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An orthologue of the *cor* gene is involved in the exclusion of temperate lambdoid phages. Evidence that Cor inactivates FhuA receptor functions

Augusto Uc-Mass[a], Eva Jacinto Loeza[b], Mireya de la Garza[a], Gabriel Guarneros[b], Javier Hernández-Sánchez[b], Luis Kameyama[b,*]

[a]*Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 14-740, México D. F. 07000, México*
[b]*Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 14-740, México D. F. 07000, México*

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Abstract

A new set of lambdoid phages (mEp) classified into different immunity groups was previously described. Phages mEp213, mEp237, and mEp410 were unable to grow in mEp167 lysogenic cells, presumably due to an exclusion mechanism expressed constitutively by the mEp167 repressed prophage. In this work, to analyze the exclusion phenomenon, we constructed a genomic library from mEp167 phage in a pPROEX derivative plasmid. A DNA fragment containing an open reading frame for a 77 amino acid polypeptide was selected by its ability to confer resistance to heteroimmune phage infection. This ORF shows high amino acid sequence identity with putative Cor proteins of phages HK022, φ80 and N15. Cells expressing the mEp167 *cor* gene from a plasmid (Cor^+ phenotype) excluded 13 of 20 phages from different infection immunity groups. This exclusion was observed in both $tonB^-$ and $tonB^+$ cells. Lambdoid mEp phages that were excluded in these cells were unable to infect cells defective in the outer membrane FhuA receptor ($fhuA^-$). Thus, Cor-mediated exclusion was only observed in $fhuA^+$ cells. Phage production after DNA transfection or the spontaneous induction of mEp prophage in Cor^+ cells was not blocked. In addition, ferrichrome uptake, which is mediated by FhuA, was inhibited in Cor^+ cells. Our results show that not only phage infection via FhuA but also a FhuA transport activity (ferrichrome uptake) are inhibited by Cor, presumably by inactivation of FhuA.

Keywords: Cor; FhuA; Phage exclusion; mEp167; λ phage

Introduction

A prophage genome, either as a plasmid or integrated in the bacterial chromosome, confers new phenotypic traits. One of these, known as immunity, is the ability to block reinfection by homoimmune phages through a prophage-encoded repressor. Temperate bacteriophages may also express gene(s) related to exclusion and superinfection exclusion phenomena which differ from phage repression systems in that they are not involved in maintenance of lysogeny and are not specific for homoimmune phages.

The lambda phage Rex system, by which T4 mutant *rII* phage is excluded by λ lysogenic cells, has been well characterized (Benzer, 1955). This system is composed of *rexA* and *rexB* λ phage gene products (Landsmann et al., 1982; Matz et al., 1982). It has been hypothesized that RexA protein interacts with RexB, forming active channels. The cell falls into a dormant state with a dramatic membrane potential loss and reduction of ATP levels (Parma et al., 1992; Sekiguchi, 1966; Snyder and McWilliams, 1989). The fraction of the cell population that recovers cellular growth after a long period of time depends on the phage/cell infection ratio (Slavcev and Hayes, 2002, 2003). T4 phage infection is also excluded by *Escherichia coli* K-12 with the defective prophage ε14. An interaction between Gol, a small peptide derived from

* Corresponding author. Fax: +52 55 5747 7100.
E-mail address: luisk@mail.cinvestav.mx (L. Kameyama).

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the T4 major head protein (Bergsland et al., 1990) and ε14-coded Lit protease causes the cleavage of elongation factor EF-Tu and has been partially associated with translation inhibition and phage exclusion (Yu and Snyder, 1994). The *prrC* gene that encodes a $tRNA^{Lys}$-specific anticodon nuclease is kept in latent form in a particular clinical *E. coli* strain. The mutant phages T4 *pnk*$^-$ and T4 *rli*$^-$, which are defective in polynucleotide kinase and RNA ligase, respectively, cannot restore the anticodon nuclease processing, and are excluded in this strain. The nuclease is activated by the phage T4-encoded DNA restriction inhibitor (reviewed by Kaufmann, 2000). HK022 lysogenic *E. coli* cells exclude lambda phage. HK022 prophage Nun protein expressed in the cell competes with lambda N protein for *boxB* NUT sites of lambda mRNA (Baron and Weisberg, 1992). Nun protein binds *boxB*, halting transcripts coming from the lambda *p*L and *p*R promoters and inhibiting the expression of downstream lambda genes (Oberto et al., 1989). There are two specific exclusion systems in the *Salmonella typhimurium* temperate phage P22. They are coded by *sieA* and *sieB*, and both exclude heteroimmune phages L, MG178 and MG40 (Susskind et al., 1971). Superinfecting phages are not able to express self-proteins in *sieA*$^+$ lysogens. It has been proposed that the membrane-associated SieA protein blocks DNA transfer of the super-infecting phage into the cell (Hofer et al., 1995; Susskind et al., 1974). Likewise, it has been proposed that T4 Imm protein may block the transfer and injection of incoming T-even phage DNA (Lu and Henning, 1994).

E. coli outer membrane protein FhuA is the transporter of ferrichrome and antibiotic-related compounds like albomycin, rifamycin CGP4832, microcin 25 and Colicin M, and it is also the receptor for phages T1, T5, φ80 and N15 (Braun et al., 1973; Hantke and Braun, 1975; Salomón and Farías, 1993; Vostrov et al., 1996; Wayne and Neilands, 1975). X-ray diffraction studies of FhuA crystals revealed a β-barrel structure made up of 22 anti-parallel β-strands (Ferguson et al., 1998; Locher et al., 1998). This structure is closed at the periplasmic side by a globular domain called "cork" or "plug" which comprises the first 159 amino-terminal residues (Ferguson et al., 1998; Locher et al., 1998) and forms a 4-strand β-sheet and 4 small α helices. Peptide mapping studies have shown that FhuA loop 4 is the binding site for phages T1, T5, and φ80 (Killmann et al., 1995).

All FhuA related activities, except infection by phage T5, are associated with the TonB energy transducing system which transduces the cytoplasmic membrane proton motive force to the FhuA receptor. The TonB system consists of (at least) the cytoplasmic membrane proteins TonB, ExbB, and ExbD (for review, see Moeck and Coulton, 1998). Indeed, outer membrane receptors like FhuA, BtuB, and other TonB-interacting proteins have a 5 residue motif, Glu-Thr-Val-Ile-Val, called the TonB box (Schramm et al., 1987). In addition, φ80 phage adsorption and other TonB-dependent activities are inhibited by an identical synthetic TonB box consensus pentapeptide (Tuckman and Osburne, 1992).

A phage protein named Cor is involved in the exclusion of the FhuA-dependent phages N15 and φ80 by either N15 or φ80 lysogenic cells (Imai and Yoshikawa, 1970; Kozyrev and Rybchin, 1987; Malinin et al., 1993; Matsumoto et al., 1985). The HK022 DNA sequence also reveals the presence of a gene orthologous to *cor* (Juhala et al., 2000). Amino acids 30 to 34 in the φ80 and N15 Cor protein sequences show homology with the consensus for the TonB box sequence. Thus, it has been proposed that Cor may interact directly with FhuA or indirectly through TonB protein in the Cor-mediated exclusion phenotype of φ80 and N15 phages (Vostrov et al., 1996).

Our laboratory previously classified a collection of mEp lambdoid phages isolated from fresh stools into 19 different immunity groups by phage–lysogen cross tests. An exclusion phenomenon by mEp167 lysogens against other members of the mEp lambdoid family was also observed (Kameyama et al., 1999). In the present work, we found that a *cor* orthologue from lambdoid phage mEp167 (cor_{mEp167}) accounts for the exclusion of FhuA-dependent lambdoid phages. Cells overexpressing Cor_{mEp167} (Cor^+ cells) excluded both TonB-dependent and TonB-independent phages, suggesting that the TonB system is not involved. The exclusion may be exerted at the phage DNA entry level, as the phage adsorption, the production of phage particles after phage DNA transfection or the spontaneous induction of lysogens in Cor^+ cells were not affected. We also present evidence that in addition to phage receptor function, a transport activity (ferrichrome uptake) of FhuA is blocked in Cor^+ cells.

Results

A cor orthologue from mEp167 phage is involved in the exclusion of a set of lambdoid phages from different immunity groups.

A set of lambdoid phages classified into different immunity groups was isolated in our laboratory. From these, phages mEp213, mEp237, and mEp410, which belong to different immunity groups, failed to grow in mEp167 lysogenic cells, possibly due to an exclusion mechanism (Kameyama et al., 1999). To identify the gene(s) responsible for exclusion, we prepared a genomic library from mEp167 phage DNA (see Experimental Procedures). One plasmid, pT41, which contains a 2743-bp mEp167 DNA fragment, excluded mEp167, mEp213, mEp237, and mEp410, but not lambda phage from W3110 (Table 1). W3110 cells harboring plasmid candidates with other genomic fragment, like pT43 (with 539 bp), and pT45 (with 144 bp), or carrying the pUC18 vector, did not exclude these phages (data not shown). These results

Table 1
Phage exclusion in *E. coli* K-12 strains

Phage	Immunity group	Strain								
		W3110	W3110 (mEp167)	W3110 [pT41; Cor$^+$]	W3110 [pKINAM; Cor$^+$]	C600 FhuA$^-$	C600 [pUCJA; FhuA$^+$]	KP1039 TonB$^-$	KP1039 [pRZ540; TonB$^+$]	KP1039 [pKINAM; Cor$^+$]
(1) λ	XVIII	+	+	+	+	+	+	+	+	+
(2) mEp167	VI	+	–	–	–	–	+	–	+	–
(3) mEp237	X	+	–	–	–	–	+	–	+	–
(4) mEp410	XII	+	–	–	–	–	+	–	+	–
(5) mEp213	IX	+	–	–	–	–	+	+	+	–
(6) mEp003	II	+	+	–	–	–	+	–	+	–
(7) mEp023	III	+	+	–	–	–	+	–	+	–
(8) mEp043	IV	+	+	–	–	–	+	–	+	–
(9) mEp174	VIII	+	+	–	–	–	+	–	+	–
(10) mEp390	XI	+	+	–	–	–	+	–	+	–
(11) mEp416	XIII	+	+	–	–	–	+	+	+	–
(12) mEp506	XV	+	+	–	–	–	+	+	+	–
(13) mEp038*	I	+	+	+	+	+	+	+	+	+
(14) mEp553	V	+	+	+	+	+	+	+	+	+
(15) mEp409	VII	+	+	+	+	+	+	+	+	+
(16) mEp460	XIV	+	+	+	+	+	+	+	+	+
(17) mEp505	XVI	+	+	+	+	+	+	+	+	+
(18) λBLK-20	XVII	+	+	+	+	+	+	+	+	+
(19) φ80	XIX	+	+	–	–	–	+	–	+	–
(20) HK022	XX	+	+	–	–	–	+	+	+	–
(21) T5*	–	+	+	–	–	–	+	+	+	–

(+) and (−): presence or absence of phage growth, as determined by infecting with a series of dilutions containing 10^2–10^8 pfu.
* Non-lambdoids phage.

indicate that plasmid pT41 containing a mEp167 DNA fragment, confers upon *E. coli* W3110 resistance similar to that seen in mEp167 lysogenic cells.

The DNA region cloned in pT41 was sequenced and analyzed to identify the gene(s) responsible for phage exclusion of heteroimmune (mEp213, mEp237, and mEp410) and homoimmune (mEp167) phages. Computational analysis using the DNA Strider program (Douglas, 1994) predicted a 234-nt ORF that codes for a 77-aa peptide. Sequence alignment analysis using Vector NTI or BLAST program with homologous sequences from the NCBI web server (http://www.ncbi.nlm.nih.gov) revealed 91% (70/77 aa) identity with the putative HK022 Cor, 61% (47/77 aa) with N15 Cor, and 74% (57/77) with φ80 Cor (Fig. 1). A 254-bp fragment carrying the putative mEp167 *cor* gene was cloned in pPROEXd (see Experimental procedures) under control of the *p*Trc promoter, yielding plasmid pKINAM. When this plasmid was expressed in an in vitro transcription–translation assay, a protein of approximately 8 kDa was observed (data not shown). As expected, this plasmid rendered W3110 cells unable to support the development of phages mEp167, mEp213, mEp237, and mEp410. No phage infection was observed even at 10 μl of a stock of phage at 1×10^8 pfu/ml spotted onto a cell lawn of W3110 [pKINAM] (Table 1). In contrast, no exclusion was observed in cells with vector pPROEXd (data not shown) or without plasmid. We further extended this analysis to members from other lambdoid immunity groups.

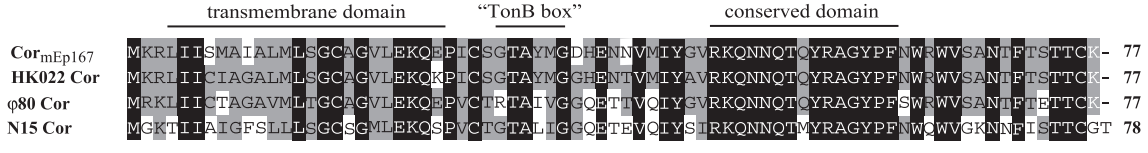

Fig. 1. Alignment of Cor protein sequence. *cor* gene product of phages mEp167, HK022, φ80 and N15 were aligned, starting from the first amino acid (Met). Identical residues in all four polypeptides are shaded in black and white letters. Similar residues are typed in black and shaded in grey. The putative transmembrane domain for Cor$_{mEp167}$ polypeptide, the putative TonB box and the conserved domain for all polypeptides are indicated by black lines above of the amino acid sequences. Identity values for Cor$_{mEp167}$ (GenBank accession no. AAT11800; and AY616010 for the corresponding gene), compared with HK022, φ80 and N15 Cor proteins (GenBank accession nos. NP037685, JN0005 (modified by Vostrov et al., 1996), and S55495) were 91%, 74% and 61%, respectively.

The mEp family members have been grouped into 19 lambdoid immunity groups and one non-lambdoid group by phage–lysogen cross tests (Kameyama et al., 1999, 2001). One member from each group was chosen randomly to analyze Cor-mediated exclusion. We found that 13 out of 20 phages, including φ80, HK022 and the four mEp phages described above, were excluded by cells overexpressing Cor from plasmid pKINAM (Cor^+ cells) (Table 1). We also tested T5 phage behavior in mEp167 lysogens and Cor^+ cells. As has also been described previously (Vostrov et al., 1996), T5 was excluded in Cor^+ cells (W3110 [pKINAM], Table 1) but not in mEp167 lysogens. A possible explanation for this behavior will be discussed below. These results suggest that a *cor* orthologue from phage mEp167 (cor_{mEp167}) is responsible for the exclusion of the phages tested.

FhuA phage receptor function is inhibited by Cor irrespective of TonB

Phage-encoded Cor proteins have also been associated with the exclusion of φ80, T1 and N15 phages by φ80 or N15 lysogens (Imai and Yoshikawa, 1970; Kozyrev and Rybchin, 1987; Malinin et al., 1993; Matsumoto et al., 1985). FhuA protein, which belongs to a family of *E. coli* outer membrane transporters, is the receptor for the above phages, in addition to being the receptor for ferrichrome, albomycin, rifamycin CGP4832, microcin 25, and colicin M (Braun et al., 1999). Therefore, we analyzed whether the mEp family individuals that were excluded by cor_{mEp167} were also able to infect C600 (*fhuA*$^-$) cells. We found that phages excluded in Cor^+ cells did not form plaques in (*fhuA*$^-$) C600 cells (Table 1). In other words, the exclusion profile of the mEp lambdoid phages tested either in *fhuA*$^-$ or Cor^+ cells was identical. To reinforce the idea that the lack of infectivity is linked to the defective *E. coli* C600 FhuA receptor and not to the genetic background of C600 cells, FhuA was re-introduced by transforming C600 (*fhuA*$^-$) cells with plasmid pUCJA, encoding the *fhuA* gene. As expected, FhuA restoration rendered C600 (*fhu*A$^-$) cells sensitive to the infection, indicating that the FhuA protein is used as a receptor by most of the mEp lambdoid phages tested (Table 1).

T1 and φ80 phage infection require both the FhuA receptor and the TonB energy transducing system. Although T5 also requires FhuA for infection, it is TonB-independent (Braun et al., 1999). Thus, we analyzed whether the FhuA-mediated infection of our lambdoid phages is associated with the TonB system. Only 3 out of 11 mEp FhuA-dependent phages infected KP1039 (*fhuA*$^+$ *tonB*$^-$) cells (Table 1). TonB-dependent phages were able to infect KP1039 cells only when they expressed *tonB* from the plasmid pRZ540, confirming the TonB-dependency of the infection in these cells regardless of the genetic background (Table 1). The capacity of some of FhuA-dependent phages that are excluded by Cor in W3110 (*fhuA*$^+$ *tonB*$^+$) cells to infect KP1039 (*fhuA*$^+$ *tonB*$^-$) cells, suggests that the TonB system may not be necessary for Cor-mediated exclusion. Therefore, we also analyzed the ability of Cor to exclude lambdoid phages in (*fhuA*$^+$ *tonB*$^-$) cells. Like T5 and HK022, TonB-independent mEp phages were excluded in (*fhuA*$^+$ *tonB*$^-$ Cor^+) cells (Table 1; compare KP1039 and KP1039 [pKINAM]). As expected, plaque formation by mEp phages, specific for other receptors (possibly LamB or OmpC) was not impaired in FhuA$^-$ or TonB$^-$ cells (Table 1; columns C600 (*fhuA*$^-$) and KP1039 (*tonB*$^-$)). These results show that Cor excludes those phages whose infection is mediated by FhuA, independently of a TonB requirement.

Iron-siderophore uptake is inhibited by Cor_{mEp167}

The uptake of ferrichrome complex is mediated by FhuA in a TonB-dependent mechanism (Hantke and Braun, 1975). The results described above indicate that the phage receptor function of FhuA could be affected by Cor irrespective of TonB. Thus, we analyzed whether ferrichrome uptake is also affected by Cor. Transport of ferrichrome was reduced to 1 pmol in Cor^+ cells as compared with approximately 23.2 and 21.1 pmol in W3110 cells without plasmid or in cells transformed with pUC18, respectively (Fig. 2). Although FhuA-mediated ferrichrome uptake is blocked by Cor, cell viability is not affected (data not shown). Thus, a single mechanism may be underlying both the inhibition of FhuA-mediated phage infection and ferrichrome transport.

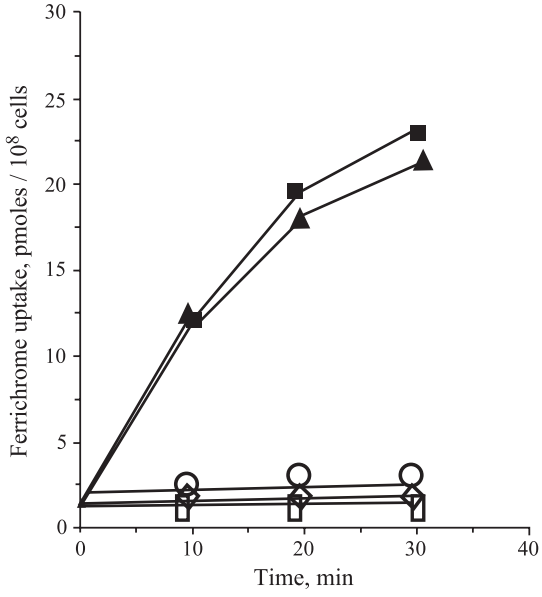

Fig. 2. Effect of Cor_{mEp167} on the uptake of [^{59}Fe]-ferrichrome. C600 (*fhuA*$^-$) (○–○), W3110 (■–■), and W3110 cells carrying pUC18 (▲–▲), pT41 (□–□) or pKINAM (◇–◇) were grown in M9 medium. Cells were harvested, washed and suspended in M9 medium plus nitrilotriacetic acid and IPTG. [^{59}Fe]-ferrichrome was added after incubation at 37 °C for 15 min. Fe uptake was measured as described in Experimental procedures.

Phage adsorption, phage DNA transfection or lysogenic induction circumvents the Cor-mediated resistance mechanism

To determine whether the phage-resistance phenotype conferred by Cor_{mEp167} was a result of inhibition at the early stages of phage infection (phage adsorption/DNA injection), cells were transfected with phage DNA in order to bypass the adsorption and DNA injection stages of infection (Garvey et al., 1996). The DNA of phage mEp237 was introduced by electroporation into Cor^+ and Cor^- cells and the number of infective center formation (EIC) was measured. The EIC formation (EIC) for Cor^+ cells was similar to the value obtained with Cor^- cells (0.76 versus 1) (Table 2A). In a conventional infection (mEp237 phage infection in Cor^+ cells), the EIC value is below the detectable limits of the assay. These observations also predict that mEp167 spontaneous phage production in mEp167 lysogenic cells should not be affected by Cor. As expected, similar numbers of plaques were obtained spontaneously from culture supernatants of lysogens with or without Cor-overexpression (Table 2B). Adsorption assay was performed as described in Experimental procedures. W3110 [pPROEXd], and W3110 [pKINAM] cells adsorbed >99% of mEp237 phages (Table 2A). These results suggest that Cor acts at the DNA injection level. These findings, added to the observed ferrichrome uptake inhibition, strongly suggest that Cor_{mEp167} impairs phage infection by inactivating FhuA and blocking DNA injection.

Discussion

In this work, we found that a Cor orthologue encoded by lambdoid phage mEp167 is responsible for the exclusion of a set of lambdoid phages from different immunity groups. In addition, we found that FhuA-mediated ferrichrome uptake is inhibited by Cor. Thus, we are the first to show that Cor inactivates not only infection via FhuA but also a FhuA transport activity. The set of lambdoid phages tested also demonstrate that Cor-mediated exclusion is independent of the TonB system.

Eleven out of the 15 mEp lambdoid phages tested in this work require FhuA (Table 1). Interestingly, only mEp lambdoid phages that infect the cell through the FhuA receptor are excluded in Cor^+ cells, indicating a FhuA-dependent mechanism of Cor-mediated exclusion. However, seven mEp phages and the unrelated phage T5 were not excluded by mEp167 lysogens but were excluded in Cor^+ cells (Table 1). Although other explanations exist, one possibility is that increasing *cor* gene dosage could affect a larger number of receptors. Phages that have a high affinity for FhuA may be able to form plaques on the lysogen, where only some receptors are inactivated. Conversely, phages that have only a low affinity for FhuA may be excluded when its availability is reduced by levels of Cor synthesized by a lysogen.

Table 2A
Adsorption percentage and EIC of mEp237 phage in Cor^+ W3110 cells after either conventional infection or electroporation of mEp237 phage DNA

Strain	% Adsorption	EIC after	
		Infection	Electroporation
W3110 [pPROEXd]	>99	1	1
W3110 [pKINAM]	>99	nd*	0.768 ± 0.089

* Not detectable.

Table 2B
Spontaneous prophage induction in Cor^+ W3110 lysogens

Phage[a]	Phage titer (pfu/ml)[b]	
	pPROEXd	pKINAM
mEp167	4×10^7	4×10^7
HK022	2×10^7	5×10^7
λ	3×10^4	2×10^4

[a] Phages obtained from W3110 lysogens.
[b] Overnight cultures from lysogenic cells, grown at 37 °C in LB broth with 100 μg/ml ampicillin and 2.5 mM IPTG (when necessary), were centrifuged and the supernatant titers were determined.

In silico analysis predicted a protein of 77 aa for mEp167 Cor, which agrees with the reported sizes of 77 aa (HK022), 77 aa (φ80) and 78 aa (N15) Cor phage proteins. Computational analysis based on SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html) predicts that Cor_{mEp167} and HK022 Cor N-terminal residues (4 to 26 aa) form a transmembrane helix (Fig. 1), as has been suggested for the N15 and φ80 Cor proteins (Vostrov et al., 1996). A transmembrane localization of Cor protein, with possible free polypeptide residues in the periplasm, may facilitate its physical interaction with FhuA. Indeed, preliminary results show that Cor is mostly distributed in the membrane (data not shown).

It has been suggested that Cor protein may interact directly with FhuA protein or indirectly via TonB (Vostrov et al., 1996). Our results favor a direct interaction of Cor with FhuA. Cor excluded only FhuA-dependent phages; including those that were excluded by Cor either TonB-dependent or TonB-independent (Table 1). In addition, phage development after phage DNA transfection or lysogenic spontaneous induction was not affected by Cor.

Outer membrane receptors FhuA, FepA, FecA, and BtuB use TonB as an energy transducer (Ferguson and Deisenhofer, 2002). FhuA contains a conserved sequence, Glu-Thr-Val-Ile-Val, named the TonB box (Schramm et al., 1987). Sequence analysis of mEp167, φ80, and HK022 Cor proteins also reveals the presence of a sequence that resembles the TonB box, amino acid residues 30 through 34. The Cor TonB box sequence for φ80 is Arg-Thr-Ala-Ile-Val, for N15 is Gly-Thr-Ala-Leu-Ile and for mEp167 is Gly-Thr-Ala-Tyr-Met. In fact, φ80 phage infection and other TonB-dependent activities are inhibited in vivo by a

synthetic TonB box consensus peptide (Tuckman and Osburne, 1992). However, the TonB box may be irrelevant to the interaction of FhuA with TonB, since in a *fhuA* mutant strain in which the TonB box was deleted, TonB-dependent functions remained unaltered (Braun et al., 1999). This implies that FhuA and TonB may interact by means of other regions in addition to the TonB box. Thus, the presence of a TonB box may be irrelevant to Cor-mediated exclusion, as indicated by the lack of infection by the TonB-independent phages mEp213, mEp416, and mEp506 in either (*fhuA*$^+$ *tonB*$^-$) or (*fhuA*$^+$ *tonB*$^+$) Cor$^+$ cells. The above observations predict that Cor could affect other FhuA receptor functions. Indeed, the transport of iron–siderophore complex was dramatically reduced in Cor$^+$ cells (Fig. 2). It should be mentioned that cell viability was not affected in Cor$^+$ cells even though ferrichrome uptake is blocked (data not shown). In fact, alternative high affinity transporters like FecA, which transports ferric citrate (Wagegg and Braun, 1981), and FepA, that transports Fe^{3+}-enterobactin (Braun and Hantke, 1981), can supply the iron demand of the cell.

These results, together with the TonB-independent phage exclusion by Cor, suggest that Cor might directly inactivate FhuA receptor function, probably before FhuA interacts with TonB or other accessory molecule, however, the possibility that Cor TonB box could be preventing the interaction of TonB with the TonB box of FhuA, as synthetic TonB box consensus peptides do, cannot be excluded. Thus, phage DNA injection may be blocked, as has been proposed for the exclusion of superinfecting phages by the membrane-associated P22 SieA protein (Hofer et al., 1995; Susskind et al., 1974) or by the lipoprotein Llp that inactivates the FhuA receptor function in the lytic conversion by T5 phage (Decker et al., 1994). This model is supported by the fact that the adsorption, lysogenic induction, or the development of plaques in Cor$^+$ cells transfected with phage DNA are not affected (Tables 2A, 2B). Preliminary observations indicate that a gene orthologous to *cor* is also present in more than 50% of the FhuA-dependent phages that were tested (data not shown). Whether *cor* expression in these phages is also linked to lambdoid phage infection exclusion, as occurs with cor_{mEp167}, is under investigation. As to a direct interaction between Cor and FhuA, we are also undertaking in vitro experiments to assess the affinity of Cor and FhuA.

Experimental procedures

Bacteria, bacteriophages, and plasmids

E. coli strains, bacteriophages, and plasmids used in this work are listed in Table 3. Strain W3110 was used to prepare phage stocks and as a control in genetic assays. Strain DH5α was used to propagate pT41, pT43, pT45, pUCJA, and pKINAM plasmids. C600 (*fhuA*$^-$) and KP1039 (*tonB*$^-$) strains were used in exclusion experiments. All bacteriophage mEp family members used in this study are from our collection (Kameyama et al., 1999). T5, λ, λBLK20, HK022, and φ80 phages were used as controls.

Table 3
Strains of *E. coli* K-12, plasmids and bacteriophages

Strain, plasmid and bacteriophage	Genotype or relevant marker	Source or reference
Bacteria		
W3110	F$^-$λ$^-$ *rph*$^-$	Bachmann, 1972; Jensen, 1993
C600	*leuB6 thi-1 lacY1 supE44 thr-1 rfbD1 fhuA21*	Appleyard, 1954
MC4100	F$^-$ *araD139 Δ(argF-lac) U169 rpsL150* (str^r) *relA1 flbB5301 deoC1 ptsF25 rbsR*	Silhavy et al., 1984
KP1039	MC4100 Δ(*trp*$^-$ *tonB*$^-$ *opp galU*$^-$ *ana*$^-$)	Roof et al., 1991
DH5α	*endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA relA1 Δ(lacIZYA-argF) U169 deoR* (*φ80dlacΔ(lacZ)M15*)	Hanahan, 1983
W3110 (mEp167)	with mEp167 prophage	This work
Bacteriophages		
λ	$imm_λ$	CSH Collection
λBLK20	pL *N*$^+$ *lacZ bla attP* imm_{21} *nin5*	Kameyama et al., 1991
φ80	$imm_{φ80}$	Matsushiro, 1963
HK022	imm_{HK022}	Dhillon and Dhillon, 1976
mEp167	$imm_{γ1}$ cor_{mEp167}	Kameyama et al., 1999
T5		cited in Killmann et al., 1995
mEp phages		Kameyama et al., 1999
Plasmids		
pUC18	*bla*	Messing, 1983
pACYC184	*cat*^{r} *tet*^{r}	Chang and Cohen, 1978
pPROEX-1	*bla lacI*^{Q} *p*Trc	Polayes and Huges, 1994
pPROEXd	*lacI*^{Q} *p*Trc Δ(80 bp)	This work
pT41	cor_{mEp167}	This work
pKINAM	*bla lacI*^{Q} *p*Trc cor_{mEp167}	This work
pUCJA	*cat*^{r} *fhuA*$^+$	This work
pRZ540	*km*^{r} *tonB*$^+$	Postle and Good, 1983

Media and growth conditions

LB, SOB, M9 and TB cultures, and TMG dilution media were prepared as previously described (Silhavy et al., 1984). Cells were grown in LB medium and phage stocks were prepared using TB. For the exclusion experiments, cells to be tested were mixed with TB soft agar and overlaid on TB

agar plates. Competent cells were prepared in SOB as described by Hanahan et al. (1991). Phage dilutions were prepared in TMG. When required, media were supplemented with ampicillin (100 μg/ml), kanamicin (50 μg/ml) or chloramphenicol (50 μg/ml). IPTG (2.5 mM) was used to induce gene expression from *p*Trc promoter and X-gal to detect β-galactosidase activity.

Electroporation procedures

Electrotransformation of phage DNA into *E. coli* W3110 [pKINAM] was done as described by Sambrook et al. (1989) with a gene pulse apparatus (Bio-Rad Corp., Richmond, CA).

Plasmid constructions

The mEp167 chromosomal DNA was restricted with *Eco*RI and 11 fragments (from approximately 14,000 to 144 bp) were produced. All these fragments were used in a shotgun experiment, and were cloned in *Eco*RI site of pUC18, and introduced into DH5α cells. From the 14 clones obtained, only three different plasmid restriction fragment phenotypes were observed. Of these, only pT41 conferred resistance to phages mEp167, mEp213, mEp237, and mEp410 infection. pKINAM contains a 254-pb cor_{mEp167} fragment. This was amplified from pT41 by PCR using the oligonucleotides PUC18f (5′-AGC GGA TAA CAA TTT CAC ACA GG-3′) and T41*Hin*dIIIr (5′-GAG GTT TGC ATT CAA AAG CTT GAG TTA TTT AC-3′). The amplified fragment was restricted with *Eco*RI and *Hin*dIII and cloned in the same sites of a pPROEXd [a derivate of pPROEX-1, which contains the *p*Trc promoter region, the 6 histidine (6× His) sequence, the cleavage site for TEV protease, and the multiple cloning sites (Polayes and Huges, 1994)]. The 80-bp DNA-segment that are missing in pPROEXd, include the first G of the GACC*AUG* initiation codon region (the first localized downstream of *p*Trc promoter), until one base more of *Nde*I site (the proceeded G of the recognition site *CATATG* G). DH5α-competent cells were transformed with ligation products and positive candidates were analyzed by *Eco*RI and *Hin*dIII restriction and confirmed by sequencing. Plasmid pUCJA contains the *fhuA* gene, which was amplified by PCR from *E. coli* W3110 chromosomal DNA using oligonucleotides FhuAf (5′-CCC AAG CTT CAG CAA GAG CAG AAA GAC AGC G-3′) and FhuAr (5′-CGG GAT CCG CAA AAG TGG TAT CGG AAT GA-3′). The amplified fragment was restricted with *Hin*dIII and *Bam*HI and cloned in the same sites of pACYC184. DH5α-competent cells were transformed with the ligation products and positive candidates were confirmed by the presence of the *fhuA* insert. The expression of FhuA was confirmed in an in vitro transcription-translation assay performed as previously described (Hernandez-Sanchez et al., 1998).

Restriction, ligation, and DNA sequencing reactions

W3110 DNA was purified following the protocol described by Sambrook et al. (1989). mEp167 Phage DNA was isolated as described by Silhavy et al. (1984). Plasmid DNA was isolated using the alkaline lysis method (Sambrook et al., 1989). Restriction and ligation were done according to manufacturer's specifications (New England Biolabs). DNA was sequenced by the method of Sanger et al. (1977), using the Perkin-Elmer ABI Big-Dye terminator cycle sequencing kit. The same primers were used for PCR amplification and DNA sequencing.

Phage experiments

For phage exclusion experiments, cell cultures were grown at 37 °C with shaking in LB broth. Overnight cultures (300 μl) were mixed with top soft agar (3 ml) and the mixture was poured on a TB-agar Petri dish to form a cell lawn. Phage stock dilutions were dropped and the plates were incubated overnight at 37 °C. For infection and electroporation tests, 2.5 mM IPTG (final concentration) was added to cell cultures. After phage DNA electroporation and conventional infection, the efficiency of infective center formation (EIC) determination was performed by plating infected cells with a sensitive indicator strain as described by Sing and Klaenhammer (1990). For spontaneous phage induction, a fresh colony of lysogen cells was taken and grown in 5 ml LB broth. Ampicillin, 100 μg/ml, and 2.5 mM IPTG were added when it was required. The overnight culture grown at 37 °C was centrifuged at 10,000 × *g* for 10 min, and supernatant phage titers were determined. The adsorption assay was made according to Garvey et al. (1996) with slightly modifications. It was determined by adding 0.5 ml of a late-log-phase culture (10^8 cfu/ml) and 50 μl of a 1M $CaCl_2$ to 0.5 ml of phages (10^7 pfu/ml). Following incubation for 15 min at room temperature, the host-phage mixture was centrifuged at 7000 × *g* for 10 min, and the supernatant was assayed for phage titer. The percentage adsorption was calculated as [(control titer − residual titer) (control titer)$^{-1}$] × 100.

[^{59}Fe]-ferrichrome transport assays

The transport assay was performed as described by Kadner and Heller (1995). Briefly, cells were grown with shaking in M9 medium supplemented with 0.5% glucose and 0.5% casamino acids. Where indicated, 2.5 mM IPTG was also added to induce *cor*. Cells were harvested by centrifugation and suspended at a density of 10^9 cells/ml in M9 medium and 0.1 mM nitrilotriacetic acid. Cells were incubated at 37 °C for 15 min. Fe–ferrichrome uptake was assayed with 0.45 μM $^{59}FeCl_3$ (Amersham, Corp.) and 0.6 μM desferri–ferrichrome (Sigma, Corp.). Fe–ferrichrome complex was made according to Fischer et

al. (1989). Samples were removed at the indicated times, filtered through Millipore membranes and washed twice with 5 ml of 0.1 M LiCl. Filters were dried and the retained radioactivity was measured in a liquid scintillation counter.

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