Review

Bacterial Virus Forcing of Bacterial O-Antigen Shields: Lessons From Coliphages

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Posted Date: 14 November 2023

doi: 10.20944/preprints202311.0846.v1

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Bacterial Virus Forcing of Bacterial O-Antigen Shields: Lessons From Coliphages

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Abstract: Presence of lipopolysaccharides (LPSs) in the outer layer of outer membranes (OMs) is an almost universal molecular signature of Gram-negative bacteria. The O-antigen or O-polysaccharide (OPS) chains attach to millions of LPS molecule to form a continuous layer on the surface of most of Escherichia coli strains. OPS structure is one of the most variable features of bacteria, with about 200 E. coli O-serotypes currently described. In this review a analyze accumulating evidence suggesting that a vast majority of these OPS types provide robust shields that restrict the access of large molecules to the OM surface. Sophisticated mechanisms employed by bacteriophages to penetrate the OPS barrier are also considered. These are initiated with specific recognition of OPS molecules by phage receptor-binding proteins (RBP), or of other cell-surface molecules that are exposed above the OPS layer. Only after can virions gain access to secondary receptors found closer to the OM surface. The mechanisms of breaking through OPS in most if not all cases appear to rely on mechanical force generated by molecular motors of processive depolymerization or deacetylation of cell surface polysaccharides by enzymatically active RBPs, or by internal rearrangement of the virion.

Keywords: bacteriophage; host range; O antigen; E. coli; bacteriophage adsorption

1. OPSs Can Serve as Physical Barriers to Phage Adsorption

1.1. Exopolysaccharides and surface polysaccharides of enterobacteria

Bacterial cells often develop impressive surface-protecting structures such as capsules protecting individual cells, sheathes surrounding several cells or covering cell chains [1,2] or biofilms with large number of the cells embedded in common matrix [3]. These protective layers are generally described as exostructures, which can be mechanically removed without compromising cell viability or integrity. Most of these layers are of a polysaccharide nature, and often referred to as capsular polysaccharide (CPS) or exopolysaccharide (EPS), though some capsule types instead consist of proteins [4] and the biofilm matrix often include multiple types of polymers, including DNA or even filamentous phage particles (reviewed in [5]) in addition to EPS. The formation of such exostructures is optional and present only in some species and strains of bacteria. The role of capsules and biofilm matrix in interactions the interaction of bacteria with their viruses, bacteriophages, were extensively studied and recently reviewed elsewhere [6–8]. In Gram-negative bacteria, the actual outer surface of cells consists of an outer membrane (OM), which in most cases is covered by additional polysaccharide structure named O-antigen or O-polysaccharide (OPS). OPS is the outmost part of the lipopolysaccharide (LPS) molecules [9] (Figure 1) that make for the bulk of the lipid material of the OM outer leaflet (while the inner OM leaflet consists mostly of phospholipids). This OPS (Figure 1) is attached to what is known as core oligosaccharide (core-OS), which in turn is attached to lipid A, the structure of which is highly conserved within bacterial species [9,10].

Structurally, OPS is a linear polysaccharide built of repetitive oligosaccharide motifs (O-units). O-unit backbone is generally 1-6 sugar residues in length. In some O-serotypes, the O-unit may contain lateral sugar residues or acetyl groups [9]. However, these lateral branches are never (up to my knowledge) extended beyond single sugar residue.

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The synthesis of both core-OS and OPS is performed by the corresponding enzymatic pathways. The variability of core-OS synthesis genes is limited. For example, in *Escherichia coli* to only five types, described as K12, R1, R2, R3, R4 [11]. At the same time, OPS variability is much greater with about 180 structural types (referred to as O-serotypes) found in different *E. coli* strains [10].

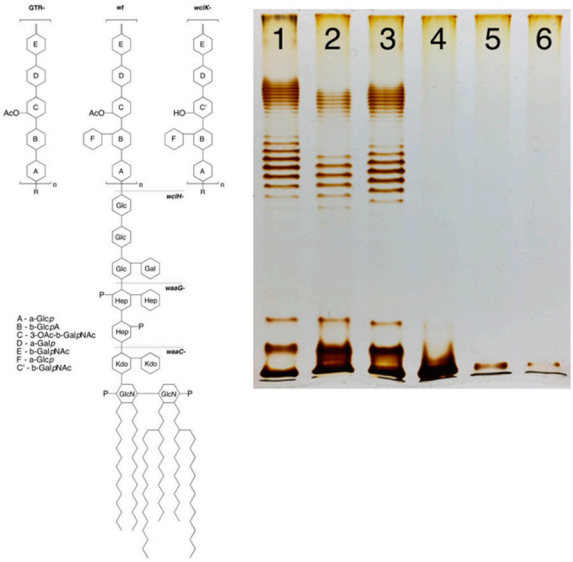

Figure 1. Structure of LPS of *E. coli* 4s strain (O22 type with additional glucosylation) and if its mutants, lacking glucosylation (GTR- mutant) or O-units O-acetylation (*wclK*-). The dashed lines indicate the LPS structure in rough mutants (as exemplified by *wclH*-) and deep-rough mutants lacking outer (*waaG* -) or most of inner core (*waaC*-). The left panel shows LPS gel profile of these strains: the wild type strain (lane 1), non-acetlated OPS *wclK*- mutant (lane 2), non-glucosylated GTR- mutant (lane 3), rough *wclH*- (lane 4) and two deep-rough mutants waaG and waaC (lanes 5 and 6). Modified from [14], Creative Commons Attribution 4.0 International License.

1.2. OPS synthesis

The mechanisms of OPS biosynthesis were extensively investigated and recently reviewed [9,10]. Most of the O antigens are synthetized via Wzx/Wzy pathway (Figure 2) In this pathway the O unit is assembled at the cytosol side of the cytoplasmic membrane (CM), being anchored in the CM by the adaptor lipid, Und-PP. The sugar residues comprising the O unit sequence are transferred to the adaptor sequentially by glucosyltransferases (GT) from the respective sugar nucleotides. Once the O unit is completed, Wzx flippase translocates the Und-PP-O-unit precursor onto the periplasmic side of CM where OPS is polymerized by Wzy polymerase. The Wzz protein controls the O-chain length. Finished OPS chains get transferred from Und-PP to vacant lipooligosacharide (lipid A + core-OS) molecule by WaaL ligase. Complete LPS is then transported to the OM by LOL transperiplasmic transportation system [12,13]. Interestingly, the number of O-units is not tightly controlled, yielding within a single cell LPS molecules with different OPS length. Interestingly, the distribution of LPS is not even and features 1-2 maximums, often in the ranges 12-17 and 25-35 O-units. This bimodal LPS distribution may be visualized by SDS-polyacrilamyde gel electrophoresis of LPS with silver staining [14] (Figure 1).

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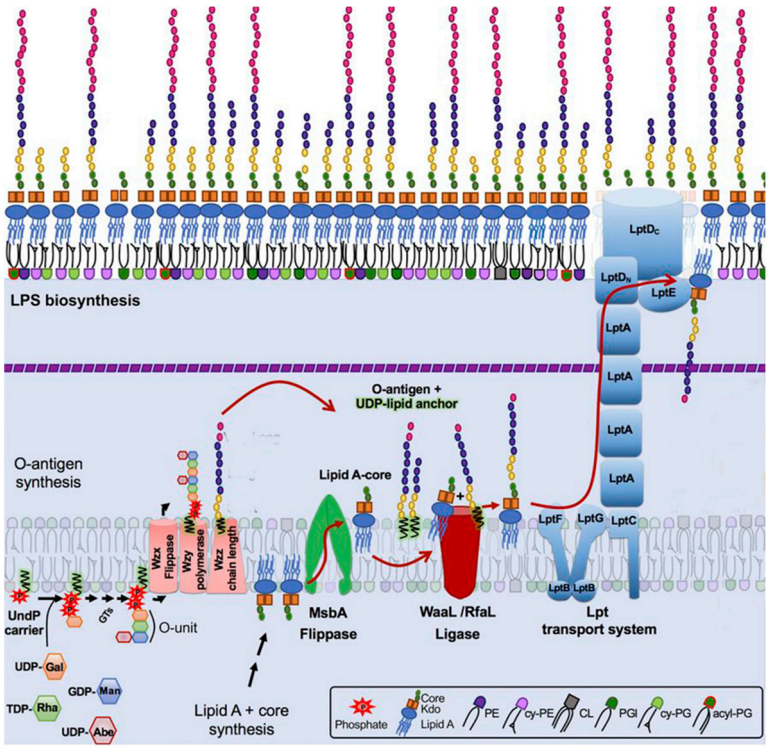

Figure 2. Model of lipopolysaccharide (LPS) synthesis with Wzy-dependent O polysaccharide (OPS) polymerization and LPS transportation to the outer membrane in enterobacteria. LPS molecules are synthesized from two precursors, lipid A-core molecules and OPS, generated by two independent pathways. Then LPS iis assembled by the ligation of O antigens to lipid A-core molecules. OPS synthesis begins at the cytosolic side of the inner membrane (IM) by a sequential transfer of monosaccharides residues from sugar-nucleotide donor molecules to the undecaprenyl phosphate carrier (Und-PP) lipid to form a Und-PP-linked O-unit. The Und-PP-O-units are then flipped to the outer IM leaflet, polymerized by the Wzy OPS polymerase under the control of Wzz protein regulating the OPS chain length. Lipid A-core biosynthesis begins in the cytoplasm and continues in parallel with OPS synthesis at the IM inner leaflet. Next, the O-antigen and lipid A-core structures are joined with WaaL also known as RfaL into one LPS superstructure. The Lpt complex spans the dual bilayers of the envelope and drives unidirectional LPS transport across the periplasm. Modified from [15], Creative Commons Attribution 4.0 International License.

A much smaller number of OPS types are synthetized by the ABC-transporter pathway. In this pathway, the linear OPS chain is assembled at the cytoplasmic side of CM and then Und-PP-OPS is transported to the periplasmic side of CM by cognate ABC transporter.

1.3. Other surface polysaccharides of E. coli

Several less abundant surface polysaccharides are found in order Enterobacteriales, which contains genus *Escherichia*. The enterobacterial common antigen (ECA) is a linear polysaccharide built of repetitive units containing three residues: N-acetyl-d-glucosamine-(α1–}-N-acetyl-d-mannosaminuronic acid(β1–}-4-acetamido-4,6-dideoxy-d-galactose [16]. The dominant surface-exposed form of ECA is ECA$_{PG}$ in which the ECA polysaccharide is linked by phospho-diester bond to the unidentified lipid anchoring it in the OM outer leaflet. This ECA form may comprise up to 0.2% of dry cell weight. In addition to ECA$_{PG}$, ECA$_{LPS}$ may present. In ECA$_{LPS}$ molecules the polysaccharide is linked to core-OS of the LPS instead of the O antigen. The abundance of ECA$_{LPS}$ is higher in rough strains, lacking the OPS biosynthesis [16]. The ECA biosynthesis is quite similar to the Wzy/Wzx dependent OPS synthesis with the repetitive units assembled at the inner CM side, ECA chain polymerized at the periplasmic CM side by WzyE polymerase under the control of the chain-length regulation WzzE protein [17] with subsequent transfer of of the ECA chain to the anchoring lipid and/or LPS molecules and translocation to the OM surface [16,18]. In addition to

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ECA_{PG} and ECA_{LPS}, the cyclic form of the ECA polysaccharide is present in the periplasmic space at least in some strains [16], however, cyclic ECA is not surface-exposed and supposedly has no influence on host recognition by bacteriophages.

Recently a new type of surface polysaccharide, NFR, was discovered in *E. coli.* This polysaccharide is secreted via the channel formed by the proteins NfrA and NfrB that earlier were known only as the phage N4 receptors [19]. Some of exopolysaccharides as above-mentioned capsular polysaccharides, bacterial cellulose [20,21] or poly-β-1,6-N-acetylglucosamine [22] may retain a link to the OM surface at the moment of their secretion or after it contributing to the phage-host interactions (see below).

2. OPS-mediated OM protection

2.1. OPS shields outer membrane surface

O antigen is highly immunogenic and extensive variability of the OPS in different strains of *E. coli* and other pathogenic enterobacteria has been interpreted as a strategy of avoidance of bacterial clearance by the immune system [23]. However, though the OPS represents an Achilles heel when facing the adaptive immune response, it effectively protects the immediate OM surface from interactions with a variety of the molecules, thereby improving the fitness of bacteria under different conditions. O-antigen, for example, was demonstrated to block the binding of antibodies targeting the structures located underneath of the OPS layer [24–26]. Interestingly, in *Salmonella* the ability of OPS to block IgG binding to OM proteins was found to depend on both protein size and the mean length of OPS chains [25]. Molecular modelling confirmed that the OPS chains in *Salmonella enterica* were too short to cover large trimers of OmpD protein while the monomeric OmpA could instead be covered by the OPS chains. This correlated well with the binding of the respective antibodies to the cell surface [25]. Noteworthy, in *S. etnerica* O4 and O9 strains used in this study the OPS chains are about 6-11 O-unit long while longer O-antigen of many E. coli strains may provide stronger protection even to large OM protein complexes. These data highlight the mechanical and non-specific protection provided by the O antigen that can be expected to be efficient against any bulky particles unless no specific mechanisms to penetrate the shield are present. In good agreement with this conclusion, O antigens provide good protection against bactericidal action of human or animal blood serum [27–29] and against the factors of innate immunity of insects [30] and plants [31]. The action of the enzymes such as lysozyme can be also inhibited by O antigens [32,33].

O antigen expression also reduces the phagocytosis of bacteria and their killing by monocytes and neutrophiles [34,35] or by amoeba [36]. The later effects are most probably mediated by blockage of the interaction of the phagocytic cells with conserved molecular signatures of bacteria because the O antigen layer is relatively thin (~20 nm) and does not mechanically prevent the engulfment of the bacterial cells. The protective features of O antigens make them important pathogenicity factors (reviewed in [10,17]).

3. Phenomenology of modulation of phage-host interactions by O antigen

Since the O antigen is able to prevent large molecules and particles from direct interaction with the OM, the structure should effectively protect the cell from bacteriophage infection. The fact that some bacteriophage and colicin receptors are efficiently blocked by O antigens of smooth strains of E. coli but exposed on their rough derivatives was demonstrated about 40 years ago [37]. However, largely neglected until now is the fact that the widely used laboratory strains of *E. coli*, such as *E. coli* B and *E. coli* K12, are rough mutants lacking OPS production. On the other hand, there is a vast literature, reporting bacteriophages using O antigen as receptors (reviewed in [38–40]).

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3.1. Strategies of the host cell recognition by bacteriophages and properties of phage resistant mutants.

Most of the tailed bacteriophages recognize at least two different receptors. The binding of the virus to the primary receptor does not trigger irreversible structural alterations of the virion. The recognition of the secondary receptor by contrast induces the genome release. In some myoviruses, such as phage T4 (see below), the receptor initially is recognized by the phage long tail fibers (LTF), which triggers the baseplate rearrangement and tail contraction.

Since the interaction of the phage receptor-binding proteins (RBP) with O antigen takes place somewhat apart of the OM surface, in most of the cases separated by the OPS. Different phages employ different host recognition strategies [38]: (I) Some phages are able to recognize the secondary receptors independently of the binding of the primary receptors. This strategy (I) is used by many siphoviruses, such as phage T5, as well as by other phage morphological groups. If the primary receptor of a phage is O antigen while the secondary receptor is an OM protein or LPS core-OS, this phage will be able to effectively infect a large variety of the rough host strains. The smooth OPS producing strains may instead be resistant or significantly less sensitive unless their OPS types are not recognized by phage RBPs. So, the phage host range may be significantly broader for rough derivatives compared to its infectivity towards parental strains.

Strategy II is employed mainly (but again not exclusively) by some podoviruses such as phage P22, in which recognition of the primary receptor is pre-requisite for the interaction with the secondary receptor. The viruses employing strategy II, and recognize the O antigen as the primary receptor, can only infect smooth strains. The rough derivatives by contrast are completely resistant to such phages and this is so even if their cognate secondary receptors are intact and present at the OM surface. The strategy used by most (again, not all) myoviruses is closer to the strategy I since the recognition of the first receptor triggers the tail contraction. Even if additional receptor is recognized after this event (like the short tail fiber (STF) receptors in T4-like phages; see below), the baseplate rearrangement and tail contraction are already irreversible and if genome delivery fails the virion will be inactivated.

Consequent to these differences, the O antigen production status of the host mutants selected for resistance to the phages employing different strategies will be expected to be different. The selective pressure from the infection if a phage uses strategy I will probably not select for altered OPS phenotypes because removal of the primary receptor does not provide the cells with protection from phage attack. For example, the T5-like bacteriophages DT57C and DT571/2 [41] recognize OPS of several types using their lateral tail fibers (LTF). These phages were found to have branched LTFs built of two proteins each harboring a receptor binding domain. So DT57C recognizes OPS of the types O22 and O81 of *E. coli* strains 4s and Hs3-104 while DT571/2 phage binds O87 and O81 types of the strains HS1/2 and HS3-104 [41]; each phage may be able to recognize additional OPS types that were not yet identified. Both phages recognize identical secondary receptor BtuB [41] and are able to grow on the rough derivatives of the aforementioned strains [28,41] as well as on many other rough strains of *E. coli* [28,41,42]. Consecutively, smooth host strains mutants selected by these phages under stringent conditions of plating onto phage agar have OPS production status indistinguishable from the parental strains (Figure 3). All these mutants were found to harbor mutations in the BtuB gene [41,43]. In theory also the secondary receptor may be somehow functionally linked to the OPS production or surface expression. For example, if the core OS serves as the secondary receptor, then the phage will select for rough mutants lacking outer core and the OPS despite the fact that the synthesis of the later may be not affected by the mutations (see Figure 1 right panel, lanes 5 and 6). However, up to my knowledge, such phage-host systems were not yet described.

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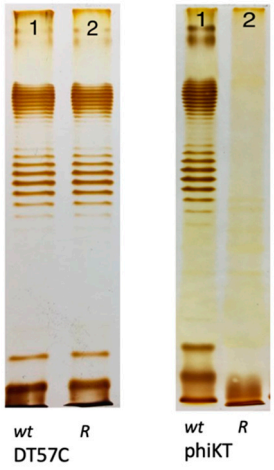

Figure 3. The LPS profiles of E. coli 4s mutants, selected for the resistance to a strategy I phage T5-like siphovirus DT57C and for a strategy II phage phiKT, a podovirus, distantly related to T7 (wt – wild type, R – resistant mutant) The images adapted from [14], Creative Commons Attribution 4.0 International License.

The O antigen-recognizing strategy II phages in contrast will select for rough phenotype. Even if the inactivation or modification of the secondary receptor may be inactivated or modified independently of the OPS (though the secondary receptor is seldom identified for such viruses), the synthesis of the later requires specific metabolic pathways that represent larger targets for spontaneous mutations compared to, for example, an OM protein gene. In accordance to this logic, the mutants resistant to several strategy II type such as phiKT, Alt63 or G7C [43,44]. Here again, some exception of the general rule may be observed. For example, G7C phage recognizes *E. coli 4s* OPS by means of enzymatically active tail spikes. But in contrast to the vast majority of bacteriophage tail spikes possessing polysaccharide depolymerizing activities (hydrolases or lyases) [39], G7C tail spike is a deacetylase [45]. Therefore, a fraction of the host mutants selected for the phage G7C resistance are true rough mutants with mutated genes of the OPS backbone synthesis, while another fraction is represented by acetylation-deficient (*wclK*-) mutants [44]. The polymerization of the OPS from non-acetylated O-units precursors is apparently less efficient and the *wclK*-showed reduced surface expression of OPS [43]. These mutants remained sensitive to some of the O antigen-dependent (strategy II) phages such as phiKT or Alt63. Later on, other phages harboring spikes with deacetylase activity towards OPS [46,47] and capsular polysaccharides of enterobacteria were discovered. These viruses may also select for two distinct phenotypes of the resistant mutants. Finally, some phages may use OPS as one of several alternative primary receptors on a particular host strain [48]so they may also select for rough resistant clones without being strategy II viruses.

The O antigen expression status of enterobacteria is easy to determine using LPS profiling by polyacrylamide gel electrophoresis. Using the silver staining procedure optimized for preferential staining of polysaccharides high quality LPS profiles (see example at Fig. } can be obtained without labor-intensive LPS purification [43]. This enables resistant clone screening as a simple procedure to reveal phage strategy of infection though, as exemplified above, the possibility of alternative explanations of observed phenotypes also should be considered. The ability of strategy II phages to select for rough mutants is likely to contribute to frequently reported decrease of the virulence of phage-resistant variants compared to the parental genotypes [49,50]. The rough mutants also gain the sensitivity to some strategy I phages that are not at all or poorly infective of the parental strain (see below).

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4. How strong is the O antigen barrier in Enterobacteria?

Until recently, the performance of the bacteriophages potentially able to infect rough strains (such as domesticated *E. coli* K12) was seldom investigated on O antigen producing strains. Bacteriophage T5 was shown to recognize the polymannose O antigen of *E. coli* F by its LTFs [51,52] to enhance the adsorption rate. Although, it was possible to obtain O antigen dependent T5 mutants that could not infect the rough strains [53], the wild type and even the *ltf* mutants were able to form plaques on both rough hosts and smooth *E. coli* F strain [51]. Therefore, the polymannose OPS did not effectively prevent the direct binding of the T5 straight tail fiber to the secondary receptor, FhuA. The same was found for the *E. coli* F5 strain, producing O28ab type of O antigen [54], which could be infected by T5 and T5-like phages and by an LTF-depleted mutant of one of these viruses. Though, in contrast to the system phage T5-*E. coli* F, the efficiency of plating (EOP) of the phages, lacking RBPs specific to the OPS of the *E. coli* F5 strain was decreased by 5-6 orders of magnitude. Interestingly, the mutant, lacking the LTF performed even slightly better compared to its parental phage, most probably because of steric constraints from bulky LTFs of the wild type which were non-functional on this specific host [54].

At the same time, the host range of the several phages of DT57C species [55] was shown to strictly depend on an ability to recognize specific OPS types [41,42]. While no plaques were detected even with high dosage of the phages applied to the lawns of heterological strains, the EOP on the lawns of rough derivatives of these strains was comparable to that on the laboratory C600 or on the strains, specifically recognizable by the LTFs of the tested bacteriophage. The same pattern was observed also for another T5-like virus Gostya9 [54,56] and another siphovirus 9g and several other phages [28,54]. Interestingly, the *E. coli* 4s strain that is sensitive to phage DT57C due to specific recognition of the O antigen by the phage LTFs becomes totally resistant to this virus upon lysogenization by an O-seroconverting temperate phage Hf4s [57] which causes additional OPS glycosylation in the lysogens. However, if rough variants of these lysogens are selected (for example, by a strategy II phage G7C), they return to sensitivity to phage DT57C despite the presence of Hf4s prophage.

The ability of OPS to restrict phage infection was recently confirmed by a systematic study by Maffei et al [58]. The authors isolated a set of 68 *E. coli* K12 phages well representing the of known coliphage diversity, several of standard model phages were also added to the study. Most of the phage types were completely or very significantly restricted on the *E. coli* K12 derivative in which the production of the O16 type O antigen was restored by precise deletion of IS element inserted into the *wbbL* gene in the conventional K12 strain(in total, 51 phage out of 74 tested in the study were severely restricted by O16 OPS). However, some phages were able to grow on the *wbbL+* strain effectively. These included all 15 of v5-like (Vequintarvirinae) isolates, phage N4, some T-even related phages and else. All but 11 of the phages examined were also blocked on the all three tested natural *E. coli* isolates producing the O antigen. Though in this assay other factors, such as host antiviral immunity systems, may be in part responsible for the host range limitation, it is noteworthy that 5 out of 16 Tevenvirinae isolates could infect al least one natural E. coli strain.

The influence of O-antigen on prophage acquisition is poorly investigated. Plaque formation on bacterial lawns requires relatively high efficacy of the infection. On the other hand, a much smaller rate of effective phage adsorption may be sufficient for formation of detectable number of the lysogens. Using the Stx-converting bacteriophage phi24B marked by an antibiotic resistance gene, James et al [59] demonstrated that the lysogenization range by this virus, determined as a set of host strains in which antibiotic resistant lysogens were formed upon phage contact with liquid cultures of bacteria, was much broader than phage lytic activity spectrum. This phenomenon may be of primary significance since lysogenization by an Stx-converting phage makes *E. coli* strain shigatoxigenic (STEC). This process is believed to be a major pathway of formation of new STEC lineages, many of which are very dangerous foodborne pathogens [60,61]. The O antigen production status of the lysogens formed under the conditions similar to those described by James et al. ([59]) was recently investigated [28]. It was found that almost all of the lysogenic clones formed out of several environmental *E. coli* strains belonging to different O serotypes turned rough. At the same time, the

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presence of phi24B prophage did not abrogate the O antigen production at least in *E. coli* 4s. These findings suggest that the lysogens were mostly formed out of naturally occurring rough mutants present in the cultures exposed to phage phi24B. Interestingly, mutants selected by lysogenization by phi24B:cat having lost the OPS shield became sensitive to a number of virulent phages that were not able to infect the parental strains [28].

The data cited above highlight the fact that the protection of cells by OPS from bacteriophage attack is potentially a non-specific effect based of physical screening of receptors that are found closer to the OM surface, such as OM proteins or LPS core-OS, from interaction with bacteriophage RBPs. Most of the environmental isolates of *E. coli* producing O antigens consequently are completely or almost completely (EOP $<10^{-5}$) protected from the phages lacking specific mechanisms to penetrate the OPS barrier. The efficacy of the O-antigen-mediated non-specific antiphage protection is poorly investigated in bacterial species, other than *E. coli*, even within Enterobacteriales order, however, given such non-specific mechanisms of protection, significant levels of expression of long enough OPS chains, especially with complex O-unit structure, should be equally effective in OM shielding with these other bacteria as it has been observed in *E. coli*.

5. Mechanisms used by bacteriophages to penetrate O antigen barrier.

The most known mechanism of OPS penetration by bacteriophage is based on virion associated enzymatically active tail spikes, affecting the OPS. The literature on phages recognizing O antigens and other surface polysaccharides of bacteria using enzymatically active tailspikes is extensive and recently reviewed elsewhere [62–67]. Most of these enzymes are polysaccharide depolymerases of hydrolyses and lyases classes. Many phages, for example the *S. enterica* temperate phage P22 [68] or related coliphage Hf4s [57], contain only one type of enzymatically active RBP (most often tailspikes), but some phages express multiple tailspikes that enable infection of hosts with different types of the CPS, EPS or OPS. These RBPs frequently form branched structures including up to 14 different depolymerase RBPs as in *Klebsiella* giant myovirus φKp24 [69]. Although the tail spike proteins with depolymerase activity were found in all three main tailed phages morphological types (myoviruses, podoviruses and siphoviruses), the way in which these proteins are involved in multi-step cell recognition and infection process may be different in different virion organization variants.

5.1. Podoviruses: cut or pull?

The most popular model of the enzyme-associated O antigen and/or capsule penetration is based on the idea of partial removal of the barrier by enzyme(s), enabling the access of RBP(s) to the secondary receptor at the OM surface. The "drilling" of a hole through the thick capsule of *E. coli* K1 cells by K1-specific podoviruses was first (up to my knowledge, as observed using electron microscopy by Lindberg as early as in 1977 (cited in [70]). This concept is supported by the fact tha purified recombinant, phage-derived tailspike proteins are able to remove the capsules or O antigens from the cell surface (see [66] for review). The enzymatic decapsulation of *Klebsiella, Acinetobacter* and some other bacteria by phage-derived proteins was even proved to be effective in treatment of experimental infections [65–67,71,72] because the removal of the capsule makes the cells more vulnerable for the immune system of the macroorganism.

Nevertheless, some known facts challenge this simple barrier-breaking theory. Many bacteriophages bearing the enzymatically active tailspikes are strategy II viruses, strictly dependent on the polysaccharide receptor for the infection. The naked OM surface fully accessible for RBPs thus cannot be recognized by these viruses. The acapsular mutants of bacteria such as *Klebsiella* frequently acquire complete resistance to the K-specific bacteriophages [71]. The nature of the conformational signal the presumably therefore must be generated upon interaction with the polysaccharide primary receptor to enable phage interaction with the OM surface (secondary receptor(s) remains unclear in most of the cases. In the P22-like *Shigella* phage Sf6 the O antigen of the Y *Shigella* serotype [73] or, less efficiently, of the $2a_2$ serotype [74] serves as a primary receptor while OmpA and OmpC are alternative secondary receptors [73]. However, at least in vitro, LPS alone or OmpA alone cannot trigger the genome DNA release form the virions but the two components added simultaneously

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cause efficient genome ejection [73]. Interestingly, the tailspikes of the phage Sf6 mutant selected for improved EOP on 2a2 host featured lower affinity to the 2a2 OPS [74]. So, although the transient interaction with the O antigen is essential for subsequent recognition of the secondary receptor but too strong binding to OPS may hinder subsequent infaction events.

The *Salmonella* phage P22 also uses O antigen as a primary receptor. In this virus, purified LPS aggregates alone were sufficient to trigger genome release, however, this was not the case with either soluble OPS or lipid A [75]. The cryo-EM study of phage P22 virion interaction with the cell surface revealed that initially the phage binds the cell obliquely through interaction of two neighboring tail spikes with the O antigen, which then brings that central tail needle into the contact with the OM surface. The later interaction induces virion re-orientation into the orthogonal position with respect to the OM and subsequent DNA release [76]. The current model for P22 infection implies a role of mechanical force created by processive ezymatic cleavage of OPS by the tail spikes that push the central tail needle into the surface of the OM (or into micelles formed by isolated LPS *in vitro*) that is sufficient to trigger virion rearrangements and then DNA ejection [75,76].

Possible function of enzymatically active tail spikes as molecular motors may be further inferred from the data on the host recognition by N4-like viruses. The coliphage G7C tail spike gp63.1 protein was shown to act as a deacetylase (esterase) instead of displaying the OPS depolymerization activity that is more common in bacteriophage RBPs. Though the OPS backbone remains intact upon gp63.1 treatment, the enzymatic activity is essential for infection of the *E. coli* 4s [45]. The indirect data on the recombinant protein binding to the cell surface indicate that this deacetylation reaction is processive [45]. The bacterial rough mutants as well as mutants lacking O-unit O acetylation are completely resistant to phage G7C. Interestingly, in phage Alt63, which is almost identical to phage G7C, the esterase moiety of gp63.1 tailspike is replaced by a classical depolymerase (lyase) domain [77]. Phage Alt63 remains a strategy II virus, but in contrast to phage G7C, phage Alt63 depends only on O antigen expression while not also on OPS acetylation. So (remarkably), the mechanisms of infection of N4-like phages G7C and Alt63 are fully compatible with both OPS degrading activity or deacetylase activity of their tail spikes without any noticeable modifications of other virion proteins.

Bacteriophage N4 has long been believed to be a strategy I virus, recognizing the NfrA protein receptor [78] using its so-called tail shaft protein, gp65 [79]. However, recently the previously unknown primary polysaccharide receptor, NGR (from ***N****4* ***g****lycan* ***r****eceptor*), was discovered [49,50]. The long-known NfrAB (from ***N*** *four* ***r****eceptor*) proteins were shown to be responsible for NGR secretion. Recognition and, probably, enzymatic degradation of this NGR polysaccharide are essential for N4 infection. Interestingly, phage N4 was shown to infect multiple host strains producing different OPS types [58]. Such a feature would be unexpected for a strategy I podovirus that depends on an OM protein for infection. The NGR receptor is present in small amounts on the cell surface since only about 45 copies of NfrA protein (equal to 5-6 complexes) are present per cell [80]. At the same time, this polysaccharide is likely to be conserved among many *E. coli* strains similarly to enterobacterial common antigen (ECA). However, interaction of one or few NGR chains should not automaticallyremove the screening of the OM surface by O antigen. Thus, mechanical force generated due to NGR interaction (degradation or deacetylation) by some N4 RBP (most probably, gp66 tail spikes) may be responsible for driving the phage tail through the OPS layer.

Summarizing the data, presented above, we speculate that the podoviruses, by possessing the enzymatically active tail spikes, penetrate the O-antigen layer largely not due to physical removal of the OPS material from the virion's way but instead by piercing this barrier using force that is generated by processive depolymerization or deacetylation of glycan receptors (Figure 4A), such as OPS itself or other polysaccharides like NGR or ECA, though which are less abundant on the cell surface are also better conserved. The later can be therefore termed "key polysaccharides" since they help some phages to penetrate through multiple different OPS types. Although this pulling mechanism seems to be seen more often in podoviruses, other phage morphotypes, especially myoviruses (below), can adopt it or combine it with other mechanisms.

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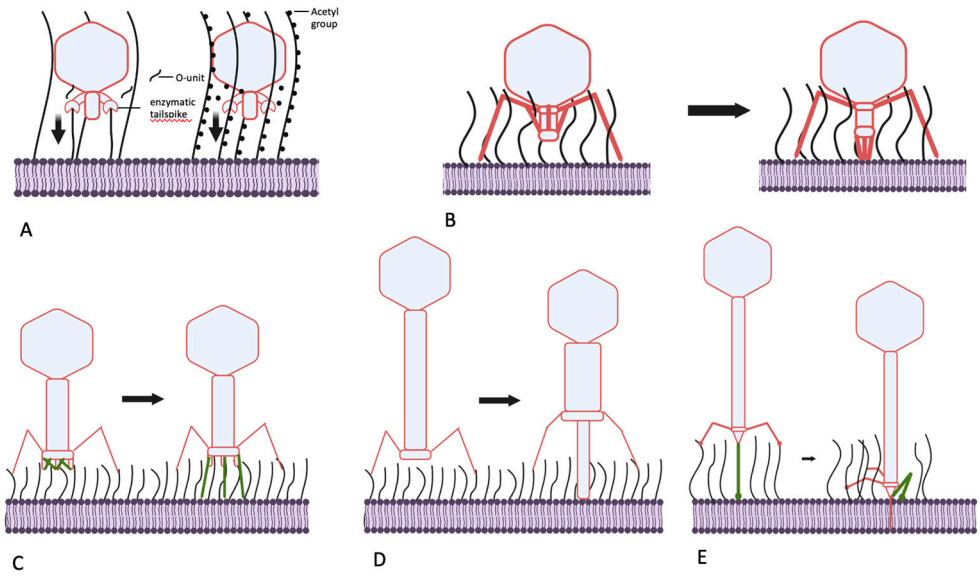

Figure 4. Mechanisms of generation of mechanical force, helping bacteriophages to penetrate O antigen shield of *E. coli.* A. Molecular motors, driven by processive polysaccharide depolymerization or deacetylation (for example, phage N4, P22-like viruses, G7C-like viruses). Vertical arrows indicate mechanical force generated; B. Movement of the tail components of some podoviruses upon primary receptor recognition (as in phage SU10); C. Deployment of the STFs of T-even related phages. D. Piercing of the OPS layer by the tube of contractile tail of long-tailed myoviruses (phage P1); E. Pulling the tail tube through the OPS by folding of the CTF in siphoviruses (for example, phage T5).

5.2. Can podoviruses push?

Interestingly, not all podoviruses that are able to infect O antigen-producing strains possess enzymatically active tailspikes. For example, some T7-related (family Autographiviridae) bacteriophages instead recognize OPS as their primary receptor and behave as strategy II viruses. Phages phiKT and PGT2 [43,81], for example, recognize O antigen using tail fibers carrying receptor binding domains (RBD) that are similar to the RBDs of the lateral tail fibers of T5-related phage DT57C. These RBDs do not have (up to our current knowledge) any enzymatic activity. In phage T7, initial cell recognition by its tail fibers is followed by interaction of the tail nozzle protein with a so-far unidentified secondary receptor and subsequent exit of the internal capsid proteins (IPs) gp15, gp15 and gp16 that extend to form a transperiplasmic conduit for the phage genomic DNA [82–84]. The proposed mechanism of this tail extension implies immersion of gp14 subunits into the OM lipid bilayer [84], which presumably requires tight contact of the distal tail tube (nozzle) protein with the OM surface. It is currently not clear how this mechanism can be adapted for "firing" from ca. 20 nm distance if the internal proteins release is activated when the nozzle remains at the surface of the OPS layer. It seems possible that IP remodeling in phiKT-like phages may be somewhat different from that of its T7 analog, forming slightly longer tail extension tube, the deployment of which creates mechanical force sufficient for OPS penetration.

Another *E. coli* podovirus SU10, a representative of *Kuravirus* genus, also possesses tail fibers depleted of any enzymatic activity. The distal domain of the SU10 long tail fibers is structurally similar to phage T4 LTF needle, recognizing the primary receptor. The receptors of SU10 were not yet identified, however the cryo-EM study revealed in detail the interactions of this virus with the host cell surface [85]. The long tail fibers are not involved in any signal transduction but the LTFs binding allows the central tail needle to interact with the OM surface, which apparently triggers structural rearrangement of the tail. The tail needle apparently interacts with the host cell surface and separates from the nozzle protein triggering the tail conformation change. Each of six nozzle subunits contain four tandemly repeated so-called nozzle domains forming a chain, folded under the nozzle. These domain chains get extended towards the cell surface. The extended nozzle domain chains

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interact with the short fibers that rotate downwards. Eventually the complex of six short tail fibers (STF) and six chains of the nozzle domains form an extended tail tube (see the Supplementary video in [85]). The rearrangement process involving rigid STF rotation, nozzle domain extension and formation of additional interactions between these domains and STFs may also provide the mechanical power necessary to push OPS molecules away from the deploying tail (Figure 4B). Noteworthy, the overall tail extension is of about 25 nm [85], which corresponds to the expected OPS layer thickness. It is not clear if the STFs of bacteriophage SU10 recognize any specific secondary receptor or instead serve exclusively as structural elements for nozzle extension. After the extended nozzle comes in touch with OM, the internal proteins are ejected to form (presumably) a transperiplasmic channel that conducts the SU10 genome into the cell.

5.3. Myoviruses – clutch and push

The contractile tail machine of myoviruses made for a paradigm of mechanical action generated by a virus particle. It is generally believed that the main function of this tail contraction is to drive the internal tail tube through the cell wall and periplasm in order to provide a conduit to phage DNA transport [86]. However, the structure of some bacteriophage contractile tails suggests that a part of their forcing action is dedicated to breaking through the external structures including throught the O antigen layer.

Bacteriophage T4 and numerous related viruses recognize their primary receptors using their LTFs. In T4, the tip (needle domain) of the LTF forms the receptor recognition center [87], while in most of other T-even related phages a monomeric RBP gp38 is present at the end of the LTF [88,89]. This LTF, otherwise reversible binding leads to a triggering of a baseplate rearrangement from hexagonal to a six-pointed star shape with simultaneous deployment of six STFs that interact with secondary receptors to tightly fix the virion at the cell surface . The initial events of the baseplate rearrangement (precisely, the widening of the central gp6-gp7 ring) already trigger the tail sheath contraction [86,90]. The tail sheath then acts as an extended spring that contracts, not only driving the tail tube down but also powering energic STF deployment. The phage T4 STFs are relatively long structures and the baseplate of the infecting phage is held at about 35 nm above the OM surface [91]. This distance appears to play no role in the infection of rough *E. coli* strains as *E. coli* B – the typical laboratory host for phage T4 cultivation. This gap nonetheless is wide enough to avoid the need to bring the large and also flat baseplate through the OPS layer to the immediate surface of the OM.

Many T-even related phages are able to infect *E. coli* strains producing O antigens of high protective ability [58,92]. Recently it has been shown that some RB49-like phages can recognize certain types of the OPS as a primary receptor and even behave on some host strains such as *E. coli* F17 as strategy II viruses though being able to infect rough derivatives of other strains [93]. These findings indirectly indicate that RB49-like viruses activate their baseplate rearrangement before any RBP contact with the immediate OM surface. Interestingly, phage Brandy49, having the broadest host range within the investigated series of RB49-like viruses, uses some different not yet identified mechanism that seems to allow its LTFs to penetrate through different types of OPS to contact some receptor at the OM surface [93]. Regardless the nature of the receptor recognized by T-even related phages at the *surface* of the O antigen producing host, the mechanical force of their STF deployment appears to be the mechanism allowing their RBPs to penetrate the OPS barrier for tight phage attachment (Figure 4C). Currently we have no consistent explanation why the LTFs Brandy49 is able to squeeze through the OPS while the LTFs of most of other T-even like phages are not. Although the host specificity of T-even like viruses is determined primarily by the monomeric RBP gp38 (except for T4 and some other viruses; see above), the internal regions of gp37, the trimeric protein of the distal LTF half, may be also involved in host range determination. Zhang and co-workers [94,95] reported that in several closely related T-even-like phages recombination with plasmids containing divergent fragments of gene 37 could swap the host specificity to that of the donor phage of the g37 sequence. Interestingly, the host ranges were tested using wild *E. coli* isolates apparently producing protective O antigens. The involvement of gp37 into the host range determination appears counter-intuitive since the genomes of the phages used in this work contain gp38 RBP homologs. Among

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possible explanations (besides of potential experimental errors) I speculate that gp37 may display several motifs able to interact with polysaccharides, though possibly with poor affinity and/or specificity. The sequential (starting from the baseplate-distal gp37 end) binding of such elements to OPS chains may be sufficient to submerge thin LTF into the O-antigen layer in a ratchet-like manner to bring gp38 at its end into the contact with some receptor at the OM surface.

Another myovirus, phage P1, is known to have a remarkably broad host spectrum [96,97]. Phage P1 has a tropism-switching mechanism with two types of the fiber genes, S and S'. At least with S' fiber, the phage recognizes some of the O antigens in *E. coli* and *Shigella* as its only receptor [97], while S fiber binds LPS core as its receptor [98]. Phage P1 has no STFs but its tail is much longer than phage T4's (ca. 245 nm vs. 114 nm) [99]. Upon interaction of its LTFs with the receptor, the tail contraction is initiated and the tail sheath shrinks from 210 to 94 nm [99,100] (Figure 3D). The phage is retained at the cell surface by extended LTFs, with the gap between the baseplate and the OM of about 100 nm [100] which is enough for the tail tube to span the OPS layer using the force of the contracting tail (Figure 4D). Apparently, the interaction of the S' LTF with the OPS receptor is strong enough to hold against the reaction force when the tail tube pierces the O antigen and other layers of the cell wall.

5.4. Siphoviruses – grab and drag.

Though the molecular architecture of long non-contractile tails is well studied (reviewed in [101,102]), the function of a siphovirus virion during host cell attack is comparatively poorly understood, including also relative to current knowledge of podovirus infection mechanisms. Up to my knowledge, the only phage for which early infection events have been well described is the bacteriophage T5. Phage T5 as well as the majority of siphoviruses infecting enterobacteria, recognizes its secondary receptor using a central tail fiber (CTF) that is equipped with a monomeric RBP, pb5 (in other siphoviruses the RBD may be located directly on the trimeric CTF protein). In case of phage T5, the secondary receptor is the OM protein FhuA, though other T5-like viruses may infect through recognition of BtuB or FepA proteins [41,103]. Phage T5 is also equipped with LTFs but these fibers are attached rigidly [104] and does not generate any conformation signal upon interaction with its OPS primary receptor (so T5 is a strategy I phage). As it was described above, many T5-like viruses rely on OPS recognition by the LTFs to infect the O antigen producing host strains. At the same time, the link between LTF binding and subsequent penetration of the phage tail tip to the OM surface remains obscure.

The sequence of structural transformations of the T5 tail during infection of rough *E. coli* cells has been recently deciphered at atomic resolution [104–106]. After the binding of the pb5 protein, located at the C end of the CTF protein pb4 trimer, to the FhuA receptor, CTF conformation changes are induced [105] leading to a sharp bending of the CTF in such a way the distal CTF part (called a spike by Linares et al.) interacts by its lateral loops with the fibronectin-like domains of the proximal part of pb4 and of distal part of the baseplate hub protein pb3 (to which pb4 is attached) [104]. This interaction results in CTF distal and proximal ends are brought together (Figure 4E) tagging the tip complex to the OM surface just next to the FhuA receptor molecule. After this event, the tape measure protein comes out of the tail to penetrate into the OM bilayer and periplasm [104]. The bending of the CTF is likely to create sufficient mechanical force to move the OPS molecules out of the way of the tail tip complex.

Summarizing all the data, we may suggest the following model of infection of an O antigen-protected host cell by T5-like phage (for example, such as *E. coli* 4s infection by DT57C virus; [41]): The LTF binds to OPS reversibly fixing the virion in an orientation that is suitable for infection. The CTF, being a narrow needle-like molecule, finds a gap in the O antigen shield taking a benefit from the Browinan motion of both the LPS molecules and the phage. After recognition of the OM protein receptor, the bending of the CTF generates mechanical force sufficient to tug the tail tip through the O antigen layer. Interestingly, in some T5-like phages, infecting capsular strains of *Klebsiella*, the CTF protein is about twice longer than in T5, an adaptation compatible with the proposed model.
Many other siphovirues of enterobacteria have similar functional relationships between the CTF and

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LTFs (reviewed in [38,102]), however the mode of action of their CTF proteins could be significantly different given the lack of sequence similarity to the T5 pb4 protein.

6. Conclusions and perspectives

The existing data indicate that O antigens of most of *E. coli* strains found in the natural habitats make robust shields protecting the OM surface from direct interaction with large molecules or molecular complexes such as bacteriophages, antibodies, complement proteins or enzymes. In the case of phages, the protection afforded by many O antigen types is sufficient to provide the cell complete resistance to the virus unless the latter is equipped with specific molecular mechanisms to penetrate this OPS barrier. Noteworthy, some phage strains, such as LTF-deficient mutants of T5-like phages, may serve as useful probe tools to test the protective function of O antigen in particular bacterial strains and/or under particular conditions. From an ecological perspective, the ability of highly variable O antigen to determine and modulate the infectivity of bacteriophages may be of major factor, influencing the diversity and dynamics of both phages and bacteria. For example, the community of commensal *E. coli* in the microbiomes of some of domestic horses include up to 1000 genetically distinct *E. coli* strains simultaneously present in a sample and having different profiles of the sensitivity to the co-occurring coliphages [107,108]. In most of the phage-host systems isolated from this source, the O antigens appears to play key roles in the phage sensitivity or resistance of the bacteria (see [43] and refs therein). The role of O antigen in controlling the spread of prophages has been recently demonstrated but the data is too scarce to estimate the real-world significance of this phenomenon [28].

Bacteriophages employ a variety of mechanisms to penetrate the OPS shield and infect the O antigen producing host strains. It has to be highlighted that despite relatively small OPS layer thickness (of about 20-30 nm), the data suggest that the protective effect of this structure has a non-specific nature and is due to mechanical shielding of the OM surface. Nevertheless, the recognition of OPS itself or any other receptor exposed outside of the OPS layer (for example, flagella, pili and conserved polysacharids such as ECA, NGR or even bacterial cellulose [109,110]) does not explain automatically how the virus penetrates through the OPS barrier. Analysis of the data of functional and structural analysis of infection mechanisms of different coliphages has, however, allowed us to speculate that the most if not all the mechanisms phages use to move through the OPS layer rely on generation of a mechanical force (Figure 4). The specific mechanisms used to generate this force, however, may be different, ranging from molecular motors powered by processive depolymerization or deacetylation of polysaccharides by enzymatically active viral RBPs to clutching to some available receptors and use of the energy of the structural rearrangement of the phage particle to get through the OPS layer.

It is possible that more variants of the force generation mechanism are still to be discovered in coliphages and in viruses of other Gram-negative bacteria. At the first glance this model contradicts to the widely accepted concept of enzymatic breaking down of bacterial surface polysaccharide layers virion associated enzymes, though the significance of removal of polysaccharide material from a patch of the cell surface may be of greater importance for penetration of thick capsules of extracellular matrix. In case of the O antigen, the force generation appears to be a more common mechanism. This mechanism employs an intrinsic weakness of the OPS barrier that is not only relatively thin but also built of fluidly moving molecules. Making an analogy to macroscopic objects, the O antigen is closer to the layer of hair of the skin rather to clothes made of a tissue. Nevertheless, successful penetration of a phage through the OPS shield should be never considered to be a trivial event. The phenomenon of a wide-spectrum phage infecting multiple different host O-serotypes should always be explained by identification of the mechanism allowing this virus to deal with a variety of structurally different but uniformly efficient barrages. Most probably, additional, elegant solutions developed by the evolution of bacterial predators will be discovered soon.

Finally, in some phage-host pairs the OPS barrier provide bacteria with lower protection yielding the cells partially resistant to the virus attack (see, for example, [52,54]) making the trajectories of the resistance development in a population of OPS-producing bacteria exposed to

phage may differ significantly from what can be observed in model systems with rough laboratory strains such as *E. coli* K-12 or B. The penetration of the OPS barrier may depend on synthesis of the "key polysaccharides" such as ECA or NGR (see Section 3) which, in turn, may be modulated by the cell physiological state and/or intercellular communications. Thus, the population-level adaptation strategies based on collective reactions of bacteria to phages (recently reviewed in [111,112]) may be also significantly impacted by the phage-OPS interplay in particular natural or experimental systems. Though, the data on the OPS mediated OM screening in other than *E. coli* bacterial species is scarce, the non-specific nature of this phenomenon allows to speculate that such anti-phage protection may be common for many different bacteria with Gram-negative cell wall type, being a major factor of bacteriophage ecology in the Biosphere.

Acknowledgments: The author is grateful to Dr. Eugene Kulikov and to Mrs. Maria Letarova from Winogradsky Institute of Microbiology RC Biotechnology RAS, Moscow, Russia for their help in the manuscript preparation and critical reading of the paper. I'm also grateful to Dr. Steve Abedon from Ohio State University, Mansfield, USA for valuable comments and linguistic editing of the manuscript.

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