მეტ სირღმისეულ შესწავლას საჭიროებს. ფაგური ტრანსდუქცია, მნიშვნელოვნად უწყობს ხელს ანტიმიკრობული რეზისტენტობის გენების გავრცელებას (Colavecchio et al. 2017). აღსანიშნავია, რომ გარემოში გავრცელებულ ბაქტერიულ შტამებში, ტრანსდუქციული პროცესები და ფაგების მიერ დონორიდან რეციპიენტისთვის ბაქტერიული დნმ-ის გადაცემა მუდმივად მიმდინარეობას. რაოდენობრივი მოდელით ზემოაღნიშნული მოვლენის შესწავლამ განისაზღვრა, რომ დაახლოებით 1.3×10^{14} ტრანსდუქციის შემთხვევას აქვს ადგილი ყოველწლიურად მაგალითად ტამპას ყურის მდინარეში (Jiang and Paul 1998), ხოლო გლობალური მასშტაბით, ფაგების მიერ გენების ჰორიზონტალური გადაცემის გლობალური მაჩვენებელი დაახლოებით 2×10^{16} /წამში შეადგენს (G. H. Wang et al. 2016).

ბაქტერიოფაგები, ბაქტერიული ვირუსები, წარმოადგენენ ყველაზე ფართოდ გავრცელებულ ორგანიზმებს: განისაზღვრა, რომ დაახლოებით 10^{31} კუდის მქონე ფაგის ვირიონია (Hendrix et al. 1999) გავრცელებული ბიოსფეროში; სხვადასხვა ეკოსისტემებს შორის, ისინი ფართოდ არიან გავრცელებული ოკეანეებში, ტბებში, ნიადაგში, ურბანული წყალარინების სისტემებში, სასმელ და ჭის წყალში (Clokie et al. 2011; Wommack and Colwell 2000; Brüssow and Hendrix 2002; Wilhelm et al. 2002; Hendrix 2002; Hambly and Suttle 2005; Suttle 2005). ფაგები, როგორც ბაქტერიულ პოპულაციებში გენების ჰორიზონტალური გადაცემის ვექტორები, სავარაუდოდ მნიშვნელოვან გავლენას ახდენენ მათ ევოლუციაზე. ფაგური ტრანსდუქცია შეიძლება განხორციელდეს მაშინაც კი, როდესაც დონორი და რეციპიენტი ბაქტერიები არ იმყოფებიან ერთსა და იმავე დროს ერთსა და იმავე ლოკაციაზე, დაშორებულ მანძილზე მიგირებული ფაგის მიერ პოტენციური რეკომბინანტისთვის გენეტიკური ლოკუსის გადაცემის გზით. გარდა ამისა, აღმოჩნდა, რომ თავისუფალი ფაგები

გარემოში უფრო ხანგრძლივად ინარჩუნებენ სიცოცხლისუნარიანობას, ვიდრე მათი მასპინძლები (Muniesa et al. 2011; Marti et al. 2014). მეტიც, რეკომბინანტში HGT შეიძლება განხორციელდეს ფაგური ტრანსდუქციით განურჩევლად მისი სასიცოცხლო ციკლისა (Chiang et al. 2019). აღწერილია მონოვალენტური ფაგების მიერ, რომლებიც მხოლოდ ერთი გარკვეული სახეობის მასპინძელი ბაქტერიის ინფიცირების უნარით გამოირჩევიან, ანტიმიკრობული რეზისტენტობის განმსაზღვრელი გენების გადაცემა *E.coli*–ის და *S. typhimurium*-ს პოპულაციებში (Colavecchio et al. 2017). უნდა აღინიშნოს ისიც, რომ მონოვალენტური ფაგების გარდა, არსებობენ ასევე პოლივალენტური ბაქტერიოფაგები, რომელთაც გააჩნიათ მასპინძელი ბაქტერიული შტამების ფართო სპექტრი, რაც შეიძლება მოიცავდეს არამარტო რომელიმე ერთ გვარში გაერთიანებულ სახეობებს, არამედ ზოგიერთ სხვადასხვა გვარის სახეობებსაც (Jensen et al. 1998; Sullivan et al. 2003; Bielke et al. 2007; Grose and Casjens 2014). შესაბამისად, გააჩნიათ რა მასპინძელის ფართო სპექტრი, პოლივალენტური ფაგები ხელს უწყობენ ბაქტერიულ პოპულაციებში რეზისტენტობის განმსაზღვრელი გენების ფართოდ მიმოცვლას როგორც სახეობათშორისო ისე გვართა შორის დონეებზე (Muniesa, Colomer-Lluch, and Jofre 2013).

ლაბორატორიულ პირობებში შესწავლილია ზოგიერთი პოლივალენტური ფაგის მიერ ანტიმიკრობული რეზისტენტობის გენების მიმოცვლა *Enterococcus-ის* (Mazaheri Nezhad Fard et al. 2011) და *Staphylococcus* (Zeman et al. 2017) გვარებში სახეობათაშორის დონეზე , . ლაბორატორიულ პირობებში ფაგური ტრანსდუქციის ფენომენი შესწავლილია ასევე *Salmonella Enterica* -ს სახეობაში (Y. Zhang and LeJeune 2008).

ჩვენს მიერ განხორციელებულმა კვლევამ (Gabashvili et al. 2020), რომელიც ეფუძნებოდა სხვადასხვა *In Silico* რეკომბინაციული მეთოდების გამოყენებას,

გამოავლინა, რომ პოლივალენტური ფაგების მიერ კერძოდ ანტიმიკრობული რეზისტენტობის გენების მიმოცვლა ბაქტერიების ბუნებრივ პოპულაციებში შეიძლება წარმოებდეს როგორც სახეობის შიგნით, ისე სახეობათაშორისო და გვართაშორის დონეებზე. ჩვენ აღმოვაჩინეთ, რომ გარკვეული ფაგების მიერ *bla*CTX-M (ფართო სპექტრის β- ლაქტამაზას მაკოდირებელი გენი), *mel* (ABC-ტიპის იფლაქს პერმეაზას მაკოდირებელი გენი) და *tet*M (რიბოსომის დამცავი ცილის მაკოდირებელი გენი) გენების ჰორიზონტალური გადაცემა შეიძლება წარმოებდეს*Escherichia, Salmonella, Streptococcus, Bacillus* და *Erysipelothrix* -ის ბუნებრივ პოპულაციებში.

ჩვენმა კვლევამ აჩვენა, რომ სალმონელას ფაგი შეიძლება წარმოადგენდეს *bla*CTX-M გენის გადამტანს არამარტო *S. enterica*-ში, არამედ ასევე *E. Coli*-ში. ამასთანავე, ჩვენი რეკომბინაციული ანალიზების შედეგების მიხედვით, რეკომბინაცია გამოვლენილია *Erysipelothrix* -ის ფაგსა, *S. suis* -სა და *E. faecium* -ს შორის, რაც მიუთითებს გვართაშორის დონეზე ამ გენის გადაცემის ფაქტებზე. *mel* გენის მიმოცვლა კი გამოვლინდა *Streptococcus* -ის ფაგს, *Erysipelothrix* -ის ფაგს, *S. pneumoniae*-სა და *B. coagulans* -ს შორის. ამასთანავე, ჩვენმა კვლევამ წარმოაჩინა *mel*-ის მიმოცვლა უშუალოდ ფაგებს შორისაც; კერძოდ, გენეტიკურად დაშორებულ, სხვადასხვა გვარის შტამებიდან გამოყოფილი *Erysipelothrix* და *Streptococcus* -ის ფაგებს შორის.

ბაქტერიებში, ანტიმიკრობული რეზისტენტობა შესაძლოა იყოს როგორც შეძენილი, ისე მისთვის ბუნებრივად დამახასიათებელი ხშირად განპირობებულისპეციფიური იფლაქს ტუმბოების არსებობითთ. (Lynch et al. 2013).. გრამდადებით და გრამუარყოფით ბაქტერიებში, იფლაქს ტუმბოს შეიძლება წარმოადგენდეს ტრანსპორტერი ცილები, რომლებიც უჯრედშიდა გარემოდან გარეთ გამოდევნის სხვადასხვა ნივთიერებას, მათ შორის ანტიბიოტიკებს და ტოქსიკურ ნაერთებს და (Webber 2003). ბაქტერიების ბუნებრივ პოპულაციებში, ეფლუქს

ტუმბოები შეიძლება მონაწილეობას იღებდნენ არამარტო ანტიმიკრობული რეზისტენტობის გენერირებაში, არამედ ბიოფილმის წარმოქმნაშიც (Poole 2007; Nikaido and Pagès 2012; Soto 2013; Alcalde-Rico et al. 2016; Alav et al. 2018). მემრანული სატრანსპორტო ცილების მაკოდირებელი გენები აღმოჩენილ იქნა ბაქტერიების გარდა, ასევე ვირუსების გენომებშიც (Greiner et al. 2018). იფლაქს ტუმბოების მაკოდირებელი გენები ვირუსებში მრავლადაა წარმოდგენილი, და მათ შორის ისინი აღმოჩენილია ბაქტერიოფაგების გენომებშიც; მაგ., ფაგურ გენომებში აღმოჩენილია K+ არხი-ის, Mg2+ ტრანსპორტერის, ნატრიუმის გლუკოზის ტრანსპორტერის, ნატრიუმ-კალციუმის სიმპორტერის და ნუკლეოტიდების ტრანსპორტერების მაკოდირებელი გენები (Greiner et al. 2018). *ასევე, A. baumannii*–ის პროფაგების გენომებში აღმოჩენილია ანტიმიკრობული რეზისტენტობის განმაპირობებელი იფლაქს ტუმბოები MFS (major facilitator superfamily) და RND ოჯახებიდან (the resistance-nodulation-division family) (Costa et al. 2018). იფლაქს გენების მატარებელი პროფაგი შეიძლება იყოს დეფექტურიც, ბაქტერიულ ქრომოსომაში ზომიერი ფაგის ინტეგრაციის პროცესში მასზე ბაქტერიული თავდაცვის სისტემების ზემოქმედების შედეგად (Canchaya et al. 2004; Pfeifer et al. 2021). ამის მიუხედავად, აღსანიშნავია, რომ ჯერ კიდევ შეუსწავლელია მონაწილეობენ თუ არა ბაქტერიოფაგები ანტიმიკრობულ რეზისტენტობასთან, განსაკუთრებით კი მულტირეზისტენტობასთან, ასოცირებული იფლაქს ტუმბოების მაკოდირებელი გენების მიმოცვლაში. მეტა-ანალიზური კვლევის ფარგლებში, ჩვენ შევისწავლეთ NCBI ვირუსულ მონაცემთა ბაზაში არსებული ფაგების გენომები. ზემოაღნიშნული ბაზა სხვა ორგანიზმების გენომებთან ერთად, მოიცავს ინფორმაციას (ნუკლეოტიდური თანმიმდევრობების ჩათვლით) ლითიური, ზომიერი და ინდუცირებულ (აქტიურ) პროფაგების, გენომების შესახებ.

ჩვენს მიერ ჩატარებულმა კვლევამ (Gabashvili et al. 2021a) ცხადჰყო, რომ ზოგიერთ ბაქტერიოფაგს შეუძლია გააჩნდეს, კერძოდ კი შეიძინოს ანტიმიკრობული

რეზისტენტობის განმსაზღვრელი იფლაქს ტუმბოების მაკოდირებელი გენები, მაგალითად როგორებიცაა MF კლასის (Major Facilitator Superfamily), ატფ-დამაკავშირებელი კასეტების (ABC ტრანსპორტიორები), resistance-nodulation-division (RND) ოჯახის ტრანსპორტერების მაკოდირებელ გენები. იფლაქს ტრანსპორტერების გენების მტარებელი ფაგების მასპინძელი შტამები შეიძლება წარმოადგენდნენ ადამიანის სხვადასხვა პათოგენებს ისეთი სახეობებიდან, როგორებიცაა *Salmonella enterica, Staphylococcus aureus, Pseudomonas aeruginosa, და Burkholderia pseudomalle*.

In silico რეკომბინაციული ანალიზებით ჩვენმა კვლევამ გამოავლინა ბაქტერიოფაგების მიერ ინდუცირებული ზემოაღნიშნული გენების შიდასახეობრივი, სახეობათაშორისო გენეტიკური რეკომბინქცია *Salmonella enterica*-ს, *Mycobacterium smegmatis*-ის, *Pseudomonas aeruginosa*-ს, *Burkholderia pseudomallei*-ს, *Staphylococcus aureus*-ს, *Staphylococcus epidermidis*-ს, *Streptococcus suis*-ს, და *Streptococcus gallolyticus*-ს ბუნებრივ პოპულაციებში.

1.2.3 ფაგების სასიცოცხლო ციკლი და მათი დივერგენცია

ფაგის მასპინძელთან და ფაგის ფაგთან კოევოლუციის ტრენდების გამოვლენა მნიშვნელოვანია თერაპიული, სურსათის უვნებლობის ან გარემოსდაცვის მიზნებისათვის განსაკუთრებით ბოლო ათწლეულებში ფაგის პრეპარატების ინტენსიური შემუშავებისა და მათი სხვადასხვა ეკოსისტემაში მზარდი გამოყენების ფონზე. როგორც ადრეული სამეცნიერო შრომების, ისე ჩვენს მიერ ჩატარებული კვლევების შედეგების მიხედვით, ანტიმიკრობული რეზისტენტობის გენებისა თუ ვირულენტობის განმსაზღვრელი გენების მტარებელი ფაგების სასიცოცხლო ციკლი, ძირითადად ზომიერი ფაგის ბუნებას უკავშირდება, (Gabashvili et al. 2020; de la Cruz

and Davies 2000; Beceiro, Tomás, and Bou 2013; Boyd 2012; Deng et al. 2019; Manaia 2017; Brown-Jaque, Calero-Cáceres, and Muniesa 2015).

მიჩნეულია რომ, ლითიურ ფაგებს, რომლებსაც პათოგენების წინააღმდეგ თერაპიული გამოყენებისათვის იყენებენ, მასპინძლის ფართო სპექტრი გააჩნიათ. ამასთანავე, უნდა აღინიშნოს, რომ ფაგების კლასიკური მეთოდებით კლასიფიცირება შესაძლოა სამომავლო გადახედვის საგნად იქცეს, რაც განპირობებულია პლაზმიდების ფორმის ფაგების აღმოჩენით (Hockenberry and Wilke 2020; Pfeifer et al. 2021). ფაგების ევოლუციაზე შესაძლოა ზეგავლენა მოახდინოს როგორც მასპინძლის ურთიერთქმედებამ, ისე მასპინძლის უჯრედში მათთან სხვა კოინფიცირებადი ფაგების ურთიერთქმედებამ, (Joseph et al. 2009; Refardt 2011; Roux et al. 2015). ზემოაღნიშნული პროცესებმა თავის მხრივ შეიძლება ზემოქმედება მოახდინოს იქონიოს მასპინძელი ბაქტერიის უჯრედის სიცოცხლის უნარიანობაზე. ამგვარად, ფაგების და მათთან მასპინძლების კოევოლუციის შესწავლა არსებითია გარემოს სხვადასხვა მიკრობიომების, მათ შორის ბაქტერიული პათოგენების ევოლუციური დივერგენციის მიმართულებების განსაზღვრისა და პროგნოზირებისთვის.

წინამდებარე კვლევა აღწერს ზომიერი და ვირულენტურ ფაგებს შორის გენომური ლოკუსების გენეტიკური რეკომბინაციის მოვლენებს, რომლებსაც, როგორც მტკიცედ ვვარაუდობთ, ადგილი ჰქონდა მათ მიერ ბაქტერიის უჯრედის კოინფიცირების პროცესსში (Gabashvili et al. 2021b). ჩვენი კვლევის შედეგების თანახმად, ფაგის მორფოგერნეზში, რეპლიკაციასა და მასპინძლის მიმართ სელექტიურ ინფიცირებაში მონაწილე ზოგიერთი გენეტიკური ლოკუსის რეკომბინაცია ზემოაღნიშნულ ფაგებს შორის შეიძლება იყოს ბი- და მულტი-ლატერული მიმართულების.

2. მეთოდები

2.1 ზღვის წყლის ნიმუშების აღება მეტაგენომური კვლევებისათვის

მწვანე კონცხისა (N 41° 41' 29.708'' E 41° 42' 15.769'') და ბათუმის ბულვარის (N 41° 39' 14.906'' E 41° 37' 36.365'') სარეკრეაციო ზოლში შეგროვებულ იქნა 2ლ ზედაპირული წყლის ნიმუშები 2018 წლის ზაფხულის (აგვისტო) პერიოდში. მშრალ ყინულზე მოთავსებული წყლის ნიმუშები გაიგზავნა Omega Bioservices Inc.-ში, (Norcross, GA 30071, აშშ) მიკრობული დნმ-ის გამოსაყოფად და სრული მეტაგენომური შოთგან სექვენირებისათვის (PhDF2016_97 გრანტის ფარგლებში) HiSeq X 10 პლატფორმის (Illumina Inc.) მეშვეობით.

2.2 მეტაგენომური მონაცემების დამუშავება და ანალიზი

- მეტაგენომური მონაცემების დასამუშავებლად გამოყენებული იყო Unix-ზე დაფუძნებული MetaWRAP-ის პროგრამაში (ვერსია v1.3.2) იმპლემენტირებული metaBAT2, CONCOCT და MaxBin2 ალგორითმები (https://github.com/bxlab/metaWRAP) (Uritskiy, DiRuggiero, and Taylor 2018), რამაც საშუალება მოგვცა აგვეწყო მაღალი ხარისხის მქონე ეგრეთწოდებული გენომური bin-ები, . FastQ (Wingett and Andrews 2018) იქნა გამოყენებული საწყისი მეტაგენომური მონაცემების სექვენსების ხარისხის კონტროლის შესამოწმებლად, ხოლო Trimmomatic (Wingett and Andrews 2018) დაბალი ხარისხის ნუკლეოტიდური თანმიმდევრობებისგან დნმ-ის მონაცემების ფილტრაციისა და შესწორებისათვის. სხვადასხვა ალგორითმით აწყობილი გენომები - bins -გაერთიანებულ იქნა Metawrap Bin დახვეწის მოდულით.

2.3 მეტაგენომებიდან ანტიმიკრობული რეზისტენტობის გენების ექსტრაქცია და იდენტიფიკაცია

metaWRAP-ით მიღებული გენომების ანოტირება და ანტიმიკრობული რეზისტენტობის გენების იდენტიფიცირება მოხდა DFAST (https://dfast.ddbj.nig.ac.jp/) (Tanizawa et al. 2016) პროგრამის მეშვეობით. გენები, DFAST ის გარდა, ასევე გაანალიზებულ იქნა RASTtk (Brettin et al. 2015) და BLAST მეშვეობით (Altschul et al. 1990), შემდგომ კი ამინომჟავური თანმიმდევრობები ფუნქციურად გაანალიზირებულ იქნა InterPro პროგრამით, რომელმაც ასევე განსაზღვრა ამ ცილების ოჯახთა კლასი (Blum et al. 2021). ARG– ების შედარებითი გავრცელება ბულვარის სანაპირო ზოლში, ბათუმის ბულვარისა და მწვანე კონცხის ნიმუშებში განისაზღვრა მეტაგენომური პროფილის სტატისტიკური ანალიზის (STAMP) გამოყენებით (Parks et al. 2014). Fisher-ის ზუსტი ტესტი, Benjamini-Hochberg FDR მიდგომით, იქნა გამოყენებული რათა შეგვედარებინა ARG გენები სხვადასხვა საიტებს შორის და განგვესაზღვრა შესწორებული ალბათობის (*p)* მნიშვნელობები.

ანოტირებული ARG გენების ნუკლეოტიდური თანმიმდევრობები წარდგენილი და გამოქვეყნებულ იქნა NCBI-ის (ბიოტექნოლოგიის ინფორმაციის ეროვნული ცენტრი - National Center for Biotechnology Information) GenBank– ში და ხელმისაწვდომია შემდეგი ნომრებით: MZ080332-MZ080385.

NCBI GenBank–ში ატვირთული ყველა ARG, რომლებიც ბლასტის ანალიზების მიხედვით გვიჩვენებდა მაღალ ჰომოლოგიას (იდენტურობა ≥ 90%) მათ კონსპეციფიებთან ზემოაღნიშნულ გლობალურ მონაცემთა ბაზაში, დაექვემდებარა რეკომბინაციულ ანალიზებს.

2.4 ბაქტერიოფაგების გენომებში ანტიმიკრობული რეზისტენტობის გენების იდენტიფიკაცია

NCBI მონაცემთა გლობალურ ბაზაში ანტიმიკრობული რეზისტენტობის გენების შემცველი ფაგების იდენტიფიკაციისათვის, გაანალიზდა CARD -ის რეზისტენტობის გენების მონაცემთა ბაზის (CARD, https: //card.mcmaster.ca) 2244 ამინომჟავური თანმიმდევრობის მონაცემი. ზემოაღნიშნულ ბაზაში, ჰომოლოგიური ცილები და მათი მაკოდირებელი გენები მოძიებული იქნა ვირუსების მონაცემთა ბაზაში შესაბამისად BLASTp და TBLASTN ალგორითმების მეშვეობით, რამაც ასევე რეზისტენტობის გენების მტარებელი ბაქტერიოფაგების იდენტიფიცირების საშუალება მოგვცა.

NCBI მონაცემთა გლობალურ ბაზაში, ანტიმიკრობული რეზისტენტობის განმსაზღვრელი იფლაქს ტუმბოების მაკოდირებელი გენების შემცველი ფაგების იდენტიფიცირებისათვის გამოვიყენეთ BacEffluxPred (http://proteininformatics.org/mkumar/baceffluxpred/) (Pandey et al. 2020) მონაცემთა ბაზაში არსებული მონაცემები (ამინომჟავური და ნუკლეოტიდური თანმიმდევრობები) რეზისტენტობასთან ასოცირებულ იფლაქს ტუმბოების გენების შესახებ. შესაბამისად, TBLASTN და BLASTp ალგორითმების მეშვეობით, მოვიძიეთ ჰომოლოგები ვირუსების მონაცემთა ბაზის მიმართ მათი დაბლასტვის მეთოდით. დაბლასტვის შედეგად ბაქტერიოფაგებში აღმოჩენილი გენების ჰომოლოგები კვლავ გაანალიზებულ იქნა BacEffluxPred (Pandey et al. 2020), InterPro (Blum et al. 2021) და BLASTP პროგრამების მეშვეობით, რომ დაგვედასტურებინა ამ გენებით განსაზღვრული ეფლუქს ტუმბოების ჩართულობა ანტიბიოტიკების ტრანსპორტირებაშიც.

ანტიმიკრობული რეზისტენტობის გენები, რომლებიც აღმოჩენილ იქნა ≥ 85 % დნმ-დნმ-ის ჰომოლოგიის საფუძველზე, შემდგომ გაანალიზდა *in silico* რეკომბინაციულ ცდებში.

2.5 ზომიერი და ლითიური ფაგების მორფოგენეზში, რეპლიკაციაში, და სპეციფიური მასპინძლების შერჩევაში მონაწილე გენების განსაზღვრა

ზომიერ და ლითიურ ფაგების ფაგების მორფოგენეზის, რეპლიკაციის, და მასპინძლის სპეციფიკის გენების იდენტიფიკაცია ასევე მოხდა NCBI-ის მონაცემთა ბაზაში ბლასტის ანალიზების გამოყენებით. ფაგის კაფსიდის, თავაკის, დნმ პოლიმერაზის, კუდის, ფაგთან ასოცირებული დნმ ჰელიკაზის, დნმ ლიგაზის, დნმ პრიმაზას, დნმ პოლიმერაზა I გენების ჰომოლოგები მოძიებულ იქნა გლობალურ მიკრობულ მონაცემთა ბაზაში ბლასტის ანალიზების მეშვეობით და ფაგებისა და ბაქტერიულ გენომებში არსებული პროფაგების გენების მაღალი იდენტობის შემთხვევაში (>85%), შერჩეული გენეტიკური ლოკუსები დაექვემდებარა რეკომბინაციულ ტესტებს.

2.6 ფაგების სასიცოცხლო ციკლისა და მასპინძელი სპექტრის განსაზღვრა

NCBI ნუკლეოტიდების მონაცემთა ბაზაში გამოვლენილი ბაქტერიოფაგების სასიცოცხლო ციკლის განსასაზღვრად გამოვიყენეთ კონკრეტული ფაგების შესახებ არსებული სამეცნიერო ლიტერატურის მონაცემები, და ასევე PHACTS (McNair, Bailey, and Edwards 2012), BACPHLIP (Hockenberry and Wilke 2020), და PhageAI (Tynecki et al. 2020) პროგრამები.

PHACTS ანალიზისთვის, თითოეული ფაგის პროტეომის ანოტაციისათვის გამოყენებულ იქნა DDBJ სწრაფი ანოტირების პროგრამა DFAST. PHACTS– ის გამოყენებისას, ფაგის სასიცოცხლო ციკლი მიჩნეულ იქნა სარწმუნოდ, თუ თითოეული ფაგის გენომისათვის პროგრამის მიერ განსაზღვრული ალბათობის მნიშვნელობები >0,5-ზე (McNair, Bailey, and Edwards 2012). PHACTS -ის გარდა, ასევე, გამოყენებულ იქნა ბაქტერიოფაგების სასიცოცხლო ციკლის ამომცნობი მანქანური სწავლების ალგორითმის ინსტრუმენტი PhageAI (Tynecki et al. 2020), და ასევე კონსერვირებული ცილების დომენების მიხედვით ფაგების სასიცოცხლო ციკის განმსაზღვრელი BACPHLIP (Hockenberry and Wilke 2020) პროგრამა.

ბაქტერიულ გენომებში პროფაგების საიდენტიფიკაციოდ და ანოტირებისათვის გამოვიყენეთ PHAge Search Tool (PHAST) (Arndt et al. 2019) პროგრამა. PHAST ანალიზებში პროფაგის რეგიონი მიჩნეულ იქნა i) არასრულად, თუ პროგრამის მიერ მისთვის მინიჭებული ქულა იყო < 60, ii) საეჭვოდ, თუ მისი ქულა იყო 60 – დან 90 – მდე ფარგლებში, და iii) ინტაქტურად, თუ მისი ქულა იყო90 – დან 100 – მდე ფარგლებში. ჩვენს ანალიზებში ვაწარმოეთ მხოლოდ ინტაქტური პროფაგის გენომების ანალიზი.

ფაგების მასპინძლის სპექტრის განსასაზღვრად გაანალიზდა მოცემულ ფაგებზე არსებული სამეცნიერო ლიტერატურა და ასევე გამოყენებული იყო ვირუს-მასპინძლის მონაცემთა ბაზა (DB) (https://www.genome.jp/virushostdb/note.html). ფაგების სავარაუდო მასპინძლების განსაზღვრისთვის გამოვიყენეთ HostPhinder (Villarroel et al. 2016) (version 1.1). . პროგრამის მიერ მასპინძელი ბაქტერიისათვის სახეობის დონეზე მინიჭებული > 0.7 ქულა , ხოლო გვარის დონეზე > 0.8, მიიჩნეოდა სარწმუნო შედეგად, თუმცა პროგრამის მიერ მინიჭებული ≥ 0.15 ქულა, თუ იგი ემთხვეოდა რეკომბინაციულ ანალიზებით მიღებულ შედეგებს, ასევე მიჩნეულ იქნა მტკიცე შედეგად.

ფაგისა და მასპინძლის ურთიერთქმედების სიგნალის (PHISs) იდენტიფიკაციისათვის გამოვიყენეთ PHISDetector (F. Zhang et al. 2019) პროგრამა, რაც კიდევ დამატებით საშუალებას გვაძლევდა გამოგვევლინა რეკომბინაციაში მონაწილე ფაგისა და ბაქტერიული შტამის ურთიერთქმედების დამატები მტკიცებულებები. უფრო კონკრეტულად, PHISDetector–ის გამოყენებით, ჩვენ გავაანალიზეთ ფაგსა და მასპინძელს შორის ურთიერთქმედების სიგნალები, რისთვისაც ბაქტერიაში გაანალიზდა მისი გენომის ნუკლეოტიდური თანმიმდევრობები , განისაზღვრა კლასტერირებული რეგულარულად ინტერფეისური მოკლე პალინდრომული განმეორებები (CRISPR), ცილებს შორისურთიერთქმედება და პროფაგების შემცველობის მახასიათებლები.. ბაქტერიოფაგმა, მის მიერ ბაქტერიის ინფიციფრებისას, შესაძლოა დატოვოს აღნიშნული ტიპის ნუკლეოტიდური თანმიმდევრობები (სიგნალები) მასპინძლის გენომში როგორც მისი კვალი., . ჩვენს ანალიზებში, PHISDetector-ით კი განსაზღვრული ალბათობის ქულები > 0.8865 მიიჩნეოდა სტატისტიკურად სარწმუნოდ და მაშასადამე მიუთითებდა ფაგსა და მასპინძელს შორის უშუალო ურთიერთქმედებაზე.

2.7 რეკომბინაციული ანალიზები

გენების ჰორიზონტალური გადაცემის შემთხვევების გამოსავლენად რეკომბინაციის დეტექციის თვისობრივად განსხვავებული ალგორითმები გამოვიყენეთ. კერძოდ, გამოყენებული იყო განტოტების დეკომპოზიციის (split decomposition) მეთოდი (Bandelt and Dress 1992) იმპლემენტირებული SplitsTree პროგრამაში (ვერსია 4.14.4)(Huson and Bryant 2006). ზემოაღნიშნული მეთოდი დნმ-ის თანმიმდევრობებში მოიძიებს იმ კონფლიქტურ სიგნალებს (გენეტიკურ ვარიაციებს), რომლებიც ამ თანმიმდევრობებში აღმოცენდება პარალელურად გენეტიკური რეკომბინაციის შედეგად. ასეთ რეკომბინაციულ მოვლენებს SplitsTree ასახავს პარარელოგრამებით წარმოდგენილი ფილოგენეტიკური ურთიერთობების

ურთიერთდაკავშირებული ქსელის სახით, განსაზღვრავს მათი თავსებადობის სარწმუნოების ერთიან ნიშნულს (fit) და ფილოგენეტიკურ ქსელში თვითოეული განტოტების და პარალელოგრამის გვერდის ბუტსტრაპის შედეგს (bootstrap). საანალიზოდ, რეკომბინაციული ტესტებისათვის, დნმ-ის თანმიმდევრობები დაექვემდებარა ელაინმენტის პროცედურებს ClustalX- ის (ვერსია 2.1) (Larkin et al. 2007) მეშვეობით. ამ ანალიზებში, SplitsTree-ს მიერ გენერირებული პარალელოგრამი განიხილებოდა მაღალსარწმუნოდ თუ მათი bootstrap – ის მნიშვნელობები ≥ 95 (1000 რეპლიკაციიდან), ხოლო fit მთლიანი განტოტების ქსელისთვის შეადგენდა ≥95. განტოტების დეკომპოზიციური მეთოდის გამოყენების შედეგად მიღებული მაღალსარწმუნო პარალელური სიგნალები ექვემდებარებოდა დამატებით გადამოწმებას ჰომოპლასის ინდექსის განსაზღვრის გზით (Pairwise Homoplasy Index [Phi]) ტესტის გამოყენებით (Bruen, Philippe, and Bryant 2006). წარმოდგენილი Phi ტესტი კონვერგენტული ევოლუციის მახასიათებლების გამოვლენის საშუალებას იძლევა, რომლებიც რეკომბინაციულ ტესტებში შეიძლება რეკომბინაციული სიგნალების მიმიკრიას ახდენდნენ.

გენეტიკური რეკომბინაციის მოვლენების და მათი ტრაექტორიების გამოსავლენად ასევე გამოვიყენეთ RDP4 პროგრამული პაკეტი (Darren P. Martin et al. 2015), რომელიც აერთიანებს რეკომბინაციის დეტექციის თვისობრივად განსხვავებულ ალგორითმებს როგორებიცაა: RDP (D. Martin and Rybicki 2000), GENECONV (Padidam, Sawyer, and Fauquet 1999), BootScan (D.P. Martin et al. 2005), MaxChi (J. Smith 1992) , Chimaera (Posada and Crandall 2001), SiScan (Gibbs, Armstrong, and Gibbs 2000) და 3Seq (Boni, Posada, and Feldman 2007). RDP4 პაკეტის გამოყენებით, ანალიზებში განისაზღვრა რეკომბინაციულ მოვლენაში მონაწილე რეკომბინანტი შტამები (რეციპიენტები)და მათი მთავარი და მინორული დონორი შტამები. RDP4–ის

მიერ გამოვლენილი ბონფერონის მიერ შესწორებული p მნიშვნელობები მიიჩნეოდა სტატისტიკურად სარწმუნოს თუ ისინი შეადგენდნენ ≤0,05.

დამატებით ასევე გამოყენებული იყო პროგრამები GARD(Kosakovsky Pond et al. 2006) და Simplot HGT– ში მონაწილე გენების გასწვრივ გენეტიკური რეკომბინაციის ცხელი წერტილების განსაზღვრის მიზნით.

3. შედეგები და დისკუსია

3.1 შავი ზღვის სანაპირო ზოლის სარეკრეაციო წყლებში მეტაგენომური ანალიზით გამოვლენილი ანტიმიკრობული რეზისტენტობის გენები გენეტიკური რეკომბინაციის გზით ბაქტერიულ პოპულაციებში შეიძლება გავრცელდეს როგორც შიდასახეობრივ და სახეობათაშორისო, ისე გვართაშორის დონეებზე

ჩვენს მიერ ჩატარებული მეტაგენომური კვლევა წარმოადგენს პირველ საპილოტე კვლევას, რომელიც აღწერს ბათუმის შავი ზღვის სანაპირო წყლებში ARG – ების გენეტიკურ მრავალფეროვნებას. მეტაგენომურ კვლევები საშუალებას იძლევა განისაზღვროს სხვადასხვა გარემოში მიკრობების ფართო სახეობრივი სპექტრი, რომლის გამოვლენა შეუძლებელია კულტურალური და სახეობის იდენტიფიკაციის სხვა ტრადიციული მეთოდებით (Singh 2020). მეტაგენომურ ანალიზს შეუძლია გამოავლინოს ბაქტერიული პოპულაციების მრავალფეროვნება და მათი განაწილება გარემოში (Panosyan et al. 2018; Gomez-Alvarez et al. 2021). აღნიშნული მიდგომა ასევე საშუალებას იძლევა გამოვავლინოთ ამ პოპულაციებში კონკრეტული სხვადასხვა ფუნქციონალური გენეტიკური ლოკუსები, მათ შორის ანტიმიკრობული რეზისტენტობის დეტერმინანტები და მათი გენეტიკური მრავალფეროვნება (Nesme et al. 2014). ამგვარად, ჩვენი კვლევა აღწერს ARG–ების გენეტიკურ მრავალფეროვნებას შავი ზღვის სანაპირო ზოლის რეკრეაციულ წყლებში და მათი ჰორიზონტალური გადაცემის ფენომენს, როგორც ერთ–ერთ გენეტიკური მექანიზმს, რომელიც თავის

მხრივ ზრდის ამ რეზისტენტობის გენების ევოლუციურ დინამიკას და ხელს უწყობს მათ გენეტიკურ დივერგენციას. რეზისტენტობის გენების მრავალფეროვნების და დინამიკის შესწავლა კი მნიშვნელოვანია, იმისთვის, რომ ვაწარმოოთ ანტიმიკრობული რეზისტენტულობის რეზერვუარების და მისი დეტერმინანტების მონიტორინგი და ამასთანავე განვსაზღვროთ ანტიმიკრობული რეზისტენტობის გენერირების ევოლუციის ტენდენციები პროგნოზირების და პრევენციული მიზნებისათვის (Matyar et al. 2008; Port et al. 2012; Chen et al. 2019). ჩვენი კვლევის ფარგლებში გაანალიზებულ შავი ზღვის მეტაგენომებში გამოვლინდა, რომ რეზისტენტულ გენებს შორის, უმეტესობა აკოდირებს იმ იფლაქს ტუმბოებს, რომლებიც განსაზღვრავენ რეზისტენტობას მრავალი სხვადასხვა ანტიბიოტიკის მიმართ, რომლებიც აქტიურად გამოიყენება ადამიანის თერაპიაში და ვეტერინარული მიზნებისთვის (Alekshun and Levy 2007). კერძოდ, ზემოაღნიშნული გენები განაპირობებენ რეზისტენტობას ტეტრაციკლინის, ქლორამფენიკოლის, ქინოლონების და აკრიფლავინის მიმართ (Gabashvili et al. 2022).

გამოვლინდა, რომ შავი ზღვის საქართველოს სანაპირო წყლების მეტაგენომიურ ნიმუშებში აღმოჩენილი რეზისტენტობის გენები ძირითადად აკოდირებს მულტირეზისტენტობის განმაპირობებელ ტრანსპორტერ ცილებს MFS-ის და Bcr/CflA-ს ოჯახებიდან; მაგ., Bcr/CflA ტრანსპორტერების ოჯახი განსაზღვრავს რეზისტენტობას ქლორამფენიკოლის, ფლორფენიკოლისა და ბიცილომიცინის მიმართ (Smith et al. 2009). გარდა ამისა, წყლის ნიმუშების მეტაგენომები შეიცავდა *emrB* გენს, *QacA* გენის ჰომოლოგს, რომელიც პასუხისმგებელია ტოქსიკური მოლეკულების ექსპორტზე ბაქტერიული და სოკოვანი (კერზოდ კი საფუარების) უჯრედებიდან (Huggins et al. 2018) და აკოდირებს ცილას, რომელიც გენერირებს რეზისტენტობას ჰიდროფობიური ქინოლონების მიმართ (Putman et al. 2000).

ჩვენს მიერ ჩატარებულმა მეტაგენომურმა კვლევებმა შავი ზღვის სანაპირო ზოლში ასევე გამოავლინა MATE ოჯახის MdtH, MdtL, MdtG და NorM-ის ტრანსპორტერების ARG-ები, რომლებიც განსაზღვრავენ რეზისტენტობას ქინოლონების მიმართ, კერძოდ ნორფლოქსაცინსა და ენოქსაცინის მიმართ (Yu et al. 2020); თუმცა ისინი ასევე განაპირობებენ რეზისტენტობას ასევე ტეტრაციკლინის და/ან სხვა ანტიბიოტიკების მიმართაც (Bao, Gao, and Wen 2020; Jebastin and Narayanan 2019; Morita et al. 2000; Brown, Paulsen, and Skurray 1999). მეტაგენომურ ანალიზებში ასევე აღმოვაჩინეთ RND ოჯახის ტრანსპორტერი ცილების მაკოდირებელი გენები, რომლებიც გრამ-უარყოფით ბაქტერიებში მნიშვნელოვან როლს ასრულებენ ანტიმიკრობული რეზისტენტობის აღმოცენებაში (Liang et al. 2016), მონაწილეობენ რა როგორც ჰიდროფილური, ისე ჰიდროფობული ქინოლონების ტრანსპორტირებაში. RND ეფლუქს ტუმბოები მოიაზრება სურსათისმიერ და ზოგიერთ სხვა ბაქტერიულ პათოგენში პათოგენურობის ერთ-ერთი ფაქტორად (Coudeyras et al. 2008; Lin et al. 2003; Guérin et al. 2016). ანალიზებში ასევე გამოვლინდა სხვა მულტირეზისტენტობის გენებიც, როგორებიცაა *HlyD*, „Small Multidrug Resistance“ და MFS მულტირეზისტენტობის გენები.

ჩვენს მიერ მეტაგენომური ანალიზებით განსაზღვრული რეზისტენტობის გენების მტარებელ ბაქტერიებს წარმოადგენდნენ ზღვის გარემოსთვის მახასიათებელ ადამიანის და ცხოველის ისეთ პათოგენურ არაპათოგენურ არაპათოგენური ორგანიზმები, როგორებიცაა: *Vibrio* (*Vibrio vulnificus*, *Vibrio fluvialis, Vibrio mimicus*), *Aeromonas* (*Aeromonas hydrophila, Aeromonas caviae*, *Aeromonas veronii,* etc), *Pseudoalteromonas, Synechococcus, Rhodobacteraceae, Gilvibacter*, *Erythrobacter*, *Altererythrobacter, Citromicrobium, Marivivens*, *Xuhuaishuia* and *Loktanella.*

ვლინდება, რომ ზემოაღნიშნული ბაქტერიები წარმოადგენენ რეზისტენტობის გენების რეზერვუარს და ხელს უწყობენ ანტიმიკრობული რეზისტენტობის გენების

ჰორიზონტალურ გადაცემას შავი ზღვის სანაპიროს სარეკრეაციო წყლების მიკრობულ პოპულაციებში. ჩვენი კვლევები ცხადყოფს, რომ ანტიმიკრობული რეზისტენტობის განმსაზღვრელი ზოგიერთი გენის გადაცემის ტრაექტორიები შეიძლება იყოს როგორც ბი- ისე მულტი-ლატერული. ჩვენს ანალიზებში ნაჩვენებია, რომ HGT-ის ცალკეულ შემთხვევებში, აღნიშნულმა ბაქტერიებმა შეიძლება "გაცვალონ" დონორული ან რეციპიენტული როლები - ერთი და იგივე შტამმა სხვა ბაქტერიული შტამთან ურთიერთქმედების დროს შეიძლება შეასრულოს ანტიმიკრობული რეზისტენტობის გენის რეცეპიენტის როლი, ხოლო სხვა შემთხვევაში, თავად გახდეს ამ დეტერმინანტის დონორი გარკვეული პოპულაციებისთვის. ამასთანავე, გამოვლინდა, რომ მაგალითად *Aeromonas media-ს, Aeromonas hydrophila-ს, Vibrio furnissii*-ს and *Vibrio fluvialis-ს ჩართულობით EmrB/QacA* (MFS) ოჯახის ტრანსპორტერის მაკოდირებელიგენის ჰორიზონტალური გენეტიკური გადაცემა შეიძლება მოხდეს გვართაშორის დონეებზე(Gabashvili et al. 2022).

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Metagenomic and Recombination Analyses of Antimicrobial Resistance Genes from Recreational Waters of Black Sea Coastal Areas and Other Marine Environments Unveil Extensive Evidence for Their both Intrageneric and Intergeneric Transmission across Genetically Very Diverse Microbial Communities

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ABSTRACT

Microbial communities of marine coastal recreation waters have become large reservoirs of AMR genes (ARGs), contributing to the emergence and transmission of various zoonotic, foodborne and other infections that exhibit resistance to various antibiotics. Thus, it is highly imperative to determine ARGs assemblages as well as mechanisms and trajectories of their transmission across these microbial communities for our better understanding of the evolutionary trends of AMR (AMR). In this study, using metagenomics approaches, we screened for ARGs in recreation waters of the Black Sea coastal areas of the Batumi City (Georgia). Also, a large array of the recombination detection algorithms of the SplitsTree, RDP4, and GARD was applied to elucidate genetic recombination of ARGs and trajectories of their transmission across various marine microbial communities. The metagenomics analyses of sea water samples, obtained from across the above marine sites, could identify putative ARGs encoding for multidrug resistance efflux transporters mainly from the Major Facilitator and Resistance Nodulation Division superfamilies. The data, generated by SplitsTree (fit $\geq$95.619; bootstrap values $\geq$ 95; Phi $p \leq 0.0494$), RDP4 ($p \leq 0.0490$), and GARD, provided strong statistical evidence not only for intrageneric recombination of these ARGs, but also for their intergeneric recombination across fairly large and diverse microbial communities of marine environment. These bacteria included both human pathogenic and nonpathogenic species, exhibiting collectively the genera of *Vibrio, Aeromonas, Synechococcus*, *Citromicrobium, Rhodobacteraceae, Pseudoalteromonas, Altererythrobacter, Erythrobacter, Altererythrobacter, Marivivens, Xuhuaishuia*, and *Loktanella.* The above nonpathogenic bacteria are strongly suggested to contribute to ARGs transmission in marine ecosystems.

1. Introduction

AMR (AMR) is one of the most persistent public health challenges (Mootapally et al., 2019), especially in the developing countries (Cuadrat et al., 2020), with multidrug-resistant bacterial pathogens causing at least 700, 000 deaths worldwide per year (Rodríguez-Verdugo et al., 2020). It has been projected that if proper preventive measures are not taken, numbers will approach 10 million deaths per year by 2050 (O'Neill, 2014; Jasovský et al., 2016), surpassing the cancer-related deaths (Tagliabue and Rappuoli, 2018; Aslam et al., 2018). Marine microbiomes can serve as complex reservoirs of antibiotic resistance genes (ARGs), representing just a part of the global resistome

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(Mootapally et al., 2019). Drug-resistant bacteria have been frequently found in waterways undergoing human influence and even in close proximity to aquaculture facilities (Alonso et al., 2001; Dang et al., 2007; Zhao and Dang, 2012; Buschmann et al., 2012). Thus, this alarming evidence triggered extensive studies to monitor ARGs especially in marine coastal recreational waters, which frequently has demonstrated the sheer abundance of these genes across the above environments (Baker-Austin et al., 2009; Port et al., 2012; Hatosy and Martiny, 2015; Du et al., 2019).

It has been suggested that the high levels of clinically relevant ARGs in these ecosystems are due to selective pressures linked to their heavy pollution by antibiotics (Kolář et al., 2001; Zhao and Dang, 2012; Du et al., 2019; Su et al., 2020). Moreover, it has been shown that ARGs gradually became more diverse in natural water bodies (Hatosy and Martiny, 2015; Su et al., 2020) partly via genetic recombination (Su et al., 2020; Gabashvili et al., 2020; Cuadrat et al., 2020) that contributes to their dissemination in this and other environments. In light of a gradual temperature increase across marine and other environments, which is due to global climate warming, genetic recombination may prompt adaptive evolution by increasing the phenotypic and genotypic variation (Payne and Wagner, 2019), and expand the fitness landscape entailing the preservation of antibiotic resistance in microbial populations (McGough et al., 2020; Rodríguez-Verdugo et al., 2020). Thus, there is a great need for a more in-depth understanding of the role of genetic recombination in the ARGs evolution and their transmission trajectories across microbial communities of aquatic environments, especially in those of marine recreational waters being exposed frequently to various anthropogenic factors.

We conducted a pilot metagenomics study to gain initial insights into a spectrum of ARGs that could be present across the City of Batumi coastal recreational waters of the Black Sea, being one of the most attractive sites of Georgia for many visitors and tourists from across the world. In addition, we applied the extensive recombinational analyses of ARGs identified putatively in this study, as well as their different alleles determined in previous other investigations to also gain the better understanding of a phenomenon of genetic recombination of AMR determinants and their transmission trajectories driven by HGT across microbial communities of the marine environment in general. In waterbodies of the above coastal site of the Black Sea, we could identify various putative ARGs encoding for multidrug efflux transporters mainly from the Major Facilitator and Resistance Nodulation Superfamilies. It is strongly suggested that generally, some of these ARGs are transmitted via HGT not only on intra- and inter-species levels, but even on inter-generic levels in marine microbial communities, involving both human pathogenic species, such as *Vibrio* and *Aeromonas*, and a broad spectrum of nonpathogenic bacteria. More specifically, these nonpathogenic were found to exhibit a fairly broad spectrum of marine bacteria from the genera of *Synechococcus*, *Citromicrobium, Rhodobacteraceae, Pseudoalteromonas, Altererythrobacter, Erythrobacter, Altererythrobacter, Marivivens, Xuhuaishuia*, and *Loktanella*. It is suggested that some of these environmentally friendly bacteria can serve as active donors and/or recipients of the above ARGs, thus contributing to the transmission of these AMR determinants across genetically very diverse microbial communities in various aquatic environments.

2. Methods

2.1. Sea water sampling

250 milliliter of surface (0–25 cm in depth) water samples were collected across four sites, being separated by ~100 m, per each coastal recreational areas of the Green Cape district (N 41° 41′ 29.708″ E 41° 42′ 15.769″) and the Boulevard (N 41° 39′ 14.906″ E 41° 37′ 36.365″) of the Batumi City, Georgia. All the sampling procedures were performed during August (sea water temperature: 25.1 °C - 27 °C). The collected water samples, for the above areas, were separately pooled (constituting a total of 1 L mixed water sample per area) and were sent on dry ice to the Omega Bioservices Inc., (Norcross, GA 30071, USA) for a total DNA extraction and the whole-metagenome (WMG) shotgun sequencing using the HiSeq X 10 platform (Illumina Inc.). Thus, the WMG shotgun sequencing was performed for the collected water samples (a total of four samples) pooled separately per each location (the Green Cape District versus the Boulevard).

2.2. Metagenome data processing and analysis

The metaWRAP (v. 1.2.1) modular pipeline (version v1.3.2) (Uritskiy et al., 2018), with the metaBAT2 (2.12.1), CONCOCT (v. 1.0.0), and MaxBin2. algorithms (https://github.com/bxlab/metaWRAP), was used for extracting high-quality draft genomes (bins) from the metagenomics data provided by the Omega Bioservices Inc. FastQC was used as a quality control tool in the analysis of the raw metagenomics data as described previously (Wingett and Andrews, 2018), including, but not limited to, determining a number of sequence reads, their minimum and maximum lengths, quality scores, a number of sequence duplicates, and ambiguous bases (N's). The pair-end fastq files were filtered and trimmed with trimmomatic (Bolger et al., 2014). The metaWRAP assembly module MEGAHIT (v. 1.1.2) was employed for assembling short reads into contigs, and the initial binning of the assembled contigs was performed using the hybrid binning approach with the metaBAT2, CONCOCT, and MaxBin2 algorithms. The co-assembled bin sets were consolidated using the metaWRAP Bin_refinement module and the consolidated bin sets were then reassembled with the metaWRAP Reassemble_bins module. The module reads were mapped to the bins applying the Burrows Wheeler Aligner (BWA, v0.7.15) strictly (no mismatches) and permissively (<5 mismatches), and were stored into their respective FastQ files. The read-pairs were pulled out even if only one read was aligned to the bin. Each read set was then reassembled with SPAdes. The evaluation of completeness and potential contamination of each of the three versions of each bin (the original bin, the "strict" re-assembled bin, and "permissive" re-assembled bin) was performed using CheckM, and the best quality version of each bin was selected.

2.3. Extraction and identification of ARGs across metagenomics data

The DNA Data Bank of Japan (DDBJ) FasT Annotation and Submission Tool, DFAST (https://dfast.ddbj.nig.ac.jp/) (Tanizawa et al., 2016) was applied for annotating the metaWRAP-assembled genomes, and identifying ARGs across these genomes. In addition to DFAST, we also employed RASTtk (Brettin et al., 2015) for reexamining the DFAST annotation results. The functional analysis of proteins was performed using InterPro, which utilizes signature models to classify them into respective families (Blum et al., 2021). The DNA sequences of the DFAST and RASTtk-identified ARG loci, as well as the amino acid sequences, obtained from their conceptual translations, were then analyzed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) respectively in the nucleotide collection (nr/nt) and protein collection (using Protein-Protein search) databases of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). For the nucleotide analyses, BLASTN, with the megablast algorithm for an optimized search, was utilized with the following default general and scoring parameters: Expected threshold - 10/; Word size - 28; Max matches in a query range - 0; Match/mismatch scores - 1,-2; Gap costs – Linear; and Extension - 2. Regions of low compositional complexity were filtered accordingly when using this algorithm. For the amino acid sequence query, we applied the BLASTP algorithm with the following default general and scoring parameters (being automatically adjusted for short input sequences): Expected threshold – 0.05; Word size – 6; The BLOSUM62 Matrix; Gap costs – Existence 11, Extension 1 with the conditional compositional score matrix adjustment. The mean identities of the top-hit BLASTN and BLASTP matches (with query coverage of

≥98%), that were respectively in the ranges of ≥85% and ≥ 90% of DNA versus amino acid sequences, were considered for validating the DFAST-derived ARG annotations. In addition, we performed the taxonomic classification of bins obtained from the metagenomics analysis, using the taxonomic classification tool, Kraken 2 (Wood et al., 2019).

The metagenomics raw datasets, generated within the scopes of this BioProject, were submitted to, and are presently available under the accession number: PRJNA772929, in the NCBI Sequence Read Archive (SRA). The DNA sequences of the conceptually-annotated ARGs, extracted from these metagenomics datasets, are available under the following accession numbers: MZ080332-MZ080385 in the NCBI GenBank.

2.4. Recombination analysis

The BLAST search-identified ARGs from the NCBI GenBank, exhibiting ≥90% of DNA-DNA identity (query coverage ≥98%) with the ARGs extracted from the above metagenomics datasets, were selected and subjected to the genetic recombination analyses. In these analyses, we applied different recombination detection algorithms to identify events of genetic recombination of the ARGs and to determine the trajectories of their transmission across microbial communities of aquatic environments. Specifically, the split decomposition method (Bandelt and Dress, 1992), implemented in the SplitsTree program (version 4.14.4) (Huson and Bryant, 2006), was used to detect and to reconstruct genetic recombination events of the ARGs within the aquatic microbiota. For the above SplitsTree analyses, the DNA sequences were aligned using ClustalX (version 2.1) (Larkin et al., 2007). In these analyses, for the SplitsTree-generated splits graphs, the bootstrap values being ≥95 (from 1000 replicates) for each node of a parallelogram, and the fit values being ≥ 95 for each splits network, were considered to be statistically significant. When identified, each recombination event was reexamined using the Pairwise Homoplasy Index (Phi) test (Bruen et al., 2006) implemented in SplitsTree in order to avoid possible false positive signals that could be due to convergent mutations, which can mimic sometimes HGT signals especially across ARGs in such analyses. The Benjamini-Hochberg correction was applied to the Phi test-generated *p* values for evaluating further the obtained results. We also employed GARD (Kosakovsky Pond et al., 2006) to determine genetic recombination hotspots across the ARGs involved in HGT.

Additionally, the trajectories of ARGs genetic recombination were determined using the RDP4 software package (Martin et al., 2015) with the following implemented algorithms: RDP (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), BootScan (Martin et al., 2005), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), SiScan (Gibbs et al., 2000), and 3Seq (Boni et al., 2007). Specifically, using RDP4, we determined representative recombinant strains (recipients) as well as the representative major and minor donors of the recombined ARGs. In these analyses, for the significant breakpoint clusters (99%), the Bonferroni-corrected *p* values only in a range of ≤0.05 were considered to be statistically significant. The default parameter – 0, the linear sequence setting, and the enabled disentangle recombination signals, were the default parameters used in the RDP4 analyses.

3. Results

3.1. Genetic diversity of ARGs across Batumi City coastal recreational waters

Here, using the metagenomics analyses, we elucidate the ARG assemblages in the Black Sea coastal recreation waters of the Green Cape district and the Boulevard of the Batumi City. 9 and 44 ARGs homologs could be detected in the analyses of a total of 81 and 139 bins derived from the metagenomics datasets respectively for the Green Cape district versus the Boulevard coastal recreational waters. A total of 9.7% and 19% of reads were mapped to the Metagenome-Assembled Genomes (MAGs) obtained respectively for the Green Cape district and the Boulevard coastal sites. Table S1 provides the detailed data on the bins, their completeness, contamination, GC content, and sizes (bps) determined in these metagenomics analyses. As shown in Fig. 1, a great majority of these putatively-identified ARGs encode for multidrug resistance efflux secondary transporters from the Bcr/CflA family being a part of the Major Facilitator superfamily (MFS). The ARGs encoding for certain proteins from the EmrB/QacA drug resistance transporter subfamily (MFS) could be also detected across the Batumi Boulevard coastal waters. Other MFS multidrug transporters, identified in the metagenomics analyses, were the genes that encode collectively for MdtH, MdtL, MdtG, and NorM proteins belonging to the multidrug and toxic extrusion (MATE) family. Among the drug extrusion translocases, we could also detect the genes encoding for the membrane fusion, acriflavine resistance, and some other proteins from the Resistance Nodulation Division (RND) superfamily (Fig. 1, Table S2). Interestingly, as shown (Fig. 1), among the RND superfamily transporter-encoding loci, the genes associated with the HlyD multidrug efflux systems were also detected (Batumi Boulevard coastal waters).

Using BLASTN and BLASTP, we determined the allelic relatedness of the ARGs homologs versus their translated amino acid sequence relatedness in the NCBI nucleotide and protein databases respectively. The results obtained from the BLASTN and BLASTP analyses are summarized in Table S2. As shown, the DNA sequences of the ARGs versus the amino acid sequences (from the conceptual DNA translations) of their coding protein products from the Bcr/CflA family shared ≥90.89% and ≥ 98.03% identities with their closest conspecifics respectively from genetically very diverse bacteria: These bacteria exhibited collectively the genera of *Pseudoalteromonas*, *Rhodobacteraceae, Gilvibacter, Qipengyuania seohaensis, Aeromonas*, and *Vibrio*.

The DNA sequences of the putatively-identified ARGs and the amino acid sequences of their protein products from the EmrB/QacA drug resistance transporter subfamily were found to share 99.23% and 99.80% identities respectively with their conspecifics of *Aeromonas schubertii* or *V. fluvialis* in the NCBI nucleotide and protein databases. The identified gene homolog, encoding for the multidrug resistance protein MdtH, shared 99.42% of DNA identity with the one carried by *A. schubertii*. However, while the acriflavine resistance-encoding gene exhibited 89.18% of the DNA identity with its homolog carried by *Yoonia vestfoldensis,* the BLAST analysis of the protein sequences of this ARG, in contrast, resulted in 99.31% of amino acid sequence identity with its conspecific encoded by the ARG associated with the other species being *Donghicola tyrosinivorans* from the above NCBI database. As further shown, the alleles of ARGs, which encode for MdtL, MdtG, and NorM, were closely related to their conspecifics from *Vibrio anguillarum, Vibrio vulnificus, V. fluvialis, Pseudoalteromonas, Sulfitobacter, Synechococcus*, and *Rhodobacteraceae*. The gene alleles, involved in encoding for the HlyD family multidrug efflux system, were most closely related to their homologs carried by either *V. vulnificus* (DNA identity: ≥ 97.20%)

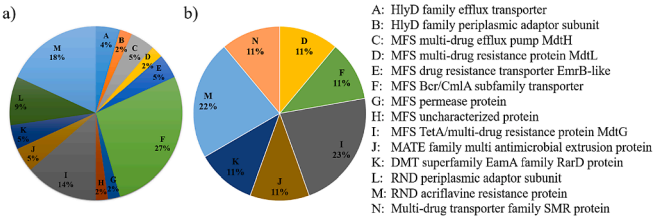

Fig. 1. A spectrum of the putative ARGs homologs identified across the coastal recreational waters of the Green Cape district and the Boulevard of the Batumi City. The ARGs homologs detected in the coastal recreational waters of the Boulevard (a) versus the Green Cape district (b); MFS - Major Facilitator Superfamily; SMR - Small Multidrug Resistance; DMT- Drug/Metabolite Transporter; MATE - Multidrug and Toxic Compound Extrusion; RND - Resistance Nodulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or *A. schubertii* (DNA identity: ≥ 97.20%). Importantly, as shown in Table S2, in 94.5% of cases, the above data exhibiting the ARGs-carrier genera or species were in a strong agreement with the ones of the bins taxonomic classification obtained using Kraken 2.

3.2. Intrageneric and intergeneric recombination of ARGs across marine microbial communities

We applied a large array of the genetic recombination detection algorithms to detect and reconstruct possible HGT events of the ARGs identified in our metagenomics study and other inversitations. Using the method of splits decomposition, the SplitsTree analyses revealed strong evidence for inter-species recombination of genetic loci encoding for the Bcr/CflA subfamily (MSF) multidrug efflux transporters in the genus of *Aeromonas*: The SplitsTree-generated split graphs shown in Fig. 2a-b, with the imbedded parallelograms exhibiting the highest fit (100) and the high bootstrapping values (varying from 81 to 100), demonstrate the genetic recombination events of these ARGs, involving *Aeromonas simiae*, *Aeromonas encheleia*, *Aeromonas caviae*, *Aeromonas veronii* and *Aeromonas jandaei*; moreover, as elucidated in Fig. 2c-d, the events of intergeneric recombination of the ARGs, which encode for the EmrB/QacA family (MFS) drug resistance transporters, were also detected, entailing *Aeromonas media*, *Aeromonas hydrophila*, *Vibrio furnissii* and *Vibrio fluvialis*. The events of intergeneric recombination of the ARGs, encoding for the RND superfamily multidrug resistance transporter periplasmic adaptor subunit, could be also identified between *Vibrio mimicus* and *Shewanella* species (Fig. 2e). In addition, we could detect the genetic exchange of the gene loci encoding for the transporter permease RarD from the EamA family (The DMT superfamily) between *V. vulnificus* and *Vibrio qinghaiendsis* (Fig. 2f; Fit: 100). Multiple events of the intra-species recombination of these and/or other genetic loci, playing their role in encoding for the multidrug resistance transporters from the HlyD family or the MATE family, could be also detected in *V. vulnificus* (Fig.S1) and *V. fluvialis* natural populations (Fig.S2).

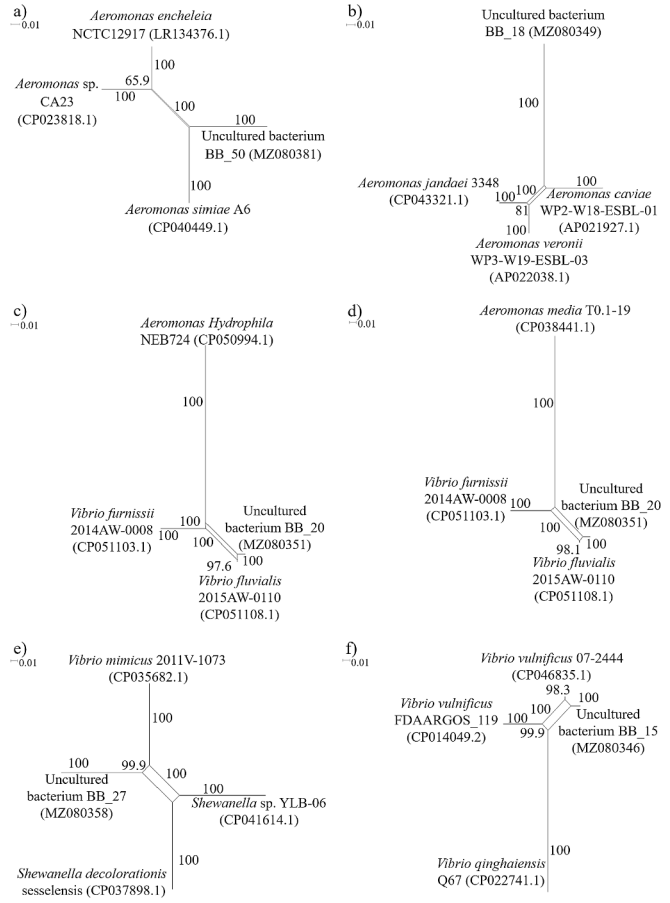

Fig. 2. Intrageneric and/or intergeneric recombination events of the putatively identified ARGs in *Vibrio* and *Aeromonas*. The SplitsTree-reconstructed events of intrageneric or intergeneric transfer of the ARGs playing roles in encoding for: Bcr/CflA subfamily (MFS) multidrug efflux MFS transporters (fit: 100) (a-b); EmrB/QacA family (MFS) drug resistance transporters (fit: 100) (c-d); EmrB/QacA family (MFS) drug resistance transporter (fit: 100) (d); RND superfamily multidrug resistance transporter periplasmic adaptor subunits (fit: 100) (e); EamA family (DMT superfamily) transporter permease RarD (fit: 100) (f). The HGT events are presented by parallelograms (with bootstrap values provided for each node) in these Splits Graphs. In parentheses, provided are the GenBank accession numbers for the DNA sequences of each strain analyzed.

It is noteworthy that due to antibiotic selective pressure, ARGs can accumulate convergent mutations (Gabashvili et al., 2020) mimicking sometimes genetic recombination in the *in-silico* analyses (Bruen et al., 2006). Hence, we applied the Phi test, which has been very instrumental in both measuring homoplasy and distinguishing recurrent mutations

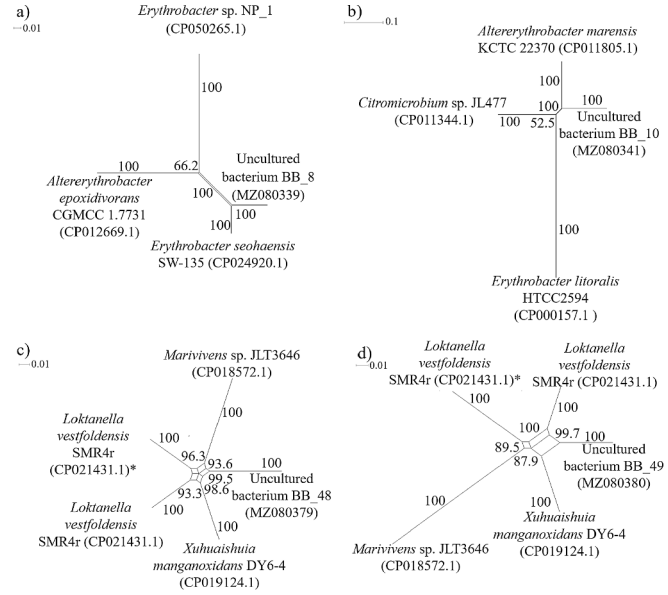

Fig. 3. Intrageneric and intergeneric recombination events of the putatively identified ARGs in human nonpathogenic bacteria of marine environment. The SplitsTree-reconstructed events of intrageneric and intergeneric transfer of the ARGs between human nonpathogenic marine bacteria, playing roles in encoding for: the Bcr/CflA subfamily (MFS) multidrug efflux transporters/MFS multidrug efflux transporters (fit: 100) (a/b); RND efflux superfamily multidrug resistance transporter permease subunit (fit: 100) (c); RND superfamily multidrug resistance protein MdtB (fit: 95.619) (d). The HGT events are presented by parallelograms (with bootstrap values provided for each node) in these Splits Graphs. In parentheses, provided are the GenBank accession numbers for the DNA sequences of each strain analyzed.

(Bruen et al., 2006; Gabashvili et al., 2020; Gabashvili et al., 2021), in order to avoid false positive results in our recombination analyses. In these analysie, the above SplitsTree-generated recombination inferences were very strongly supported by the Phi test results (p: ≤0.0494), which are detailed in Table 1 and Table S3. Besides, for gaining further insights into the above-observed HGT events, we reexamined the same DNA sequence subsets of the ARGs, using GARD. In this analysis, the GARD-generated data reflected a large number of recombination hotspots across the ARGs examined (Table S4).

3.3. Trajectories of genetic recombination events involving ARGs in marine environments

We applied RDP4 to identify donors versus recipients (recombinants) of the recombining ARGs for determining the directions of the above-observed HGT events across the microbial communities. As shown (Table 2), using RDP4, we could identify the uncultured bacterium BB_2 (MZ080333), associated most closely with *A. schubertii* (CP039611.1) (Table S2) and *A. encheleia* (LR134376.1), serving respectively as a major donor and a minor donor of the Bcr/CflA subfamily multidrug efflux transporters-encoding gene loci for *A. simiae* (CP040449.1) in two independent HGT events ($p \leq 0.0286$); in addition, during two independent HGT events ($p \leq 0.0447$), *A. caviae* (AP021927.1) and *A. veronii* (AP022038.1) appeared to have switched their donor versus recombinant roles, exchanging these efflux transporters-encoding genetic loci with *A. jandaei*, which itself was the minor donor for the *A. veronii* recombinant strain; similarly, another *A. schubertii*-associated uncultured bacterium BB_18 (MZ080349), identified in our metagenomic analysis, was shown to be the donor for *A. caviae* ($p \leq 0.0447$); the RDP4 analysis showed that the uncultured bacterium BB_20 (MZ080351), which was genetically linked most closely to *V. fluvialis* (CP014035.2) and *V. furnissii* (CP051103.1), was the major donor of the EmrB/QacA family drug resistance transporter-encoding loci for *V. fluvialis* (CP051108.1) ($p \leq 0.0000000224$); another uncultured bacterium BB_15 (MZ080346), linked most closely to one of the *V. vulnificus* strains (CP019121. 1), could be identified to be the major donor of the EamA family transporter permease RarD-encoding gene loci for *V. vulnificus* (CP014049.2) and *V. qinghaiensis* (CP022741.1) ($p \leq 0.000582$); and vice versa, *V. qinghaiensis* (CP022741.1), as well as the above uncultured bacterium BB_15 (MZ080346), appeared to be the donors of the RarD-encoding gene loci for *V. vulnificus* (CP014049.2) ($p \leq 0.0412$).

Using RDP4, we could identify multiple donors and/or recipients from different other species/genera as well, donating or acquiring the ARGs encoding for multidrug efflux transporters from the MF and the RND superfamilies (Table 3, Table S5): certain *Altererythrobacter* and *Erythrobacter* species could be found to serve as the donor/recipient when exchanging with each other the Bcr/CflA subfamily multidrug efflux transporters-encoding genetic loci ($p \leq 0.0479$); the uncultured bacterium BB_10 (MZ080341), serving as the *Altererythrobacter marensis* and *E. litoralis* donor of this subfamily multidrug efflux transporter-encoding gene loci ($p \leq 0.0479$), was found to be most closely linked to *Erythrobacter flavus* (AP019389.1) (Table S2); besides, the acquisition of the above gene by *A. marensis* (CP011805.1) from *Citromicrobium* could be also revealed, exhibiting an another independent HGT event ($p \leq 0.0479$); the intergeneric recombination events involving the genes that play a role in encoding for the RND superfamily efflux transporters could be also detected between *X. manganoxidans* and *Loktanella vestfoldensis* ($p \leq 0.049$); in addition, *Marivivens* appeared to be the minor donor of the RND superfamily efflux transporters-encoding loci for L. *vestfoldensis* ($p \leq 0.0396$); the RND superfamily efflux transporter-encoding gene of the uncultured bacterium GC_1 (MZ080332), the major donor in these HGT events, exhibited the closest DNA identity with that of L. *vestfoldensis*, suggesting intra-species recombination in this species. Figs. S1-S4 and Tables S3-S5 provide collectively additional strong evidence for the intra-species recombination of the ARGs in natural populations of this and several other species.

Table 1
The results of the Phi test examining homoplasy across the putatively identified ARGs involved in HGT events reconstructed by the method of splits decomposition.

SplitsTree Fig.	Gene	Phi test-generated p value	Benjamini-Hochberg adjusted p value
Fig. 2a	Bcr/CflA subfamily (MFS) multidrug efflux transporter	1.01E-04	0.000101
Fig. 2b	Bcr/CflA subfamily (MFS) multidrug resistance transporter	1.58E-02	0.0158
Fig. 2c	EmrB/QacA family (MFS) drug resistance transporter	8.10E-11	8.1e-11
Fig. 2d	EmrB/QacA family (MFS) drug resistance transporter	1.16E-10	1.16e-10
Fig. 2e	RND superfamily multidrug resistance transporter periplasmic adaptor subunit	9.76E-03	0.00976
Fig. 2f	EamA family (The DMT superfamily) transporter permease RarD	0.0	0*
Fig. 3a	Bcr/CflA subfamily (MFS) multidrug efflux transporter	4.94E-02	0.0494
Fig. 3b	MFS multidrug efflux transporter	1.88E-06	1.88e-06
Fig. 3c	RND efflux superfamily multi-drug-resistance transporter permease subunit	5.55E-17	5.55e-17
Fig. 3d	RND superfamily multidrug resistance protein MdtB	0.0	0*

* Positive for recombination, Phi test yielded $p \leq 0.05$.

4. Discussion

It is noteworthy that in marine environments, frequencies of ARGs appear to be higher nearshore (Port et al., 2012) than other areas, suggesting the presence of anthropogenic factors, e.g., such as coastal runoffs of drug-resistant bacteria from terrestrial sources, and the selection of these organisms emerging due to antibiotic runoffs in these sites (Hatosy and Martiny, 2015). As a result of the above selection, certain bacterial assemblages appear to be the reservoirs of ARGs. However, it is not entirely clear how these microbial assemblages contribute to the transmission of these AMR determinants via HGT across other bacterial populations that reside in these environments. Moreover, it is also noteworthy that in microbes, the frequency of HGT can accelerate when the temperatures increase at degrees (Walsh et al., 2011; Rodríguez-Verdugo et al., 2020). Thus, in light of the increased sea surface temperatures providing ideal growth conditions for bacteria (Johnson et al., 2012; Joint and Smale, 2017), which is due to global climate change, it can be thought that this phenomenon can promote genetic recombination of ARGs across microbial populations in marine environments. More specifically, it can be thought that while contributing clearly to the emergence of various communicable and other types of diseases (Andersson and Ekdahl, 2006; Stewart et al., 2008; Lindgren et al., 2012; Foote et al., 2017), the increased sea surface temperatures also promote AMR in natural populations of waterborne human and/or animal bacterial pathogens. Hence, it is highly important to constantly monitor ARGs and to determine their genetic diversity and transmission trajectories across microbial communities of these environments, for our better understanding of all possible sources and global trends of the AMR transmission especially in light of global warming.

4.1. ARGs assemblages across Batumi City recreational waters

The previous metagenomic study by et al. Jaiani et al. (2020) (Jaiani et al., 2020), exploring microbial communities of water bodies of the Black Sea at the Poti and Gonio coastal sites (located respectively at 73.5

Table 2
The representative recombinant strains, the representative major and minor donor strains of the putatively identified ARGs, and the trajectories of interspecies recombination in *Vibrio* and *Aeromonas*, determined by RDP4.*

Recombinant Strain	Representative of major donor	Representative of minor donor	Gene product	Beginning and end breakpoints (99% Cl*)	RDP4-generated *p* value	SplitsTree Fig.
Aeromonas simiae A6 (CP040449.1)	Uncultured bacterium BB_50 (MZ080381)	*Aeromonas encheleia* NCTC12917 (LR134376.1)	Bcr/CflA subfamily (MFS) multidrug efflux transporter	2280 (1–3099) - 2430 (1–3099)	RDP: 8.32E-04 GENECONV: 2.86E-02 Bootscan: 2.05E-03 Maxchi: 8.86E-04 Chimaera: 4.48E-04 SiSscan: 1.60E-02 3Seq: NS	Fig. 2a
A. simiae A6 (CP040449.1)	Uncultured bacterium BB_50 (MZ080381)	*A. encheleia* NCTC12917 (LR134376.1)	Bcr/CflA subfamily (MFS) multidrug efflux transporter	1019(UD) - 1162(UD)	RDP: 7.09E-03 GENECONV: NS Bootscan: 2.36E-03 Maxchi: 4.15E-03 Chimaera: NS SiSscan: NS 3Seq: NS	Fig. 2a
Aeromonas caviae WP2-W18-ESBL-01 (AP021927.1)	*Aeromonas veronii* WP3-W19-ESBL-03 (AP022038.1)	Unknown [Uncultured bacterium BB_18 (MZ080349)]	Bcr/CflA subfamily (MFS) multidrug efflux transporter	662 (628–809) - 784 (628–809)	RDP: NS GENECONV: NS Bootscan: NS Maxchi: 3.16E-07 Chimaera: 3.82E-02 SiSscan: NS 3Seq: 4.47E-02	Fig. 2b
A. veronii WP3-W19-ESBL-03 (AP022038.1)	*Aeromonas jandaei* 3348 (CP043321.1)	*A. caviae* WP2-W18-ESBL-01 (AP021927.1)	Bcr/CflA subfamily (MFS) multidrug efflux transporter	83(UD) – 242 (91–482)	RDP: 5.41E-03 GENECONV: NS Bootscan: NS Maxchi: 4.03E-02 Chimaera: NS SiSscan: NS 3Seq: 6.15E-03	Fig. 2b
Vibrio fluvialis 2015AW-0110 (CP051108.1)	Uncultured bacterium BB_20 (MZ080351)	*Vibrio furnissii* 2014AW-0008 (CP051103.1)	EmrB/QacA family (MFS) drug resistance transporter	178 (151–194) - 362 (325–383)	RDP: NS GENECONV: 1.04E-12 Bootscan: 5.93E-15 Maxchi: 7.57E-09 Chimaera: 1.80E-08 SiSscan: 2.24E-08 3Seq: 1.28E-13	Fig. 2c-d
Vibrio qinghaiensis Q67 (CP022741.1)	Unknown [Uncultured bacterium BB_15 (MZ080346)]	*Vibrio vulnificus* FDAARGOS_119 (CP014049.2)	EamA family (DMT superfamily) transporter permease RarD	639 (566–648) - 692(UD)	RDP: NS GENECONV: NS Bootscan: NS Maxchi: NS Chimaera: 5.82E-04 SiSscan: 1.06E-04 3Seq: NS	Fig. 2f
V. vulnificus FDAARGOS_119 (CP014049.2)	Uncultured bacterium BB_15 (MZ080346)	Unknown [*Vibrio qinghaiensis* Q67 (CP022741.1)]	EamA family (DMT superfamily) transporter permease RarD	853(UD) - 871(UD)	RDP: 1.64E-02 GENECONV: 4.12E-02 Bootscan: NS* Maxchi: NS* Chimaera: NS* SiSscan: 2.93E-25 3Seq: NS*	Fig. 2f

* NS – not significant; UD – Undetermined; Cl-breakpoint cluster.

Table 3
The representative recombinant strains, the representative major and minor donor strains of the putatively identified ARGs, as well as the trajectories of intergeneric HGT in nonhuman pathogenic marine bacteria, determined by RDP4.

Recombinant Strain	Representative of major donor	Representative of minor donor	Gene product	Beginning and end breakpoints (99% Cl)	RDP4-generated *p* value	SplitsTree Fig.
Altererythrobacter epoxidivorans CGMCC 1.7731 (CP012669.1)	*Erythrobacter seohaensis* SW-135 (CP024920.1)	Unknown [*Erythrobacter* sp. NP_1 (CP050265.1)]	Bcr/CflA subfamily (MFS) multidrug efflux transporter	902 (868–1044) - 987 (868–1044)	RDP: NS GENECONV: NS Bootscan: NS Maxchi: 5.50E-04 Chimaera: 1.08E-02 SiSscan: NS 3Seq: NS	Fig. 3a
Erythrobacter sp. isolate NP_1 (CP050265.1)	Unknown [*A. epoxidivorans* CGMCC 1.7731 (CP012669.1)]	*E. seohaensis* SW-135 (CP024920.1)	Bcr/CflA subfamily (MFS) multidrug efflux transporter	68 (UD) - 214 (137–467)	RDP: 1.65E-03 GENECONV: NS Bootscan: 1.05E-02 Maxchi: NS Chimaera: 4.01E-02 SiSscan: 4.37E-03 3Seq: NS	Fig. 3a
Altererythrobacter marensis KCTC 22370 (CP011805.1)	Uncultured bacterium BB_10 (MZ080341)	Unknown [*Citromicrobium* sp. JL477 (CP011344.1)]	MFS multidrug efflux transporter	14(UD) - 593(377–678)	RDP: 3.32E-03 GENECONV: NS Bootscan: 3.19E-02 Maxchi: 4.85E-06 Chimaera: 2.94E-04 SiSscan: 4.79E-02 3Seq: 2.07E-02	Fig. 3b
Erythrobacter litoralis HTCC2594 (CP000157.1)	Unknown [*A. marensis* KCTC 22370 (CP011805.1)]	Uncultured bacterium BB_10 (MZ080341)	MFS multidrug efflux transporter	485(UD) - 529(UD)	RDP: NS GENECONV: NS Bootscan: NS Maxchi: 1.11E-03 Chimaera: 2.03E-02 SiSscan: NS 3Seq: NS	Fig. 3b
Uncultured bacteriumBB_48 (MZ080379)	*Xuhuaishuia manganoxidans* DY6–4 (CP019124.1)	Unknown [*Loktanella vestfoldensis* SMR4r (CP021431.1)]	RND efflux superfamily multi-drug-resistance transporter permease subunit	264(UD) - 2246 (2224–2367)	RDP: 1.43E-06 GENECONV: 3.54E-05 Bootscan: 1.31E-02 Maxchi: 2.83E-07 Chimaera: 3.37E-03 SiSscan: 4.94E-14 3Seq: 6.93E-05	Fig. 3c
Xuhuaishuia manganoxidans DY6–4 (CP019124.1)	*L. vestfoldensis* SMR4r (CP021431.1)	Uncultured bacteriumBB_48 (MZ080379)	RND efflux superfamily multi-drug-resistance transporter permease subunit	2244 (UD) - 2329 (2253–2482)	RDP: 9.49E-03 GENECONV: NS Bootscan: 2.43E-03 Maxchi: 2.11E-02 Chimaera: 2.03E-02 SiSscan: NS 3Seq: NS	Fig. 3c
L. vestfoldensis SMR4r (CP021431.1)*	*Xuhuaishuia manganoxidans* DY6–4 (CP019124.1)	Unknown [Uncultured bacteriumBB_48 (MZ080379)]	RND efflux superfamily multi-drug-resistance transporter permease subunit	84(1–2504) - 233(1–2504)	RDP: 1.52E-02 GENECONV: NS Bootscan: 4.90E-02 Maxchi: 4.17E-03 Chimaera: 5.92E-04 SiSscan: NS 3Seq: NS	Fig. 3c
L. vestfoldensis SMR4r (CP021431.1)	Uncultured bacterium BB_49 (MZ080380)	*Marivivens* sp. JLT3646 (CP018572.1)	The RND superfamily multidrug resistance protein MdtB	366(256–409) - 424(409–458)	RDP: 1.61E-04 GENECONV: 2.82E-02 Bootscan: NS Maxchi: 3.31E-04 Chimaera: 6.38E-03	Fig. 3d

(*continued on next page*)

Table 3 (*continued*)

Recombinant Strain	Representative of major donor	Representative of minor donor	Gene product	Beginning and end breakpoints (99% Cl)	RDP4-generated *p* value	SplitsTree Fig.
L. vestfoldensis SMR4r (CP021431.1)	Uncultured bacterium BB_49 (MZ080380)	*Xuhuaishuia manganoxidans* DY6–4 (CP019124.1)	The RND superfamily multidrug resistance protein MdtB	544(463–680) - 630(463–680)	SiSscan: NS 3Seq: 9.02E-03 RDP: NS GENECONV: NS Bootscan: NS Maxchi: NS Chimaera: 3.55E-02 SiSscan: NS 3Seq: 4.29E-02	Fig. 3d
L. vestfoldensis SMR4r (CP021431.1)*	Uncultured bacterium BB_49 (MZ080380)	*Marivivens* sp. JLT3646 (CP018572.1)	RND superfamily multidrug resistance protein MdtB	281(UD) - 490(UD)	RDP: 3.96E-02 GENECONV: NS Bootscan: NS Maxchi: 1.42E-02 Chimaera: 7.25E-03 SiSscan: 2.48E-11 3Seq: NS	Fig. 3d

*NS – not significant; UD – Undetermined; Cl-breakpoint cluster. The genome of L. *vestfoldensis* strain SMR4r (CP021431.1) carried two copies of the gene encoding for RND superfamily multidrug resistance protein MdtB; *The second copy of L. *vestfoldensis* SMR4r (CP011431.1) gene (coordinates: c1106987–1,103,886) encoding for the RND superfamily multidrug resistance protein MdtB.

and 13.8 km from Batumi), has provided some preliminary and important information on multidrug efflux transporters genes present in these geographical sites. The City of Batumi, which is located at the Black Sea coast, represents one of the most crowded resorts in Georgia, attracting a large number of visitors including many tourists from around the world especially during a summer season. In this pilot study, we offer preliminary insights into the spectrum and genetic diversity of ARGs in the recreational coastal waters of this City.

As shown, most of the putatively identified ARGs, detected in the above recreational waters, represent the AMR determinants that encode for the Bcr/CflA family multidrug resistance efflux secondary transporters from the MFS superfamily described previously (Pao et al., 1998; Law et al., 2008). The MFS unite 74 families of multidrug transporters, which translocate a wide variety of substrates, e.g., amino acids, peptides, antibiotics, sugars, phosphates nucleosides and ions, across the cytoplasmic membrane (Reddy et al., 2012). Some of these transporters of the Bcr/CflA family confer resistance to chloramphenicol, florfenicol and bicyclomycin (Smith et al., 2009). The other ARGs, detected in these recreational waters, encode for the multidrug efflux pumps from the EmrB/QacA transporter subfamily. These AMR determinants, including the *emrB* gene, which is a *QacA* gene homolog, were found to encode for the membrane transport proteins (Paulsen et al., 1996) responsible for the export of toxic molecules from bacteria and yeast (Huggins et al., 2018), conferring resistance to hydrophobic quinolones (Putman et al., 2000). Besides, the certain genes, that we could identify in this study, encode collectively for MdtH, MdtL, MdtG, and NorM proteins, conferring resistance respectively to quinolones, such as norfloxacin and enoxacin (Yu et al., 2020), tetracycline and/or some other antibiotics (Brown et al., 1999; Morita et al., 2000; Jebastin and Narayanan, 2019; Bao et al., 2020). The above proteins belong to the MATE family, which do not exhibit significant similarity with any other MFS members (Brown et al., 1999).

The recreational waters of the above coastal areas were also found to contain the ARGs coding for the drug extrusion translocases from the RND superfamily, which, in gram-negative bacteria, play a vital role in generating AMR (Liang et al., 2016) by transporting both hydrophilic and hydrophobic quinolones (Putman et al., 2000). Importantly, in certain foodborne and other bacterial pathogens, the RND efflux pumps are thought to contribute also to pathogenicity (Lin et al., 2003; Coudeyras et al., 2008; Guérin et al., 2016). Overall, coupled with the previous findings (Jaiani et al., 2020), the above results strongly suggest that the multidrug efflux transporters are fairly abundantly present across these Georgian coastal waters of the Black Sea.

4.2. The genetic relatedness of ARGs identified in Batumi recreational waters

As compared to the developed countries, the countries with lower-middle and upper-middle incomes are thought to accumulate more community-acquired drug-resistant infections (Malik and Bhattacharyya, 2019). However, especially in the developing countries, the transmission sources of AMR have not been clearly understood yet. In Georgia, there has been a great lack of the understanding of AMR genetic determinants of AMR and their relatedness in respect to those bacterial pathogens from aquatic environments that can cause waterborne and foodborne infections. In this study, we attempted to determine also the genetic relatedness of the identified ARGs across the global microbial populations, using both the DNA BLAST analyses in the NCBI nucleotide database and the Taxonomic classification tool Kraken 2. As shown, a fairly large number of these putatively identified ARGs, encoding for the multidrug resistance transporters from the Bcr/CflA and some other families, were most closely related to the ones carried by *Vibrio, Aeromonas, Pseudoalteromonas*, *Synechococcus*, *Rhodobacteraceae, Gilvibacter,* and some other genera. A great majority of these organisms (e.g., *Synechococcus*, *Alteromonas*, *Pseudoalteromonas*, *Vibrio* and *Aeromonas*) were found to be abundantly present in the Poti and Gonio coastal waters of the Black Sea in Georgia (Jaiani et al., 2020). Among the identified *Vibrio* species from the above subset, *V. fluvialis,* being commonly found in coastal environments (Ramamurthy et al., 2014), is an emerging human pathogen, while *V. vulnificus* has been one of the leading causes of seafood-associated infections and mortality in many countries including the United States (Elmahdi et al., 2017). Besides, the certain ARGs, encoding for multidrug efflux transporters from the MF and RND superfamilies, were most closely associated with the ones carried by *A. schubertii*. This species is pathogenic mainly to snakehead fish (Liu and Li, 2012) and shrimp (Sangpo et al., 2020), although, it has been isolated sometimes from abscesses, wounds, skin, pleural fluids, and blood of patients, causing infections also in humans (Hickman-Brenner et al., 1988; Carnahan et al., 1989). Coupled with the previous findings (Jaiani et al., 2020), thus, it is reasonably safe to suggest that the above zoonotic and/or foodborne pathogenic *Vibrio* and *Aeromonas* species, which are commonly present in marine environments, may represent a part of the microbial resistome existing in the targeted recreational waters of the Black Sea.

Along with the *Vibrio* and *Aeromonas* species, *P. piscicida*, being also frequently found in various marine environments, may appear to be an another potential ARG-carrier serving as an alternative reservoir of some of these genes in the above recreational waters. This species demonstrates bactericidal properties toward *V. vulnificus* and *Vibrio parahaemolyticus* (Richards et al., 2017), which can be suggestive of their antagonistic interactions possibly existing in these and other aquatic environments. The *Synechococcus* genus, which was also predicted to be a potential ARG-carrier in the above explored marine sites, reflects unicellular cyanobacteria that are wildly present, contributing to the biogeochemical cycles, in the aquatic ecosystems globally (Callieri, 2017; Lee et al., 2019). Other predicted ARG-carriers, e.g., such as *Rhodobacteraceae* (Alphaproteobacteria) (Dogs et al., 2017), *Gilvibacter* (Onda et al., 2015), *Q. seohaensis* (Xu et al., 2020), *Y. vestfoldensis* (Johansson et al., 2019), *D. tyrosinivorans* (Sung et al., 2015), and *Sulfitobacter* (Song et al., 2019), also belong to the endosymbiotic microflora frequently present in marine environments. Thus, it is thought that the Batumi coastal waters of the Black Sea may contain a fairly large spectrum of the marine microbial resistome, although, more in-depth and extensive metagenomic analyses and studies (with a significantly larger sample size) are planned to strongly support the above suggestions.

4.3. Intrageneric and intergeneric recombination of ARGs and their transmission trajectories

The surveillance data show that the acquisition and transmission of ARGs across the natural populations of bacterial pathogens is expected to become increasingly severe, gathering a significant attention to the controlling of these organisms, and to the deciphering of their AMR determinants (Abe et al., 2020). The ARGs have been detected extensively not only in various marine environments (Rahman et al., 2008), but also ocean-open sites (Hatosy and Martiny, 2015) including even some polar areas (Rahman et al., 2015) not exposed to antibiotic selective pressures. Unlike chemical pollutants, the genetic pollutants, e.g., such as ARGs, originating from both natural and man-made settings, can replicate and increase in their frequency partially via HGT (Abe et al., 2020). Recent studies suggest that HGT plays some important role in the ARGs transmission in various marine ecosystems (Dong et al., 2019; Chen et al., 2020). Thus, along with elucidating the patterns of the AMR development, which has long become a significant focus of the public health attention (Malik and Bhattacharyya, 2019), determining the HGT-mediated transmission trajectories of ARGs is another key approach to generate the additional important information for our better understanding of the AMR global trends. It is noteworthy that some of the previous findings highlight the tendency, according which, HGT occur more commonly at certain increased temperatures (Walsh et al., 2011; Rodríguez-Verdugo et al., 2020). Thus, as the global warming continues to rise, deciphering the trajectories of ARGs genetic recombination across marine microbial communities becomes highly imperative to identify possible unknown sources contributing to AMR transmission.

Here, we provide strong statistical evidence for both intrageneric and intergeneric recombination events of multiple putatively identified genes encoding for multidrug efflux transporters of the MFS, and for those of the RND and DMT subfamilies, in several human and/or animal pathogenic species of *Aeromonas* and *Vibrio*. Generally, in the natural populations of bacteria, HGT events occur more commonly on intraspecies levels, as opposed to the above modes of genetic recombination, which is due to species specific, host specific, and possibly other constraints that can exist in these organisms. It can be strongly suggested that among the *Aeromonas* species, *A. hydrophila, A. caviae, A. veronii, A. media*, and *A. jandaei*, causing collectively gastroenteritis and extraintestinal manifestations (Overman and Janda, 1999; Fernández-Bravo and Figueras, 2020) sometimes with the exhibited AMR (Overman and Janda, 1999; Ramadan et al., 2018; Mokhtar et al., 2020), are actively involved in such HGT events. *A. simiae* has been isolated from various sources including some animals (Fontes et al., 2010), their faeces (Harf-Monteil et al., 2004), as well sewage (Chen et al., 2019). This species has the ability to develop resistance at least to some β-lactams (Chen et al., 2019). Among the above ARG-recombining vibrios, coupled with *V. fluvialis* and *V. qinghaiensis*, the latter being not pathogenic to humans (Ramamurthy et al., 2014; Gong et al., 2018), both *V. furnissii* (Ballal et al., 2017) and *V. mimicus* (Campos et al., 1996) are regarded to be the emerging pathogens. Importantly, it is suggested that *V. mimicus* has the ability to recombine the gene that encode for the RND superfamily multidrug resistance transporter periplasmic adaptor even with genetically very distinct bacteria from the other genus, such as *Shewanella*. Being most frequently associated with aquatic environments, *Shewanella* was found to cause human infections and to develop resistance to antibiotics (Yousfi et al., 2017). Interestingly enough, *Shewanella decolorationis* from the above genus has demonstrated an unusual ability (without apparent modifications of its metabolism) to grow under temperatures varying from 24 °C to 40 °C (Lemaire et al., 2019). Hence, it can be thought that, due to the increased global warming conditions, *Shewanella* can thrive even more and become a larger reservoir for certain ARGs in aquatic environments.

It is strongly suggested that the modes of intrageneric and intergeneric recombination of the multidrug resistance efflux transporter-encoding loci can be both be- and multi-directional not only in *Aeromonas* and *Vibrio*, but also across the *Erythrobacter*, *Altererythrobacter, Altererythrobacter*, *Citromicrobium, Marivivens*, *Xuhuaishuia* and *Loktanella,* representing collectively a fairly large and diverse microbial resistome (Van Trappen et al., 2004; Li et al., 2016; Wang et al., 2016; Xu et al., 2018). As the donor and/or the recipient of ARGs, the above genera clearly seem to play via HGT some important role in the transmission of at least multidrug resistance efflux transporters of the MFS, and the RND and DMT superfamilies.

5. Conclusion

The recreational coastal waters that are contaminated by ARGs can promote the emergence and transmission of drug-resistant strains of many zoonotic and/or waterborne pathogens especially in light of the increased sea surface temperatures promoting their growth. The archiving deeper insights into the genetic diversity of these ARGs, and into the molecular genetic mechanisms underlying their divergence and transmission in marine environments can contribute significantly to determining and predicting the existing and future evolutionary trends of AMR respectively. The recreational coastal waters of the Batumi City exhibited mainly the presence of multidrug resistance transporters belonging to the MFS, as well as to the RND and DMT superfamilies. We suggest that, in the above marine environment, their presence could be explained most likely by the physiological multifunction of these AMR determinants in bacteria, although, this can partially be associated as well with some antibiotic selective pressures that may exist in these coastal areas. Besides, it can be also suggested that the large microbial assemblages that include mainly, but not limited to, *Vibrio*, *Aeromonas*, *Pseudoalteromonas, Altererythrobacter, Erythrobacter,* and *Loktanella*, serve as the active reservoirs of the ARGs that encode for these multidrug resistance transporters, thus contributing to their transmission via HGT in various marine environments. In such HGT events, many members of these genera seem to readily switch their roles of the donor and the recipient of these ARGs. Moreover, it also suggested that the HGT-mediated transmission mode of these AMR determinants can be both bi- and multi-directional across the above microbial genera in marine environments in general, exhibiting not only evidence of intra- and inter-species recombination, but also that of intergeneric recombination of these ARGs.

Availability of data and material

DNA sequence data, generated in this study, are publicly available in GenBank Database of the National Center for Biotechnology Information (NCBI).

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Credit author statement

EG was responsible for conducting the sampling, and the major metagenomics and recombination analyses. SK was responsible for the BLAST analysis, and contributed to the quality assessment of the DNA sequence data generated in the metagenomics analyses. TC provided her assistance in the selection of DNA sequences of ARG homologs in the NCBI database. LT performed the validation analysis of the BLAST-selected ARG homologs for recombination analyses. RT provided her assistance in the DNA sequence alignment analyses, and the extraction of ARGs from the metagenomics data. KD provided her expertise in the DNA sequence data compilation. SK revised a draft of the manuscript, contributing to the formulation of its final version for submission. MK's role in this study was the research conceptualization and supervision, as well as the formulation of the manuscript and its submission to the Journal.

Declaration of Competing Interest

The authors declare that they have no competing interests. Stylianos Koulouris is employed by the European Food Safety Authority (EFSA). The present article is published under the sole responsibility of the author and may not be considered as an EFSA scientific output. The positions and opinions presented in this article are those of the author alone and do not necessarily represent the views or scientific works of EFSA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.margen.2021.100916.

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3.2 ფაგური ტრანსდუქცია ჩართულია ანტიმიკრობული რეზისტენტობის გენების *bla*CTX-M, *mel*, და *tetM* ლოკუსების სახეობათშორის გავრცელებაში ბაქტერიათა ბუნებრივ პოპულაციაში

ფაგები, მათ შორის პოლივალენტური ფაგები, შესაძლოა მონაწილეობენ ანტიმიკრობული რეზისტენტობის განმაპირობებელი გენების ჰორიზონტალურ გადაცემაში (Gabashvili et al., 2020). კვლევის ფარგლებში შევისწავლეთ NCBI გლობალურ ბაზის 2239 ფაგის გენომები და გამოვავლინეთ 19 ბაქტერიოფაგი შემენილი რეზისტენტობის გენით (ARGs). ჩვენმა კვლევამ წარმოაჩინა პოლივალენტური ფაგების ჩართულობა ანტიმიკრობული რეზისტენტობის გენების ჰორიზონტალურ გადაცემაში და უჩვენა, რომ გარკვეული პოლივალენტური ზომიერი ბუნების მქონე ბაქტერიოფაგები მონაწილეობას ღებულობენ *bla*CTX-M, *tet*M, *mel* გენების სახეობათშორის მიმოცვლაში *E. coli, S. enterica, S. pneumoniae, S. sonnei, S. suis, E. rhusiopathiae,* და *B. coagulans* -ის ბუნებრივ პოპულაციებში. ჩვენი კვლევების თანახმად *Salmonella*-ს ფაგი შესაძლოა წარმოადგენდეს *bla*CTX-M გენის ვექტორს *E. coli* - სა და *S. enterica* -ს შორის, ხოლო *tet*M რეზისტენტობის გენის გავრცელება *S. suis* -ის ბუნებრივ პოპულაციაში შესაძლოა უკავშირდებოდეს *Erysipelothrix* -ის ფაგთან და *E. faecium*-ის შტამთან მის გენეტიკურ რეკომბინაციას. *Streptococcus* -ის ფაგი, *Erysipelothrix* -ის ფაგი, და *S. pneumoniae*- ისა და *B. coagulans* -ის ბუნებრივი პოპულაციები კი ჩართული არიან *mel* გენის მიმოცვლაში. ასევე აღსანიშნავია რომ *mel* გენის მიმოცვლა შეიძლება მოხდეს ისეთ ორგანიზმებს შორის, როგორებიცაა *Streptococcus* -ის და *Erysipelothrix* -ის ფაგები. ჩვენს მიერ მიღებულმა შედეგებმა ასევე გამოავლინეს პოლივალენტური ფაგების ჩართულობა *bla*CTX-M, *tet*M, და *mel* გენების ჰორიზონტალურ მიმოცვლაში სახეობათშორისო დონეზე.

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Phage Transduction is Involved in the Intergeneric Spread of Antibiotic Resistance-Associated bla_{CTX-M}, *mel,* and *tetM* Loci in Natural Populations of Some Human and Animal Bacterial Pathogens

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Abstract

The horizontal genetic transfer (HGT) of antibiotic resistance genes (ARGs) mediated by species-specific bacteriophages contributes to the emergence of antibiotic-resistant strains in natural populations of human and animal bacterial pathogens posing a significant threat to global public health. However, it is unclear and needs to be determined whether polyvalent bacteriophages play any role in the intergeneric transmission of ARGs. In this study, we examined the genome sequences of 2239 bacteriophages from different sources for the presence of ARGs. The identified ARG-carrying bacteriophages were then analyzed by PHACTS, PHAST, and HostPhinder programs to determine their lifestyles, genes coding for bacterial cell lysis, recombinases, and a spectrum of their potential host species, respectively. We employed the SplitsTree, RDP4 and SimPlot software packages in recombination tests to identify HGT events of ARGs between these bacteriophages and bacteria. In our analyses, some ARG-carrying bacteriophages exhibited temperate and/or polyvalent patterns. The bootstrap values (97–100) for the SplitsTree-generated parallelograms, fit values (97–100) for splits networks, Phi *P* values ($< 10^{-17}$ to 3.9×10^{-16}), RDP4 *P* values ($\leq 7.8 \times 10^{-03}$), and the SimPlot results, provided strong statistical evidence for the phage transduction events of bla_{CTX-M}, *mel*, and *tetM* loci on inter-species level. These events involved several host species such as *Escherichia coli, Salmonella enterica, Shigella sonnei, Streptococcus pneumoniae* and *Bacillus coagulans.* HGT of *mel* loci between *Erysipelothrix* and *Streptococcus* phages were also detected. These results firmly suggest that certain bacteriophages possibly with temperate properties induce the intergeneric dissemination of bla_{CTX-M}, *mel* and *tetM* in the above species.

Introduction

Antibiotic resistance remains a major global health concern in light of increasing prevalence of this phenomenon, with ca. 700,000 instances of human mortality annually attributed to resistant bacterial infections [1]. Clonal expansion of drug-resistant strains, chromosomal mutations conferring resistance to drugs, and HGT of ARGs between strains are the mechanisms responsible for generating and amplifying antibiotic resistance in human and animal infections. HGT is largely driven by mobile genetic elements, such as plasmids and transposons, and bacteriophages across different bacterial communities [1, 30, 45, 48]. Along with transformation and conjugation involving these genetic elements,—the latter being previously regarded as the most efficient mechanism for the exchange of genetic material among bacteria [10]—phage transduction is thought to be one of the mechanisms that contributes to the emergence of antibiotic-resistant strains [8, 32]. Each phage has its own

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particular host range, and while some can only infect one or a few bacterial strains, other phages can infect many strains and species [16, 39]. Previous studies provide ample evidence that the transduction of antibiotic resistance determinants is mainly mediated by species-specific (monovalent) phages [8]. In contrast, only a very few attempts have been made to determine whether polyvalent phages—those that can infect different species—contribute to inter-species dissemination of ARGs: the bacteriophage-mediated transduction of ARGs between several *Enterococcus* species was demonstrated under laboratory conditions [29], besides, the transfer of antibiotic resistance by bacteriophages between *Staphylococcus* species has been also elucidated [50]. These findings deliver preliminary evidence to assume that polyvalent phages play at least some role in the spread of antibiotic resistance between species.

In this study, we provide strong statistical evidence for inter-species recombination of $bla_{\text{CTX-M}}$ (the extended-spectrum β-lactamase-encoding gene), *mel* (the ABC-type efflux permease-encoding gene) and *tetM* (ribosomal protection protein-encoding gene) mediated by bacteriophage across multiple bacterial genera. Herein, we also track events of genetic exchange of *mel* between bacteriophages from distinct host species. In addition, inter-species recombination of $bla_{\text{CTX-M}}$, and events of both inter- and intra-species recombination of *tetM* in foodborne and other bacterial pathogens, are elucidated.

Materials and Methods

Identification of ARG-Carrying Bacteriophages

A total of 2244 amino acid sequence records for ARGs available in the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca) were included in the analysis. Using these amino acid sequences, we performed a homology search of these genes across the genomes of 2239 bacteriophages annotated in the protein and nucleotide databases of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) to identify ARG-carrying bacteriophage. In this analysis, the amino acid sequences for each ARG were blasted against the NCBI nucleotide and protein databases using the TBLASTN and BLASTp algorithms, respectively. In the TBLASTN analysis, we applied 10 as a default value for the expected number of chance matches using a random model.

Classification of the ARG-Carrying Phages Lifestyle and Their Host Prediction

We performed both proteome and genome analyses of the ARG-carrying bacteriophages to predict their lifestyle (virulent versus temperate) and a spectrum of their potential host species. For classifying the lifestyle (temperate versus lytic) of ARG-carrying bacteriophages identified in the NCBI nucleotide database, we used both the corresponding references and the online program PHACTS with the Random Forest algorithm (https://edwards.sdsu.edu/PHACTS/index.php). For the PHACTS analysis, a proteome of each ARG-carrying phage was obtained using the DDBJ Fast Annotation and Submission Tool, DFAST (https://dfast.nig.ac.jp), with GHOSTX as default aligner. When using PHACTS [31], we considered the predicted phage lifestyle to be reliable if generated probability values were > 0.5. We applied the PHAge Search Tool (PHAST) [51] (https://phast.wishartlab.com) to identify bacterial cell lysis and recombinase genes in the ARG-carrying bacteriophages for better understanding of the genomic features of these organisms.

For the ARG-carrying phages, we searched for the corresponding references and other relevant information on their actual hosts in the NCBI database and the Virus-Host Database (DB) (https://www.genome.jp/virushostdb/note.html). Besides, HostPhinder (version 1.1) [44] (https://cge.cbs.dtu.dk/services/HostPhinder/) was applied to predict putative hosts for these phages. The coverage values being > 0.7 for species, and 0.8 for genus, were interpreted as strong indicators for host species prediction, while predictions based on a coverage value below 0.1 were only correct for 47% (species) and 63% (genus) of the phages as predetermined previously [44]. Given that, in our analysis, the coverage values ≥ 0.1 were still considered to be valuable indicators.

Recombination Analyses of ARGs

We applied the method of split decomposition [2] implemented in the SplitsTree program (version 4.14.4) to detect genetic recombination of ARGs between bacteriophages and bacterial strains. Bootstrap values ≥ 95 (from 1000 replicates) for each node of the SplitsTree parallelograms, and fit ≥ 95 for each splits network were considered statistically significant. Each splits network was reexamined using the Phi (Pairwise Homoplasy Index) test [5] to confirm evidence of putative recombination events, and to avoid false positive results that sometimes could be due to convergent mutations that may exist across the ARGs. We applied RDP [28], GENECONV [35], BootScan [27], MaxChi [41], Chimaera [37], SiScan [12] and 3Seq [3] implemented in the RDP4 software package (Beta 4.96) [26] to confirm recombination events, and to identify major and minor parents (donors) of the recombined loci, as well as recombinant strains (recipients). For detecting genetic recombination, RDP4 executes recombination analyses without any need for predefined sets of non-recombinant reference sequences, which makes it more generally applicable than many other available recombination analysis tools [26]. The above fast and powerful

heuristic recombination detection methods implemented in RDP4 sequentially test every combination of three sequences in an input alignment to identify both recombinant and its possible parental DNA sequences. The default parameter – 0, the linear sequence setting as well as enabled disentangle recombination signals were applied for the number of permutation in all RDP4 analyses. Bonferroni-corrected P values ≤ 0.05 predetermined (RDP4 Instruction Manual), for significant breakpoint clusters (99%) across the ARGs DNA sequence alignments, were considered statistically significant in these analyses. The putative recombination hotspots identified by RDP4 in the ARGs DNA alignments were reexamined using the SimPlot v3.5.1 program [25]. The window size and the step size were set to 200 bp and 20 bp, respectively, in this analysis.

Results and Discussion

Classification and Host Ranges of ARGs-Carrying Bacteriophages

It is important to indicate that ARG-carrying bacteriophages have been isolated not only from bacterial strains that cause human or animal infections [11], but also from river, sewage water [46], activated sludge [36], marine environment [6], and human fecal samples [38]. The lifestyle of such phages and a spectrum of their host species remain largely unclear and need to be determined in order to gain essential insights into a magnitude of the dissemination of ARGs via phage transduction in natural populations of infectious bacterial agents.

We identified nineteen ARGs-carrying bacteriophages among 2239 phages examined in NCBI, using the BLASTp and TBLASTN searches for the amino acid sequences of the ARGs from the CARD (Table S1). These ARGs-carrying bacteriophages (except the unclassified *Streptococcus* phage, Javan 630) belonged to one of the families of the order of *Caudovirales* such *Myoviridae*, *Siphoviridae* and *Podoviridae* (Table S2). The results of the HostPhinder analysis with ≥ 0.15 coverage values predict that some of these phages could infect more than one host species (Table S2). Thus, based on these findings, we reasoned that phages might operate on both intra- and inter-species levels in natural populations of bacteria. Interestingly, the HostPhinder analysis with the coverage value 0.59 strongly predicted *E. coli* as a putative host for *Salmonella* phage SJ46 (KU760857.1). However, the obtained coverage values being 0.039, 0.0037, and 0.0035 exhibited a very weak association of the above *Salmonella* phage strain with both its actual host species, *S. enterica* and two putative alternative host species, *Shigella sonnei* and *Shigella flexneri*, respectively (Table S3). We believe that such ambiguous results could be because HostPhinder predicts host species of a phage only by searching for the most genetically similar phages in a database of reference phages with known hosts, for which it uses the number of co-occurring *k*-mers (DNA sequences of length *k*) as a measure of genetic similarity. Hence, when applying this method HostPhinder disregards a highly mosaic structure of the phage genomes. The splitting of entire phage genomes into overlapping *k*-mers was proposed recently as a solution for the above problem [18, 24], although, this approach has not been employed yet. It must be also noted that a number of sequenced genomes of phages and the information on their host species is still very limited in the NCBI and other databases, making a comprehensive host prediction analysis even more difficult.

The increasing number of genome sequences deposited in databases foster the identification of temperate phages by marker genes [19]. Examining the prophage and other marker genes, PHACTS predicted confidently a temperate lifestyle for a majority of these phages (Table S4). These results provide additional evidence to the view widely supported by the previous findings [15], implying that the ARGs are most frequently transmitted by temperate phages when phage transduction is implicated in the dissemination of these genes. Using PHAST, we could identify recombinase-coding genes (Table S5) in eleven bacteriophages from this subset, at least some of which play an important role in facilitating homologous recombination as reported previously [34]. The genes responsible for bacterial cell lysis could be also identified by PHAST across some of these bacteriophages genomes (Table S5). These findings can be of a great value to the future studies aimed at the better understanding of the genomic features of the ARG-carrying bacteriophages.

Genetic Recombination of ARGs Between Bacteriophages and Their Host Species

Horizontal transfer of ARGs mediated by bacteriophages appears to be relatively common than previously believed, contributing to the dissemination of resistance to antibiotics [8]. Our hypothesis assumed that there exist polyvalent phages, which contribute to the intergeneric spread of ARGs in natural populations of bacterial infectious agents. The consensus results obtained from our analyses using different recombination detection algorithms and methods implemented in the SplitsTree, RDP4 and SimPlot software packages revealed strong evidence for phage-mediated intergeneric spread of three ARGs, $bla_{\text{CTX-M}}$, *mel* and *tetM* across various human and animal bacterial pathogens. CTX-M confers resistance against penicillins, oxyimino-cephalosporins (i.e., cefuroxime, cefotaxime, ceftriaxone, cefpodoxime, ceftazidime, ceftiofur, cefquinome) and monobactams [4]. The ABC-type efflux permease-encoding gene, *mel* is a homolog

of the ATP-binding cassette gene *msrA* [40], conferring macrolide resistance in various Gram-positive bacteria. The *tetM* gene, which is widely present in many species across eight gram-negative and eighteen gram-positive genera [7], leads to resistance to tetracycline.

Generally, plasmids are considered as the main contributors to the $bla_{\text{CTX-M}}$ transfer across various species of bacteria [30]. However, the high prevalence of $bla_{\text{CTX-M}}$ in the genomes of phages recovered from soil and water, and the generalized transduction of this gene mediated by some members of *Myoviridae* (specifically, T4-like *E. coli* phages) may be suggestive of at least some role of these organisms in the spread of $bla_{\text{CTX-M}}$—associated antibiotic resistance [9, 32]. In our recombination analyses, SplitsTree generated a parallelogram (Fig. 1a), which involved the *Salmonella* phage (KU760857.1), *S. enterica* (MF418176.1), *E. coli* (AB900900.1) and *S. sonnei* (AB284167.1), exhibiting events of genetic recombination of $bla_{\text{CTX-M}}$ between these organisms.

To date, it is unknown whether the phage transduction plays any role in the dissemination of *mel* in natural populations of human and animal bacterial pathogens. The results

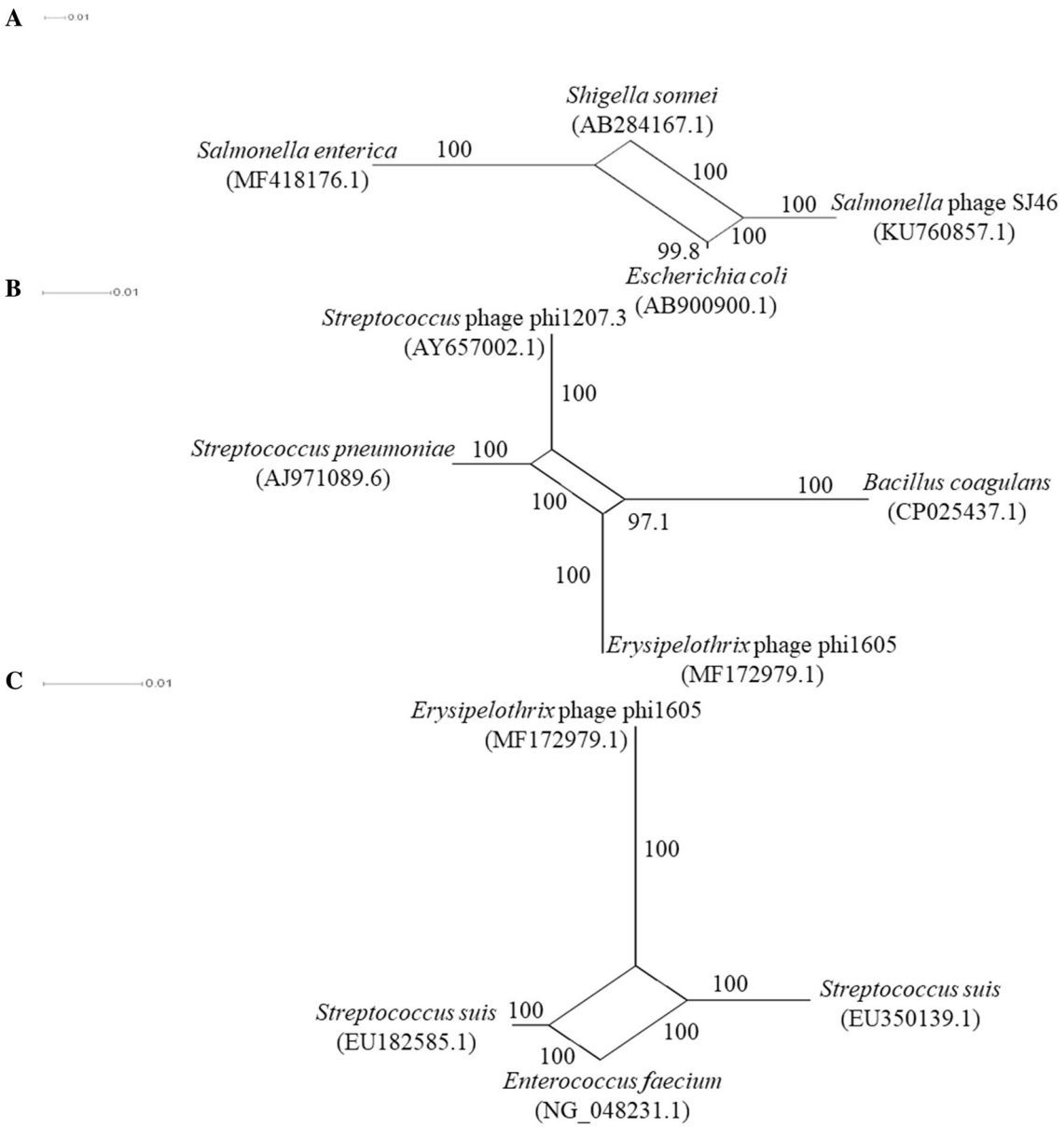

Fig. 1 Splits Graphs constructed by SplitsTree, exhibiting the genetic recombination of $bla_{\text{CTX-M}}$ (fit: 99.5) (**a**), *mel* (Fit: 100) (**b**), and *tetM* (Fit: 100) (**c**) loci between various bacteriophages and bacterial species strains. The genetic recombination events are presented by parallelograms (with bootstrap values provided for each node) in these Splits Graphs

of our SplitsTree analysis (Fig. 1b) revealed the HGT events of *mel* between the *Streptococcus* phage (AY657002.1), *Erysipelothrix* phage (MF172979.1), *S. pneumoniae* (AJ971089.6) and *B. coagulans* (CP025437.1).

HGT has been also one of the evolutionary forces that drive the dissemination of *tetM* loci via conjugation and genetic transformation in bacterial communities [45]. The transfer of tetracycline resistance was achieved via the phage transduction in some *Enterococcus* [29] and *Streptococcus* [43] species under laboratory conditions. In our analyses, Splitstree allowed us to detect events of the inter-species recombination of *tetM* loci between bacterial species from different genera, involving also the bacteriophage (Fig. 1c). More specifically, we observed the genetic exchange of *tetM* loci between the *Erysipelothrix* phage (MF172979.1), *S. suis* (EU182585.1) and *E. faecium* (NG_048231.1).

It is important to note that, while searching for parallel genetic changes that particularly exhibit conflicting evolutionary signals across genetic loci, SplitsTree may generate false positive results due to the presence of convergent mutations particularly in genetic loci that undergo selective pressure, sometimes mimicking genetic recombination events. For such instances, the Phi test [5] can be one of the best approaches that allow detecting homoplasy and distinguishing recurrent mutations from recombination. The split networks (Fig. 1a–c) generated in our SplitsTree analysis were highly supported by the results of the Phi test with the *P* values being $< 10^{-17}$ for $bla_{\text{CTX-M}}$, 3.9×10^{-16} for *mel*, and 5.6×10^{-17} for *tetM*, additionally demonstrating statistically significant evidence of genetic recombination.

Even though SplitsTree has been a very useful tool for studying intra- and inter-species recombination among both bacteria [22, 23] and viruses [33], when visualizing putative recombination events in a capacity of quartet networks, it has no ability to distinguish between donors and recipients of the exchanged genetic loci. Thus, we applied the RDP4 software package to reexamine the genetic recombination events of $bla_{\text{CTX-M}}$, *mel*, and *tetM* determined by SplitsTree, and to identify donors and recipients among the phages and bacterial strains involved in these events. The RDP4 analysis provided strong evidence for the horizontal genetic transfer of $bla_{\text{CTX-M}}$, *mel*, and *tetM* loci between the same bacteriophages and these bacterial species strains, thus highly agreeing with the results obtained from the SplitsTree analysis. Table 1 summarizes the recombinant strains, their major and minor donors as well as the recombination beginning and end breakpoints across the ARGs determined by RDP4.

Very importantly, displaying the highly significant *P* values, the generated results were consistent across a great majority of recombination tests conducted using this software package. RDP4 has been successfully applied alone [26] or in combination with SimPlot [49] to study a phenomenon of genetic recombination between different organisms. In this study, the results obtained additionally from the SimPlot analysis of $bla_{\text{CTX-M}}$, *mel* and *tetM* revealed the presence of recombination hotspots across all these loci for the same subset of the bacteriophages and bacterial species strains (Fig. 2).

The consensus results of the above recombination detection analyses, strongly suggest that the *Salmonella* phage SJ46 could serve as a donor of $bla_{\text{CTX-M}}$ for both *E. coli* and *S. sonnei* strains. It can be also strongly suggested that certain $bla_{\text{CTX-M}}$-carrying *S. enterica* strains could be sometimes donors of this gene to *E. coli* and *S. sonnei*. This scenario will appear more plausible if we consider the results obtained from the HostPhinder analysis, which predicted *E. coli* as a potential host species for the *Salmonella* phage SJ46.

The consensus results of the recombination analyses also provided strong statistical evidence for (i) the acquisition events of *mel* by the *Erysipelothrix* phage from two genetically distant genera such as *Streptococcus* and *Bacillus*, and (ii) the genetic exchange of *mel* between *Erysipelothrix* and *Streptococcus* phages. In particular, the results obtained from the SplitsTree, Phi, the RDP4 and SimPlot analyses (Table 1, Figs. 1, 2) collectively demonstrate two events of *mel* acquisition by the *Erysipelothrix* phage (MF172979.1) separately from *S. pneumoniae* (AJ971089.6) (major donor) and *B. coagulans* (CP025437.1) (minor donor)—the latter being a probiotic species [21]. This finding adds another layer of evidence to the growing concerns associated with the facts of acquisition of *mel* and other ARGs via HGT by probiotic species used in both livestock and human applications, thus, representing a serious safety threat [14, 20]. Importantly also, the above results (Table 1, Figs. 1, 2) showing the event of HGT of *mel* between the *Erysipelothrix* phage (MF172979.1) and the *Streptococcus* phage (AY657002.1) is strongly in agreement with the earlier suggestion that genetic recombination takes place between bacteriophages [17]. While it remains unknown whether genetic recombination occur between temperate phages and virulent (lytic) phages, this possible scenario still should not be excluded. Thus, the above findings call for an urgent need for more extensive studies on phage evolution, especially in terms of potential long-term consequences of various phage applications for food processing, veterinary and medical therapeutic purposes. The most recent studies on human gut microbiota introduce a new concept of bacteriophages as human pathogens, suggesting that these organisms sometimes can be implicated in developing some diseases via their interplay with gut microbiota and the human host [13, 42]. Hence, the above findings allow us to pose a legitimate and important question: How can HGT between bacteriophages as well as among these and their hosts alter and shape gut microbiota (which may lead to short- and/or long-term adverse health consequences) in humans and animals, especially when

Table 1 The donor and recipient strains of $bla_{\text{CTX-M}}$, *mel* and *tetM*, and the statistics for the recombination beginning and end breakpoints that were determined by the RDP4 analyses

ARG	$bla_{\text{CTX-M}}$ (ARO: 3,001,889)		*mel* (ARO: 3,000,616)		*tetM* (ARO: 3,000,186)	
Recombination event	# 1	# 2	# 1	# 2	# 1	# 2
Recombinant	*Shigella sonnei* (AB284167.2)	*Escherichia coli* (AB900900.1)	*Erysipelothrix* phage phi1605 (MF172979.1)	*Erysipelothrix* phage phi1605 (MF172979.1)	*Streptococcus suis* (EU350139.1)	*Streptococcus suis* (EU182585.1)
Major donor	*Salmonella enterica* (MF418176.1)	*Salmonella* phage SJ46 (KU760857.1)	*Streptococcus pneumoniae* (AJ971089.6)	*Streptococcus* phage phi1207.3 (AY657002.1)	*Streptococcus suis* (EU182585.1)	*Enterococcus faecium* (NG_048231.1)
Minor donor	*Salmonella* phage SJ46 (KU760857.1)	*Salmonella enterica* (MF418176.1)	*Bacillus coagulans* (CP025437.1)	Unknown	*Erysipelothrix* phage phi1605 (MF172979.1)	*Erysipelothrix* phage phi1605 (MF172979.1)
Beginning breakpoint (position in alignment)	99% Cl 184–209	99% Cl 553–575	99% Cl 877–1067	99% Cl 1–47	99% Cl 1535–1637	99% Cl 209–449
End breakpoint (position in alignment)	99% Cl 655–680	Undetermined	99% Cl 1330–1451	99% Cl 352–789	Undetermined	99% Cl 520–640
P values						
RDP	–	–	1.963×10^{-03}	2.193×10^{-03}	1.239×10^{-09}	–
GENECONV	9.630×10^{-26}	2.406×10^{-29}	–	–	4.511×10^{-09}	2.241×10^{-03}
BootScan	1.391×10^{-31}	8.738×10^{-32}	7.291×10^{-04}	3.401×10^{-03}	2.683×10^{-10}	7.572×10^{-06}
MacChi	1.249×10^{-21}	4.690×10^{-18}	3.284×10^{-05}	8.758×10^{-04}	8.523×10^{-07}	1.902×10^{-06}
Chimaera	–	$3.223\times\times10^{-18}$	4.726×10^{-07}	9.849×10^{-04}	7.226×10^{-07}	1.322×10^{-04}
Siscan	–	1.7324×10^{-25}	3.334×10^{-09}	1.051×10^{-02}	1.781×10^{-06}	5.029×10^{-05}
3Seq	5.925×10^{-55}	1.096×10^{-46}	1.725×10^{-07}	7.785×10^{-03}	1.079×10^{-08}	8.460×10^{-05}

Cl breakpoint cluster

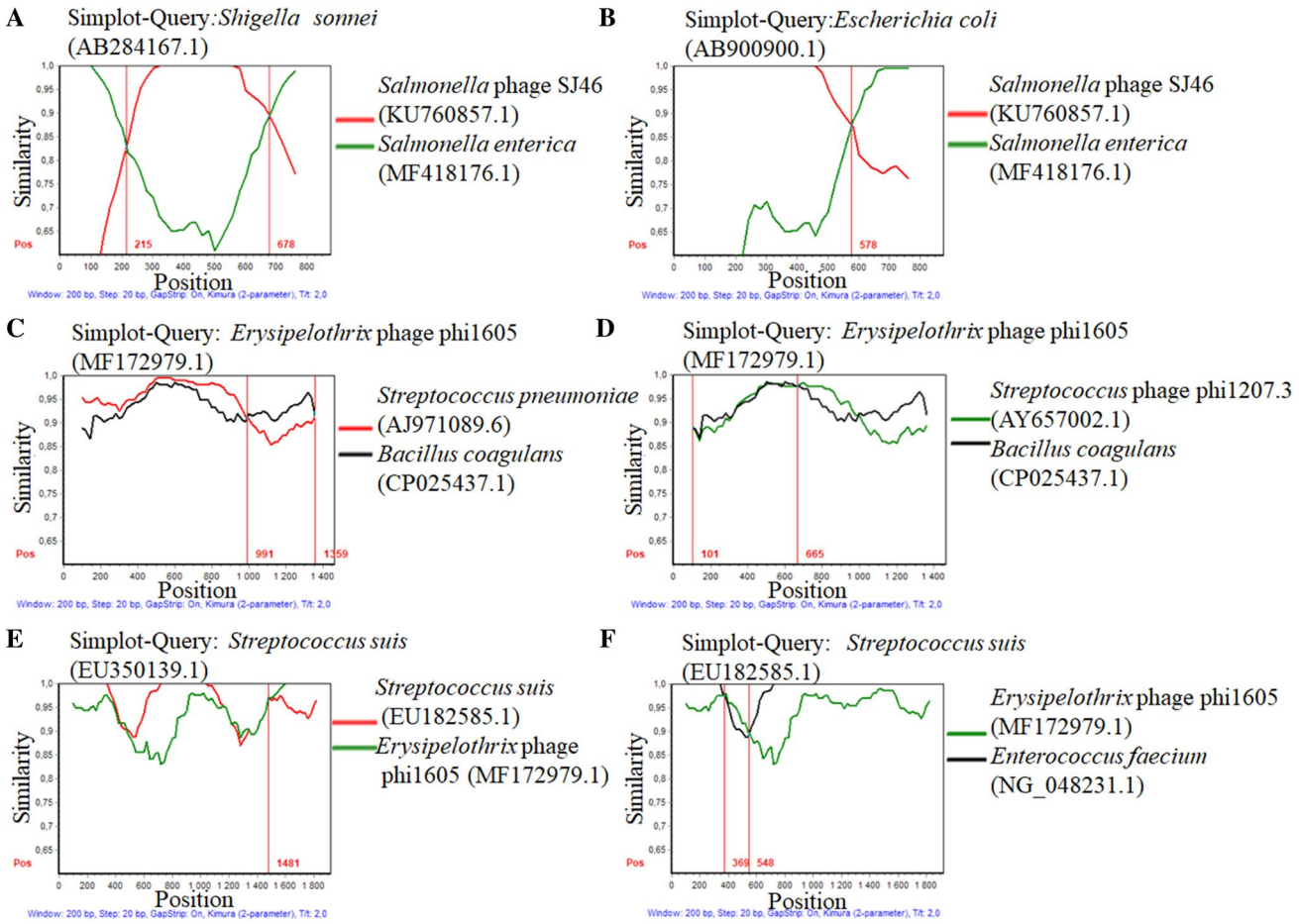

Fig. 2 The similarity plot analysis providing evidence for genetic recombination of the bla_{CTX-M}, *mel,* and *tetM* loci between the ARG-carrying bacteriophages and human and/or animal pathogenic bacterial species. **a** The recombination beginning and end breakpoints in the alignment of the bla_{CTX-M} DNA sequences of the *Salmonella* phage (KU760857.1), *S. sonnei* (AB284167.2), and *S. enterica* (MF418176.1). **b** The recombination breakpoint in the alignment of the bla_{CTX-M} DNA sequences of the *Salmonella* phage (KU760857.1), *E. coli* (AB900900.1) and *S. enterica* (MF418176.1). **c** The recombination beginning and end breakpoints in the alignment of the *mel* DNA sequences of the *Erysipelothrix* phage (MF172979.1), *S. pneumonia* (AJ971089.6), and *B. coagulans* (CP025437.1). **d** The recombination hotspots in the alignment of the *mel* DNA sequences of the *Erysipelothrix* phage (MF172979.1), the *Streptococcus* phage (AY657002.1), and *B. coagulans* (CP025437.1). **e** The recombination hotspots in the alignment of the *tetM* DNA sequences of *Erysipelothrix* phage (MF172979.1) and two *S. suis* strains (EU350139.1 and EU182585.1). **f** The recombination beginning and end breakpoints in the alignment of the *tetM* DNA sequences of the *Erysipelothrix* phage (MF172979.1), *S. suis* (EU182585.1), and *E. faecium* (NG_048231.1). In this analysis, we used the RDP4-identified recombinant strains as query

applying phages excessively in attempts to replace antibiotics for therapy or food processing? Besides, the acquisition of *mel* by the *Erysipelothrix* phage (MF172979.1) from *S. pneumonia* (AJ971089.6) pinpoints to a role of *Erysipelothrix* phages in the transmission of this gene in natural populations of the above important pathogen. Furthermore, we also suggest that some *Erysipelothrix* phages promote the dissemination of *mel* among *E. rhusiopathiae* strains, which are pathogenic for both humans and animals. We found that the same *Erysipelothrix* phage also carried *tetM*, and that this bacteriophage could be a putative donor of this gene for *S. suis* strains (EU350139.1 and EU182585.1). The *tetM* gene is thought to be widely present in the environmental isolates of *E. rhusiopathiae* resistant to tetracycline [47]. With the given scenario, it is entirely plausible that some *Erysipelothrix* phages have ability to acquire this gene from their immediate host species and then, transmit it to even other bacterial genera, e.g., such as *Streptococcus*. Interestingly enough, as reported previously, the *tetM* loci of *E. faecalis* and *E. rhusiopathiae* shared 99% of DNA–DNA similarity [47]. However, the results of our BLAST analysis show that different alleles of this gene generally exhibit comparatively much less nucleotide sequence similarity (≤ 93%) across different genera (unpublished data). In the present study, the consensus results provide strong statistical evidence for inter-species recombination of *tetM* involving

E. faecium (NG_048231.1) and the above *Erysipelothrix* phage as the putative major and minor donors, respectively, for the *S. suis* strain (EU182585.1). The genetic exchange of *tetM* between the *S. suis* strains was also elucidated in the recombination analyses. Thus, we suggest that intra-species recombination contributes to the dissemination of resistance to tetracycline in the natural populations of this species.

Conclusions

We strongly suggest that certain bacteriophages possibly with temperate lifestyle contribute to the intergeneric spread of $bla_{\text{CTX-M}}$, *mel*, and *tetM* in natural populations of bacteria including not only human and/or animal pathogens (e.g., such as *E. coli*, *S. enterica*, *S. pneumoniae, S. sonnei*, *S. suis*, and *E. rhusiopathiae*) but also the probiotic species, e.g., such as *B. coagulans*. The genetic exchange of *mel* is thought to occur also between some *Streptococcus* and *Erysipelothrix* bacteriophages. Besides, we strongly suggest that certain *Salmonella* phages can serve as vectors promoting the inter-species recombination of $bla_{\text{CTX-M}}$ loci between *E. coli* and *S. enterica*. In the natural populations of *S. suis*, *tetM* loci can be sometimes introduced and disseminated via intergeneric recombination mediated by some strains of *Erysipelothrix* phages and *E. faecium*.

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Compliance with Ethical Standards

Conflict of interest Stylianos Koulouris is employed by the European Food Safety Authority (EFSA). The present article is published under the sole responsibility of the authors and may not be considered as an EFSA scientific output. The positions and opinions presented in this article are those of the authors alone and do not necessarily represent the views or scientific works of EFSA.

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3.3 ბაქტერიულ პოპულაციებში ბაქტერიოფაგებით ინდუცირებული ანტიბიოტიკების იფლაქსის ცილების მაკოდირებელი გენების გენეტიკური რეკომბინაცია სახეობათაშორისო და გვართაშორის დონეებზე

ჩვენ ასევე გამოვავლინეთ ბაქტერიოფაგების როლი ანტიბიოტიკების იფლაქსის ტუმბოების მაკოდირებელი გენების მიმოცვლაში (Gabashvili et al. 2021a). NCBI მონაცემთა ბაზაში არსებული ფაგური და ბაქტერიული გენომების ანალიზის მეშვეობით, ჩვენმა კვლევამ გამოავლინა ბაქტერიოფაგები, რომელთა გენომებიც მოიცავდნენ ერთ ან რამოდენიმე ეფლუქს ტუმბოების მაკოდირებელ გენს, მათ შორის MF ოჯახის, ABC ოჯახის, და RND ოჯახის ტრანსპორტერების მაკოდირებელ გენებს. აღნიშნული ბაქტერიოფაგების მასპინძელ ბაქტერიებს წარმოადგენენ *Salmonella enterica, Staphylococcus aureus, Pseudomonas aeruginosa, Burkholderia pseudomallei, Bacillus thuringiensi, Mycobacterium smegmatis*.

In Silico რეკომბინაციული ანალიზების შედეგების მიხედვით, აღნიშნული ფაგები წარმოადგენენ ამ გენების დონორებს ბაქტერიების ბუნებრივ პოპულაციებში როგორც შიდასახეობრივ და სახაობათაშორისო, ისე ზოგიერთ გვართაშორის დონეზეც. ჩვენ აღმოვაჩინეთ მულტირეზისტენტული ABC გადამტანი პერმეაზას მაკოდირებელი გენის ლოკუსის სახეობათაშორისო რეკომბინაციის მოვლენები, რომლებშიც ჩართული იყვნენ მოიცავდნენ *B. thuringiensis* -ის ფაგი და *Bacillus anthracis-ის*, *Bacillus cereus*-ის და *Bacillus tropicus* -ის ბუნებრივი პოპულაციები. (Fig. 2b). ჩვენმა კვლევამ დაახასიათა MFS ტრანსპორტერის გენის ბაქტერიოფაგებით გამოწვეული გვართა შორის დონეზე რეკომბინაციის მოვლენებიც, სადაც ჩართულები იყვნენ *M. smegmatis* -ის ფაგი და *S. harbinensis-ის*, *S. chartreusis*-ის და *Actinomadura-ს* ბაქტერიული შტამები. ამგვარად, ჩვენი კვლევების თანახმად, ბაქტერიოფაგების მიერ გამოწვეული რეკომბინაცია ხელს უწყობს ანტიბიოტიკების იფლაქსის ცილების მაკოდირებელი გენების გავრცელებას ბაქტერიების სხვადასხვა სახეობებში.

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GENES AND GENOMES

Bacteriophage-Mediated Risk Pathways Underlying the Emergence of Antimicrobial Resistance via Intrageneric and Intergeneric Recombination of Antibiotic Efflux Genes Across Natural populations of Human Pathogenic Bacteria

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Abstract

Antimicrobial resistance continues to be a significant and growing threat to global public health, being driven by the emerging drug-resistant and multidrug-resistant strains of human and animal bacterial pathogens. While bacteriophages are generally known to be one of the vehicles of antibiotic resistance genes (ARGs), it remains largely unclear how these organisms contribute to the dissemination of the genetic loci encoding for antibiotic efflux pumps, especially those that confer multidrug resistance, in bacteria. In this study, the in-silico recombination analyses provided strong statistical evidence for bacteriophage-mediated intra-species recombination of ARGs, encoding mainly for the antibiotic efflux proteins from the MF superfamily, as well as from the ABC and RND families, in *Salmonella enterica, Staphylococcus aureus, Staphylococcus suis, Pseudomonas aeruginosa,* and *Burkholderia pseudomallei*. Events of bacteriophage-driven intrageneric recombination of some of these genes could be also elucidated among *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus tropicus* natural populations. Moreover, we could also reveal the patterns of intergeneric recombination, involving the MF superfamily transporter-encoding genetic loci, induced by a *Mycobacterium smegmatis* phage, in natural populations of *Streptomyces harbinensis* and *Streptomyces chartreusis*. The SplitsTree- (fit: 100; bootstrap values: 92.7–100; Phi $p \leq 0.2414$), RDP4- ($p \leq 0.0361$), and GARD-generated data strongly supported the above genetic recombination inferences in these in-silico analyses. Thus, based on this pilot study, it can be suggested that the above mode of bacteriophage-mediated recombination plays at least some role in the emergence and transmission of multidrug resistance across a fairly broad spectrum of bacterial species and genera including human pathogens.

Keywords Bacteriophage · Pathogenic bacteria · Antibiotic efflux genes · Genetic recombination

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Introduction

The ongoing spread of antimicrobial resistance (AMR) exhibits a growing trend towards a rapid emergence of drug-resistant (DR) and multi-drug resistant (MDR) bacterial pathogens, posing a global threat to human, animal, and environmental health [1–3]. The plausible causes of AMR, otherwise termed as "the global resistome", have been mainly due to the irrational and excessive use of antibiotics in both public health and food industries. These factors, coupled with release of nonmetabolized antibiotics or their residues into different environments, contribute to the genetic selection pressures especially for the emergence of MDR strains globally [1]. In natural populations of both human and animal pathogenic bacteria, AMR exhibits two following mechanisms: acquired resistance and intrinsic resistance, the latter associated with the carriage of specific inherent structural and/or functional characteristics including efflux pumps [4].

In Gram-positive and -negative bacteria, the efflux pumps represent transport proteins involved in the extrusion of toxic substrates (including antibiotics) from within their cells into the external environment [5]. The bacterial efflux systems that confer clinical levels of resistance to antimicrobials fall into the following five classes: the major facilitator (MF) superfamily, the ATP (adenosine triphosphate) -binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family belonging to the drug/metabolite transporter (DMT) superfamily, and the multidrug and toxic compound extrusion (MATE) family. These efflux pumps were found to be increasingly involved in the generation of AMR across natural populations of bacteria [6, 7]. While drug-specific efflux mechanisms are encoded mainly by mobile elements, the multidrug efflux pumps are generally chromosomally encoded in these organisms [6, 8]. In addition to the clonal expansion of DR and MDR strains [4], the dissemination of these and other resistance determinants across various bacteria, via horizontal gene transfer (HGT), also contributes significantly to the emergence of AMR infections in humans and animals. Thus, the determining of transmission mechanisms of antibiotic resistance genes (ARGs) that are involved in antibiotic efflux in these organisms has been one of the critical steps towards our in-depth understanding of the global trends and dynamics of AMR evolution [9, 10].

Bacteriophages represent the most abundant organisms in biosphere [11], contributing markedly to the transmission of ARGs across natural populations of various human and animal pathogenic bacteria in different environments [10, 12, 13]. It is noteworthy that the global rate of phage-mediated HGT events was estimated to be approximately 2×10^{16} per second [14], although, further in-depth studies are needed to gain more precise insights into the above estimate. In this scenario, a phenomenon of the phage-transduction-driven transfer of antibiotic efflux-associated genes and its trajectories, across natural populations of bacteria, have been very poorly understood. A body of evidence from the previous studies, in this respect, has been mainly limited to very scarce in vivo and in vitro findings [12, 15] and to viral genomic and metagenomic data [16–19], demonstrating only the carriage of these ARGs by the phages form different environments. Intriguingly enough, however, it is suggested that phage-driven transfer of some genetic loci (*mel* and *tetM*) involved in antibiotic efflux can stretch even beyond the species-specific boundaries in bacteria [10].

In this study, we identified the genetic loci that encode for bacterial efflux pumps across the phage genomes, and performed extensive in-silico recombination analyses to elucidate their phage-mediated transfer and their transmission trajectories across various bacterial species and genera. In addition, we attempted to determine a lifestyle of the ARGs-recombining phages in these analyses. It was revealed that certain *Streptococcus, Leptospira, Gardnerella,* and *Erysipelothrix* phages carried in their genomes multiple, and sometimes functionally different, antibiotic efflux-associated bacterial genes. Phage-mediated intra-species recombination of some of these genes could be determined in natural populations of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Streptococcus suis*. Here, we provide also strong statistical evidence for events of inter-species recombination of the antibiotic efflux genetic loci in *Staphylococcus* and *Bacillus,* and for even their intergeneric recombination between *Mycobacterium* and *Streptomyces*, all being mediated by various phages.

Methods

Selection of bacterial efflux-associated ARGs across phage genomes

The amino acid sequences datasets of bacterial efflux-conferring antibiotic resistance proteins (ARPs) were obtained from the BacEffluxPred database (http://proteininformatics.org/mkumar/baceffluxpred/). The amino acid sequences of these ARPs were then analyzed in the protein database of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) using the BLASTP algorithm to identify their homologs encoded across the phage genomes available in the NCBI viral database. The following BLASTP-search default general and scoring parameters were applied in this analysis: Expected threshold — 0.05; Word size — 6; The BLOSUM62 Matrix; Gap costs — Existence

11, Extension 1 with the conditional compositional score matrix adjustment. The above parameters were automatically adjusted for short input sequences. Each homologous amino acid sequences, identified in the predicted phage proteome using the BLASTP analysis in the NCBI protein database, were subsequently examined by the machine-learning based two-tier in-silico tool, BacEffluxPred [20] and InterPro [21] in order to confirm or to predict the bacterial efflux-conferring ARPs. The ARPs amino acid sequences, selected in the BacEffluxPred- and InterPro-derived protein data consensus analyses, were then blasted against the NCBI protein database using the above BLASTP-search default general and scoring parameters to identify their homologous ARPs encoded across bacterial genomes available in the NCBI bacterial database. The efflux-associated ARGs identified in bacteria, the encoded proteins of which shared ≥ 85% of amino acid sequence homology with those of the ARPs determined across the phage genomes, were then included together with the phage ARGs in the in-silico recombination analyses.

Recombination analyses

ClustalX (version 2.1) [22] was applied for aligning the DNA sequences of the antibiotic efflux-associated ARGs. A large panel of the recombination detection algorithms, implemented in the SplitsTree [23], RDP4 [24], and GARD [25] software packages, were used to identify events of genetic recombination of the ARGs, and to determine their transmission trajectories between phages and bacteria. In particular, the split decomposition method [23], imbedded in SplitsTree (version 4.14.4), was applied to reconstruct genetic recombination events of the ARGs between these organisms. Fit values ≥ 95 for the SplitsTree-generated splits networks, and bootstrap values ≥ 95 (from 10 000 replicates) for the nodes of parallelograms from the above networks, were considered to be statistically significant. RDP [26], GENECONV [27], BootScan [28], MaxChi [29], Chimaera [30], SiScan [31], and 3Seq [32] algorithms, implemented in RDP4, were applied to identify ARGs donor and recipient (recombinant) strains of the phages and bacteria, thus determining the trajectories of HGT events across their natural populations. Bonferroni-corrected p values in a range of ≤ 0.05 were considered to be statistically significant for the significant breakpoint clusters (99%) in these analyses, performed using the RDP4 default parameters, its linear sequence setting and enabled disentangle recombination signals. Additionally, GARD was employed to detect a possibly larger spectrum of genetic recombination hotspots than it could be detected by RDP4 across the recombining ARGs of the phages and bacteria. The Pairwise Homoplasy Index (Phi) test [33] was used to examine homoplasy in the identified recombining ARGs in order to avoid false positive signals that may sometimes emerge in the in-silico recombination analyses.

Phage-host interaction tracing and phage lifestyle prediction analyses

We applied PHISDetector [34], screening for phage-host interaction signals (PHISs), in order to explore additional evidence for interactions specifically between the ARGs donor and recombinant phages and bacterial strains identified in the above recombination analyses. More specifically, Using PHISDetector, we analyzed the PHISs, including sequence compositions, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), protein–protein interaction and/or prophage features, in these organisms. The PHISDetector-predetermined probability scores being ≥ 0.8865, for phage-host interactions, were considered to be statistically significant in these analyses.

The information on the actual lifestyle (temperate versus virulent) for most of the identified ARG-recombining phages was not available. Thus, the Bacteriophage Lifestyle Predictor tool, BACPHLIP [35], PHACTS (https://edwards.sdsu.edu/PHACTS/index.php) [36], and the Bacteriophage Life Cycle Recognition tool with Machine Learning algorithm, PhageAI (https://phage.ai/) [37], were applied in the analysis of these phages to predict their lifestyle. A PHACTS-predicted phage lifestyle was accepted as true when respective probability values were > 0.5 as determined previously. We used a stringent approach also while using PhageAI: a phage lifestyle was considered as true positive if the PhageAI-generated prediction accuracy values were ≥ 97%.

Results

Bacterial efflux-associated ARGs across phages genomes

In the analysis of over 8000 phage genomes available in the NCBI GenBank database, a total of 21 phages were found to carry a bacterial efflux-associated ARG(s) using the BLASTP algorithm (Table S1). As shown, most of these ARGs were determined to encode for the ARPs from the MF superfamily, and the ABC and RND families. The host species of the ARG-carrier phages were associated with various human and/or animal pathogens including, but not limited to, *Salmonella enterica, Mycobacterium smegmatis, Pseudomonas aeruginosa, Burkholderia pseudomallei, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus suis*, and *Streptococcus gallolyticus*. In addition, the phage proCM3 (KF296717.1) recovered from human nonpathogenic host species, *Bacillus thuringiensis,* could be also found to carry the gene encoding for the ABC transporter

ATP-binding protein. Certain phages from other host species, such as *S. suis, G. vaginalis, E. rhusiopathiae,* and *Leptospira* were determined to carry multiple ARGs in their genomes (Table 1).

The BacEffluxPred- and InterPro-generated data were mainly in consensus with the BLASTP-hits, although exhibiting different levels of resolution in the ARPs annotations (Table S1). As shown, the BLASTP search-resulted hits for the amino acid sequences of these bacterial efflux-associated ARPs, encoded across the phage genomes, were reflecting the closest amino acid identities (99.26–100%) with the homologs of the ARPs encoded by their host species genomes (and occasionally on their host genus levels). In these analyses, only two exceptions were identified: the *Mycobacterium smegmatis* phage Enkosi (NC_028936.1) genome-encoded MFS transporter type protein shared 99.56% of the amino acid sequence identity with *Flavimobilis soli* MFS DHA2 family lincomycon resistance transporter (PFG35418.1); the *S. suis* phage phi-SC181 (MK359990.1) genome-encoded ABC transporter ATP-binding protein shared 100% of the amino acid sequence identity with the *Lactobacillales* ABC-F type ribosomal protection protein OptrA (WP_131648058.1).

Phage-driven genetic recombination of bacterial efflux-associated ARGs

Using the method of splits decomposition, the SplitsTree analysis of bacterial efflux ARGs could identify events of both inter-species and intergeneric recombination of some of these genetic loci, involving the phages and their host species (Fig. 1). In particular, as shown in Fig. 1a, we could identify events of inter-species recombination of the lantibiotic protection ABC transporter ATP-binding protein-encoding gene loci between the *S. aureus* phage (MG543995.1) and the strains of *S. aureus* and *S. argenteus*. We could also determine events of interspecies recombination of the multidrug ABC transporter permease-encoding gene loci between the *B. thuringiensis* phage (KF296717.1) and the strains of *Bacillus anthracis*, *bacillus cereus*, and *Bacillus tropicus* (Fig. 1b). Figure 1c and d provide collectively statistical evidence for events of the phage mediated intra-species recombination of the ABC transporter ATP-binding protein-encoding gene loci in *S. suis* and *P. aeruginosa* strain populations. Very importantly, in Fig. 1e, we elucidate the events of both interspecies and intergeneric recombination of the MFS efflux transporter-encoding gene loci that could be determined between the *M. smegmatis* phage Enkosi (NC_028936.1), *Streptomyces harbinensis*, *Streptomyces chartreusis* and *Actinomadura* species. The highest fit (100) and the very robust bootstrap values (92.9–100), generated for each splits network and the parallelograms respectively (Fig. 1a–e), provide additional strong evidence for the above HGT events.

In addition, using RDP4, we determined the donor and recipient strains of the ARGs in these HGT events, as well as the transmission trajectories of and the recombination beginning and end breakpoints across these genetic loci. The results obtained from the RDP4 analysis are summarized in Table 2. As shown, certain *S. aureus*, *S. suis* and *P. aeruginosa* phages could serve as the donor and/or the recipient of some these ARGs across the above-described events of intra-species and inter-species recombination. More importantly, however, the *B. thuringiensis* phage (KF296717.1) was determined to be the recipient of the multidrug ABC transporter permease-encoding gene loci for *B. tropicus* (the major donor) *B. cereus* (the minor donor) during independent events of inter-species recombination; and vice versa, this phage was determined to be also the minor donor of the above gene for the both species; even more importantly, the *B. anthracis* strain HDZK-BYSB7(CP026608.1) was determined to be the representative major donor of the same gene for the *B. cereus* strain FDAARGOS_781 (CP053991.1); the *M. smegmatis* phage Enkosi (NC_028936.1) appeared to be the recombinant strain having acquired the MFS efflux transporter-encoding gene loci from *S. chartreusis* and *S. harbinensis* (being respectively the major and minor donors), collectively demonstrating events of intergeneric recombination. It must be indicated that the RDP4-generated *p* values were ≤ 0.0361 providing very strong statistical evidence for the recombinant and donor strains and the HGT trajectories determined in these analyses. Using GARD, we could identify additional multiple recombination hotspots across all the above ARGs subsets (Fig. 2a–e), unveiling another strong evidence for genetic recombination of the bacterial efflux-associated loci in these organisms.

With the Phi test, screening for specific mutations linked to the convergent evolution, we reexamined the DNA sequences of the recombined ARGs to further determine the validity of the recombination hotspots. The Phi test-generated data are presented in Table 3, which displays the statistically very strong *p* values (≤ 0.2414) that highly support the results obtained from the above SplitsTree, RDP4 and GARD analyses.

Phage-host interaction and phage lifestyle

PHISDetector was applied to screening for the phage-host interaction signals (PHISs) specifically among the recombinant and donor strains of the phages and bacteria involved in the HGT events elucidated in the above genetic recombination analyses. The PHISDetector-generated probability scores, obtained from this analysis, are presented in Table 4. As shown, the strong phage-host interaction signals ($p \geq 0.8865$) were received for the recombinant and donor strains of the phages and bacteria

Table 1 Phages carrying multiple bacterial efflux-associated genes, and the NCBI GenBank-determined coordinates of these genetic loci across the phage genomes

Phage (GenBank accession no.)	Bacterial efflux-associated genes and their coordinates in phage genome*				
Erysipelothrix rhusiopathiae phage phi1605 (MF172979.1)	ABC efflux; ATPase component of ABC transporter (71,078–72,541)	MFS efflux; macrolide-efflux protein (69,740–70,957)			
Gardnerella vaginalis phage vB_Gva_AB1 (MW387018.1)	Lantibiotic transport ATP-binding protein (44,242–45,132)	MFS efflux; ATP-binding cassette domain-containing protein (41,857–43,866)			
Leptospira phage vB_LbrZ_5399-LE1 (KF114879.1)	RND efflux; RND transporter, Hydrophobe/Amphiphile Efflux-1 (HAE1)/Heavy Metal Efflux (HME) family, permease protein (46,879–49,968)	RND efflux; RND transporter, Hydrophobe/Amphiphile Efflux-1 (HAE1)/Heavy Metal Efflux (HME) family, permease protein (49,965–52,856)	RND efflux; HlyD family secretion protein (45,364–46,854)		
Leptospira phage LinZ_10 (KF114880.1)	RND efflux; RND transporter, Hydrophobe/Amphiphile Efflux-1 (HAE1)/Heavy Metal Efflux (HME) family, permease protein (65,491–68,382)	RND efflux; RND transporter, Hydrophobe/Amphiphile Efflux-1 (HAE1)/Heavy Metal Efflux (HME) family, permease protein (68,379–71,468)	RND efflux; HlyD family secretion protein (71,493–72,983)		
Streptococcus suis phage phiNJ3 (KT336320.1)	ABC efflux; ABC transporter (5637–7100)	ABC efflux; macrolide-efflux protein (4300–5517)			
Streptococcus suis phage phiSC070807 (KT336321.1)	ABC efflux; macrolide-efflux protein (2305–3522)	ABC efflux; ABC transporter (3642–5105)			
Streptococcus suis phage phi-SC181 (MK359990.1)	ABC efflux; ABC transporter ATP-binding protein (11,514–13,481)	ABC efflux; macrolide efflux MFS transporter (3818–5035)			
Streptococcus suis phage phi-SsuZKB4_rum, complete genome (MN270279.1)	Lantibiotic transport ATP-binding protein SrtF (39,930–40,631)	ABC efflux; ABC-F type ribosomal protection protein (54,777–56,240)	ABC efflux; ATP-binding cassette domain-containing protein (45,737–47,545)	MFS efflux; MFS efflux pump (53,440–54,657)	MFS efflux; Tetracycline resistance, MFS efflux pump (15,630–16,850)
Streptococcus suis phage phi-SsuYTJ2_rum, partial genome (MN270277.1)	ABC efflux; ABC-F type ribosomal protection protein OptrA (322–2235)	MFS efflux; Tetracycline resistance, MFS efflux pump (48,590–49,777)			
Streptococcus suis phage phi-SsuNJ2_rum (MN270271.1)	ABC efflux; ABC-F type ribosomal protection protein (5641–7104)	MFS efflux; MFS efflux pump (4303–5520)			

*ARG coordinates, determined by GenBank across a phage genome, are presented in parenthesis following a gene designation

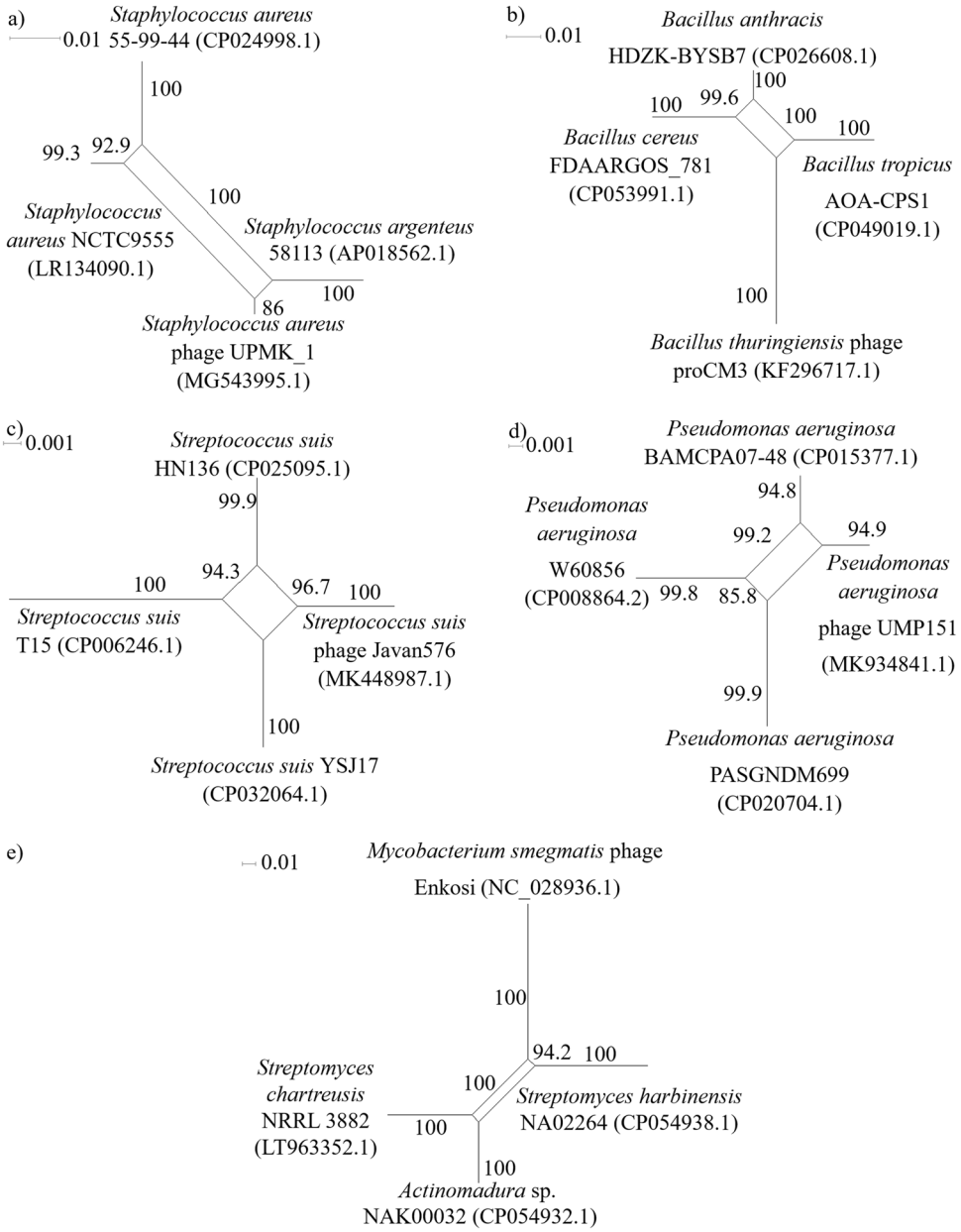

Fig. 1 SplitsTree-generated splits graphs displaying the phage-mediated genetic recombination events of genetic loci involved in antibiotic efflux in different bacterial genera. a Intra- and inter-species recombination events of the lantibiotic protection ABC transporter ATP-binding protein-encoding gene, involving the *S. aureus* phage and the strains of *S. aureus* and *S. argenteus* (fit: 100); b inter-species recombination events of the multidrug ABC transporter permease-encoding gene, involving the *B. thuringiensis* phage and the strains of *B. tropicus*, *B. anthracis*, *B. cereus* (fit: 100); c intra-species recombination events of the ABC efflux encoding gene, involving the *S. suis* phage Javan576 and the *S. suis* strains (fit: 100); d intra-species recombination events of the MFS transporter-encoding gene, involving the *P. aeruginosa* phage UMP151 and *P. aeruginosa* strains (fit: 100); e intergeneric recombination events of the MFS transporter-encoding gene, involving the *M. smegmatis* phage and the strains of *S. harbinensis, S. chartreusis,* and *Actinomadura* (fit: 100). In each splits graph (a-e), the HGT events are presented by a parallelogram with bootstrap values for its every node. A GenBank accession number, for each strain, is provided in the parentheses following the strain descriptions in these splits graphs

involved in these HGT events. The only exception was the HGT event exhibiting an indirect interaction between *M. smegmatis* phage Enkosi (NC_028936.1) and its putative host, *Actinomadura* NAK00032 (CP054932) (Fig. 1e), with no relative record of interaction identified in the analysis.

There was no information available on the actual lifestyles of the recombinant and donor phages, involved in the SplitsTree- and RDP4-resolved HGT events, except the *B. thuringiensis* phage proCM3 (KF296717.1) clearly with the temperate lifestyle shown in the previous study [38]. Thus, we applied

Table 2 The efflux-associated ARGs donor and recipient strains of the phages and their putative host species, as well as the recombination beginning and end breakpoints across their recombined ARGs as determined by RDP4

Recombinant strain	Representative major parent	Representative minor parent	ARG-encoded protein product	Beginning and end breakpoints (99% Cl)	RDP4 algorithm-generated *p* values	SplitsTree Fig
S. aureus phage UPMK_1 (MG543995.1)	*S. aureus* strain NCTC9555 (LR134090.1)	*S. argenteus* strain 58,113 (AP018562.1)	Lantibiotic ABC transporter ATP-binding protein	19 (Undetermined) - 535 (514–564)	GENECONV3.11E-02 Bootscan 2.24E-03 Maxchi 7.48E-06 Chimaera 4.72E-03 SiSscan 4.34E-09 3Seq 6.00E-08	Figure 1a
B. thuringiensis phage proCM3 (KF296717.1)	*B. tropicus* strain AOA-CPS1 (CP049019.1)	*B. cereus* strain FDAAR-GOS_781 (CP053991.1)	Multidrug ABC transporter permease	1244 (1–1575) - 1412 (1–1575)	SiSscan2.66E-02 3Seq 3.61E-02	Figure 1b
B. cereus FDAAR-GOS_781 (CP053991.1)	*B. anthracis* strain HDZK-BYSB7 (CP026608.1)	Unknown [*B. thuringiensis* phage proCM3 (KF296717.1)]	Multidrug ABC transporter permease	1170 (1–1181) - 1429 (Undetermined)	Maxchi2.09E-02 Chimaera 9.51E-03 SiSscan 4.29E-04	Figure 1b
B. tropicus AOA-CPS1 (CP049019.1	*B. cereus* strain FDAAR-GOS_781 (CP053991.1)	*B. thuringiensis* phage proCM3 (KF296717.1)	Multidrug ABC transporter permease	648 (Undetermined) - 723 (Undetermined)	RDP 1.06E-02 GENECONV 1.76E-02	Figure 1b
S. suis HN136 (CP025095.1)	*S. suis* strain *T15* (CP006246.1)	*Streptococcus* phage Javan576 (MK448987.1)	ABC transporter ATP-binding protein (ABC efflux)	539 (Undetermined) - 1011 (Undetermined)	Bootscan 1.31E-02 Maxchi 6.94E-03 Chimaera 1.01E-02 SiSscan 8.01E-07	Figure 1c
P. aeruginosa W60856 (CP008864.2)	*P. aeruginosa* strain BAM-CPA07-48 (CP015377.1)	Unknown [*P. aeruginosa* phage UMP151 (MK934841.1)]	MFS transporter	36 (Undetermined) - 633 (1–747)	Bootscan 1.45E-02 Maxchi 6.27E-03 3Seq 3.87E-03	Figure 1d
M. smegmatis phage Enkosi (NC_028936.1)	Unknown [*S. chartreusis* strain NRRL 3882 (LT963352.1)]	*S. harbinensis* strain NA02264 (CP054938.1)	MFS transporter	317 (Undetermined) - 348 (Undetermined)	Chimaera 3.21E-02 SiSscan7.29E-04	Figure 1e

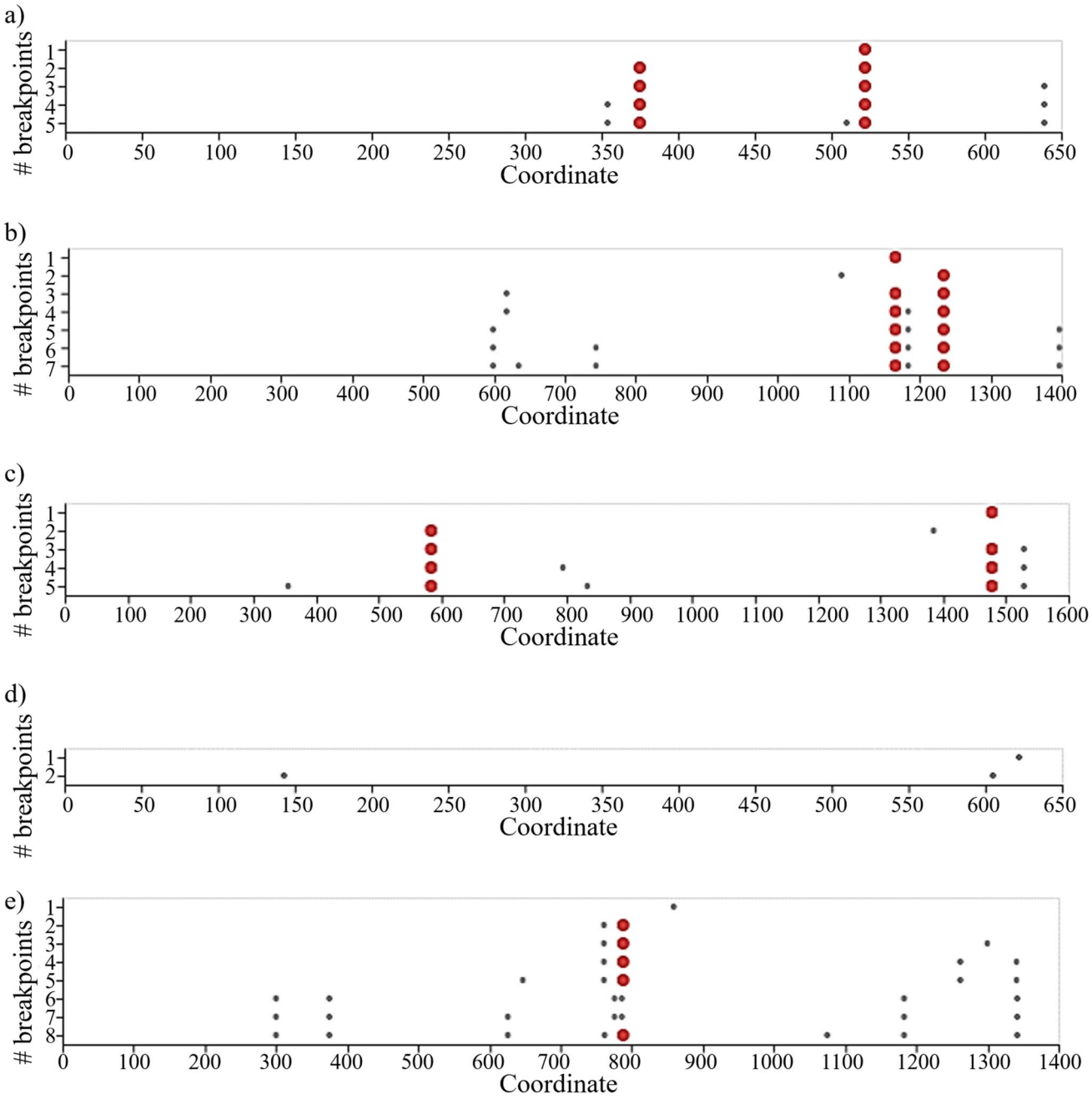

Fig. 2 The genetic recombination hotspots determined by GARD across the DNA sequence alignments of the antibiotic efflux-associated ARGs of the recombining phages and bacteria identified in the SplitsTree and RDP4 analyses. a The recombination hotspots detected by GARD in the DNA sequence alignment of the lantibiotic ABC transporter ATP-binding protein-encoding gene; b the recombination hotspots detected in the DNA sequence alignment of the multidrug ABC transporter permease-encoding gene; c the recombination hotspots detected in the DNA sequence alignment of the ABC transporter ATP-binding protein-encoding gene; d the recombination hotspots detected in the DNA sequence alignment of the MFS transporter-encoding gene. The red dots indicate significant recombination breakpoints determined by GARD

three different phage lifestyle prediction statistical tools, BACPHLIP, PHACTS, and PhageAI, to gain preliminary insights into and to predict whether these organisms represented virulent or temperate phages. The results received from this analysis are displayed in Table 5. As shown, BACPHLIP, PHACTS, and PhageAI performed differently providing conflicting resolutions for the lifestyle of these phages. For example, PhageAI and PHACTS predicted respectively confidently and non-confidently the virulent (lytic) lifestyle, whereas BACPHLIP predicted the temperate (lysogenic) lifestyle, for

Table 3 Phi test-generated *p* values measuring homoplasy across the recombined ARGs

Strain subset (SplitsTree Fig.)	ARG	Phi test-generated *p* value
Figure 1a	Lantibiotic ABC transporter ATP-binding protein	7.208E-5
Figure 1b	Multidrug ABC transporter permease	1.325E-4
Figure 1c	ABC transporter ATP-binding protein (ABC efflux)	0.04294
Figure 1d	MFS transporter	0.2414
Figure 1e	MFS transporter	0.03142

the *B. thuringiensis* phage proCM3 (KF296717.1); similarly, PhageAI and PHACTS predicted respectively confidently and non-confidently the temperate lifestyle, whereas BACPHLIP predicted the virulent lifestyle, for the *P. aeruginosa* phage UMP151 (MK934841.1); failing unambiguously in their predictive abilities, both PhageAI and PHACTS predicted the virulent lifestyle for the *B. thuringiensis* phage proCM3 (KF296717.1) with the actual temperate lifestyle [38].

Discussion

A global public health crisis has been exacerbated as antibiotics frequently fail to eradicate human and animal pathogenic bacteria due to the continued emergence of AMR in their natural populations. Among other mechanisms, HGT contributes significantly to the rapid spread of AMR via natural transformation, conjugative plasmids, and phage transduction, occurring even between genetically unrelated pathogens [3]. Growing evidence suggests that phages may play a more significant role in the transmission of ARGs than previously thought [39]. However, it still remains largely unclear how these organisms contribute to the dissemination of ARGs that are involved in encoding for antibiotic efflux proteins across different bacterial species and genera. In the intervening periods, numerous plasmid- and chromosome-encoded bacterial efflux mechanisms have been identified as important AMR determinants in various human and animal pathogens [6].

Acquisition and carriage of antibiotic efflux genes by phage genomes

The studies using the viral metagenomic shotgun sequencing data, obtained from the analyses of clinical or environmental samples, suggest that antibiotic efflux genes can be fairly frequently present in phage genomes [16–18]. However, these viral metagenomic analyses have been mainly limited to identifying these ARGs rather than determining and classifying specifically the phages that carried these genes. Here, we show that while the antibiotic efflux genes can be predominantly carried by the phages infecting human and animal pathogenic bacteria, they can be also acquired and transmitted by the phage(s) from human nonpathogenic species, e.g., such as *B. thuringiensis* being used widely as an insect biocontrol agent [40]. Here, we also show that among these ARG-carrier phages from the NCBI viral genome database, a great majority of them were recovered from *Streptococcus species* such as *S. suis* and S. *gallolyticus* being human pathogens [41, 42]. Other ARG-carrier phages, in this list examined, were reflecting collectively a fairly broad spectrum of the host species, including, but not limited to, *S. enterica*, *S. aureus, S. epidermidis*, *P. aeruginosa, B. pseudomallei*, *M. smegmatis*, and *G. vaginalis*, also representing important human pathogens [43–47].

Our study shows that these phages from the above host species carried ARGs encoding mainly for the antibiotic efflux proteins from the MF superfamily, and the ABC and RND families. More importantly, it is also shown that certain phages, infecting at least *E. rhusiopathiae*, *G. vaginalis, S. suis*, and *Leptospira*, carried multiple such genetic loci, sometimes of different functions. For example, a genome of the *S. suis* phage (MN270279) was found to possess five genes encoding for the antibiotic efflux proteins from the MF superfamily and the ABC family. The MFS and ABC types of antibiotic efflux transporters are thought to be the most abundant and largest drug efflux systems in prokaryotic organisms [48, 49]. The MF superfamily encompasses efflux transporters, discriminated into 74 families, translocating a wide variety of substrates, e.g., antibiotics, peptides, amino acids, sugars, phosphates nucleosides and ions, across the cytoplasmic membrane [50]. This superfamily includes efflux transporters that confer resistance to chloramphenicol, florfenicol and bicyclomycin [51], hydrophobic quinolones [52], norfloxacin and enoxacin [53], tetracycline and/or some other antibiotics [54–57]. In human pathogenic bacteria, certain ABC transporters confer multi-drug resistance [58, 59], including resistance to nisin, bacitracin, and several beta-lactam antibiotics [60]. Similarly, the RND efflux pumps confer resistance to multiple structurally distinct classes of antimicrobials, also contributing to multi-drug resistance [61].

Table 4 Phage-host interactions identified by PHISDetector, and the lifestyles of the recombinant and donor phages as predicted by PhageAI, BACPHLIP, and PHACTS

Phage	Lifestyle			Bacterium	PHISDetector-resolved score/interaction		SplitsTree Fig
	PhageAI (%)	BACPHLIP	PHACTS (Averaged Probability ± Standard Deviation)		Score	Interaction	
Staphylococcus aureus phage UPMK_1 (MG543995.1)	Temperate (75.29)	Temperate (0.975)	Confidently temperate (0.68 ± 0.042)	*S. aureus* strain NCTC9555 (LR134090)	1	Yes	Figure 1a
				S. argenteus strain 58,113 (AP018562)	1	Yes	
				S. aureus strain 55–99-44 (CP024998)	0.9423	Yes	
Bacillus thuringiensis phage proCM3 (KF296717.1)*	Virulent (91.69)	Temperate (0.925)	Non-confidently lytic (0.52 ± 0.04)	*B. tropicus* strain AOA-CPS1 (CP049019)	0.9246	Yes	Figure 1b
				B. cereus strain FDAARGOS_781 (CP053991)	0.8948	Yes	
				B. anthracis strain HDZK-BYSB7 (CP026608)	0.924	Yes	
Streptococcus suis phage Javan576 (MK448987.1)	Temperate (75.03)	Virulent (0.95)	Confidently temperate (0.529 ± 0.024)	*S. suis* strain HN136 (CP025095)	0.9129	Yes	Figure 1c
				S. suis strain YSJ17 (CP032064)	1	Yes	
				S. suis strain T15 (CP006246)	0.9121	Yes	
Pseudomonas aeruginosa phage UMP151 (MK934841.1)	Temperate (98.34)	Virulent (0.8)	Non-confidently temperate (0.526 ± 0.046)	*P. aeruginosa* strain W60856 (CP008864)	1	Yes	Figure 1d
				P. aeruginosa strain BAMCPA07-48(CP015377)	0.9299	Yes	
				P. aeruginosa strain PASGNDM699 (CP020704)	1	Yes	
Mycobacterium smegmatis phage Enkosi (NC_028936.1)	Temperate (62.86)	Temperate (0.975)	Non-confidently temperate (0.505 ± 0.054)	*S. harbinensis* strain NA02264 (CP054938)	0.8865	Yes	Figure 1e
				S. chartreusis strain NRRL 3882 (LT963352)	0.9379	Yes	
				Actinomadura strain NAK00032 (CP054932)	No relative records		

While the RND complexes are generally chromosomally encoded, some of these drug resistance are carried and transferred by some plasmids [62, 63].

Thus, the carriage of the drug efflux transporters-encoding genes by the phages, identified in our study, pinpoints to some important role of these organisms in the dissemination of antibiotic efflux pumps in strain populations of the above described host species. Besides, it must be noted that the MF superfamily efflux pumps [64, 65], ABC transporters [66], RND efflux pumps [61, 63], with their wide substrate range, appear to be involved in bacterial virulence, with some entailing also biofilm development [43, 64]. Therefore, it can be strongly suggested that via genetic recombination of the ARGs involved in bacterial efflux, the phages contribute to the emergence of virulence and environmental fitness in their hosts natural populations even more significantly than previously envisaged [67, 68].

Phage-driven intrageneric and intergeneric recombination of bacterial efflux-associated ARGs

Early empirical evidence for the phage mediated in vivo transduction of the efflux pump-encoding gene in *Streptococcus pyogenes*, conferring resistance to various antibiotics, suggested that the phages could potentially spread specifically such ARGs in natural environments [12, 15]. The recent metagenomic findings [16–19], revealing the presence of bacterial efflux-associated ARGs in phages, provide another strong empirical evidence for their role in the dissemination of these genes in aquatic and other environments. Nevertheless, as indicated above, these previous findings exhibited only the facts that at least certain bacterial efflux-associated ARGs can be carried and disseminated by phages across their host species. However, the specificity and trajectories of such phage-induced HGT events remain largely unclear.

Here, the results of our recombination analyses provide additional and solid evidence for the presence of phage-driven HGT events contributing to the transmission of the bacterial efflux-associated genetic loci in human pathogens on the intra-species levels. Specifically, our recombination analyses unveil past HGT events reflecting collectively the acquisition and transmission of the ABC transporter ATP-binding protein-encoding and the MF superfamily transporter-encoding genetic loci by the phages within the natural populations of *S. aureus*, *S. suis*, and *P. aeruginosa*. Also, importantly, we provide strong statistical evidence for inter-species recombination of the multidrug ABC transporter permease-encoding genetic loci driven by one of the *B. thuringiensis* phage representatives serving as the donor for *B. cereus*, and as the recombinant for both *B. cereus* and *B. tropicus*.

The earlier study [10] reported on evidence for the intergeneric transfer of two ARGs, *mel* and *tetM* loci, mediated by an *Erysipelothric* phage acting as the recombinant for *Streptococcus pneumoniae* and *Bacillus coagulans*, and as the donor for *S. suis*. Here, we provide another strong statistical evidence for the phage transduction-driven intergeneric transfer of bacterial efflux-associated ARGs. Specifically, the outcomes of the recombination detection analyses strongly suggest that certain *M. smegmatis* phages can have the ability to acquire and transmit the MF superfamily transporter-encoding genetic loci within natural populations of *S. harbinensis* and *S. chartreusis*. These findings were consistent with the previous report in terms of phage-mediated intergeneric recombination involving different bacterial genera [69]. As also shown in this study, the robust results of the recombination analyses were backed up by the PHISDetector-generated data, supporting strongly the above elucidated HGT interactions between the phages and bacteria. Regrettably however, due to a lack of consensus between the PhageAI, BACPHLIP, and PHACTS resolutions, the lifestyles for most of the ARGs-recombining phages could not be unambiguously determined in our experiments. As indicated above, among these phages, the actual lifestyle was known only for the *B. thuringiensis* phage proCM3 (KF296717.1) characterized in the previous study [38] demonstrating its temperate nature.

Conclusion

A role of the phages in the transmission of bacterial efflux-associated ARGs, in natural populations of human and animal pathogenic bacteria, is poorly understood and need to be clearly determined. Here, the phages are suggested to contribute to the dissemination of these drug-resistance and multi-drug resistance determinants involving a large number of pathogenic species. These species include, but are not limited to, *Salmonella enterica, Mycobacterium smegmatis, Pseudomonas aeruginosa, Burkholderia pseudomallei, Staphylococcus aureus, Staphylococcus epidermidis,* Streptococcus suis, and *Streptococcus gallolyticus*. Certain phages that infect human nonpathogenic species, *B. thuringiensis*, are suggested to serve as the donor of the multidrug ABC transporter permease-encoding gene loci for *B. cereus* and *B. tropicus*. Besides, it is also strongly suggested that some *M. smegmatis* phages can acquire and transfer the MF superfamily efflux transporter gene loci even on the intergeneric levels within the natural populations of *S. chartreusis* and *S. harbinensis*.

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Data availability The information on the DNA sequence data, described in this study, is publicly available in the GenBank Database of the National Center for Biotechnology Information (NCBI).

Code availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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3.4 პოლივალენტური ფაგების ბუნებრივ პოპულაციებში ბი- და მულტი-ლატერული გენების გადაცემა

მიჩნეულია, რომ ლითიური სასიცოცხლო ციკლის მქონე ფაგებს გააჩნიათ ფართო გამოყენების პერსპექტივა ვეტერინარულ მედიცინაში, სამედიცინო თერაპიასა და სურსათის უვნებლობაში. თუმცა, იმისათვის, რომ თავიდან აცილებულ იქნას ბაქტერიოფაგების მასშტაბური გამოყენებით მიღებული შესაძლო გაუთვალისწინებელი და არახელსაყრელი შედეგები, საჭიროა სირღმისეულად განვსაზღვროთ ზომიერ და ლითიური ფაგებს შორის ურთიერთქმედებები (Kupczok et al. 2018). ჩვენი კვლევების ფარგლებში გამოვიკვლიეთ რეკომბინაციის ფენომენი და მისი ტრაექტორიები ზემოაღნიშნული ბუნების ფაგების ბუნებრივ პოპულაციებში. ამჯერადაც, რეკომბინაციული ანალიზების შედეგების თანახმად, რომლებიც ერთობლივად წარმოაჩენენ SplitsTree, RDP4, GARD, Simplot პროგრამების პაკეტების გამოყენებით მიღებულ მყარ მონაცემებს (fit: 100; $P \leq 0.014$),განისაზღვრა, რომ ფაგების მორფოგენეზის, მასპინძლის შერჩევის სპეციფიურობის, და ფაგურ რეპლიკაციის პროცესებში ჩართული გენები მიმოიცვლება ზოგიერთ ზომიერ და ლიტიურ ფაგს შორის, სავარაუდოდ ბაქტერიის მათ მიერ კოინფიცირებისას. ამასთანავე, განისაზღვრა ისიც, რომ გენეტიკური რეკომბინაციის ეს მოვლენები შესაძლოა მოხდეს იმ ფაგებს შორისაც, რომელთა მასპინძლები წარმოადგენენ ბაქტერიების სხვადასხვა სახეობას განსხვავებული გვარებიდან. კერძოდ, ასეთი ფაგების მასპინძელი სახეობები აღმოჩნდა ისეთი ორგანიზმები, როგორებიცაა*Salmonella enterica*, *Escherichia coli*, Staphylococcus *aureus*, და *Shigella flexneri*. აღსანიშნავია ისიც, რომ ჩვენს მიერ გამოვლენილია ფაგის მთავარი კაფსიდის მაკოდირებელი გენის (რომელიც გამოყენებულია მანამდე ფაგების კლასიფიკაციის ერთ-ერთ გენეტიკურ მარკერად) რეკომბინაციაც ამ ფაგებს შორის (Gabashvili et al. 2021b). ამგვარად, ეს მოვლენა ბადებს

შეკითხვას, ხომ არ ცვლის გენეტიკური რეკომბინაცია ამ გენის ნუკლეოტიდურ თანმიმდევრობას იმ დონეზე, რა დონეზეც აკუმულირებული გენეტიკური ვარიაცია შეიძლება სახეობის გენეტიკური მახასიათებლის მხოლოდ მიმიკრიას ახდენდეს კლასიფიკაციის ტესტს დაქვემდებარებულ ორგანიზმში.

კვლევის ფარგლებში რეკომბინაციულმა ანალიზებმა ცხადყო, რომ გენეტიკური რეკომბინაცია უმეტესად ხდება ვირულენტურ ფაგებს შორის, ან მხოლოდ ზომიერ ფაგებს შორის, მაგრამ, როგორც ზემოთ აღინიშნა, იგი ასევე შესაძლებელია მოხდეს, თუმცა შედარებით იშვიათად, ვირულენტურსა და ზომიერ ფაგს შორის (Gabashvili et al. 2021b). მაგალითად, ჩვენს რეკომბინაციულ ანალიზებში განისაზღვრა DNA პოლიმერაზა I მაკოდირებელი გენის მიმოცვლა ზომიერ *V. parahaemolyticus* და *V. vulnificus* ფაგებსა და ლითიურ *V. parahaemolyticus* VP06 ფაგს შორის. ასევე, ამავე ანალიზებით ნაჩვენები იქნა დნმ რეპლიკაციის და გენების მიმოცვლა ლითიურ *E.coli* ფაგებსა და *E. faecium* ბაქტერიის პროფაგს შორის. რეკომბინაციის მოვლენები ასევე დაფიქსირდა *E. coli*-ის და *S. boydii*-ის სპეციფიკურ ფაგებსა და *E. faecium*-ის პროფაგს შორის. მიღებული შედეგი გარკვეულწილად ემთხვევა აქამდე არსებულ ხედვას იმის შესახებ, რომ მასპინძლის კოინფიცირებისას რეკომბინაცია შესაძლოა მოხდეს: 1.ზომიერ ფაგებს შორის; 2. ზომიერ ფაგსა და მასპინძლის პროფაგს შორის; 3. ლითიურ ფაგებს შორის; და ასევე 4. ლითიურ ფაგებსა და პროფაგებს შორის (Kupczok et al. 2018). ამგვარად, რეკომბინაცია შესაძლებელია მოხდეს როგორც ზომიერ ფაგებში (Lynch et al. 2010, Gabashvili et al. 2021b) ისე ლითიურ ფაგებშიც (Gabashvili et al. 2021b).

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ORIGINAL PAPER

Bi- and Multi-directional Gene Transfer in the Natural Populations of Polyvalent Bacteriophages, and Their Host Species Spectrum Representing Foodborne Versus Other Human and/or Animal Pathogens

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Abstract

Unraveling the trends of phage-host versus phage-phage coevolution is critical for avoiding possible undesirable outcomes from the use of phage preparations intended for therapeutic, food safety or environmental safety purposes. We aimed to investigate a phenomenon of intergeneric recombination and its trajectories across the natural populations of phages predominantly linked to foodborne pathogens. The results from the recombination analyses, using a large array of the recombination detection algorithms imbedded in SplitsTree, RDP4, and Simplot software packages, provided strong evidence (fit: 100; $P \leq 0.014$) for both bi- and multi-directional intergeneric recombination of the genetic loci involved collectively in phage morphogenesis, host specificity, virulence, replication, and persistence. Intergeneric recombination was determined to occur not only among conspecifics of the virulent versus temperate phages but also between the phages with these different lifestyles. The recombining polyvalent phages were suggested to interact with fairly large host species networks, including sometimes genetically very distinct species, such as e.g., *Salmonella enterica* and/or *Escherichia coli* versus *Staphylococcus aureus* or *Yersinia pestis*. Further studies are needed to understand whether phage-driven intergeneric recombination can lead to undesirable changes of intestinal and other microbiota in humans and animals.

Keywords Bacteriophage · Genetic recombination · Horizontal gene transfer · Intergeneric recombination · Foodborne pathogens · Host species

Introduction

Bacteriophage, otherwise known as phage, represents the most abundant biological entity, with more than an estimated 10^{31} tailed phage virions (Hendrix et al. 1999), residing in Earth's biosphere (Wommack and Colwell 2000; Brüssow and Hendrix 2002; Wilhelm et al. 2002; Hendrix et al. 2003; Hambly and Suttle 2005; Suttle 2005). Particularly over the last three decades, interest in the use of phages in a number of domains has increased. These applications include those for human therapy (Kutter et al. 2010; Wright et al. 2009; Sarker et al. 2012), veterinary purposes (Gigante and Atterbury 2019; Fernández et al. 2018; Akmal et al. 2020), and food safety (Moye et al. 2018; Woolston et al. 2013; Abuladze et al. 2008; Grose and Casjens 2014). Pros and cons of these phage applications have been widely contemplated, focusing heavily not only on their efficacy but, importantly, also on the safety of their projected long-term use towards

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humans and animals (Cisek et al. 2017; Loc-Carrillo and Abedon 2011; Endersen et al. 2014; Henein 2013; Principi et al. 2019).

A long record of evidence demonstrates that most phage are host specific (Grose and Casjens 2014; Wichels et al. 1998; Holmfeldt et al. 2007), i.e., infect-specific bacterial strains, serovars, biovars, subspecies, or species. While the monovalent phages infect strains within a single host species, polyvalent ones can have the ability to infect bacteria not only from a single genus, but also across different genera (Grose and Casjens 2014; Sullivan et al. 2003; Jensen et al. 1998; Bielke et al. 2007). To date and influenced by safety precautions, only the application of virulent phages has been considered to be appropriate whereas the temperate phages are largely ignored. This is due to the fact that the phage can sometimes acquire and transmit various antibiotic resistance genes (ARGs) and virulence determinants, contributing, respectively, to the emergence of drug-resistant and highly virulent strains in natural populations of human and animal bacterial pathogens (Gabashvili et al. 2020; de la Cruz and Davies 2000; Beceiro et al. 2013; Boyd 2012; Deng et al. 2019; Manaia 2017; Brown-Jaque et al. 2015). However, recent advances in phage research reflect ever-growing evidence suggesting strongly the dysbiotic nature of certain phage populations against some mammals including humans (Tetz and Tetz 2016, 2018; Tetz et al. 2017; Manrique et al. 2017). It was reported that, via their interplay with the gut microbiota or specific cells of humans (Gogokhia et al. 2019), the phages can worsen (Bollyky and Secor 2019) or even cause some human diseases (Tetz and Tetz 2018), such as, e.g., dysbiosis (Lepage et al. 2008) and inflammatory bowel disease (Lepage et al. 2008; Norman et al. 2015). In this scenario, it becomes highly imperative to gain a more in-depth understanding of host coinfections caused by different phage and that of phage–phage interactions as part of their influence on the larger microbiomes (Reyes et al. 2010; Minot et al. 2011; Pride et al. 2012). Being heavily driven by horizontal gene transfer (HGT) (Kupczok et al. 2018; Casjens 2005; Cicin-Sain et al. 2005; Dang et al. 2004; Worobey and Holmes 1999), the phage coevolutionary interactions, which seem to be prevalent in various environments (Roux et al. 2014; Flores et al. 2011; Díaz-Muñoz 2017; Kupczok et al. 2018), can sometimes modify the phage genome evolution (Roux et al. 2015; Turner and Chao 1998; Moineau et al. 1994; Joseph et al. 2009; Refardt 2011) and even dictate the fate of their hosts. In this light, a deeper understanding of HGT and its trajectories that collectively involve virulent and temperate phage populations, and their host species networks is highly desirable. This will enable us to predict and assess human and animal health risks associated with undesirable microbiome changes that can possibly occur due to these genetic interactions. This is especially true, when phages, in increasingly large quantities, are artificially and extensively introduced across various microenvironments including intestines of the above organisms.

In this study, we provide strong evidence for both intra- and intergeneric recombination within and between the natural populations of monovalent versus polyvalent phages with different lifestyles, involving fairly broad host species networks representing some important foodborne and other human and/or animal pathogens. These host species networks collectively included but were not limited to, *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, *Shigella boydii*, *Vibrio vulnificus,* and *Vibrio parahaemolyticus.* The results obtained from our recombination tests suggest also the possibility of simultaneous exchange of multiple genetic loci that, as a whole, play important roles in phage morphogenesis, virulence, replication, and host specificity. Herein, we elucidate as well the intergeneric recombination events between some actual or predicted virulent and temperate phages, where these organisms exchanged sometimes their roles of a donor versus that of a recipient of some of these genetic loci. Certain limitations of the freely available programs designed specifically for the phage lifestyle prediction are also revealed and discussed in this study.

Materials and Methods

Selection of Phage and Prophage Genomes

For the recombination analyses, we selected the phage genomes available in the nucleotide database of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). In the above database, we initially randomly selected the phage strains recovered from foodborne pathogens, such as *E. coli*, *S. enterica*, *Listeria monocytogenes*, *Campylobacter jejuni*, *V. vulnificus,* and *V. parahaemolyticus*. Subsequently, the genome DNA sequences of these phages as query were then blasted against the NCBI nucleotide database to search for their intra- and inter-genome homologs. In this analysis, the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) with the megaBLAST algorithm was applied. The megaBLAST algorithm was used with the following default general and scoring parameters: Expected threshold—10/; Word size—28; Max matches in a query range—0; Match/mismatch scores—1,-2; Gap costs—Linear; and Extension—2. Regions of low compositional complexity were filtered accordingly when using this algorithm. In the BLAST analysis, we selected the phage and prophage genomes that, with respect to the query sequences, exhibited ≥ 80% DNA similarity, considering that HGT most frequently occur among closely related phage, which share

a high DNA homology in their genomes (Hendrix et al. 1999; De Paepe et al. 2014; Martinsohn et al. 2008).

We applied also PHASTER (PHAge Search Tool-Enhanced Release, http://phaster.ca/) as reported previously (Arndt et al. 2016; Zhou et al. 2011) to identify prophages across bacterial genomes, and/or to annotate/reannotate phage and prophage genetic loci when needed. Alternatively, the RAST tool kit (RASTtk) (Brettin et al. 2015) was additionally employed for the gene annotation procedures to enhance their precision and validity.

Recombination Analyses of Phage Genes

We used the split decomposition method (Bandelt and Dress 1992) implemented in the SplitsTree program (version 4.14.4) (Huson and Bryant 2006) to detect and reconstruct recombination events between the selected phages, as well as among some of these phages and the prophages. For the SplitsTree analyses, the DNA sequences were initially aligned using ClustalX (version 2.1) (Larkin et al. 2007). The SplitsTree-generated bootstrap values being ≥ 95 (from 1000 replicates) for each node of a parallelogram, and the fit values being ≥ 95 for each splits network, were considered to be statistically highly significant. When detected, the SplitsTree-inferred recombination events were reexamined using the Phi (Pairwise Homoplasy Index) test (Bruen et al. 2006) to avoid false-positive HGT signals, which could be due to convergent mutations that sometimes mimic HGT in recombination analyses.

The SplitsTree-identified HGT events were reexamined further using RDP (Martin and Rybicki 2000), GENECONV (Padidam et al. 1999), BootScan (Martin et al. 2005), MaxChi (Smith 1992), Chimaera (Posada and Crandall 2001), SiScan (Gibbs et al. 2000), and 3Seq (Boni et al. 2007) imbedded in the RDP4 software package (Beta 4.96) (Martin et al. 2015)). Using RDP4, we identified recombinant strains (recipients) as well as their representative major and minor parents (donors) of the recombined genetic loci, thus, determining trajectories of each recombinational event. In these RDP4 analyses, for the number of permutations, we used the default parameter—0, the linear sequence setting, and the enabled disentangle recombination signals. Besides, we applied a very stringent approach: When identified, for the significant breakpoint clusters (99%), only the predetermined Bonferroni-corrected P values in a range of ≤ 0.05 were considered to be statistically significant in the above analyses. If recombination hotspots could not be detected by RDP4, the same DNA alignments were then reexamined employing the SimPlot v3.5.1 program (Lole et al. 1999). In the SimPlot analyses, the window size and the step size were set respectively to 200 bp and 20 bp.

Prediction of Lifestyle and Host Species of Recombining Phage

For the lifestyle classification of the recombining phages (virulent versus temperate), we analyzed their genomes using PHACTS with the Random Forest algorithm (https://edwards.sdsu.edu/PHACTS/index.php). In the PHACTS analysis, a predicted phage lifestyle was considered as true if respective probability values were > 0.5 as determined previously (McNair et al. 2012). In addition, we applied PhageAI (https://phage.ai/), a new online-supported software platform, which is an alternative to PHACTS, utilizing the advanced machine learning and natural language processing techniques for the phage lifestyle prediction. We applied a stringent approach in this analysis also by determining a phage lifestyle as true if the PhageAI-generated prediction accuracy values were $\geq 97\%$. The obtained PHACTS and the PhageAI data were then compared with the information on the actual lifestyle of each phage described in the corresponding references when available in various scientific literature and the NCBI database. Besides, we applied HostPhinder (version 1.1) (https://cge.cbs.dtu.dk/services/HostPhinder/) (Villarroel et al. 2016) to predict other potential host species for the recombining phages. In the HostPhinder analysis, the coverage values in a range of ≥ 0.1 were accepted as statistically significant for the host species prediction, considering the previous host prediction estimates from the earlier study (Villarroel et al. 2016).

Results and Discussion

Genetic Recombination Between Phages from Different Host Species

The homology-driven recombination between phages (Hendrix et al. 2003; Sandmeier et al. 1992; De Paepe et al. 2014; Costa et al. 2018; Martinsohn et al. 2008; Hendrix 2002) as well as among these organisms and prophages (De Paepe et al. 2014; Costa et al. 2018; Martinsohn et al. 2008) can be considered to be a predominant mode of HGT in phage natural populations. However, there is still a lack of systematic understanding of how polyvalent phages associated with foodborne and other pathogens contribute to this evolutionary process.

In this study, using a large panel of recombination detection algorithms, we performed multivariate analyses of the genomes of these organisms to detect and elucidate events of homologous recombination in their natural populations linked predominantly to foodborne pathogens. Initially, for the recombination tests, a total of 860 phages associated with different host species were selected in the NCBI nucleotide database, applying the megaBLAST algorithm. Using

the method of splits decomposition (Bandelt and Dress 1992) with the SplitsTree analyses, we could detect multiple events of both intra- and intergeneric recombination between the phages, as well as between these organisms and a fairly large number of prophages, involving collectively the genes associated with phage head or tail morphogenesis, phage DNA synthesis/replication, or DNA repair and recombination. All the recombining phages and prophages identified in this study are provided, respectively, in Tables 1S and 2S. Specifically, the events of intra- and intergeneric recombination between the phages recovered from *E. coli*, *S. enterica*, *S. aureus,* and *S. flexneri* were detected in these analyses, entailing the genes that encode for the major capsid or prohead proteins, or the prohead protease (Fig. 1a–b). Besides, the genetic exchange of the prohead protein-encoding gene loci between the phages isolated from *V. campbellii*, *V. harveyi,* and *V. parahaemolyticus* could be also determined (Fig. 1c). The SplitsTree-generated parallelograms (Fig. 1a–c) were supported by the highly robust bootstrap values (95.1–100) and by the highest fit (100) exclusively, providing strong evidence for all these recombination events. It is noteworthy that, in some phage populations, the genes associated with the procapsid synthesis were determined to evolve with little intrusion of the genetic information from other phage types (Casjens and Thuman-Commike 2011). The results obtained from our SplitsTree analysis shown that the phages even from different families, such as *Siphoviridae* and *Myoviridae* can exchange sometimes the prohead protease-encoding gene loci (Fig. 1b), thus, supporting the assumption that HGT can occur between unrelated phage species (Montag et al. 1989). Apart from that, these results add another layer to explanatory evidence for the genome mosaics of *E. coli* and *S. aureus* phages (Martinsohn et al. 2008), which clearly seem to be formed partially due to intergeneric recombination. Besides, it should be noted that the major capsid protein-coding gene has been used as a genetic marker for phage classification (Grose and Casjens 2014; Smith et al. 2013; Costa et al. 2018) or the preliminary assignment of phages to specific groups (Born et al. 2019). On this note, the results of our study raise an important question as to whether the genetic alterations that accumulate due to intergeneric recombination across this gene loci can obscure phage phylogenies when the above locus is applied singly as the genetic marker for the phage classification and/or phage clustering analyses. Nevertheless, given that, generally, the analysis of a single gene can bear this and some other important disadvantages, the whole genome sequencing of phages is a reliable alternative for both the classification of these organisms and determining their genetic relatedness.

Interestingly enough, (Fig. 1c) the parallelogram was based on the DNA alignment of the prohead protease- and prohead protein-coding genes of the *Vibrio* phages that

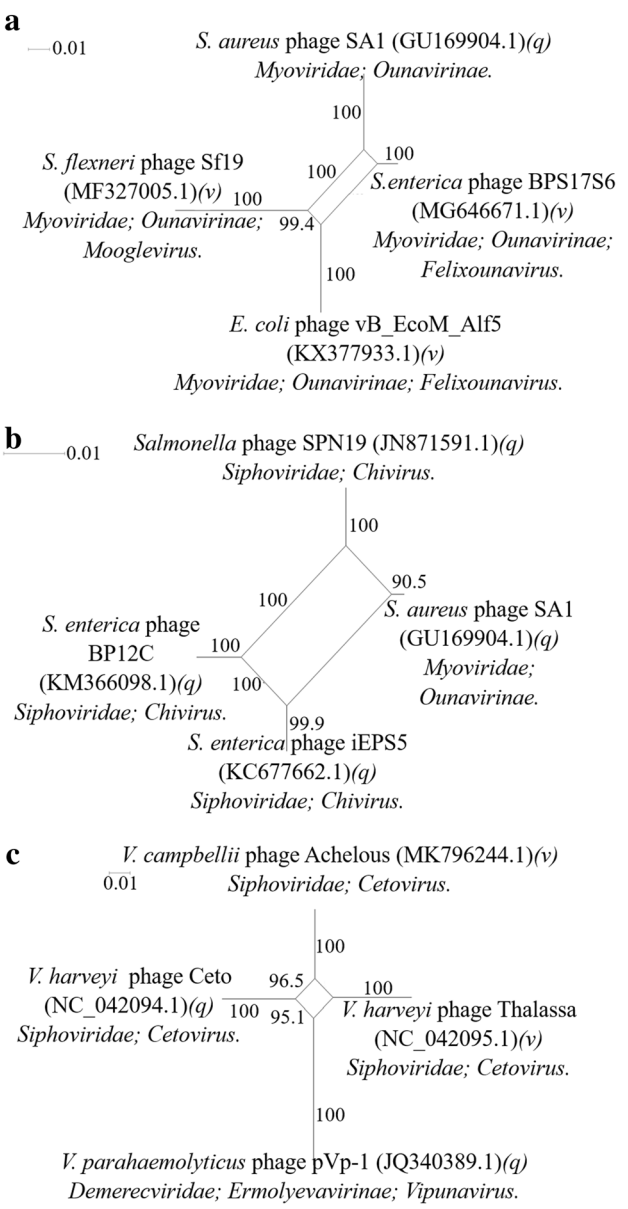


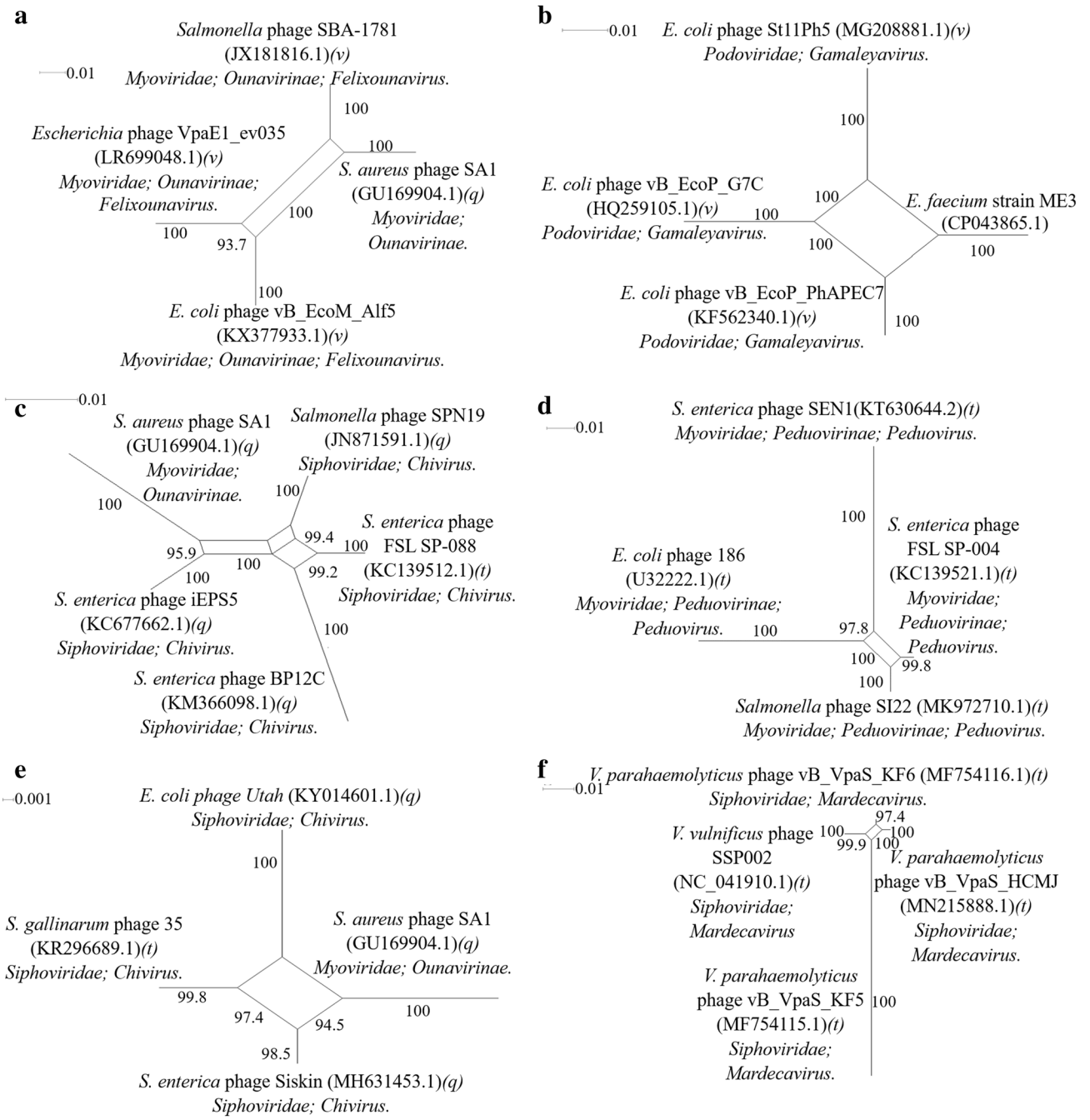
Fig. 1 SplitsTree-generated splits graphs display the intergeneric recombination events between the phages from different host species, entailing the genes encoding major capsid and prohead proteins or prohead protease. In the splits graphs **a**–**c**, the HGT events are presented as the parallelograms with the bootstrap values. A GenBank accession number, for each strain, is provided in the parentheses following the strain description in these splits graphs. $^{(v/t/q)}$—Virulent/temperate/questionable lifestyle as determined in the analyses (Table S4 of Supplemental material). **a** Intergeneric recombination between *E. coli*, *S. enterica*, *S. aureus,* and *S. flexneri* phage strains, involving the major capsid-encoding gene (fit: 100). The major capsid-encoding gene allele of the *S. enterica* phage BPS17S6 (MG646671.1)$^{(v)}$ is representative for the same gene allele of the *S. enterica* phage BPS17W1 (NC_042097.1)$^{(v)}$, and the *S. enterica* phage BPS15S6 (MG646670.1)$^{(v)}$; **b** Intergeneric recombination between *S. enterica* and *S. aureus* phage strains, involving the prohead protease- and prohead protein-encoding genes (fit: 100); **c** Intergeneric recombination between *V. campbellii*, *V. harveyi,* and *V. parahaemolyticus* phage strains, involving the prohead protein-encoding gene (fit: 100). The prohead protein-encoding gene allele of the *V. campbellii* phage Achelous (MK796244.1)$^{(v)}$ is representative for the same gene allele of the *V. campbellii* phage Brizo (MK895508.1)$^{(v)}$ and *V. harveyi* phage Bennett (MN958086.1)$^{(v)}$

shared the DNA–DNA identities in a range of 87.62–100% (coverage: 100%) (Table S1). With the above DNA identity values, we reasoned that, while all these genes contribute to the phage capsid morphogenesis (Maurer et al. 2020; Black et al. 1994), they are very unlikely to encode for these different protein products in the above phages. Hence, we analyzed the same DNA sequences using alternatively RASTtk—one of the most efficient tools for the accurate annotation of microbial genomes (Brettin et al. 2015)—in order to resolve the above ambiguity with the gene functions. In contrast, the RASTtk analysis resulted in a significantly more plausible scenario, according which, conceptually, the both genetic loci represent a single gene that encodes for a capsid and scaffold protein playing a role in the viral head morphogenesis (Ahamed et al. 2019). Also, this gene assists the assembly of the outer shell, dissociating it from the phage capsid during the subsequent packaging (Shen et al. 2012). Thus, hereafter in this study, the above genetic loci of these *Vibrio* phage are referred singly as the capsid and scaffold protein-encoding gene. It is noteworthy that in GenBank, at least some phage genome annotations appeared to be misleading, suggesting inability of the gene callers to capture a full complexity of the phage genomes (McNair et al. 2019).

It is reported that the tail genes (other than the receptor-binding domain 9b of tailspike) of certain phage lineages are rarely affected by HGT from other phage types (Casjens and Thuman-Commike 2011). These genes play an essential role in host specificity (Zhang et al. 2018; Miernikiewicz et al. 2016), because almost all phage recognize host receptors via their tail (Chaturongakul and Ounjai 2014). It was thought that very high variability of the receptor specificity being driven largely by HGT would increase even further and become nearly limitless in phage populations (Montag et al. 1989). In our study, the Splits decomposition analyses generated the robust parallelograms (Fig. 2a–e) exhibiting collectively multiple events of intra- and intergeneric recombination of various tail genes between phages recovered from *S. enterica*, *E. coli*, and *S. aureus* as well as among some of these phages and prophages carried by certain strains of *S. enterica* and *E. faecium.* Coupled with some previous findings (De Paepe et al. 2014; Roux et al. 2012; Costa et al. 2018; Durmaz and Klaenhammer 2000; Bouchard and Moineau 2000; Baker et al. 1991), our results also strongly suggest that HGT between different phage and prophages/defective prophages may appear to be a fairly frequent phenomenon. Events of intrageneric recombination of the tail-encoding loci between the phages of *Vibrio parahaemolyticus* and *Vibrio vulnificus* were also detected in our experiments.

The phage tail proteins play a role in the assembly of the tail tube and shield the structure exterior exposed to the environment (Auzat et al. 2008). The tail tape measure protein has some critical functions associated with the virion assembly, morphology, and infection (Mahony et al. 2016), dictating the tail length, and facilitating DNA transit to the cell cytoplasm during infection (Mahony et al. 2016; Cumby et al. 2015). It was demonstrated that specific genetic alterations in the tail tape measure protein-encoding gene can lead to a reduction in the infective efficiency of phage (Mahony et al. 2016), and that the transfer of the above locus between phage of diverse evolutionary origins is very limited (Smith et al. 2013). Here, our SplitsTree analyses show that intergeneric recombination of the phage tail tape measure protein-encoding gene could occur between the *S. aureus* and *S. enterica* phages from different families (*Myoviridae*, *Ounavirinae* versus *Siphoviridae*, *Chivirus,* respectively) (Fig. 2c). Extensive events of intergeneric recombination of this gene between phages or prophages of *S. enterica* and *E. coli* phages were also revealed in the Splits decomposition analyses (Fig. S1). To date, it was unknown whether the tail sheath protein-encoding gene was involved in HGT across phage populations. The tail sheath protein initiates viral DNA injection in the host cell (Lopes et al. 2014; Kurochkina et al. 2018), thus, being an important component of the molecular machinery that facilitates very high viral infection efficiency (Aksyuk et al. 2009). In some phage, this protein is thought to function also as a receptor-binding protein interacting with the outer membrane receptor (NfrA) of a bacterial host (Z. Zhang et al. 2018). The results of the above splits decomposition analyses (Fig. 2d) are strongly suggestive of at least intrageneric transfer of this gene loci not only between some *Peduovirus* phages of *E. coli* and *S. enterica*, but also possibly between these phages and many *S. enterica* prophages. It is important to note that the tail tape measure protein- and the tail sheath protein-encoding genes were used singly as the phylogenetic markers for the classification and phylogenetic inferences of various phage (including *E. coli* phage) or prophage (Smith et al. 2013; Adriaenssens and Cowan 2014; Costa et al. 2018). Whether the intergeneric recombination of the above genetic loci can change generic-specific patterns in these phage still needs to be determined.

In Fig. 2e, a single parallelogram displays intergeneric recombination events of the tail fiber protein-encoding gene loci between the *Siphoviridae* phages of *S. enterica*, *E. coli*, and the *Myoviridae* phage of *S. aureus*. The tail fiber protein mediates adsorption of a phage particle to bacterial host cells by binding to specific cell surface receptors (Dunne and Loessner 2019). It is noteworthy that host recognition is moderated through a reversible interaction of the long tail fibers with lipopolysaccharides or with a specific outer membrane porin protein (Yu and Mizushima 1982), often determining phage-host range (Dunne and Loessner 2019). It is thought that chimeric structures of the tail fiber gene, which exhibits extensive DNA sequence variability



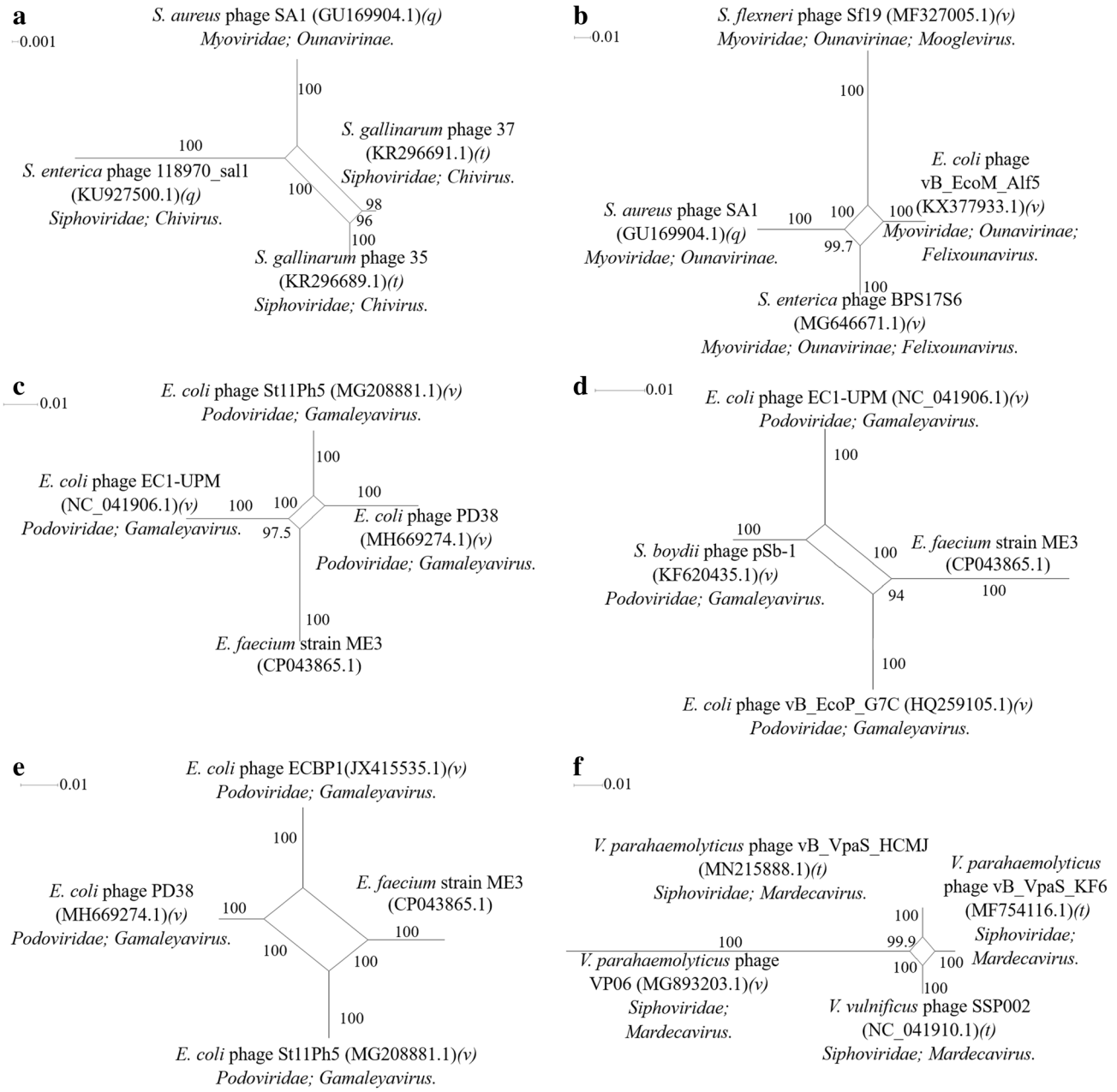
(Miernikiewicz et al. 2016), could be due to HGT events between genetically distinct phage (Sandmeier et al. 1992). Interestingly, while the ancient transfer of this gene in a large group of unrelated temperate and virulent phage strains was documented (Montag et al. 1989; Sandmeier et al. 1992; Haggård-Ljungquist et al. 1992), to date, there was a lack of evidence for its recent genetic transfer in modern phage populations (De Paepe et al. 2014). Based on the output of our splits decomposition analyses (Fig. 2e), we assume that the genetic exchange of the tail fiber genes occurs within modern phage populations as well, although, more in-depth studies are needed to strongly support the above assumption still. Considering the functions of these tail morphogenesis genes, we believe that both intrageneric and intergeneric recombination of some of these genetic loci between the phages from different host species causing foodborne and other diseases can have some important impact on the phage evolution. In particular, we strongly suggest that HGT can sometimes alter and subsequently increase not only virulence and pathogenic potential of the recombining phages, but also their infective abilities allowing them to broaden the host spectrum and to occupy new niches including possibly

◂**Fig. 2** SplitsTree-generated splits graphs display the genetic recombination events of genes involved in the tail morphogenesis (i) between phage strains from different host species that cause foodborne and/or other diseases, and (ii) among some of these phages and the *E. faecium* and *S. enterica* prophages. In the splits graph, the HGT events are presented by the parallelograms with bootstrap values provided for each node. A GenBank accession number, for each strain, is provided in the parentheses following strain description in these splits graphs. $^{(v/t/q)}$—Virulent/temperate/questionable lifestyle as determined in the analyses (Table S4 of Supplemental material). **a** Intergeneric recombination of the putative tail protein-encoding gene between phage strains of *S. enterica*, *E. coli,* and *S. aureus* (fit: 100); **b** Intergeneric recombination of the putative tail protein-encoding gene between *E. coli* phage and *E. faecium* prophage (fit: 100). The tail protein-encoding gene allele of the prophage of *E. faecium* strain ME3 (CP043865.1) is representative for the same gene allele of *E. coli* phage PGN829.1 (MH733496.1)$^{(v)}$; **c** Intergeneric recombination of the gene encoding the phage tail tape measure protein between phage strains of *S. aureus* and *S. enterica* (fit: 97.786); **d** Intergeneric recombination of the major tail sheath protein-encoding gene between phage of *E. coli* and *S. enterica* (fit: 100). The major tail sheath protein-encoding gene allele of *S. enterica* phage FSL SP-004 (KC139521.1)$^{(t)}$ is representative for the same gene allele of the prophage (genome coordinates: 3085027..3115622) of *S. enterica* strain FORC93 (CP032304.1), the prophage (genome coordinates: 220875..251470) of *S. enterica* strain ATCC BAA-664 (CP034773.1), and the prophage (genome coordinates: 4025253..4055848) of *S. enterica* strain SA20026289 (CP022490.1). The major tail sheath protein-encoding gene allele of the *Salmonella* phage SI22 (MK972710.1)$^{(t)}$ is representative for the same gene allele of the *Salmonella* phage SW9 (MK972711.1)$^{(t)}$, the prophage (genome coordinates: 1235879..1270114) of *S. enterica* strain FORC098 (CP030029.1), the prophages (genome coordinates: 1239866..1298455 and 4749550..4789908) of *Salmonella* strain USDA-ARS-USMARC-1913 (CP025278.1), the prophages (genome coordinates: 1200402..1258991 and 4710353..4750711) of the *S. enterica* strain 10TTU468x (CP032814.1), the prophages (genome coordinates: 1200394..1258983 and 4710362..4750720) of *S. enterica* strain 11TTU1590 (CP032817.1), the prophage (genome coordinates:1249465..1283699) of *S. enterica* strain CFSAN070643 (CP024168.1), the prophage (genome coordinates: 1298745..1332980) of *S. enterica* strain CFSAN070645 (CP024164.1); **e** Intergeneric recombination of the tail fiber protein-encoding gene between phages of *S. enterica*, *E. coli,* and *S. aureus* (fit: 100). The tail fiber protein-encoding gene allele of the *S. aureus* phage SA1 (GU169904.1)$^{(q)}$ is representative for the same gene allele of the *S. enterica* phage FSL SP-030 (KC139519.1)$^{(t)}$ and the *S. enterica* phage FSL SP-039 (KC139514.1)$^{(t)}$; (f) Intrageneric recombination of the putative tail protein-encoding gene between phages of *V. parahaemolyticus* and *V. vulnificus* (fit: 100)

beneficial or environmentally friendly bacteria. It can be thought that the frequency of such phage–phage interactions will increase significantly as phage in large quantities, especially, the polyvalent ones, are introduced artificially in certain microenvironments including intestines of humans and animals. In turn, this can easily lead to at least some important microbiome alterations in these domains, the long-term outcomes of which are extremely hard (if not impossible) to predict from environmental, human, and animal health perspectives.

Implying the splits decomposition method, we could also detect extensive events of intrageneric and intergeneric recombination across the genetic loci that are linked to the phage DNA synthesis, DNA replication, and DNA repair and recombination (Fig. 3). Figure 3a displays the parallelogram that elucidates intergeneric recombination of the DNA helicase-encoding gene between the *Myoviridae* phage of *S. aureus* and some *Siphoviridae* phages of *S. enterica*. In phage, the DNA helicase-encoding gene stimulates DNA replication and DNA recombination (Jones et al. 2001; Yonesaki 1994; Gauss et al. 1994). Importantly also, in this gene, certain genetic changes can lead to a delay in the phage DNA synthesis (Gauss et al. 1994) and reduced survival (Yonesaki 1994). We could identify also intergeneric recombination events of the DNA ligase-encoding gene between various phages isolated from *E. coli*, *S. enterica*, *S. flexneri*, and *S. aureus* (Fig. 3b). The DNA ligases play critical roles in DNA replication, as well as in DNA repair and DNA recombination (J. Wang et al. 2019a) and are specifically essential to the induction of DNA ends relaxation (Rossi et al. 1997), the sealing of gaps in duplex DNA (Mueser et al. 2010), oligomerization of bacteriophage (Cherepanov et al. 2001; Nilsson and Magnusson 1982), ligation of DNA with base pair mismatched (Yuan et al. 2007), and nick closing (Ciarrocchi et al. 1993). Thus, it is safe to suggest that some HGT-driven allelic changes of these genes can easily affect ecological fitness and survival of phage.

Interestingly, the splits decomposition analyses could identify the HGT events between the *E. faecium* prophage and the *E. coli Podoviridae* phages (Fig. 3c) or among this prophage, and some *Podoviridae* phages of both *E. coli* and *S. boydii* (Fig. 3d), involving the genetic loci, that in GenBank, are, respectively, annotated as the DNS protein-encoding gene and the single-stranded DNA (ssDNA)-binding protein-encoding gene. In contrast to the ssDNA-binding protein, which, in phage, protects ssDNA intermediates during replication, repair and recombination (Cernooka et al. 2017; Kazlauskas et al. 2016; Shereda et al. 2008), the DNS protein is exclusively a bacterium-produced DNase enzyme with broad functions. Specifically, this protein contributes to biofilm formation, detachment of bacterial cells from biofilms, nutrient acquisition by bacteria (Seper et al. 2011), and reduction of their natural transformability (Blokesch and Schoolnik 2008; Gaasbeek et al. 2009). Thus, the presence of this gene in a phage genome would be explained solely by illegitimate recombination that can occur sometimes not only between genetically very distinct phage (Haggård-Ljungquist et al. 1992; Martinsohn et al. 2008; Bobay et al. 2013b), but also among phages and bacteria (Bobay et al. 2013a; Menouni et al. 2015). Interestingly, in the GenBank database, this recombined gene of the *E. faecium* prophage (CP043865.1), sharing the fairly high DNA identities (92.69–92.93%) with the DNS protein-coding



genes of three *E. coli* phages (NC_041906.1, MH669274.1, and MG208881.1) (Table S1), is annotated as the DUF3987 domain-containing protein. However, in contrast to the GenBank conceptual annotation, in our analysis, using RasTtk, the DNS protein-coding genes of all the above organisms appeared to encode the phage DNA primase (as referred further in this study), which is a site-specific RNA polymerase. This enzyme initiates the DNA synthesis (Kato et al. 2004), catalyzing specifically the synthesis of the oligoribonucleotides required for the initiation of lagging strand DNA synthesis in phage (Lee et al. 2010). It also acts a molecular brake in DNA replication (Lee et al. 2006). It was found that certain alterations (which may sometimes occur due to HGT) in some subdomains of this gene can result in loss of selective DNA binding (Lee et al. 2010). Thus, our study raises additional awareness of the need for reexamining the phage genome annotations presented in GenBank.

HGT of DNA polymerases-coding genes between viruses and their hosts was previously suggested (Filée et al. 2002). In our SplitsTree analyses, the *E. faecium* prophage (CP043865.1) was found to recombine the DNA polymerase-encoding gene with the *E. coli* phages (Fig. 3e), whereas HGT events of this genetic locus between the phages isolated from *Vibrio parahaemolyticus* and *Vibrio vulnificus*

◂**Fig. 3** SplitsTree-generated splits graphs display the intergeneric recombination events of the genes involved in phage DNA synthesis, DNA replication, and DNA repair and recombination, between (i) phages from different host species causing foodborne and/or other diseases, and (ii) among some of these phages and the *E. faecium* prophage. In the splits graph, the HGT events are presented by the parallelograms with bootstrap values provided for each node. A GenBank accession number, for each strain, is provided in the parentheses following strain description in these splits graphs. $^{(v/t/q)}$—Virulent/temperate/questionable lifestyle as determined in the analyses (Table S4 of Supplemental material). **a** Intergeneric recombination of the DNA helicase-encoding gene between *S. enterica* phages and the *S. aureus* phage (fit: 100). The gene allele of the *S. enterica* phage118970_sal1 (KU927500.1)$^{(q)}$ is representative for the same gene allele of *S. enterica* phage BP12C (KM366098.1)$^{(q)}$; **b** Intergeneric recombination of the DNA ligase-encoding gene between phages recovered from *E. coli*, *S. enterica*, *S. flexneri,* and *S. aureus* (fit: 100). The gene allele of the *S. enterica* phage BPS17S6 (MG646671.1)$^{(v)}$ is representative for the same gene allele of *S. enterica* phage BPS17W1 (NC_042097.1)$^{(v)}$ and *S. enterica* phage BPS15S6 (MG646670.1)$^{(v)}$; **c** Intergeneric recombination of the DNS protein-encoding gene between the *E.coli* phages and the *E. faecium* prophage (fit: 100). The gene allele of the prophage of the *E. faecium* strain ME3 (CP043865.1) is representative for the same gene allele of *Escherichia coli* phage PGN829.1 (MH733496.1)$^{(v)}$; **d** Intergeneric recombination of the ssDNA-binding protein gene between the *E.coli phages*, the *S. boydii phage,* and the *E. faecium* prophage (fit: 100). The gene allele of the prophage of the *E. faecium* strain ME3 (CP043865.1) is representative for the same gene allele of *Escherichia coli* phage PGN829.1 (MH733496.1)$^{(v)}$; **e** Intergeneric recombination of the DNA polymerase-encoding gene between the *E.coli* phages and the *E. faecium* prophage (fit: 100). The gene allele of the prophage of the *E. faecium* strain ME3 (CP043865.1) is representative for the same gene allele of *Escherichia coli* phage PGN829.1 (MH733496.1)$^{(v)}$; **f** Intrageneric recombination of the putative DNA polymerase I-encoding gene between the phages recovered from *V. parahaemolyticus* and *V. vulnificus* (fit: 100)

were also evident in our experiments (Fig. 3f). The phage DNA polymerases are essential for the viral genome replication, DNA recombination and repair (Bedford et al. 1997; Hollis et al. 2001; Rezende et al. 2003). Interestingly enough, the alleles of the DNA primase-, ssDNA-binding protein, and DNA polymerase-encoding genes of the *E. faecium* prophage (CP043865.1) were found to be identical to those of some recombining *E. coli* phages involved in our analyses (Fig. 3c–e). Hence, it remains unclear whether such an allelic identity is linked solely to HGT, which could occur between the above organisms, or alternatively, to a possible common ancestral origin of these *E. coli* phages and the *E. faecium* prophage.

The recombination tests can generate false-positive signals when misinterpreting convergent mutations as HGT patterns (Bobay and Ochman 2017; Bruen et al. 2006; Gabashvili et al. 2020). For such instances, the Phi test (Bruen et al. 2006) has been considered to be an extremely powerful tool to detect recombination and to validate the results by measuring homoplasy (White et al. 2013; Cangi et al. 2016; Gabashvili et al. 2020). Thus, using the Phi test, we reexamined the same recombining phage subsets in order to validate the results obtained from the splits decomposition analyses. The Phi test-generated *P* values were significantly less than 0.05 (Table 3S), strongly supporting the HGT inferences (Figs. 1, 2, 3, and 1S) by the splits decomposition analyses.

Trajectories of Intergeneric Recombination Across Phage Natural Populations

Even though the HGT phenomenon with respect to different phage populations has been studied more or less extensively, the information on the trajectories of both intrageneric and intergeneric recombination between phages associated with the foodborne and other human pathogens is still very limited. For example, the events of genetic exchange between phages infecting host species from the genera of *Salmonella*, *Escherichia,* and *Shigella* could be previously elucidated to some extent (Casjens and Thuman-Commike 2011), while its directions across these and other phage populations remained largely unknown.

In our study, we applied the RDP4 recombination detection algorithms that allow determining specifically HGT trajectories by identifying a donor and a recipient of the recombined genetic loci, as well as recombination hotspots and a size of the recombined DNA material. Thus, for the above purposes, using RDP4, we analyzed exactly the same allies of the recombining phage genes being a part of the HGT events determined by the SplitsTree analysis. By utilizing RDP4, we reexamined and validated at the same time the SplitsTree-generated HGT inferences. The results obtained from the RDP4 analyses of the genes involved in the phage major capsid or head morphogenesis are presented in Table 1. As shown, the *S. flexneri* phage (*Mooglevirus*) (MF327005.1) could serve as the major donor of the major capsid protein-encoding gene loci for the *E. coli* phage (KX377933.1) (*Felixounavirus*). Besides, some *Salmonella enterica* phages (NC_042097.1, MG646671.1, and MG646670.1) from the genus *Felixounavirus* appeared to be the minor and major donors of this gene, respectively, for the same *E. coli* phage and the *S. aureus phage* (GU169904.1) from *Ounavirinae* representing a subfamily within *Myoviridae*. Two events of recombination of the major capsid protein-encoding gene loci were also identified by RDP4, where the same *S. aureus and S. flexneri* phages appeared, respectively, to be the minor vs major donors for the *Escherichia coli* phage (KX377933.1) (*Felixounavirus*). It is also shown that phages of *S. enterica* and *S. aureus* could exchange their roles of the recipient and donor of the capsid and scaffold protein-encoding gene loci. As demonstrated in Table 1, it is strongly suggested that, as the donor or the recipient, certain *Vibrio* phages, including *Vibrio parahaemolyticus* phage (JQ340389.1), even from the different families, e.g., such as *Demerecviridae* and *Siphoviridae*, could exchange the prohead protein-coding gene loci. The RDP4-derived

Table 1 Representative recombinant phages, and their major and minor donor phages of the genetic loci encoding the major capsid or prohead proteins, and the prohead protease, as well as the recombination hotspots, as determined by RDP4

Recombinant strain	Representative major parent	Representative minor parent	Gene product	Beginning and end breakpoints in a gene DNA alignment (99% Cl)	RDP4-generated *P* value	SplitsTree-identified HGT (Fig. citation)
E. coli phage vB_EcoM_Alf5 (KX377933.1)$^{(v)}$ *Myoviridae; Ounavirinae; Felixounavirus*	*S. flexneri* phage Sf19 (MF327005.1)$^{(v)}$ *Myoviridae; Ounavirinae; Mooglevirus*	*S. enterica* phage BPS17S6 (MG646671.1)$^{(v)}$ * *Myoviridae; Ounavirinae; Felixounavirus*	Major capsid protein	497 (478–509) – 801 (731–1110)	RDP—1.78E-02 GENECONV—3.41E-05 Bootscan—NS Maxchi—7.03E−10 Chimaera—3.43E−07 SiScan—4.73E−17 3Seq—1.69E−05	Figure 1a
S. aureus phage SA1 (GU169904.1)$^{(}$q) *Myoviridae; Ounavirinae*	*S. enterica* phage BPS17S6 (MG646671.1)$^{(v)}$ * *Myoviridae; Ounavirinae; Felixounavirus*	Unknown, closest = *S. flexneri* phage Sf19 (MF327005.1)$^{(v)}$ *Myoviridae; Ounavirinae; Mooglevirus;*	Major capsid protein	507 (443–524) – 754(652–785)	RDP—6.08E−04 GENECONV—NS Bootscan—NS Maxchi—7.83E−07 Chimaera—1.28E−05 SiScan—3.32E−08 3Seq—7.86E−06	Figure 1a
E. coli phage vB_EcoM_Alf5 (KX377933.1)$^{(v)}$ *Myoviridae; Ounavirinae; Felixounavirus*	*S. flexneri* phage Sf19 (MF327005.1)$^{(v)}$ *Myoviridae; Ounavirinae; Mooglevirus*	*S. aureus* phage SA1 (GU169904.1)$^{(q)}$ *Myoviridae; Ounavirinae*	Major capsid protein	38 (Undetermined) – 116 (93–431)	RDP—NS GENECONV—3.49E−04 Bootscan—NS Maxchi—1.35E−03 Chimaera—2.32E−02 SiScan—NS 3Seq—1.71E−02	Figure 1a
E. coli phage vB_EcoM_Alf5 (KX377933.1)$^{(v)}$ *Myoviridae; Ounavirinae; Felixounavirus*	*S. flexneri* phage Sf19 (MF327005.1)$^{(v)}$ *Myoviridae; Ounavirinae; Mooglevirus*	*S. aureus* phage SA1 (GU169904.1)$^{(q)}$ *Myoviridae; Ounavirinae*	Major capsid protein	988 (Undetermined) – 1089 (Undetermined)	RDP—4.41E−03 GENECONV—4.94E−02 Bootscan—NS Maxchi—8.24E−03 Chimaera—NS SiScan—3.33E−12 3Seq – NS	Figure 1a
S. enterica phage BP12C (KM366098.1)$^{(q)}$ *Siphoviridae; Chivirus*	Unknown, closest = *S. aureus* phage SA1 (GU169904.1)$^{(q)}$ *Myoviridae; Ounavirinae*	*S. enterica* phage iEPS5 (KC677662.1)$^{(q)}$ *Siphoviridae; Chivirus*	prohead protease ClpP	796 (188–869) – 1167 (Undetermined)	RDP—5.19E-08 GENECONV—1.15E-05 Bootscan—2.90E−08 Maxchi—3.58E−09 Chimaera—NS SiSscan- NS 3Seq—1.78E−12	Figure 1b
S. aureus phage SA1 (GU169904.1) $^{(q)}$ *Myoviridae; Ounavirinae*	*Salmonella* phage SPN19 (JN871591.1)$^{(q)}$ *Siphoviridae; Chivirus*	*S. enterica* phage iEPS5 (KC677662.1)$^{(q)}$ *Siphoviridae; Chivirus*	Prohead protease ClpP	2 (Undetermined) – 303 (Undetermined)	RDP – NS GENECONV—2.69E−03 Bootscan—4.12E−03 Maxchi—1.66E−03 Chimaera—1.55E−03 SiSscan- 3.80E−06 3Seq—1.05E−04	Figure 1b

Table 1 (continued)

Recombinant strain	Representative major parent	Representative minor parent	Gene product	Beginning and end breakpoints in a gene DNA alignment (99% Cl)	RDP4-generated *P* value	SplitsTree-identified HGT (Fig. citation)
V. harveyi phage Thalassa (NC_042095.1)$^{(v)}$ *Siphoviridae; Cetovirus*	*V. parahaemolyticus* phage pVp-1 (JQ340389.1)$^{(q)}$ *Demerecviridae; Ermolyevavirinae; Vipunavirus*	*V. campbellii* phage Achelous (MK796244.1)$^{(v)}$ * *Siphoviridae; Cetovirus*	Capsid and scaffold protein-encoding gene	1 (Undetermined) – 164(130–239)	RDP—9.05E−05 GENECONV-2.99E−05 Bootscan—3.39E−03 Maxchi—6.55E−06 Chimaera—4.25E−05 SiScan—7.61E−09 3Seq—4.55E−02	Figure 1c

GenBank accession number, for each strain, is provided in the parentheses following the strain description

NS No significant *P* value could be obtained for the identified recombination event using the above algorithm(s)

$^{(v/t/q)}$ Virulent/temperate/questionable lifestyle

*Representatives of the phage strains/prophages that have the identical recombining gene alleles as described in Fig. 1a–c, respectively

data provide additional strong evidence (Table 2) for both the intrageneric and intergeneric recombination of the phage tail genes across the phage natural populations infecting different foodborne and other human and/or animal pathogens. It is clear that these phages even from different genera (e.g., such as *Felixounavirus*, *Gamaleyavirus*, *Peduovirus*, *Chivirus*, and *Mardecavirus*) can readily switch the roles of the donor versus recipient for the exchange of some tail-encoding loci.

As indicated above, several previous studies (De Paepe et al. 2014; Roux et al. 2012; Costa et al. 2018; Durmaz and Klaenhammer 2000; Bouchard and Moineau 2000; Baker et al. 1991) report on the HGT events between phages and prophages or defective prophages. Interestingly enough, in our RDP4 analyses, the *E. faecium* prophage ME3 (CP043865.1) appeared to be the minor donor of the putative tail protein gene for the *E. coli* phage strain (HQ259105.1) (Table 2), while sharing this gene allelic identity with that of another *E. coli* phage (MH733496.1). In addition, the RDP4 data strongly suggest that, during the independent HGT events, the *E. coli* phage (U32222.1) could act as the recombinant strain acquiring the phage tail tape measure protein gene loci from both the *S. enterica* phages (MK972710.1, KC139521.1) within the same genus *Peduovirus* and the prophages of multiple genetically different *S. enterica* strains (CP030029.1, CP025278.1, LR134140.1, CP032814.1, etc.) At the same time, this *E. coli* phage (U32222.1) and the prophages of some *S. enterica* strains (CP032304.1, CP034773.1, and CP022490.1) appeared to be, respectively, the minor vs major donors of the major tail sheath protein-coding gene loci for the *S. enterica* phage (KT630644.2).

The results of the RDP4 analyses (Table 3) provide additional and strong statistical evidence for both intrageneric and intergeneric recombination of the genetic loci associated with the phage DNA synthesis, DNA replication, and DNA repair and recombination between the phages from the above host species. As shown, in these HGT events, certain phages (e.g., GU169904.1, MG646671.1, MH669274.1) from the different genera and/or the different host species could share the roles of recombinant versus recipient of some of these loci. Moreover, some of the HGT events entailed the same *faecium* prophage ME3 (CP043865.1), where it served as the minor donor or the recipient (recombinant) of the DNA primase gene loci, and exclusively as the minor donor of the DNA polymerase-encoding gene loci, for some *E. coli* phages from the genus *Gamaleyavirus*. Our study revealed the highly recombining *Ounavirinae* phages from different host species (e.g., the *S. aureus* phage [GU169904.1], *S. flexneri* phage [MF327005.1], *E. coli* phages [KX377933.1 and KX377933.1]), which might have exchanged simultaneously some of the above described loci (Figs. 1, 2, 3, and Tables 1, 2, 3). Besides, as also shown in Tables 1, 2, 3, displaying the recombination beginning and end breakpoints determined by RDP4, it can be suggested that both the intrageneric and intergeneric recombination events frequently entail internal loci of the genes. This was consistent with the previous study assuming that some chimeric structures of the tail fiber genes could be due to the genetic exchange of their short domains between unrelated phages (Sandmeier et al. 1992).

In our analysis, RDP4 could not clearly determine the direction of, and the recombination beginning and end breakpoints across the ssDNA-binding protein-coding loci recombined between the *E. coli* and *S. boydii* phages and *E. faecium* prophage. Thus, alternatively, we analyzed the DNA sequence alignment of the ssDNA-binding protein-coding

Table 2 Representative recombinant phages, and their major and minor donor phages or prophages of the genetic loci encoding the phage tail proteins, as well as the recombination hotspots, as determined by RDP4

Recombinant strain	Representative major parent	Representative minor parent	Gene product	Beginning and end breakpoints in a gene DNA alignment (99% Cl)	RDP4-generated *P* value	SplitsTree-identified HGT (Fig. citation)
Salmonella phage SBA-1781 (JX181816.1)[v] *Myoviridae; Ounavirinae; Felixounavirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	*Escherichia* phage VpaE1_ev035 (LR699048.1)[q] *Myoviridae; Ounavirinae; Felixounavirus*	Putative tail protein	138 (Undetermined) – 666 (643–733)	RDP 1.21E−05 GENECONV 1.11E−04 Bootscan 1.92E−05 Maxchi 2.95E−10 Chimaera 1.91E−07 SiScan 4.20E−05 3Seq 6.23E−08	Figure 2a
E. coli phage vB_EcoM_Alf5 (KX377933.1)[v] *Myoviridae; Ounavirinae; Felixounavirus*	*Escherichia* phage VpaE1_ev035 (LR699048.1)[q] *Myoviridae; Ounavirinae; Felixounavirus*	Unknown, closest = *Salmonella* phage SBA-1781 (JX181816.1)[v] *Myoviridae; Ounavirinae; Felixounavirus*	Putative tail protein	803(679–803) – 862(854–878)	RDP 6.25E−03 GENECONV 1.35E−02 Bootscan NS Maxchi 6.48E−03 Chimaera 5.46E−04 SiScan 1.36E−05 3Seq NS	Figure 2a
E. coli phage vB_EcoM_Alf5 (KX377933.1)[v] *Myoviridae; Ounavirinae; Felixounavirus*	*Escherichia* phage VpaE1_ev035 (LR699048.1)[q] *Myoviridae; Ounavirinae; Felixounavirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	Putative tail protein	26 (Undetermined) – 711(681–734)	RDP 4.00E−04 GENECONV 1.41E−03 Bootscan 9.37E−04 Maxchi 1.17E−02 Chimaera 2.83E−03 SiScan 8.05E−11 3Seq 3.19E−06	Figure 2a
E. coli phage vB_EcoP_G7C (HQ259105.1)[v] *Podoviridae; Gamaleyavirus*	Unknown, closest = *E. coli* phage St11Ph5 (MG208881.1)[v] *Podoviridae; Gamaleyavirus*	*E. faecium* strain ME3 (CP043865.1)*	Putative tail protein	357 (Undetermined) – 552 (Undetermined)	RDP 1.32E−02 GENECONV NS Bootscan NS Maxchi 4.77E−02 Chimaera 2.50E−02 SiScan 7.18E−03 3Seq 2.97E−03	Figure 2b
E. coli phage 186 (U32222.1)[t] *Myoviridae; Peduovirinae; Peduovirus*	Unknown, closest = *Salmonella* phage SI22, (MK972710.1)[t] *Myoviridae; Peduovirinae; Peduovirus*	*S. enterica* phage FSL SP-004 (KC139521.1)[t] *Myoviridae; Peduovirinae; Peduovirus*	Phage tail tape measure protein	747 (1–770) – 1601 (1575–2396)	RDP 4.08E−22 GENECONV 5.35E−17 Bootscan 5.68E−13 Maxchi 2.45E−20 Chimaera 1.79E−09 SiScan 3.40E−34 3Seq 3.55E−15	Figure 2b
S. enterica phage BP12C (KM366098.1)[q] *Siphoviridae; Chivirus*	*Salmonella* phage SPN19 (JN871591.1)[q] *Siphoviridae; Chivirus*	Unknown, closest = *S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	Phage tail tape measure protein	1386(796–1407) – 3305 (2689–3510)	RDP 1.37E−20 GENECONV 4.19E−26 Bootscan 4.07E−29 Maxchi2.05E−18 Chimaera 2.36E−06 SiSscan3.91E−26 3Seq 2.20E−31	Figure 2c

Table 2 (continued)

Recombinant strain	Representative major parent	Representative minor parent	Gene product	Beginning and end breakpoints in a gene DNA alignment (99% Cl)	RDP4-generated *P* value	SplitsTree-identified HGT (Fig. citation)
Salmonella phage SPN19 (JN871591.1)[q] *Siphoviridae; Chivirus*	*S. enterica* phage FSL SP-088 (KC139512.1)[t] *Siphoviridae; Chivirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	Phage tail tape measure protein	1502 (1436–1749) – 2338 (2231–2454)	RDP 9.63E−23 GENECONV 4.67E−21 Bootscan 1.27E−22 Maxchi1.04E−12 Chimaera 1.14E−12 SiSscan2.98E−10 3Seq 3.80E−24	Figure 2c
S. enterica phage iEPS5 (KC677662.1)[q] *Siphoviridae; Chivirus*	*S. enterica* phage FSL SP-088 (KC139512.1)[t] *Siphoviridae; Chivirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	Phage tail tape measure protein	1188 (835–1221) – 3913 (3526–4157)	RDP 6.64E−03 GENECONV 9.23E−07 Bootscan 3.33E−03 Maxchi3.71E−10 Chimaera 6.06E−07 SiSscan2.53E−04 3Seq 3.33E−15	Figure 2c
S. enterica phage FSL SP-088 (KC139512.1)[t] *Siphoviridae; Chivirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	*S. enterica* phage BP12C (KM366098.1)[q] *Siphoviridae; Chivirus*	Phage tail tape measure protein	1989 (1457–2019) – 2246 (2144–2454)	RDP 6.35E−11 GENECONV 1.36E−10 Bootscan NS Maxchi1.54E−09 Chimaera 7.81E−10 SiSscan4.56E−08 3Seq NS	Figure 2c
S. enterica phage FSL SP-088 (KC139512.1)[t] *Siphoviridae; Chivirus*	*Salmonella* phage SPN19 (JN871591.1)[q] *Siphoviridae; Chivirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	Phage tail tape measure protein	3837 (3737–3879) – 4015 (3935–4219)	RDP NS GENECONV 1.19E−07 Bootscan 1.39E−08 Maxchi 3.16E−04 Chimaera 3.02E−04 SiSscan1.73E−04 3Seq 4.88E−06	Figure 2c
S. enterica phage FSL SP-088 (KC139512.1)[t] *Siphoviridae; Chivirus*	Unknown, closest = *S. enterica* phage iEPS5 (KC677662.1)[q] *Siphoviridae; Chivirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	Phage tail tape measure protein	1508 (1435–1644) – 1987 (1928–2457)	RDP 1.41E−05 GENECONV 8.76E−04 Bootscan NS Maxchi2.34E−06 Chimaera 7.29E−07 SiSscan5.64E−13 3Seq 5.45E−06	Figure 2c
S. enterica phage iEPS5 (KC677662.1)[q] *Siphoviridae; Chivirus*	*S. aureus* phage SA1 (GU169904.1)[q] *Myoviridae; Ounavirinae*	*S. enterica* phage FSL SP-088 (KC139512.1)[t] *Siphoviridae; Chivirus*	Phage tail tape measure protein	2412 (1454–2454) – 3178 (2651–3492)	RDP 2.08E−06 GENECONV 4.29E−05 Bootscan NS Maxchi7.58E−04 Chimaera 1.22E−04 SiSscan2.53E−04 3Seq 5.96E−06	Figure 2c

Table 2 (continued)

Recombinant strain	Representative major parent	Representative minor parent	Gene product	Beginning and end breakpoints in a gene DNA alignment (99% Cl)	RDP4-generated *P* value	SplitsTree-identified HGT (Fig. citation)
S. aureus phage SA1 (GU169904.1)$^{(q)}$ *Myoviridae; Ounavirinae*	Unknown, closest = *Salmonella* phage SPN19 (JN871591.1)$^{(q)}$ *Siphoviridae; Chivirus*	*S. enterica* phage iEPS5 (KC677662.1)$^{(q)}$ *Siphoviridae; Chivirus*	Phage tail tape measure protein	154(77–162) – 375(188–462)	RDP NS GENECONV 1.44E−04 Bootscan 2.58E−05 MaxchiNS Chimaera NS SiSscan4.02E−17 3Seq 3.05E−03	Figure 2c
E. coli phage 186 (U32222.1)$^{(t)}$ *Myoviridae; Peduovirinae; Peduovirus*	*Salmonella* phage SI22 (MK972710.1)$^{(t)}$ * *Myoviridae; Peduovirinae; Peduovirus*	*S. enterica* phage FSL SP-004 (KC139521.1)$^{(t)}$ *Myoviridae; Peduovirinae; Peduovirus*	Phage tail tape measure protein	2351 (2197–2402) – 2439 (Undetermined)	RDP 5.70E−03 GENECONV 1.89E−05 Bootscan NS Maxchi 2.95E−03 Chimaera 1.99E−03 SiScan 5.82E−05 3Seq 2.13E−03	Figure S1
S. enterica phage SEN1 (KT630644.2)$^{(t)}$ *Myoviridae; Peduovirinae; Peduovirus*	*S. enterica* phage FSL SP-004 (KC139521.1)$^{(t)}$ * *Myoviridae; Peduovirinae; Peduovirus*	Unknown, closest = *E. coli* phage 186 (U32222.1)$^{(t)}$ *Myoviridae; Peduovirinae; Peduovirus*	Major tail sheath protein	202(119–233) – 542(505–593)	RDP 9.06E−08 GENECONV 4.09E−07 Bootscan 5.33E−08 Maxchi 1.79E−14 Chimaera 5.92E−13 SiScan 1.69E−14 3Seq 2.66E−5	Figure 2d
Salmonella gallinarum phage 35 (KR296689.1)$^{(t)}$ *Siphoviridae; Chivirus*	*E. coli* phage Utah (KY014601.1)$^{(q)}$ *Siphoviridae; Chivirus*	*S. enterica* phage Siskin (MH631453.1)$^{(q)}$ *Siphoviridae; Chivirus*	Tail fiber protein	75(1–269) – 610(523–641)	RDP NS GENECONV NS Bootscan 4.90E−02 Maxchi3.32E−03 Chimaera NS SiSscan7.90E−08 3Seq2.89E−04	Figure 2e
V. vulnificus phage SSP002 (NC_041910.1)$^{(t)}$ *Siphoviridae; Mardecavirus*	Unknown, closest = *V. parahaemolyticus* phage vB_VpaS_KF5 (MF754115.1)$^{(}$q) *Siphoviridae; Mardecavirus; unclassified Mardecavirus*	*V. parahaemolyticus* phage vB_VpaS_HCMJ (MN215888.1)$^{(t)}$ *Siphoviridae; Mardecavirus; unclassified Mardecavirus*	Putative tail protein	45 (Undetermined) – 2076 (1222–2220)	RDP NS GENECONV 1.69E−02 Bootscan NS Maxchi7.21E−04 Chimaera 9.19E−05 SiSscan 4.91E−43 3Seq 2.71E−03	Figure 2f

GenBank accession number, for each strain, is provided in the parentheses following the strain description

NS No significant *P* value could be obtained for the identified recombination event using the above algorithm(s)

$^{(v/t/q)}$ Virulent/temperate/questionable lifestyle

*Representatives of the phage strains/prophages that have the identical recombining gene alleles as described in Fig. 2a–f, respectively

loci using SimPlot. It plots similarity versus position by calculating and plotting the percent identity of the query sequence to a batch of reference sequences in their DNA alignment (Lole et al. 1999) for identifying HGT events and their hotspots. SimPlot has been successfully used for studying the HGT phenomenon in viruses (Paraskevis et al. 2020; Wang et al. 2019b; Zhang et al. 2013; Yuan et al. 2017; Adiputra et al. 2019) including phages (Gabashvili et al. 2020). In our analysis, SimPlot could identify five recombination hot spots (nucleotide positions: 240, 288,

Table 3 Representative recombinant phages, and their major and minor donor phages or prophages of the genetic loci involved in the phage DNA synthesis, DNA replication, and DNA repair and recombination, as well as the recombination hotspots, as determined by RDP4

Recombinant strain	Representative major parent	Representative minor parent	Gene product	Beginning and end breakpoints in a gene DNA alignment (99% Cl)	RDP4-generated *P* value	SplitsTree-identified HGT (Fig. citation)
S. aureus phage SA1 (GU169904.1)$^{(q)}$ *Myoviridae; Ounavirinae*	*S. enterica* phage 37 (KR296691.1)$^{(}$t) *Siphoviridae; Chivirus*	*S. enterica* phage 118970_sal1 (KU927500.1)$^{(q)}$ * *Siphoviridae; Chivirus*	DNA helicase, phage-associated	1136(898–1193) – 1452 (Undetermined)	RDP 5.07E−03 GENECONV NS Bootscan 9.24E−03 Maxchi1.86E−05 Chimaera 1.02E−04 SiSscan1.54E−08 3Seq 6.89E−03	Figure 3a
S. enterica phage BPS17S6 (MG646671.1)$^{(v)}$ *Myoviridae; Ounavirinae; Felixounavirus*	*S. aureus* phage SA1 (GU169904.1)$^{(q)}$ *Myoviridae; Ounavirinae*	Unknown, closest = *S. flexneri* phage Sf19 (MF327005.1)$^{(v)}$ *Myoviridae; Ounavirinae; Mooglevirus;*	DNA ligase	368(323–458) – 607(556–636)	RDP 1.53E−05 GENECONV 2.46E−03 Bootscan NS Maxchi 4.38E−10 Chimaera 1.51E−06 SiScan 4.95E−19 3Seq 3.67E−07	Figure 3b
E. coli phage vB_EcoM_Alf5 (KX377933.1)$^{(v)}$ *Myoviridae; Ounavirinae; Felixounavirus*	*S. aureus* phage SA1 (GU169904.1)$^{(q)}$ *Myoviridae; Ounavirinae*	*S. flexneri* phage Sf19 (MF327005.1)$^{(v)}$ *Myoviridae; Ounavirinae; Mooglevirus;*	DNA ligase	358(0–380) – 711(671–1101)	RDP 2.64E−05 GENECONV 2.62E−03 Bootscan 2.78E−03 Maxchi 1.37E−12 Chimaera 1.69E−04 SiScan 9.22E−14 3Seq 1.51E−13	Figure 3b
S. aureus phage SA1 (GU169904.1)$^{(}$q) *Myoviridae; Ounavirinae*	*S. enterica* phage BPS17S6 (MG646671.1)$^{(v)}$ *Myoviridae; Ounavirinae; Felixounavirus*	*S. flexneri* phage Sf19 (MF327005.1)$^{(v)}$ *Myoviridae; Ounavirinae; Mooglevirus;*	DNA ligase	922(883–968) – 1121 (Undetermined)	RDP NS GENECONV 3.73E−03 Bootscan NS Maxchi 1.62E−02 Chimaera NS SiScan 4.07E−21 3Seq 1.55E−03	Figure 3b
E. faecium strain ME3 (CP043865.1)*	*E. coli* phage EC1-UPM (NC_041906.1)$^{(v)}$ *Podoviridae; Gamaleyavirus*	*E. coli* phage St11Ph5 (MG208881.1)$^{(v)}$ *Podoviridae; Gamaleyavirus*	DNA primase gene	86(54–176) – 409 (Undetermined)	RDP NS GENECONV 8.22E−03 Bootscan 8.52E−03 Maxchi2.18E−02 Chimaera 6.73E−06 SiScan3.35E−09 3Seq 1.09E−05	Figure 3c
E. coli phage PD38 (MH669274.1)$^{(v)}$ *Podoviridae; Gamaleyavirus*	*E. coli* phage St11Ph5 (MG208881.1)$^{(v)}$ *Podoviridae; Gamaleyavirus*	Unknown, closest = *E. faecium* strain ME3 (CP043865.1)*	DNA primase gene	413(413–449) – 916 (Undetermined)	RDP NS GENECONV NS Bootscan 3.46E−02 Maxchi2.77E−02 Chimaera 8.29E−03 SiScanNS 3Seq 9.84E−04	Figure 3c
E. coli phage St11Ph5 (MG208881.1)$^{(v)}$ *Podoviridae; Gamaleyavirus*	*E. coli* phage PD38 (MH669274.1)$^{(v)}$ *Podoviridae; Gamaleyavirus*	*E. faecium* strain ME3 (CP043865.1)*	DNA polymerase I	188(1–788) – 616(1–788)	RDP 3.57E−02 GENECONV NS Bootscan NS Maxchi 1.90E−03 Chimaera 6.81E−03 SiScan 1.96E−10 3Seq 2.92E−03	Figure 3e

Table 3 (continued)

Recombinant strain	Representative major parent	Representative minor parent	Gene product	Beginning and end breakpoints in a gene DNA alignment (99% Cl)	RDP4-generated *P* value	SplitsTree-identified HGT (Fig. citation)
V. parahaemolyticus phage vB_VpaS_KF6 (MF754116.1)$^{(t)}$ *Siphoviridae; Mardecavirus; unclassified Mardecavirus*	*V. parahaemolyticus* phage vB_VpaS_HCMJ (MN215888.1)$^{(t)}$ *Siphoviridae; Mardecavirus; unclassified Mardecavirus*	V. vulnificus phage SSP002 (NC_041910.1)$^{(}$t) *Siphoviridae; Mardecavirus*	Putative DNA polymerase I	296(1–619) – 2181 (1903–2309)	RDP5.91E−04 GENECONV 4.83E−04 Bootscan 3.40E−05 Maxchi1.03E−06 Chimaera 3.36E−03 SiSscan1.06E−07 3Seq 2.41E−07	Figure 3f

GenBank accession number, for each strain, is provided in the parentheses following strain description

Strain designation, for each phage, is specified according to its actual host species designation

NS No significant *P* value could be obtained for the identified recombination event using the above method

$^{(v/t/q)}$ Virulent/temperate/questionable lifestyle

*Representatives of the phage strains/prophages that have the identical recombining gene alleles as described in Fig. 3a–f, respectively

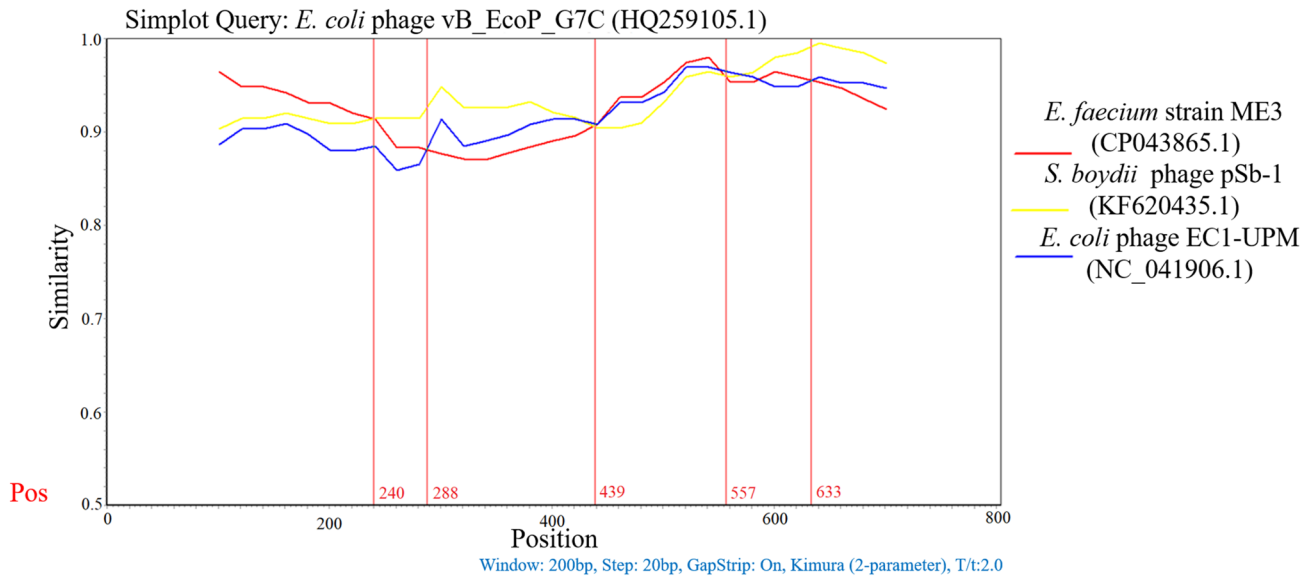


Fig. 4 Simplot-determined recombination hotspots across the ssDNA-binding protein-encoding genes of the *E. coli* and *S. boydii* phages, and the *E. faecium* prophage

439, 557, and 633) across the ssDNA-binding protein-encoding gene (Fig. 4).

Lifestyles and Host Species Spectra of Recombining Phages

The phages seem to evolve within two general evolutionary modes differing notably in the extent of HGT. In particular, it was demonstrated previously that temperate phage distributes into high- and low-gene flux modes, whereas virulent phage shares only the lower gene flux mode (Mavrich and Hatfull 2017). Moreover, these evolutionary modes were suggested to be a function of bacterial host, and that the temperate and lytic phage were distributed in either modes depending on the host phylum (Mavrich and Hatfull 2017). Here, we report on the actual or putative lifestyles and host species spectra of the recombining phages examined in our study, adding another layer of knowledge to the above findings.

Presently, PHACTS and PhageAI are the only freely available software packages designed specifically for classifying the phage lifestyle. While PHACTS has been used

for predicting the phage lifestyle in several more studies (McNair et al. 2012; Jia et al. 2015; Yuan et al. 2014; Cui et al. 2017; Czajkowski et al. 2014; Gabashvili et al. 2020), there has been no information available on the PhageAI performance in the peer-reviewed scientific reports. In our study, PHACTS preformed very poorly generating mainly very weak predictive values (Table S4) and failing in the lifestyle (temperate versus virulent) prediction in four cases out of 11 cases when compared with the actual lifestyles of the recombining phages available very limitedly in the previously published literature. PHACTS predicts the phage lifestyle by examining the amino acid sequences of the phage genes, which fairly frequently result in 0.5–0.6 averaged probability (i.e., unsatisfactory) values (McNair et al. 2012). In contrast, analyzing the DNA sequence data, PhageAI-generated strong predictive signals in most cases (Table S4), although it failed once when falsely predicting the temperate lifestyle for the *V. parahaemolyticus* phage (MG893203.1) classified previously as virulent (Wong et al. 2019). In contrast, however, in our analyses, the PhageAI-produced 3D visualization of the phage distribution plot constructed on the basis of the genome clustering, including 184 temperate and 285 virulent phages, showed that the *V. parahaemolyticus* phage was more closely related to the virulent ones (Fig. S2). Hence, whereas PhageAI, being a repository of knowledge of ~ 10,000 bacteriophages and simultaneously a tool for the phage genome analysis with the utility of Artificial Intelligence support (https://phage.ai/), is clearly a more promising software package than PHACTS, it still requires significant improvements, for robust phage classification.

Our understanding of the HGT-driven genetic interactions between temperate and virulent phages, including how these phenomena impact the evolution of these organisms, is far from comprehensive, which is primarily due to a very scarce information available in this respect. For example, even though the ancient gene transfer between a large group of unrelated virulent and temperate phages was reported (Montag et al. 1989; Haggård-Ljungquist et al. 1992; Sandmeier et al. 1992), no other HGT events among the phages with different lifestyles were elucidated. It was only hypothesized that genetic recombination between virulent and temperate phages—even among those from different host species (Sousa et al. 2020)—can take place (Bailly-Bechet et al. 2007), whereas substantial evidence was still lacking to date. Here, consolidating solely the statistically highly significant data obtained across our analyses of the phage lifestyle (Table S4) and the identified HGT events (Figs. 1, 2, 3, and S1, and Tables 1, 2, 3 and S3), we suggest that both intrageneric and intergeneric recombination mainly occur within the conspecific groups of the virulent versus temperate phage strains or between these organisms and the prophages. This is somehow similar to the view that, during coinfection, temperate phage can recombine with prophages in the host genome, whereas lytic phages can recombine with other lytic types or with prophages (Kupczok et al. 2018). However, it can be thought that the above evolutionary trends do not exhibit a clear and exhaustive picture of the phage–phage and phage–prophage interactions, and may appear sometimes even misleading due to very limited data that are presently available on a role of HGT in the evolutionary divergence of phage populations. More specifically, because of a high demand of various phage industries for the genome sequencing of virulent phage, the number of their sequenced genomes is significantly higher compared with that of the sequenced temperate phage genomes, thus, reducing the chances of HGT detection in the populations of the latter. Moreover, the HGT events at least between the virulent phages and the prophages, identified in both our study and other investigations, may not always exhibit genetic recombination between these organisms. Instead, some of these cases can easily be associated with the earlier HGT events that took place between the virulent phages and the prophage progenitors, i.e., the temperate phages before their switching to a prophage state. In this respect, one clear example can be the HGT event between the prophage of the *E. faecium* strain (CP043865.1) and the *E. coli* virulent phage (HQ259105.1) identified in our recombination analyses (Fig. 2b and Table 2). In particular, if we consider the above scenario as a true HGT event, then *E. faecium* should be accepted to be the most plausible environment for such a prophage–phage interaction. Interestingly, however, the outcomes of the genome analysis of the *E. coli* virulent phage (HQ259105.1) with HostPhinder rejected this scenario, predicting strongly only *S. boydii* (not *E. faecium*) as an alternative host species for this phage (Table S4). However, this finding highly supported the results of the SplitsTree and Simplot analyses (Figs. 3d and 4, respectively) providing strong evidence for the genetic exchange of the ssDNA-binding protein-encoding gene loci between the above prophage and the *E. coli* and *S. boydii* phages. Thus, for these particular independent HGT events, *S. boydii* and possibly *E. coli* could serve as the host species, i.e., the most plausible environments for such genetic interactions. We analyzed further the intact prophage of the *E. faecium* strain (CP043865.1) using HostPhinder. In this analysis, only *E. coli* was strongly predicted by HostPhinder to be an alternative host species for the progenitor (a temperate phage) of the *faecium* prophage (CP043865.1) (Table S4). It is noteworthy that the above prophage is identical to the *E. coli* phage PGN829.1 (*Gamaleyavirus*) (MH733496.1), while sharing > 93% of the genome-wide DNA identities with several other *E. coli* phages (MG208881.1, MH669274.1, KJ135004.2, etc.) from the genus *Gamaleyavirus*. Importantly, this prophage does not share close genetic similarity to any other prophage or phage of *E. faecium* in the GenBank database. Therefore, it can be thought that a putative progenitor of this prophage

could easily be a temperate phage of *E. coli*, which infected *E. faecium* and integrated into its genome. With this in mind, therefore, one can suggest that the reconstructed HGT event between the prophage of the *E. faecium* strain (CP043865.1) and the *E. coli* virulent phage (HQ259105.1), entailing the putative tail protein-encoding gene (Fig. 2b and Table 2), most likely point to the earlier event of intrageneric recombination among the *E. coli* polyvalent temperate phage and the *E. coli* virulent phage within the genus *Gamaleyavirus*. The same observation can be applied to some other HGT events, elucidated in our recombination analyses (Figs. 2b, 3c and e; Tables 2 and 3) that involve this prophage and the *E. coli* phages. Our study raises an important question as to whether or not HGT can naturally transform virulent phages into temperate ones and/or vice versa, and which needs to be determined from human, animal, and environmental health points.

Phage significantly outnumbers their hosts (Díaz-Muñoz 2017; Weinbauer 2004; Suttle 2007; Rohwer and Barott 2013), which led to the suggestion that half of these hosts have a potential to be infected (i.e., be cross-infective) or are actually infected by an average of > 2 phage (Díaz-Muñoz 2017). During the host coinfections, phage–phage interactions not only involve HGT (Kupczok et al. 2018; Casjens 2005; Cicin-Sain et al. 2005; Dang et al. 2004; Worobey and Holmes 1999), but sometimes can even alter the genetic exchange in phage populations (Dang et al. 2004; Worobey and Holmes 1999; Turner and Chao 1998; Cicin-Sain et al. 2005). Hence, it is imperative to determine the entire spectrum of the phage-host species for the better understanding of the complex bacterial networks that are subjected to and provide an adequate environment for these coinfections, promoting HGT-driven phage–phage and phage–prophage interactions especially on intergeneric levels. This will allow us to predict undesirable microbiome changes as well as the negative evolutionary allometry and/or other potential problematic consequences possibly driven by the above interactions in various microenvironments, e.g., human and animal intestine. The main rationale for this is the renewed knowledge on phage, with the ever-growing concerns that, as previously indicated, at least some of these organisms can contribute to some diseases in mammals including humans (Tetz and Tetz 2016,2018; Tetz et al. 2017; Manrique et al. 2017; Gogokhia et al. 2019; Bollyky and Secor 2019; Lepage et al. 2008; Norman et al. 2015). Importantly, the phage-associated evolutionary pathways leading to the risks of diseases to humans and animals remain largely unclear and need to be urgently determined. At the same time, prediction of the phage-host species spectra is an essential step towards rationalizing effective and safe phage formulations.

The results obtained from the phage lifestyle analysis (Table S4), using Hostphinder, strictly showed that, among two phages from different host species, at least one of them was of polyvalent nature in every single HGT event determined in our experiments (Tables 1, 2, 3). This was in a strong agreement with the concept that, for two phages to recombine their genetic loci on an intergeneric level, at least one of these phages needs to be polyvalent with the ability to infect the host species of the other phage. Moreover, as shown in Table S4, Hostphinder predicted that a great majority of the phages, involved in the intrageneric recombination events, were polyvalent. Very importantly also, some of these phages were predicted by Hostphinder to have the ability to infect two species from distinct genera, other than its actual host genus. For example, Hostphinder predicted that certain *E. coli* phages can infect both *S. enterica* and *S. aureus* or *S. boydii*, whereas some *S. enterica* phages can invade *E. coli* and *S. aureus* or even *Y. pestis* being the ethological agent of plague. The results obtained from the Hostphinder analyses also suggest that there exist *S. flexneri* phages that can infect three different other host species, such as *E. coli*, *S. enterica*, and *S. aureus.*

Increased prevalence of Bacteriophages has been linked to aggravated intestinal inflammation and colitis (Gogokhia et al. 2019; Duerkop et al. 2018), as well as to dysbiosis in inflammatory bowel disease cases (Lepage et al. 2008). In the human gut environment, specific interplays of viruses and bacteria (Wang et al. 2015), as well as some certain alterations in both the enteric virome (Norman et al. 2015) and general microbiota (Tetz and Tetz 2016), were suggested to be among the mechanisms that can lead to these and possibly some other diseases. As already indicated, our analysis, exploring phage lifestyle by Hostphinder, clearly shows that at least some *S. enterica*, *S. flexneri*, *S. aureus*, and *S. boydii* phages with the polyvalent properties can infect *E. coli*. These bacterial species include not only pathogenic strains, but also those that thought to be essential to, and are part of the normal microbiota of human and animal intestines. Thus, it can be thought that such phages can contribute to both the short- and long-term evolutionary changes of the microbiota across the above microenvironments, especially when significantly large quantities are ingested orally through microbially contaminated drinking water or food. Interestingly, none of the virulent or temperate phages, demonstrating polyvalent properties, could be predicted by Hostphinder to infect any other environmentally friendly or beneficial bacteria. The HostPhinder prediction abilities are limited to the analysis of the co-occurring k-mers between the query phage genome and the genomes of reference phages with known hosts (Villarroel et al. 2016). Thus, while such a lifestyle of these phages may appear true at a cursory glance, the above observed tendency with the host selection (confined solely to foodborne and other pathogens) could be partly due to the extremely scarce data available on the phage genomes of beneficial bacteria available in the nucleotide databases. On this note, it is clear that the phages from the beneficial

and environmentally friendly bacteria have been largely disregarded in the global phage research been driven largely by the commercial interests to industrialize these organisms for human and animal therapies as well as for food and environmental safety. Very regrettably, this ever-intensified trend of the phage research is pursued in light of an extremely limited understanding of long-term evolutionary safety of various phage applications across different microenvironments.

Conclusions

We strongly suggest that intergeneric recombination can be both bi- and multi-directional across the natural populations of polyvalent phages that infect at least foodborne and some other human and/or animal pathogenic bacteria. It is also suggested that certain events of intergeneric recombination can simultaneously involve the multiple genetic loci collectively linked to phage morphogenesis, pathogenicity, virulence, replication, and environmental persistence. There is a great need for more in-depth studies to determine whether the phage-driven intergeneric recombination contributes to the undesirable changes of the beneficial microbiota that are constantly observed at least in human and animal intestines.

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Compliance with Ethical Standards

Conflict of interest Stylianos Koulouris and Tobin Robinson are employed by the European Food Safety Authority (EFSA). The present article is published under the sole responsibility of the authors and may not be considered as an EFSA scientific output. The positions and opinions presented in this article are those of the authors alone and do not necessarily represent the views or scientific works of EFSA.

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3.5 ლითიური და ზომიერი ფაგები: გენეტიკური განსხვავება და მსგავსება

მწირი სამეცნიერო ლიტერატურისა და ბაქტერიოფაგების სასიცოცხლო ციკლის განმსაზღვრელი სხვადასხვა პროგრამებით მიღებული (PHACTS, BACPHLIP და PhageAI) არაკონსისტენტური შედეგების გამო, ზოგიერთ შემთხვევაში, რთული იყო განგვესაზღვრა ჰორიზონტალური გენების გადაცემაში ჩართული ბაქტერიოფაგების სასიცოცხლო ციკლი.

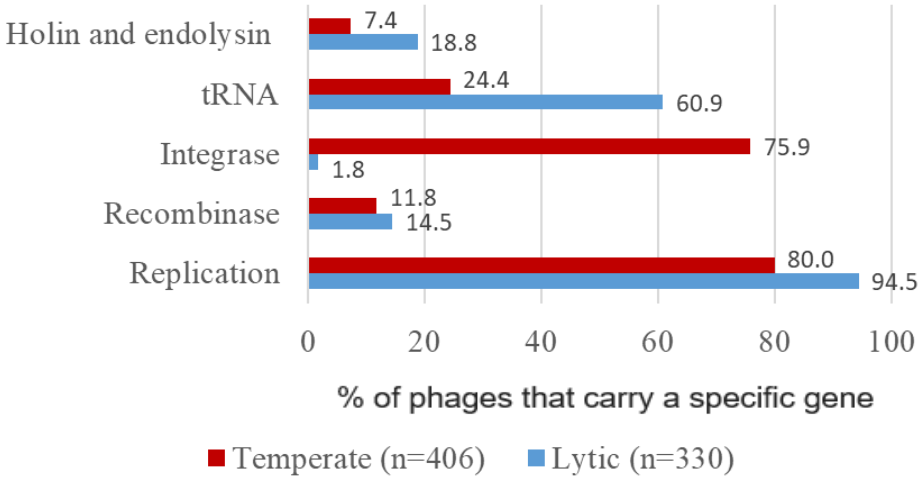
ფაგების სასიცოცხლო ციკლის განსაზღვრა და მათთვის ლითიური თუ ზომიერი ბუნების მიკუთვნება კომპლექსური საკითხია, ვინაიდან თუნდაც ლითიური ფაგის ლითიური უნარები შეიძლება განსხვავდებოდეს მისი მასპინძლების მიხედვით (Niu et al. 2014; Petrov et al. 2010), რაშიც იგი მრავლდება. ამასთანავე, ზოგიერთ ფაგს, რომლებიც გარკვეული პერიოდი მიიჩნეოდა ლითიურად, აღმოჩნდა, რომ გააჩნდათ ზომიერი ფაგის ბუნება, თუმცა მასპინძელ ბაქტერიებში ისინი ვერ ახდენდნენ სტაბილურ ლიზოგენურ ფაგად გარდაქმნას (Gill et al. 2011). შესაბამისად, დავინტერესდით განგვესაზღვრა ის გენეტიკური მახასიათებლები, რომელიც განასხვავებს ლითიურ და ზომიერ ფაგებს ერთმანეთისაგან.

სამეცნიერო მონაცემების მიხედვით ლითიური ფაგებს გააჩნიათ საკუთარი რეპლიკაციის ცილების გენები (Li et al. 2012); ექსპერიმენტალური მონაცემების მიხედვით გაირკვა, რომ ფაგის გენებით კოდირებული ჰოლინი და ენდოლიზინი შესაძლოა იწვევდნენ სინერგიულ ლითიურ აქტივობას გარკვეულ ბაქტერიოფაგებში (Lee et al. 2015). ამასთანავე, ცნობილია, ისიც, რომ ლითიური და ზომიერი ფაგების გენომების GC შემადგენლობა შესაძლებელია იყოს ერთ-ერთი განმასხვავებელი ფაქტორი ამ სხვადასხვა სასიცოცხლო ციკლის მქონე ფაგებს შორის (Lynch et al. 2010). გარდა ამისა, ლითიური ფაგები უფრო მეტ tRNA– ს გენებს შეიცავს, ვიდრე ზომიერი ფაგები (Delesalle et al. 2016; Bailly-Bechet, Vergassola, and Rocha 2007), ხოლო

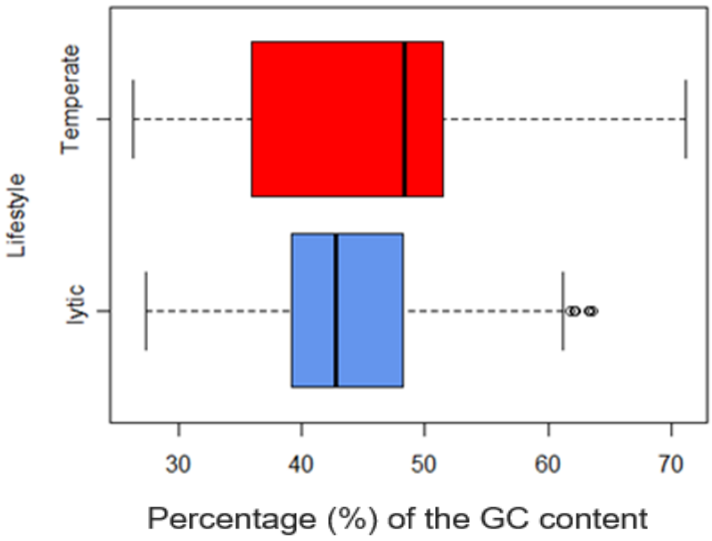
ინტეგრაზა/რეკომბინაზა ცილების მაკოდირებელი გენები ძირითადად ზომიერი ფაგების მახასიათებელია (Ganz et al. 2014). აღნიშნული ფაქტორები გამოვიკვლიეთ ჩვენს ანალიზებში და ზემოაღნიშნული მახასიათებლები შევადარეთ ლითიურ და ზომიერ ფაგებს შორის.

არსებული სამეცნიერო ლიტერატურის მიხედვით კვლევის ფარგლებში წინასწარ მოვახდინეთ ზომიერი და ლითიური ფაგების იდენტიფიცირება, რომელთა გენომების ნუკლეოტიდური თანმიმდევრობების შესახებ ინფორმაცია ხელმისაწვდომი იყო ა NCBI-ს გლობალურ მონაცემთა ბაზაში. აღნიშნული ფაგების გენომების ანალიზის საფუძველზე შევეცადეთ გადაგვემოწმებინა ამ ფაგების სასიცოცხლო ციკლი, რისთვისაც გამოყენებული იყო ფაგების სასიცოცხლო ციკლის განმსაზრელი პროგრამები როგორებიცაა Bacphlip, PhageAI და Phacts. პროგრამებით. ის ფაგები, რომელთა სასიცოცხლო ციკლი, პროგნოზირებული ზემოაღნიშნულიპროგრამებით, არ დაემთხვა ლიტერატურაში ასახულ მათ სასიცოცხლო ციკლს, არ იქნა გათვალისწინებული შემდგომი ანალიზებისთვის. საბოლოოდ შევარჩიეთ ისეთი ლითიური და ზომიერი ფაგები, რომელთა სასიცოცხლო ციკლი დასტურდებოდა, როგორც ლიტერატურული წყაროების მიხედვით, ისე ჩვენს მიერ ჩატარებული ბიოინფორმატიული ანალიზების შედეგებით. შერჩეული ლიტიური და ზომიერი ფაგების გენომები შევადარეთ მათი G+C და tRNA შემცველობის მიხედვით. ფაგების გენომები ასევე გაანალიზდა მათში ჰოლინის, ენდოლიზინის, tRNA-ის, ინტეგრაზას, რეკომბინაზას და რეპლიკაციის ცილების მაკოდირებელი გენების შემცველობის განსაზღვრის მიზნითაც. შედეგები წარმოდგენილია სურათ 1-ში.

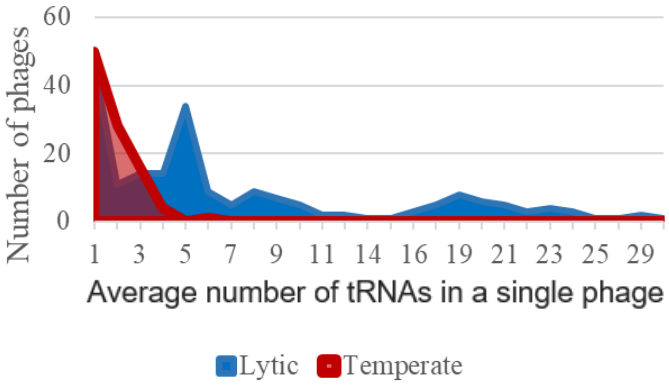
A)


Holin and endolysin
7.4
18.8
tRNA
24.4
60.9
Integrase
75.9
1.8
Recombinase
11.8
14.5
Replication
80.0
94.5
0
20
40
60
80
100
% of phages that carry a specific gene
Temperate (n=406)
Lytic (n=330)

B)


Lifestyle
Temperate
lytic
30
40
50
60
70
Percentage (%) of the GC content

C)


Number of phages
60
40
20
0
1
3
5
7
9
11
14
16
19
21
23
25
29
Average number of tRNAs in a single phage
Lytic
Temperate

სურათი 1. ლითიური და ზომიერი ფაგების მახასიათებელი გენები

a) ჰოლინის და ენდოლიზინის, tRNA, ინტეგრაზას, რეკომბინაზას, რეპლიკაციის (რეპლიკაციის, პრიმაზას, დნმ პოლიმერაზას და ჰელიკაზას) ცილების მაკოდირებელი გენების შეცველი ზომიერი და ლითიური ბაქტერიოფაგების პროცენტული რაოდენობა;

b) GC შემცველობა (%) ზომიერ და ლითიურ ფაგებში;

c) tRNA გენების შემცველობა (%) ზომიერ და ლითიურ ფაგებში

სურათი 1 -ის მიხედვით, tRNA - ის გენების სიჭარბე (5-ზე მეტი tRNA-ის გენი) და ინტეგრაზა გენების შემცველობა ლითიურ და ზომიერ ფაგებს განასხვავებს ერთმანეთისაგან, თუმცა არ არის გამორიცხული რომ ძირითადად ზომიერი ფაგების მახასიათებელი ინტეგრაზას გენი, ან tRNA -ების გენების სიმწირე ასევე არსებობდეს ლითიურ ფაგშიც. მსოფლიოში, წარმოდგენა ფაგების ევოლუციაზე და ლითიურსა და ზომიერ ფაგებს შორის ჰორიზონტალური გენების მიმოცვლის შესახებ, ჯერ კიდევ მწირია. ზომიერ და ლითიურ ფაგებში ზოგიერთი თითქმის იდენტური ნუკლეოტიდური თანმიმდევრობის მქონე გენების ალელების არსებობას გენეტიკურ რეკომბინაციას უკავშირებენ (Moura de Sousa et al. 2021). ლითიურ და ზომიერ ფაგების დიდ ჯგუფებს შორის გენების გადაცემის ვარაუდები იქნა გამოთქმული სხვადასხვა კვლევებში (Montag et al. 1989; Haggård-Ljungquist et al. 1992; Sandmeier et al. 1992). ჩვენი კვლევები (Gabashvili et al. 2021b) უჩვენებს, რომ გენების ჰორიზონტალური მიმოცვლა შესაძლოა მოხდეს ლითიურ და ზომიერ ფაგებს, მათ მიერ მასპინძლის კოინფიცირების პერიოდში და რომ გენეტიკური რეკომბინაცია ასევე შესაძლებელია ასევე ამ ორგანიზმებსა და პროფაგებს შორის.

არსებობს ბაქტერიების ფაგებით კოინფიცირების მაღალი ალბათობა, ვინაიდან ფაგების რაოდენობა მნიშვნელოვნად აჭარბებს მათი მასპინძლების რაოდენობას

(Díaz-Muñoz 2017; Weinbauer 2004; Suttle 2007; Rohwer and Barott 2013); შესაბამისად, ვარაუდობენ, რომ მასპინძელი ბაქტერიები სავარაუდოდ ინფიცირდება საშუალოდ 2 ან მეტი ფაგით (Díaz-Muñoz 2017). მასპინძელი შტამის კოინფექციების დროს კი ფაგებს შორის ურთიერთქმედებისას ხდება შეიძლება ადგილი ჰქონდეს HGT-ს (Kupczok et al. 2018; Casjens 2005; Cicin-Sain et al. 2005; Dang et al. 2004; Worobey and Holmes 1999); ამ მოვლენამ ცხადია შესაძლოა გამოიწვიოს ფაგური პოპულაციის გენომის შეცვლაც კი (Dang et al. 2004; Worobey and Holmes 1999; Turner and Chao 1998; Cicin-Sain et al. 2005). ჩვენმა კვლევამ (Gabashvili et al. 2021b) აღწერა მრავალი ფაგი, რომლებიც მონაწილეობს მორფოგენეზის, მასპინძლის სპეციფიკაციისა და რეპლიკაციის ცილების მაკოდირებელი გენების რეკომბინაციაში. მეტი სიღრმისეული კვლევებია საჭირო ფაგების მასპინძლების სრული სპექტრისა და ფაგების მიერ ბაქტერიების კოინფიცირების ხელშემწყობი გარემოს დასადგენად; ეს კვლევები საშუალებას მოგვცემდნენ განგვესაზღვრა ის ევოლუციური ცვლილებები როგორც ბაქტერიულ მიკრობიოტაში, ისე ფაგებში, რომლებიც თან შეიძლება ახლავდეს ფაგებს შორის და ფაგსა და პროფაგს შორის ურთიერთქმედებას, რისი ხდომილების რისკები შეიძლება გაიზარდოს ფაგების მასიურად გამოყენებისას როგორც მინიმუმ თერასპიული მიზნებისთვის, განსაკუთრებით კი, კუჭ-ნაწლავის ტრაქტის დაავადებების მკურნალობისთვის.

4. დასკვნა

შავი ზღვის აუზში, მწვანე კონცხსა და ბათუმის (ბულვარის) სარეკრეაციო წყლების მიკრობიოტა შეიცავს MFS-ის, MATE-ის, DMT-ის და RND-ის ოჯახების იფლაქს ტუმბოების ან) მაკოდირებელ გენებს, მათ შორის მულტირეზისტენტობის განმსაზღვრელი იფლაქს ტრანსპორტერების მაკოდირებელი გენებს. ზემოაღნიშნული ანტიმიკრობული რეზისტენტობის გენები უმეტესად ასახავენ რეზისტენტობის ბუნებრივ მექანიზმებს როგორც ადამიანის და/ან ცხოველის პათოგენებში (*Vibrio vulnificus, Vibrio mimicus, Vibrio fluvialis, Aeromonas hydrophila* და სხვ.) ისე არაპათოგენურ ბაქტერიებში, როგორებიცაა *Synechococcus, Citromicrobium, Rhodobacteraceae, Pseudoalteromonas, Altererythrobacter, Erythrobacter, Altererythrobacter, Marivivens, Xuhuaishuia* და *Loktanella*. ამასთანავე, ზოგიერთი ამ სახეობების/გვარის შტამში, ისინი ზოგჯერ შეიძლება გენეტიკური რეკომბინაციით ინდუცირებულ შეძენილ რეზისტენტობასაც ირეკლავდნენ. ბაქტერიოფაგებს გააჩნიათ გარკვეული როლი რეზისტენტობის, მათ შორის მულტირეზისტენტობის, განმსაზღვრელი იფლაქს ტრანსპორტერების (MFS, ABC, RND) მაკოდირებელი გენების, შეძენილი რეზისტენტობის განმაპირობებელი გენების (მაგ., blaCTX-M, mel, და tetM) ჩათვლით, გავრცელებაში ბაქტერიების ბუნებრივ პოპულაციებში. ფაქტერიოფაგებით ინდუცირებულ ზოგიერთი ამ ანტიმიკრობული რეზისტენტობის დეტერმინანტების გენეტიკურ რეკომბინაციას ადგილი შეიძლება ჰქონდეს როგორც შიდასახეობრივ და სახეობათაშორისო დონეებზე, ისე გვართაშორის დონეზეც. მიკრობულ პოპულაციებში, ამ გენების გენეტიკური რეკომბინაციის ტრაექტორიები ირეკლავს როგორც ბი-, ისე მულტილატერული გადაცემის გზებს. როგორც ერთნაირი, ისე სხვადასხვა სასიცოცხლო ციკლის მქონე ფაგებს შორის გენეტიკური რეკომბინაცია შესაძლებელია მასპინძლის უჯრედში მათ მიერ

კოინფიცირებისასრამაც თავის მხრივ შეიძლება ხელი შეუწყოს ზემოაღნიშნული გენეტიკური ლოკუსების ევოლუციურ დივერგენციას ამ ორგანიზმებში.

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