Arch Virol (2008) 153:1271–1280
DOI 10.1007/s00705-008-0111-0

ORIGINAL ARTICLE

# Analysis of some phenotypic traits of feces-borne temperate lambdoid bacteriophages from different immunity groups: a high incidence of *cor*+, FhuA-dependent phages

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Received: 19 December 2007 / Accepted: 2 April 2008 / Published online: 31 May 2008


**Abstract** A group of previously isolated heterogeneous mEp lambdoid phages (43) from 19 different immunity groups for phage infection was further characterized to gain insight into some phenotypic traits and to assess their relationship with phage λ. Interestingly, the FhuA host receptor was required by the majority of mEp phages (37 out of 43; ~85%). The *cor* gene, which has been reported to be involved in FhuA-dependent exclusion of lambdoid phages, was also found in most of the FhuA-dependent phages. Accordingly, no *cor* amplification by PCR was obtained among the six FhuA-independent mEp lambdoid phages. In contrast, it was found that around 25% of the population (10 out of 43 phages) required the specific and essential λ N antitermination function, and the λ site-specific DNA recombination function was observed only in two members (4.6%). Thus, a larger proportion of phages require the FhuA receptor for infection, and this is frequently correlated with the *cor* gene.

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## Introduction

Lambda is among the most studied bacteriophages at all levels [1]. Lambda and the related lambdoid bacteriophages share a common genomic organization, in which genes are arranged in ordered clusters involved in recombination, gene expression regulation, phage exclusion, DNA replication, cell lysis, and the head and tail structure. Indeed, this conserved gene organization has facilitated recombination among lambdoid phages, leading to a large number of hybrid phages in their natural environment [10, 11, 21, 33, 36, 57, 59]. Lambdoid bacteriophages also have distinctive functional properties that set them apart from other bacteriophage families. After infection, λ can follow either the lytic or the lysogenic pathway. In the lysogenic pathway, the λ chromosome is integrated into that of *E. coli* via specific attachment sites. To date, all of the lambdoid phages that have been described use antitermination as a positive regulation mechanism. In λ phage, *E. coli* RNA polymerase, the λ N gene product (N), host Nus factors (NusA, B, E and G), and the mRNA *nut* site form the transcription elongation complex, which allows the expression of genes, bypassing several *t*R and *t*L terminators [22, 58].

Unlike λ, which requires the LamB receptor for infection, some lambdoid phages (N15, HK022, and $\phi$80) and also unrelated phages such as T1 and T5 have been reported to infect cells using the FhuA receptor [9, 29, 49, 55, 56]. The *E. coli* FhuA protein is also the receptor for $Fe^{3+}$-ferrichrome, colicin M, microcin 25, albomycin and rifamycin SGP4832 antibiotics [8]. With the exception of T5 phage, FhuA receptor activity depends on the TonB–ExbB–ExbD protein complex [8]. These proteins integrate the TonB energy transduction system from the inner to the outer membrane [42]. The exclusion of FhuA-dependent

N15 and $\phi$80 phages by either N15 or $\phi$80 lysogenic cells has been indistinctly linked to phage protein Cor [34, 40, 43, 45]. Thus, HK022 DNA sequence analysis reveals the presence of a "*cor*-like" gene [36]. It has been proposed that Cor may interact directly with FhuA or indirectly through the TonB protein in the Cor-mediated exclusion phenotype of $\phi$80 and N15 phages [55]. FhuA has also been identified as the receptor for mEp167 phage. This is part of a collection of mEp lambdoid phages isolated from fresh stools of the same region and classified into 19 different immunity groups by phage-lysogen cross tests [39]. An exclusion phenomenon by mEp167 lysogens against other members of the mEp lambdoid family was also observed. A *cor* orthologue from one of the lambdoid phages (mEp167) accounted for the exclusion of FhuA-dependent lambdoid phages [54]. Cells overexpressing Cor (Cor + cells) excluded both TonB-dependent and TonB-independent phages, suggesting that the TonB system is not involved. Furthermore, the exclusion is exerted at the phage DNA entry level since phage adsorption, production of phage particles after phage DNA transfection and the spontaneous induction of lysogens in Cor + cells were not affected. In addition to the phage FhuA receptor function, a transport activity (ferrichrome uptake) was also inhibited by Cor, suggesting a direct blockage of FhuA [54].

Lambdoid phages have been isolated from different regions and from different sources including fresh feces or sewage and *E. coli* strain collections [15, 32 and references therein, 33, 41, 50]. However, there are relatively few reports on isolation and characterization of lambdoid and non-lambdoid phage families, even though several reports indicate a high frequency of different virus-like particles ($10^7$ pfu/ml) in lakes or bay sediments, not to mention sewage and disposal water [4, 44, 61]. We previously isolated and characterized a group of lambdoid phages by UV light induction, anti-$\lambda$ antibody recognition, DNA hybridization tests, etc. [39]. To analyze their relatedness with $\lambda$ phage, this heterogeneous group of pages was further analyzed for lambda-specific functions like N antitermination, site-specific integrative recombination, the receptor required for *E. coli* infection and the presence of the *cor* gene. Interestingly, most of the phages required FhuA receptor for infection. In addition, *cor* moron [36] was always associated with FhuA-dependent phages. In contrast, it was found that 25% of the population required the $\lambda$ N antitermination function, and the $\lambda$ integrative-specific recombination function was only present in two members out of 43. Thus, although some heterogeneity was observed in the lambdoid features of the analyzed phages, a conserved feature (FhuA receptor specificity) and its frequent association with *cor* was common among phages from nineteen different infection immunity groups.

## Materials and methods

### Bacteria, bacteriophages and media

Genotypes and relevant markers for bacteria, bacteriophage and plasmids are shown in Table 1. The mEp lambdoid bacteriophages used in this work were those reported by Kameyama et al. [39]. Bacteriophages were usually expanded in *E. coli* strain W3110, and strain C600 was used for testing FhuA specificity. MCR106 is derivative of MC4100 with the Δ(*lam*B)106 mutation, and MH760 is a derivative of MC4100 containing the *omp*R472 mutation, which confers an $OmpF^+$, $OmpC^-$ phenotype [52]. N6171 cells transformed with pGH006 plasmid were employed for the $\lambda$ N equivalent protein complementation assay (for details, see below). Strain LE289 was used for the red-plaque assay on tetrazolium medium complemented with galactose [17]. Plaques were observed after incubation for 36 h at 37°C. Luria-Bertani (LB), tetrazolium (TTC), tryptone broth (TB) or semi-solid media, and TMG dilution media were prepared as described previously by Silhavy et al. [52]. Antibiotic was added at 100 μg/ml ampicillin, 50 μg/ml X-gal, and 5 g/l galactose when required.

### pGH006 plasmid construction

For pGH006 plasmid construction, $\lambda$ *int*'-'*lac*Z' $cI_{857}$ $cII_{68}$ phage [25] was digested with *Hin*dIII, and the ~12.5 kb DNA fragment spanning a region from the *o*L *p*L promoter to *int*'-'*lac*Z including *sib* was purified and ligated in pBR322 *Hin*dIII. The new plasmid, pKG-3, of ~16.8 kb, was digested with *Sph*I and *Sma*I, and the ~7.3 kb fragment containing *int*'-'*lac*Z, was cloned in the *Hpa*I and *Sph*I sites of plasmid pKC-30 [51]. From the resulting plasmid (pGH004), the 839 bp *sib* region was eliminated by partial digestion with *Bam*HI, yielding pGH006. This construct contains *o*L *p*L sites, a $\lambda$N leader region including the *nut* site (boxA and boxB) to which $\lambda$N and Nus host factors bind to modify RNA polymerase, the RNase III processing region, the AUG of N until the *Hpa*I site (~1/3 of the N gene was ligated to the *Sma*I site of the *exo* gene). The *int*'-'*lac*Z fusion is located downstream of $tL_3$ which is found immediately downstream of the *Sma*I site (Fig. 4).

### Phage exclusion

Phage propagation was performed according to Kameyama et al. [38]. C600 (*fhu*$A^-$) cells were resuspended in TB top agar and plated out in TB solid medium on a petri dish. Phage dilutions ($10^{-2}$, $10^{-4}$, $10^{-6}$ and $10^{-8}$) prepared from ~$10^{-10}$ PFU/ml stocks were dropped onto the lawn. When the drops dried, plates were incubated overnight at 37°C.

**Table 1** Strains, bacteriophages and plasmids

| Strains, bacteriophages and plasmids | Genotypes or relevant markers | Sources or references |
|---|---|---|
| Bacteria | | |
| W3110 | $F^{-}\lambda^{-}rph^{-}$ | [3, 35] |
| C600 | *leuB6 thi-1 lacY1 supE44 thr-1 rfbD1 fhuA21* | [2] |
| MC4100 | $F^{-}$ *araD139 Δ(argF-lac)U169 rpsL150* ($str^{r}$) *relA1 flbB5301 deoC1 ptsF25 rbsR* | [12] |
| MCR106 | MC4100 Δ(*lam*B)106 | [16] |
| MH760 | MC4100 *omp*R472 (*omp*$F^{+}$, *omp*$C^{-}$ phenotype) | [27] |
| LE289 | *sup*$F^{-}$ *gal*$T^{-}$ (*pg* 1-*att*BOB́-*bio*) Δ | [17] |
| N6171 | N4903 *lac*Z21 ($\lambda$ $cI_{857}$ $N_{am7am53}$ ΔBAMH1) | [28] |
| TAP159 | W3110 *nus*B5 | [39] |
| TAP176 | W3110 *nus*E71 *zhb*511::Tn10 | [39] |
| TAP180 | W3110 *nus*A1 | [39] |
| TAP189 | W3110 *nus*D026 | [39] |
| W3110(HK022) | Lysogen for HK022 | This work |
| Bacteriophages | | |
| $\lambda$ | $imm_{\lambda}$ | CSH Collection |
| $\lambda$ *int*'-'*lac*Z | *int*'-'*lac*Z $cI_{857}$ $cII_{68}$ | [25] |
| λBLK20 | *p*L *N*'-'*lacZ bla attP* $imm_{21}$ *nin5* | [38] |
| $\phi$80 | $imm_{\phi 80}$ | [46] |
| HK022 | $imm_{HK022}$ | [15] |
| mEp phages | | [39] |
| Plasmids | | |
| pUCJA | *cat fhuA*$^{+}$ | [54] |
| pBR322 | *bla tet* | [5] |
| pKC-30 | *bla p*L *nut*L N | [51] |
| pKG-3 | *bla p*L until *int*'-'*lac*Z *sib* | This work |
| pGH004 | *bla p*L *nut*L N′HpaI/SmaI *t*L3 *p*I *int*'-'*lac*Z *sib* | This work |
| pGH006 | *bla p*L *nut*L N′HpaI/SmaI *t*L3 *p*I *int*'-'*lac*Z | This work |
| pCWB27 | *bla mal*K5′ Δ*160-lam*B (*lam*$B^{+}$) | [7] |
| pRAM1006 | *bla omp*$C^{+}$ | [47] |

Plaque development was observed and quantified to assess the exclusion phenotype.

Red-plaque assay for $\lambda$ site-specific recombination

A red-plaque assay was done according to Enquist and Weisberg [17]. They engineered a galK gene with the *att*B and *att*P integration region inserted. When $\lambda$ integrase (Int) was present, intragene recombination occurred, deleting the extra insert, reconstituting the galK gene and restoring galactokinase activity.

Genetic assay to determine $\lambda$ N specific antitermination function

The genetic system for determining the $\lambda$ N-specific antitermination function consisted basically of three elements: (a) lysogenic *lac*$Z^{-}$ bacteria with a cryptic prophage containing $\lambda N_{am7am53}$ and $cI_{857}$ (thermosensitive repressor), (b) a plasmid bearing the *o*L *p*L promoter region, a *nut* site, the transcription terminator $tL_3$ and *p*I promoter upstream the reporter *int*'-'*lac*Z fusion gene, and (c) an infective phage that produces blue plaques when producing a $\lambda$-like N able to complement the antitermination function at the permissive temperature in the presence of X-gal.

N6171 cells harboring pGH006 plasmid were grown overnight in LB broth supplemented with ampicillin (100 µg/ml); 3 ml soft agar and 200 µl X-gal (20 mg/ml) and were layered onto LB-agar plates containing the same concentration of ampicillin and X-gal. Once the agar solidified, different phage dilutions were dropped onto the lawn and incubated overnight at 31°C, 37°C or 42°C. The development of blue plaques was observed the next day.

PCR conditions for *cor* gene amplification and DNA sequencing

Phage DNA used for *cor* gene amplification was obtained as follows: 1 ml lysate ($\sim 10^{10}$ PFU/ml) was spun at 10,000×g for 10 min. Then 0.5 ml of cleared lysate was transferred into a new tube and was heated 10 min in boiling water (this process releases DNA by denaturing the protein and breaking the phage capsid). Design of primers containing the conserved *cor* regions (FwCor: 5′-CGG GAT CCG GAT ACT TTC AAA ATG AAA AGG CTA ATT AT-3′, the underlined ATG is the first initiation codon, and the RvCor′: 5′-CGG GAT CCA CCC AGC GCC AGT TGA AGG GAT A-3′) was done by aligning HK022, N15, $\phi$80 and mEp167 *cor* DNA sequences using Oligo program 4.1. PCR conditions were: 1 µl of DNA, 0.25 µl of (2.5 U/100 µl) Taq polymerase (Roche), 2.5 µl of 5 µM primer solution, 5 µl of 10× PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM of each dNTPs, and sterilized water to 50 µl total volume. This mixture was spun down at 8,000×g before thermocycling (Perkin-Elmer model 2400), 40 cycles of 94°C/30 s; 65°C/30 s and 70°C/30 s. The amplified DNA was analyzed in 1.2% agarose gels stained with ethidium bromide. For DNA sequencing, thermocycling conditions were: 30 cycles of 94°C/20 s; 53°C/20 s and 60°C/20 s using the Applied Biosystems BigDye® XTerminator™ Purification kit and the FWCor primer.

## Results

FhuA receptor is required for infection by the majority of lambdoid phages from diverse immunity groups

Forty-three lambdoid phages isolated from human faeces were classified into nineteen different infection immunity groups according to the phage-lysogen cross test [39]. The heterogeneity of these phages was indicated by UV light induction, anti-$\lambda$ antibody recognition and DNA hibridization tests [39] and by their enzyme restriction pattern (data not shown). In concordance with the heterogeneity of the phage infection immunities, as much as 50% (9 out of 19) of the immunity groups were represented by only one member (Fig. 1). This contrasts with the high frequency of phages requiring FhuA receptor for infection (around 85% of the members, 37 out of 43). They were unable to infect *E. coli* strain C600 (*fhu*A$^-$) unless the cells were transformed with a FhuA-expressing plasmid [54] (Table 2). Regarding the receptor specificity of the remaining phages, only 3 (mEp409, mEp414 and mEp553) were unable to infect MCR106 (Δ*lam*B) cells (Table 2) even though they were transformed with pCWB27 (*lam*B$^+$), indicating that

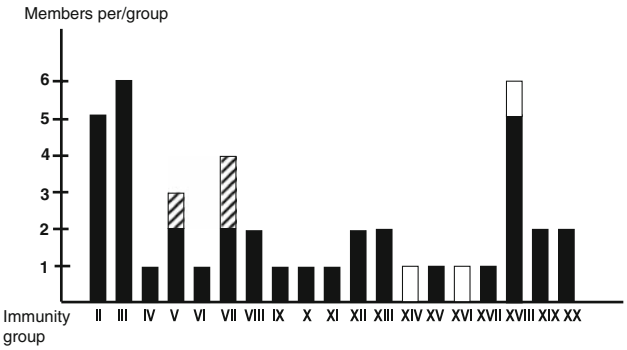


**Fig. 1** Frequency distribution of nineteen lambdoid phage-infection immunity groups. Phages were classified into nineteen immunity groups following the phage-lysogen cross test as previously reported by Kameyama et al. [39]. Phage groups were classified according to phage number per group. *Black bars* represent FhuA-receptor dependent phages; *striped* and *white bars* are phages that were unable to grow in MCR106 and MH760 cells, respectively

unlike $\lambda$, other factors may be responsible for their exclusion in Δ*lam*B cells. Likewise, mEp332, mEp460 and mEp505 phages did not grow in MH760 (*omp*C$^-$) (Table 2), but only mEp332 was able to infect MH760 cells complemented with pRAM1006 (*omp*C$^+$).

The *cor* gene is present in half of the lambdoid phage population

Phage-encoded Cor protein is involved in the exclusion of T1, T5, $\phi$80, N15, HK022 and mEp167 phages, which require FhuA receptor for infection [40, 43, 45, 54]. Indeed, the *cor* gene and the FhuA-binding $\lambda$ *J* homolog (gene *p23* for HK022, or *p21* for N15 phages) are separated by two ORFs in the tail fiber cluster region [60]. In addition, Cor-mediated exclusion has only been observed in FhuA + cells. Thus, it was relevant to test for the presence of the *cor* gene in the lamboid phages. The ~155 bp intragene *cor* region, as analyzed by PCR, was found in 25 out of 43 phage DNAs (~58%) (PCR results from 10 phages are shown in Fig. 2, Table 2). For control purposes, negative or positive amplification resulted from $\lambda$ (*cor*-) and mEp167 (*cor*+) phages, respectively, when tested under the same conditions. PCR-DNA fragments from 4 randomly selected phages were sequenced (mEp123, mEp147, mEp234 and mEp502). DNA sequence alignment analysis revealed an almost identical matching with the HK022 *cor* gene, except for one base in the mEp123 and mEp502 *cor* genes (Fig. 3). We have reported that mEp167 prophage containing a *cor* gene excludes some FhuA-dependent phages and that the number of excluded phages increases when the mEp167 *cor* gene is expressed from multi-copy plasmids [54]. Although positive *cor* amplification by PCR was observed with mEp003, mEp023 and mEp174 prophages, they did not exclude at least 8 FhuA-dependent

**Table 2** Analysis of FhuA-specificity, *cor* gene presence, λN antitermination and λatt integration of 43 mEp lambdoid phages

| Phage | Immunity group | Strain | | | | | PCR (*cor*-like) | λ-like N antitermination | λ-like integration |
|---|---|---|---|---|---|---|---|---|---|
| | | W3110 | C600 (*fhu*A$^-$) | C600 (pUCJA; FhuA$^+$) | MCR106 (Δ*lam*B) | MH760[a] (*omp*C$^-$) | | | |
| mEp003 | II | + | − | + | + | + | + | + | − |
| mEp141 | II | + | − | + | + | + | + | + | − |
| mEp147 | II | + | − | + | + | + | + | + | − |
| mEp450 | II | + | − | + | + | + | + | + | − |
| mEp468 | II | + | − | + | + | + | + | + | − |
| mEp023 | III | + | − | + | + | + | + | − | − |
| mEp145 | III | + | − | + | + | + | + | − | − |
| mEp498 | III | + | − | + | + | + | − | − | − |
| mEp504 | III | + | − | + | + | + | − | − | − |
| mEp534 | III | + | − | + | + | + | − | − | − |
| mEp543 | III | + | − | + | + | + | − | − | − |
| mEp043 | IV | + | − | + | + | + | − | − | − |
| mEp064 | V | + | − | + | + | + | + | + | − |
| mEp263 | V | + | − | + | + | + | − | + | − |
| mEp553 | V | + | + | + | − | + | − | + | − |
| mEp167 | VI | + | − | + | + | + | + | − | − |
| mEp173 | VII | + | − | + | + | + | + | − | − |
| mEp409 | VII | + | + | + | − | + | − | − | − |
| mEp414 | VII | + | + | + | − | + | − | − | − |
| mEp524 | VII | + | − | + | + | + | + | − | − |
| mEp174 | VIII | + | − | + | + | + | + | − | − |
| mEp539 | VIII | + | − | + | + | + | − | − | − |
| mEp213 | IX | + | − | + | + | + | + | − | − |
| mEp237 | X | + | − | + | + | + | + | − | − |
| mEp390 | XI | + | − | + | + | + | − | − | − |
| mEp410 | XII | + | − | + | + | + | + | − | − |
| mEp502 | XII | + | − | + | + | + | + | − | − |
| mEp416 | XIII | + | − | + | + | + | + | − | − |
| mEp457 | XIII | + | − | + | + | + | + | − | − |
| mEp460 | XIV | + | + | + | + | − | − | − | − |
| mEp506 | XV | + | − | + | + | + | + | − | − |
| mEp505 | XVI | + | + | + | + | − | − | + | + |
| mEp235 | XVII | + | − | + | + | + | + | − | − |
| mEp120 | XVIII (λ) | + | − | + | + | + | + | − | − |
| mEp123 | XVIII (λ) | + | − | + | + | + | + | − | − |
| mEp155 | XVIII (λ) | + | − | + | + | + | + | +(?) | − |
| mEp234 | XVIII (λ) | + | − | + | + | + | + | − | − |
| mEp332 | XVIII (λ) | + | + | + | + | − | − | + | + |
| mEp500 | XVIII (λ) | + | − | + | + | + | + | − | − |
| mEp533 | XIX | + | − | + | + | + | − | − | − |
| mEp266 | XIX | + | − | + | + | + | − | − | − |
| mEp138 | XX | + | − | + | + | + | − | − | − |
| mEp144 | XX | + | − | + | + | + | − | − | − |
| λ | XVIII | + | + | + | − | + | − | + | + |
| φ80 | XIX | + | − | + | + | + | + | − | − |

**Table 2** continued

| Phage | Immunity group | Strain | | | | | PCR (*cor*-like) | λ-like N antitermination | λ-like integration |
|---|---|---|---|---|---|---|---|---|---|
| | | W3110 | C600 (*fhu*A$^-$) | C600 (pUCJA; FhuA$^+$) | MCR106 (Δ*lam*B) | MH760[a] (*omp*C$^-$) | | | |
| HK022 | XX | + | − | + | + | + | + | − | − |

[a] MH760 strain bears *omp*R472 mutation, which confers the OmpF$^+$, OmpC$^-$ phenotype [52]

+ Phage growth and/or PCR amplification and/or blue or red plaque production

(?) Caused by N and/or CI

lambdoid phages [39]. Whether these genes are also able to exclude superinfecting phages when expressed from a multi-copy plasmid remains to be determined.

## λ N antitermination is conserved by a quarter of the lamboid population

Equivalent λ N antitermination function of the lambdoid phages was determined using the pGH006 plasmid construct and the N antitermination assay described in Materials and methods (Fig. 4). In this assay the white plaque phenotype at 32°C indicates the lack of N antitermination. Since *p*I is also present upstream of the *int*'-'*lac*Z fusion in the pGH006 construct, the white-plaque phenotype at the same temperature also rules out the possibility of CII activity from the infecting phage. The blue-plaque phenotype at 32°C is indicative of CII activity, and the production of blue plaques at 37 or 42°C but white ones at 32°C is the result of N antitermination. Around 75% of the whole phage population produced white plaques at the three temperatures, indicating a lack of N antitermination and CII activity. Nine out of the remaining 12 phages presented the blue-plaque phenotype either at 37 or 42°C and white plaques at 32°C, revealing the presence of N antitermination (Table 3). However, mEp141 and mEp147 produced pale blue plaques at 32°C, which could be due to a residual CII-like activity. As expected, the blue-plaque phenotype of mEp155, mEp332 and λ phages from immunity group XVIII or imm λ was only observed at 42°C (Table 3). Interestingly, Degnan et al. [14] reported that mEp332 contains λ-like CII and N; therefore, the mEp332 blue-plaque phenotype obtained could be caused by both activities. However, it is not easy to discern whether the mEp155 blue plaque phenotype comes from CII and/or N activities. In the particular case of mEp502 phage, blue plaques are produced at 32°C, indicating λ CII-like activity. On the other hand, none of the mEp phages indicated in Table 3, with the exception of mEp502, grew in *nus*B5 and *nus*E71 mutants at 37°C as λ did. They had a limited growth in the *nus*A1 mutant, and most of them grew well in *nus*D026 mutant. This suggests that these phages undergo a similar mechanism for the formation of the antitermination complex. All of the representative phages (8) belonging to immunity groups II and V, which are heterogeneous, as the phage-DNA restriction pattern indicates (data not shown), presented N antitermination. This may indicate a close genetic relationship in the immunity region of groups II, V and λ phage.

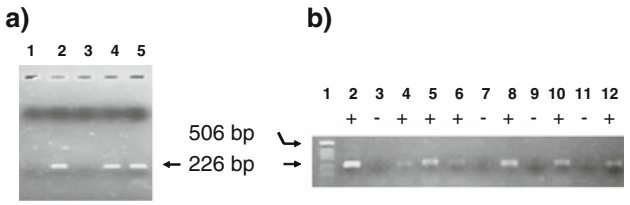


**Fig. 2** PCR analysis of lambdoid phage *cor* regions. The ~165 bp DNA intra-gene *cor* region was amplified by PCR. **a** *Lane 1*, DNA amplification using *cor*-specific primers without DNA template; *lanes 2–4*, amplification from mEp167, mEp093, HK022 and φ80 phage-DNAs, respectively. As positive controls, DNA from mEp167, HK022 and φ80 phages containing the *cor* gene was used. **b** *Lane 1*, molecular marker (GIBCO BRL 1 kb DNA Ladder), *lanes 2–12*, DNA amplification of mEp-524, -534, -543, -064, -409, -500, -263, -023, -533 and -410, respectively. +, amplification; −, no amplification

## Two out of 43 lambdoid phages possessed the λ site-specific recombination system

λ Integrase is essential in the mechanism by which λ DNA integrates into the host chromosome and is expressed from the *p*I promoter [57]. mRNA from *p*L cannot express integrase because a *sib* site located beyond *int* gene is sequentially processed by RNase III and PNPase exonuclease [24, 26]. λ integrase, IHF host factors and HU protein are involved in the specific recombination [19, 20]. To analyze whether the specific λ integrative recombination system is present in the lambdoid phages, we used the red-plaque assay as has been reported previously [17]. After incubating plates overlaid with LE289 bacteria for 36 h at 37°C, only mEp332 and mEp505 phages produced red plaques (Table 2). This result agrees with our previous report in which it was shown that these phages recombined very efficiently with λBLK20 [39], producing hybrid progenies, presumably by site-specific recombination.

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HK022cor  1 CTCTGGATACTATCAAAATGAAAAGGCTAATTATCTGCATAGCGGGCGCTTTAATGCTGTCTGGTTGCGCTGGTGTACTTGAGAA
mEp123cor´                                                          ----C-----------------------
mEp147cor´                                                          ----------------------------
mEp234cor´                                                          ----------------------------
mEp502cor´                                                          ----------------------------
mEp167cor   -----------T-----ATG------------------------AT--------------------T-----------------

HK022cor    ACAGAAACCGATTTGCAGCGGCACGGCATATATGGGTGGCCATGAGAATACCGTTATGATTTACGCCGTTCGAAAGCAAAACAAC
mEp123cor´  --------------------------------------------------------------------------------
mEp147cor´  --------------------------------------------------------------------------------
mEp234cor´  --------------------------------------------------------------------------------
mEp502cor´  --------------------------------------------------------------------------------
mEp167cor   ----G----T----------------------------A-----------A--------------G---------A---------

HK022cor    CAGACGCAATACCGGGCCGGATATCCCTTTAACTGGCGCTGGGTAAGTGCGAACACATTCACAAGCACGACATGTAAATAA 251
mEp123cor´  ---------------------
mEp147cor´  ---------------------
mEp234cor´  ---------------------
mEp502cor´  -------------A-------
mEp167cor   -----T----------------------C--------T--------------------------------------TAA
```

**Fig. 3** Alignment of *cor* gene segment sequences. HK022 and mEp167 *cor* gene (GenBank accession no. AF069308 and AY616010, respectively) were aligned with *cor* gene segments of mEp123, mEp147, mEp234 and mEp502 phages. *Dashes* show identical nucleotide residues compared to HK022 *cor* gene sequence. *Arrows* under the sequences denote the primers, and ATG and TAA in *bold* indicate the initiation and termination codons

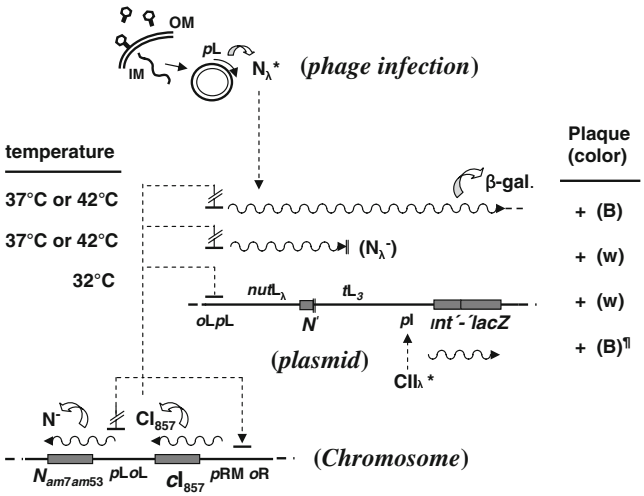


**Fig. 4** Genetic assay to determine λN-specific antitermination function. The assay consists of three components: **a** a lysogenic *lacZ*$^-$ bacterium with a cryptic prophage containing $\lambda N_{am7am53}$ and $cI_{857}$, **b** a plasmid (pGH006) containing the *o*L *p*L promotor region, the *nut* site and the transcription terminator $tL_3$ upstream of the reporter *int*'-'*lac*Z fusion gene; and **c** the *λN*-like producing phage. Phage growth is indicated by +, (B) and (W) indicate blue and white plaques, respectively, "*" means equivalent to N or CII of *λ*, and "¶" indicates growth and blue plaque formation at any temperature

Although integration is not an essential function for phage development, it is a fundamental process for maintaining *λ* prophage stage. Thus, the low number of phages showing *λ* integration (2 out of 43) may be indicative of a higher degree of variation of a non-essential gene.

## Discussion

In this work, we found a high frequency (~85%) of FhuA-dependent phages in a collection of heterogeneous lambdoid phages from different immunity groups. In addition, *cor* moron was present in 50% of the phages tested and was always detected in FhuA-dependent phages. This contrasted with the frequency of *λ* N antitermination function, which was required by around 25% of the population, and the *λ* integrative-specific recombination function, which was only present in two members out of 43. Thus, FhuA receptor specificity and its frequent association with *cor*+ predominated among the phages analyzed even though they came from different infection immunity groups.

The group of mEp phages used in this work was isolated using the derivative *E. coli* K-12 strain LE392 ($r^-$, $m^+$) [52]. The exclusion of 37 out of 43 (~85%) mEp lambdoid phages in strain C600 and the infection when the cells were supplemented with *fhu*A$^+$ (pUCJA) indicated that the deficiency of FhuA was the main cause for the exclusion. Thus, FhuA receptor was required for infection by most of the phages tested. Although a bias towards the selection of *nus*-dependent phages was imposed on the original selection of the phages tested, this was independent of phage receptor specificity, and therefore, nus-dependence does not depend on FhuA specificity. For example, we isolated nus-dependent lamboid phages with receptor specificities other than FhuA. On the other hand, HK022 is a nus-independent lambdoid phage that requires FhuA for

**Table 3** $\lambda$ N antitermination, *nus*-host mutant growth and HK022-mediated exclusion of mEp lambdoid phages

| Phage | Immunity group | W3110 (HK022) | *nus*A1 | *nus*B5 | *nus*D026 | *nus*E71 | N6171/pGH006 | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 32°C | 37°C | 42°C |
| mEp003 | II | − | +/− | − | +/− | − | + (w) | +/− (B) | − |
| mEp141 | II | − | +/− | − | + | − | + (pb) | + (B) | − |
| mEp147 | II | − | +/− | − | + | − | + (pb) | + (B) | + (B) |
| mEp450 | II | − | +/− | − | + | − | + (w) | + (B) | +/− (B) |
| mEp468 | II | − | − | − | + | − | + (w) | + (B) | +/− (B) |
| mEp064 | V | − | − | − | + | − | + (w) | + (B) | − |
| mEp263 | V | − | +/− | − | + | − | + (w) | + (B) | − |
| mEp553 | V | − | − | − | + | − | + (w) | + (B) | − |
| mEp505 | XVI | − | − | − | + | − | + (w) | + (B) | + (B) |
| mEp155 | XVIII | − | + | − | + | − | − | − | + (B) |
| mEp332 | XVIII | − | +/− | − | +/− | − | − | − | + (B) |
| $\lambda$ | XVIII | − | + | − | + | − | − | − | + (B) |
| $\phi$80 | XIX | + | + | +/− | + | + | + (w) | + (w) | +/− (w) |
| HK022 | XX | − | + | + | + | + | + (w) | + (w) | + (w) |
| mEp502 | XII | + | + | + | + | − | + (B) | + (pb) | + (pb) |

*B* blue plaques, *w* white plaques, *pb* pale blue plaques

\+ plaque production at any dilution

+/− $10^2$–$10^4$ fold the wt (W3110) strain exclusion rate

− $>10^5$ fold the wt exclusion

infection. Therefore, the results presented here suggest that FhuA receptor specificity is widely distributed in the lamboid group. Consequently, the frequency of FhuA-specific phages has probably been underestimated due to the extended use of C600 (*fhu*A$^-$) strain in numerous lambdoid phage studies.

Following the high frequency of FhuA-specific phages is the number of *cor*+ phages as detected by PCR: 25 out of 43 (around 50%). The frequency of *cor*+ phages may have been enhanced because of mutations within this region in FhuA-dependent phages. However, this is less likely, since the primers were designed from the highly conserved regions of *cor* gene. In addition, the size of the PCR-amplified band (226 bp) corresponded to the reported *cor* sequences [54, 55]. The high frequency of *cor*+ phages and its association with FhuA specificity strongly indicates that the Cor-mediated exclusion of FhuA-dependent phages is broadly distributed within the lambdoid phage group. The linkage of the FhuA-specific $\lambda$ *J* homolog and the presence of the *cor* gene and the frequency of *cor* in the lamboid phages (50%) is suggestive of a scenario where *cor* moron may have been integrated before LamB- or OmpC-specific *J* holomologs were acquired. A relatively large DNA region containing FhuA-specific *J* homolog and *cor* genes could have recombined illegitimately with another region containing a $\lambda$ *J*-like gene, resulting in a LamB-dependent phage. This is in agreement with genetic mosaicism and the modular theory of phage diversity [6, 53]. Whatever the mechanism may be, the *cor* moron insertion renders the host resistant to re-infection through FhuA receptor, and it was not surprising that no amplification was obtained from 6 FhuA-independent mEp lambdoid phage DNAs. Around 15% of the phages tested required a receptor different from FhuA. Thus, the essential FhuA-specific *J* homolog stands out among other lambdoid features. It is noteworthy that within the N15 phage genome sequence there is a high homology between its left arm (upstream *p*16 gene) and the $\lambda$ genome, and between its right arm (downstream *p*18 gene) and the HK022 genome [37, 48]. This is an example of the contribution of the recombination process in evolution and is in agreement with the mosaicism observed in the majority of the tailed phages analyzed [30, 31].

Before the report of Kameyama et al. [39], the Nus host factor requirement was only associated with the lambdoid phage group. This encouraged us to apply the inverse genetic system with isogenic *nus* mutants to identify Nus-dependent phages. The percentage of Nus-dependent phages showing $\lambda$ N antitermination (10 out of 43) suggests that this essential system was less conserved than FhuA specificity. A double amber mutant phage $\lambda\, N_{\mathrm{am7am53}}\, nin5$, which removes *t*R2 and lacks a $\lambda$ N active protein, grows well in wild-type *E. coli* [13]. Possibly, some equivalent mutants in the environment, with deletions or mutations of the transcription terminator *t*R2, might partially explain this minor divergence. HK022 Nun protein binds to $\lambda$ *box*B

of the mRNA *nut* site as $\lambda$ does. The exclusion analysis for phages producing blue plaques carried out in HK022 lysogen showed that all phages except $\phi$80 and mEp502 were unable to grow in HK022 lysogen (Table 3). Although HK022 also contains the *cor* gene, its exclusion action could be disregarded when compared with Nun, as more than 50% (20 out of 37) of FhuA-dependent phages can grow (data not shown). This behaviour parallels mEp167 lysogen, where *cor* in multi-copy plasmids excludes more FhuA-dependent phages than the lysogen [54]. For the three FhuA-independent (mEp332, mEp505 and mEp553) phages, exclusion can be attributed to Nun. Although exclusion for the other eight FhuA-dependent phages is uncertain, direct analysis of blue plaques suggests N function. Nun action is antagonistic to $\lambda$ N as it blocks transcription elongation, producing a truncated transcript [18]. Whether divergence of these functions occurred and how they became antagonists remains unknown.

Lambda integrase as a member of the tyrosine recombinase family is involved in site-specific recombination. These members are structurally diverse and are responsible for many important biological processes [23]. Lambda *int* gene is not essential, and its product, the $\lambda$ integrase, only recognizes its own *att* sites for recombination. In addition, the homology between different integrases and the attachment sequences is low [23]. Thus, the low frequency of the non-essential function of $\lambda$ site-specific recombination (two out of 43 members) could suggest a high divergence within the members of the lambdoid group. Although all the phages tested could lysogenize strain W3110, direct evidence of integration is not available.

The FhuA receptor requirement by a group of phages from different immunity groups and the wide distribution of FhuA specificity in the group tested suggest that these phages are part of a broader population from which different phages, including $\lambda$, might have diverged. Alternatively, these phages may have independently acquired this characteristic during their evolution. This work was carried out mainly with phages isolated from human faeces collected in Morelos state (Mexico); however, other lambdoid phages such as $\phi$80, N15 or HK022, derived from other geographical areas, are also FhuA-dependent. Preliminary results with phages from other geographic areas also reveal a high percentage of FhuA-specific phages and their frequent association with the *cor* gene. This supports the idea of a wide distribution of FhuA-dependent phages and the Cor-mediated exclusion mechanism in the lambdoid group.

**Acknowledgments** We are very grateful to Dr. Thomas J. Silhavy, who kindly provided us MCR106, MH760 strains and pCWB27 and pRAM1006 plasmids, and to Dr. Maxwell E. Gottesman for providing strain N6171. We acknowledge V. Ramirez Nieves for clerical and M. Sc. G. Aguilar González for technical assistance. During this work, L.F., E.M. and A.B. were granted with fellowships from CONACyT. This work was supported in part by grant 50598/24241 from CONACyT.

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