Applied and Environmental Microbiology, Nov. 2010, p. 7310–7313 Vol. 76, No. 21
0099-2240/10/$12.00 doi:10.1128/AEM.02721-09

Bacteriophage PhiX174's Ecological Niche and the Flexibility of Its *Escherichia coli* Lipopolysaccharide Receptor▽

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Received 9 November 2009/Accepted 31 August 2010

To determine bacteriophage PhiX174's ecological niche, 783 *Escherichia coli* isolates were screened for susceptibility. Sensitive strains are diverse regarding their phylogenies and core lipopolysaccharides (LPS), but all have rough phenotypes. Further analysis of *E. coli* K-12 LPS mutants revealed that PhiX174 can use a wide diversity of LPS structures to initiate its infectious process.

PhiX174 belongs to the *Microviridae* family of bacteriophages (12). It is a small, icosahedral, nontailed virus with a circular single-stranded DNA. From its isolation in 1935 up to now, PhiX174 has been used in many landmark experiments because of its small genome size (5,386 nucleotides [nt]) and nonpathogenic status. Furthermore, since PhiX174 is a coliphage, it can be used as an indicator of viral or fecal contamination in aquatic environments (International Organization for Standardization, ISO 10705-2) (5).

In 1974, Suzuki et al. found that while phage adsorption is restricted to bacteria which possess a specific receptor, the replication of PhiX174 DNA can be supported by different *Escherichia coli* strains and distantly related bacteria, such as *Pseudomonas aeruginosa* (28). These findings imply that the limiting step for PhiX174 infection is entry and not replication or lysis. The commonly used PhiX174 host is the laboratory-derived strain *E. coli* C, which has a specific rough lipopolysaccharide (LPS) recognized as the receptor (13).

The LPS is a major component of the outer membrane of Gram-negative enterobacteria, which is involved in interactions with both biotic and abiotic factors in the environment. It is composed of a lipid A anchored in the membrane and an oligosaccharide core and can have a polysaccharide (O antigen) bound to this core. The inner part of the core LPS is highly conserved within the Gram-negative bacteria (1), whereas the outer-core biochemical structure of the LPS is more diverse. In the *E. coli* species, five outer-core types have been described: R1, R2, R3, R4, and K-12 (1). *E. coli* C exhibits an R1 core type. The study of its recently published sequence reveals that its core LPS is fully functional but that the O antigen is affected by an IS insertion in the *rfb* locus that generates its rough phenotype (GenBank accession number CP000946). Interestingly, a similar IS insertion is found in K-12 (4), creating a rough phenotype, but the bacterium is still resistant to PhiX174, which suggests that the exposed R1 core might be critical for PhiX174 infection. Among the *E. coli* Reference Collection (ECOR), which is representative of the genetic diversity of the entire *E. coli* species (1, 20), up to 70% of isolates are of the R1 type (1). However, only 3% (8/291) of *E. coli* strains isolated from sewage, stools, drinking water, or the laboratory have been found to be sensitive to PhiX174 (19).

To better define the molecular determinants affecting the ecological niche of the model virus PhiX174, we did the following: (i) screened a large collection of natural *E. coli* isolates for PhiX174 susceptibility, (ii) characterized the identified sensitive strains based on their phylogenetic group, serotype, and LPS core type, and (iii) studied the susceptibility to PhiX174 of LPS mutants of *E. coli* K-12. Our analysis revealed that PhiX174 sensitivity is a phenotypic convergence with diverse molecular origins.

Restricted niche for PhiX174 among natural *E. coli* isolates. A collection of 783 *E. coli* strains, isolated from human or animal stools, was screened for PhiX174 susceptibility: (i) 72 ECOR strains, encompassing commensal and pathogenic strains and strains from human and animal hosts and found in different countries (20); (ii) 372 human commensal strains from healthy volunteers in France (186), Colombia (28), Benin (46), Croatia (57), and Mali (55) (9, 10); and (iii) 339 strains isolated from French wild or domestic animals (11). Five-microliter droplets of overnight cultures from each isolate were added onto soft agar (LC medium supplemented with agar and calcium [25]), containing 10^7 PFU/ml PhiX174 (DSMZ4497), and incubated at 33°C. The bacteria were categorized as sensitive to PhiX174 when their droplets did not form a circular and opaque colony. The resistant strain *E. coli* K-12 and the sensitive strain *E. coli* C (DSMZ13127) were used as negative and positive controls respectively. While droplets of *E. coli* K-12 form circular and opaque colonies, droplets of *E. coli* C do not generate any sign of bacterial growth. The suspected sensitive strains were then grown overnight and plated on a layer of soft agar on which 10 μl droplets of PhiX174 stock solution was added (19). The isolates sustaining the phage growth, defined as formation of clear lysis plaques where phage droplets had been added, were considered susceptible.

Only six *E. coli* strains, or 0.8%, were found to be sensitive to PhiX174 (Table 1). This result is consistent with findings of previous studies (19). The sensitive strains found are from all over the world (France, Mali, and Croatia); however, it appears that they are all commensal strains from human origin (Fisher's exact test, $P = 0.04$). This observation is quite interesting, since human *E. coli* strains normally can be found in

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▽ Published ahead of print on 10 September 2010.

TABLE 1. Characterization of *E. coli* strains sensitive to PhiX174

Strain	Country	Host	*E. coli* group/ subgroup[a]	O type[b]	LPS core type(s)[c]
E. coli C (DSMZ13127)	France	Sewage	A_0	Autoagglutinating	R1
M1402	Mali	Human	B1	Autoagglutinating	R3
C4741	Croatia	Human	A_0	Autoagglutinating	R1
VDG388	France	Human	A_1	Autoagglutinating	R2
VDG401	France	Human	A_1	Autoagglutinating	R1 and R2
ROAR340	France	Human	$B2_3$	Autoagglutinating	R1
ROAR375	France	Human	A_1	Autoagglutinating	R2

[a] *E. coli* phylogenetic group and subgroups were determined as described previously (6, 10).
[b] O types were determined by O antiserum autoagglutination.
[c] LPS core types were determined by PCR amplification as described in reference 1.

other animals (30), apart from one subclone, recently discovered, which appears to be strictly human (7, 11). Yet the fact that all PhiX174-sensitive *E. coli* bacteria are from human sample suggests some different selective pressures between human and animal hosts that will have to be further investigated.

Diversity of the natural *E. coli* isolates sensitive to PhiX174. To further investigate the requirement for sensitivity to PhiX174, we then looked for genotypic or phenotypic traits that would explain their common sensitivity. O types of the sensitive strains were obtained with O-antiserum agglutination (Statens Serum Institute, Copenhagen, Denmark), and all appeared to autoagglutinate (Table 1), confirming that the PhiX174-sensitive strains are rough, with no O-antigen bound to their core LPS.

The phylogenetic groups and subgroups were assigned to the sensitive *E. coli* strains using the triplex PCR phylotyping method (6, 10). They belong to the A_1, A_0, B1, and $B2_3$ groups/subgroups (Table 1). The reference PhiX174-sensitive *E. coli* C strain belongs to the A_0 subgroup. This implies that sensitive strains are not monophyletic and that the O rough phenotype appeared independently several times. This further suggests that the O rough characteristic is a requirement for PhiX174 susceptibility. Moreover, this result supports the idea that entry is the limiting step in PhiX174 propagation, since very different *E. coli* bacteria can sustain the phage growth as soon as they are O rough. It also implies that sensitivity to PhiX174 occurs through this phenotypic convergence. Given that O rough strains have the core of their LPS exposed, we looked at whether the sensitive strains share a core LPS.

Diversity of the core LPS structure of natural *E. coli* isolates sensitive to PhiX174. Many enzymes participate in the synthesis of the LPS. The genes encoding the biosynthesis of the core LPS are mainly in the *rfa* (or *waa*) locus, organized into three operons, *gmhD*, *waaQ*, and *waaA* (29), with three more genes, *rfaE*, *lpcA*, and *gmbB*, distributed further on the genome. Five outer core LPS types of *E. coli* (R1, R2, R3, R4, and K-12) that correspond to different compositions and sequences of the *waaQ* operon have been described. A PCR method with primers from the *rfa* locus was used to differentiate the five LPS core types (1). The natural *E. coli* isolates sensitive to PhiX174 were found to have an R1-, R2-, or even R3 LPS-core-type LPS (Table 1). For the VDG401 strain, both the R1 and R2 PCRs were positive. This could correspond to an undescribed LPS core type.

Once more, those sensitive strains are remarkable for their diversity. Given that they have different *rfa* operons, different mutations could explain their common rough phenotype and thus their sensitivity to PhiX174. Yet since the inner-core LPS is highly conserved, it remains possible that all these strains expose a similarly truncated core LPS and that, despite their genetic diversity, they all share the same LPS phenotype. We therefore studied the impact of the knockouts of different genes involved in the core LPS synthesis of the model bacterium *E. coli* K-12 on its susceptibility to PhiX174.

Susceptibility to PhiX174 of *E. coli* K-12 core LPS mutants. *E. coli* K-12 exhibits a K-12 LPS core type, has a rough phenotype, and is resistant to PhiX174. The susceptibility to PhiX174 of the single gene knockout *rfaB*, *rfaC*, *rfaD*, *rfaE*, *rfaF*, *rfaG*, *rfaH*, *rfaI*, *rfaJ*, *rfaL*, *rfaP*, *rfaQ*, *rfaS*, *rfaT*, *rfaY*, *rfaZ*, *gmhB*, and *lpcA* mutants was tested using the KEIO collection (2). They were all resistant to lysis by PhiX174, except for the *rfaB*, *rfaC*, *rfaD*, *rfaE*, *gmhB* and *lpcA* mutants. According to a previous report, the deletions of *rfaC*, *rfaE*, and *lpcA* confer a "deep rough" phenotype to the bacteria, preventing the hanging of the first heptose of the inner core LPS (3). Similarly, the enzyme GmhB participates in the biosynthesis of the first heptose. Nevertheless, its activity could be partially complemented by that of another enzyme (17). The deletion of *rfaD* generates a heterogeneous LPS, either heptoseless or with an incorrect form of the heptose (23). The *rfaB* mutation modifies the outer core.

It is interesting to note that thanks to these *E. coli* K-12-sensitive mutants, the bacterium-bacteriophage interactions between *E. coli* and PhiX174 can now be studied in great depth with the enormous amount of knowledge accumulated on *E. coli* K-12.

Flexibility of the LPS of strains sensitive to PhiX174. Complementation of the sensitive *rfaB*, *rfaC*, *rfaD*, *rfaE*, *gmhB*, and *lpcA E. coli* K-12 knockout mutants with plasmids expressing the inactivated genes (the respective ASKA plasmids [16], induced by isopropyl-β-thiogalactoside [final concentration of 0.1 μM]) restored the resistance phenotype. However, when they were electroporated and induced in the *E. coli* isolates we had found to be sensitive to PhiX174, none of the isolates became resistant. This implies that their susceptibility can't be explained by a single deleterious mutation in one of these genes. Furthermore, it suggests that the more diverse part of the core LPS is exposed in each of these isolates and nevertheless supports PhiX174 adsorption. Thus, the susceptibility

to PhiX174 can be sustained by a large range of different molecular defects affecting the LPS, either in *E. coli* K-12 or in *E. coli* natural isolates.

This flexibility in the recognition by PhiX174 has already been described. Different mutations on sensitive O rough R1-type LPS strains (derived from *E. coli* C) had different impacts in its recognition by PhiX174. For instance, the deletion of the terminal galactose turns the bacterium resistant (13), whereas the deletion of the terminal glucose (*rfaV*) doesn't, but the latter deletion decreases its sensitivity (13, 22). Similarly, the deletion of the third heptose of the inner core of O rough R1 LPS (*rfaQ*) doesn't affect sensitivity to PhiX174 (21). Thus, the outer core of O rough R1 LPS can tolerate a few mutations without turning the bacterium resistant. Besides, *Salmonella enterica* serovar Typhimurium can be sensitive to PhiX174 despite its non-R1 core LPS, which bears different hexoses and branching (15). From all these observations, it has been suggested that the PhiX174 recognition and adsorption process is dependent on the global conformation of the LPS rather than on its precise composition (13). The recognition of potential hosts by PhiX174 hence has some flexibility. Here we show that this flexibility is even larger, since we show that R2 or R3 core LPS type and "deep-rough" *E. coli* K-12 mutants can also be sensitive to PhiX174 and thus the core LPS can be either complete, modified, or even totally truncated.

Evolutionary implications. Different susceptible strains can be recognized by PhiX174: *E. coli* C, the reference strain, but also, as we have shown here, O rough *E. coli* strains exposing diverse core LPS and even "deep-rough" *E. coli K-12* mutants. Hence, PhiX174 can adsorb to a wide range of molecular structures. The fact that even deep-rough strains can be infected opens the interesting perspective that strains from diverse Gram-negative bacterial species which share a similar defect could be infected *in vivo*. Hence, the ecological niche of PhiX174 may not be as restricted as previously thought.

In *E. coli*, no phylogenetic subgroups have been found having an O rough or "deep-rough" phenotype as a group characteristic, which suggests that the sensitive clones are short-lived on an evolutionary time scale. They may emerge *de novo* many times, as our data suggest, due to mutations and some short-term evolutionary selective pressure such as the ones imposed by the immune system or phage predation, but their LPS impairment is surely counterselected in the long term. The balance of benefit and cost is quite clear for the deep-rough mutants. While deep-rough mutants provide resistance to some phages, they are characterized *in vitro* by a reduced growth rate and a hypersensitivity to detergents and hydrophobic antibiotics (26). Moreover, they are affected in their motility (14) and their ability to conjugate (24, 27) and they have an attenuated virulence phenotype (18).

Whether large or not, the ecological niche of PhiX174 appears to be based on the emergence of mutants with various LPS phenotypes and different genetic backgrounds that may even extend to different species. As such, PhiX174 has presumably evolved to be a generalist since specializing too much to any of these different structures may restrict its host range and long-term chance of survival. Experimental evolution supports such a view since it has consistently shown that most adaptation to a host occurs in the capsid genes (12) and may result quite rapidly in a reduced host range (8).

We thank the National BioResource Project (NIG, Japan) for the KEIO collection and for the ASKA plasmids used in this study.

A.M. was supported by a fellowship from the French Research Ministry. E.D. was partially supported by the Fondation pour la Recherche Médicale. O.T. was supported by the Agence Nationale de la Recherche (ANR-05-JCJC-0136-0).

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