Temperate bacteriophages collected by outer membrane vesicles in *Komagataeibacter intermedius*

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Abstract

The acetic acid bacteria have mainly relevance for bacterial cellulose production and fermented bio-products manufacture. The purpose of this study was to identify temperate bacteriophages in a cellulose-producing bacterial strain *Komagataeibacter intermedius* IMBG180. Prophages from *K. intermedius* IMBG180 were induced with mitomycin C and nalidixic acid. Transmission electron microscopy analysis exhibited tailed bacteriophages belonging to *Myoviridae*. A PCR assay targeting the capsid gene of the myoviruses proved phylogenetic position of induced phages. Nalidixic acid was poor inducer of prophages, however, it induced the OMV-like particles release. Size of OMVs depended on an antibiotic applied for phage induction and varied in the range of 30–80 and 120–200 nm. Inside some of them, tails of phages have been visible. Under conditions, inducing prophages, OMVs acted as the collectors of formed phage particles, using outer membrane receptors for phage detection (in this case, outer membrane siderophore receptor), and fulfilled therefore "a cleaning," as well as defensive functions, preventing bacteriophage spread outside population. This is the first description of myoviruses affiliated to *K. intermedius*, as well as outer membrane vesicles interaction with phages within this host.

Abbreviations

BC - bacterial cellulose
BLAST - basic local alignment search tool
OMV - outer membrane vesicle
ORF - open reading frame
PCR - polymerase chain reaction

Introduction

The acetic acid bacteria like *Komagateibacter intermedius* (former *Gluconacetobacter*) have been mainly employed for BC production [1]. Due to unique structural and mechanical properties as compared with higher plant cellulose, BC is becoming a commodity material in various fields. Its biocompatibility, hydrophilicity, transparency, and non-toxicity make it an attractive candidate for biomedical and materials engineering fields [2]. Another area of

acetic acid bacteria application is a production of fermented foods. The tendency to vegetarian life-style implies consuming of non-dairy nutraceuticals and calls for designing of new safe health-keeping fermented foods [3]. Many fermented products, including beverages such as kombucha, where *K. intermedius* is one of microbial community-member, act as probiotics/synbiotics essential for people, which work in stressful conditions [4]. Commercial value of *K. intermedius* is also in bioconversion of waste materials to nanofibrillated cellulose [5]. Considering the industrial potency of *K. intermedius*, selection of robust strains for industry is recognized as a primary task.

Bacteriophages occur in high numbers in environmental ecosystems and are thus significant mediators of microbial survival and activities. Meanwhile, the infection of bacterial industrial cultures by bacteriophages, as well as the induction of prophages leads to serious problems, including a spoilage or complete loss of the desired bio-products. Temperate prophages can be induced under the impact of agents, which interrupt host genomic DNA replication, and generate complete or defective particles, e.g., tails. In spite of an increasing interest in *K. intermedius*, there is no information available about bacteriophages hosted by this bacterium. Genomic studies of the *Komagateibacter* genus representatives (*K. xylinus*, *K. hansenii*) have revealed that they may own prophages [6-8]. For example, putative tailed bacteriophage has been reported in the *K. xylinus* NBRC 3288 genome [7]. Microbial diversity of the kombucha (a tea fungus') ecotype accommodated in Ukraine has been examined, using both culture-dependent and culture-independent approaches [9]. Within this multi-microbial culture the cellulose-producing *K. intermedius* population was recognized as a species formed up to 5.0% of the community. The purpose of this study was to isolate cellulose-producing bacterium and to evaluate the presence of inducible bacteriophages in the isolated strain.

Materials and methods

The cellulose-producing bacterial culture has been isolated from kombucha beverage on HS medium [10] and identified by using 16S rDNA sequencing and analysis (sequence deposited in Genbank, NCBI, under accession number KF908876). The purpose of this study was to recognize conditions, which induce temperate bacteriophages in *K. intermedius* IMBG180, and to identify type of phages capable to infect this organism. Prophages from IMBG180 were induced in the log-phase growing culture in HS medium with mitomycin C (1.0 μg/ml) and nalidixic acid (20 μg/ml). After appropriate treatment, the culture was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and further ultra-centrifuged at 100,000 g for 1 h at 4°C (Beckman Instruments Inc., L8M, rotor 55.2 Ti). The resulting pellet was re-suspended in sterile 0.9% saline. Further on tailed phage-like particles were viewed in samples by transmission electron microscope JEM-1400 (Jeol Inc., Japan). Formvar coated copper grids were dipped into a sample and contrasted with 2.0% solution of uranylatcetate within 30 s.

Results and discussion

PCR assay targeting putative prophage in the genome of *K. intermedius* IMBG180 by primers designed to the gene coding for capsid protein of the myoviruses [11] generated a product with a homology to the gene for outer membrane siderophore receptor. Within this sequence, a small fragment possesses homology to a wide range of *Myoviridae* phage sequences deposited to GenBank (NCBI). BLASTN analysis of the sequences collected in the database of genomic islands—Pre_GI (Uni Pretoria, SU)—showed that similar short sequences existed in complete genomes of many bacterial species. Pre_GI houses genomic islands predicted by

the SeqWord Genomic Island Sniffer program [12], using oligonucleotide signatures of the genomic fragments. It should be noted, that genomic islands possess many putative genes including IS-elements and phage-derived modules such as integrases, transposases, etc. [13]. On the other hand, BLASTX showed that the PCR product generated by primers to the *Myoviridae* phage capsid gene can be translated in six ORFs resembling outer membrane siderophore receptor gene, mainly of acetic acid bacterial strains. It was reported elsewhere that bacteriophages were able to mimic the ferric siderophores during adsorption. In Gram-negative bacteria, high-affinity iron acquisition requires outer membrane-localized proteins,—porins—that bind iron chelates at the cell surface [14]. At the same time, ligand-gated porins show broad multifunctionality by also acting as receptors for bacteriophages, as well as toxins and antibiotics [15].

Efficiency of induction of prophages in the *K. intermedius* varied, depending on the used induction agents, e.g., after the nalidixic acid treatment of the liquid culture we observed defective phage-like particles, which had heads. Induction of bacterial culture by mitomycin C resulted in the expected release of bacteriophages with heads and contractile tails that could be assigned morphologically to the family *Myoviridae* (Fig. 1a and b). The study of properties of isolated bacteriophages was complicated due to their low titre in the samples. From our point of view, the primary reason of such inefficient prophage induction was the peculiarities of host bacteria, which form a tight aggregation within a 3D cellulose network during cultivation. These aggregations protect inner bacterial cells from the inducers influence. Another putative reason could lie in the impossibility of propagation and amplification of isolated bacteriophages due to the innate resistance, i.e., resistance of lysogenic cells to re-infection by the same or related temperate phages.

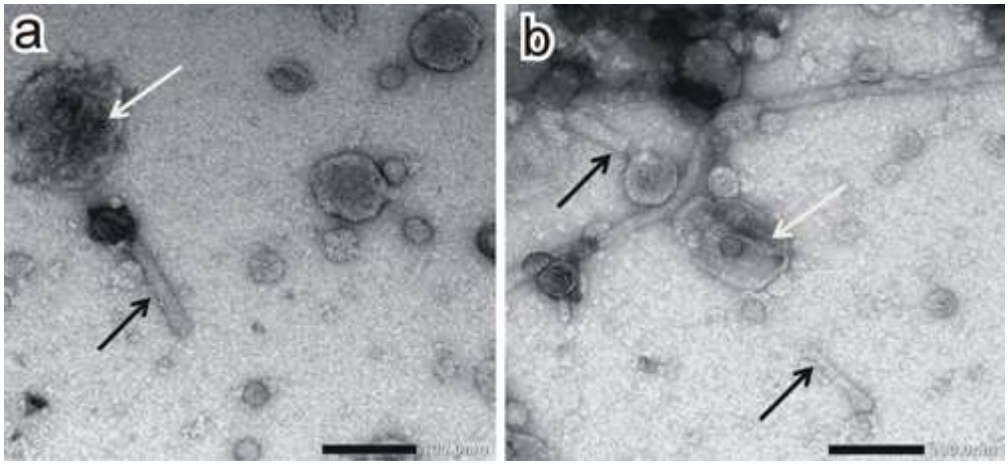

Figure 1. Electron micrograph illustrating morphology of temperate bacteriophage/s detected in *K. intermedius* IMBG180 after treatment with mitomycin C (a, b, black arrows) and phage tails location within outer membrane vesicles (a, b, white arrows). Samples were contaminated with co-precipitating bacterial appendages sheared from the live bacteria, e.g., bacterial cellulose threads (an intermittent arrow). Scale bars are 100 nm.

In the lysed cells preparations, OMVs have been revealed after ultracentrifugation (Fig. 2). OMV are membrane-derived particles surrounded by a (phospho) lipid bilayer that are released by bacterial cells. In microbial world, the release of vesicles has been demonstrated for both pathogenic and nonpathogenic organisms (reviewed in [16] living in diverse econiches, including biofilms [17]. In stressful conditions, e.g., under heat shock [18], upon infection with bacteriophage [19], cells release OMVs in increased numbers. With transmission electron micrographs we were able to resolve heterogeneity of vesicles in

lysates of the *K. intermedius* IMBG180 culture. Sub-populations of OMV in the range from 30 to 80 nm and from 100 to 200 nm have been observed (see Figs. 1a, b and 2 c, d). Vesicles induced with mitomycin C showed low size distribution and averaged near 156 nm in diameter as revealed by a dynamic light scattering (Zetasizer 3000HSA, Malvern Instruments). Different OMVs size populations were observed in a study by Fulsundar et al. [20], where size depended on the type of stressor. Most vesicles possessed single membrane, however, vesicles with double membranes were observed also (see Fig. 2c and d) as described earlier by [21]. Inside the vesicles released after treatment with mitomycin C, tail-like particles morphologically resembling phage tails were discovered (see Fig. 1a and b). Absorbtion of phage particles by OMVs could be one of the reason of their low titre in the cell lysates.

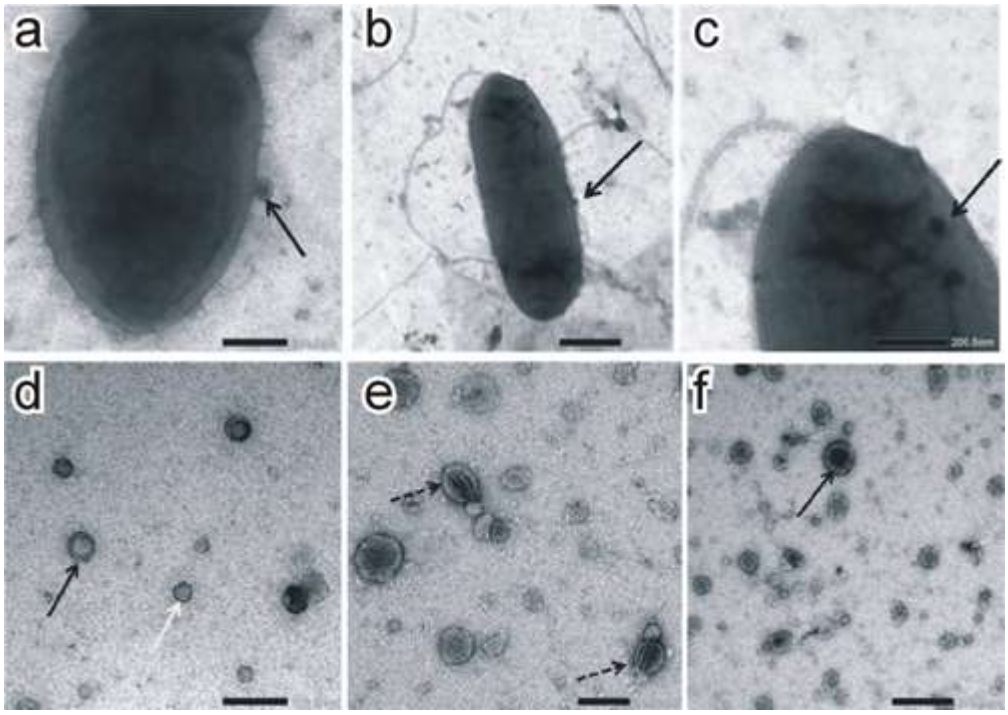

Figure 2. Outer membrane vesicles production by *Komagataeibacter intermedius* after a treatment with antibiotics. Negative-stained general view of bacterial cell in the process of electron-dense vesicles budding (a, b); macroscopic view a bacterial cell with a cellulose thread on the pole and an electronic-dense network, supporting budding vesicles (c); vesicles of different sizes were observed in preparations of lysed bacterial cells treated with mitomycin C (d–f). Vesicles had single membrane (a white arrow), two membranes (a black arrow), or a creast-like creature on the surface (e, an intermittent arrow). Scale bars are: a, c, f-200 nm; b-500 nm; d, e-100 nm.

Budding of the electron-dense OMVs was clearly visible on the surface of the bacterial cell pole producing a nanocellulose thread (Fig. 2b and c) and also in dividing cells (Fig. 2a). Remarkably, an electron-dense cytoskeletal network was observed below the emerging vesicles. Similar observation was made by Velimirov and Hagemann [22]. While many species are known to release vesicles, this phenomenon has not been studied before in the cellulose-producing bacteria. In this study, our results support the concept of Manning and Kuehn [23] and Biller et al. [24], which anticipated the vesicles to form complexes with phages and therefore to prevent phage attack by acting as decoys. OMVs derive from bacterial outer membrane and may confer nearly identical outer membrane receptors for bacteriophages. In such a way, vesicles probably interact with phages and restrain their

dissemination outside bacterial cell. In our case it was observed that OMVs acted as scavengers of formed phage particles.

Concluding remarks

Morphologically resemble to temperate *Myoviridae* phage particles, mainly defective ones, induced by the stressor agents in *K. intermedius* IMBG180 have been revealed. Low numbers were controlled by OMVs, which engulfed these particles and kept inside. Further research will be directed on the determination of the potential role of temperate bacteriophages in host-phage interactions under harsh conditions and biofilm formation. Isolation of highly purified OMVs will be indispensable to better understand the role of OMVs in preservation of bacterial host population.

Acknowledgments

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Conflict of interest

The manuscript does not contain human studies and experiments using animals.

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