

## Short-Tailed Stx Phages Exploit the Conserved YaeT Protein To Disseminate Shiga Toxin Genes among Enterobacteria<sup>∇</sup>

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Received 25 May 2007/Accepted 17 July 2007

**Infection of *Escherichia coli* by Shiga toxin-encoding bacteriophages (Stx phages) was the pivotal event in the evolution of the deadly Shiga toxin-encoding *E. coli* (STEC), of which serotype O157:H7 is the most notorious. The number of different bacterial species and strains reported to produce Shiga toxin is now more than 500, since the first reported STEC infection outbreak in 1982. Clearly, Stx phages are spreading rapidly, but the underlying mechanism for this dissemination has not been explained. Here we show that an essential and highly conserved gene product, YaeT, which has an essential role in the insertion of proteins in the gram-negative bacterial outer membrane, is the surface molecule recognized by the majority (ca. 70%) of Stx phages via conserved tail spike proteins associated with a short-tailed morphology. The *yaeT* gene was initially identified through complementation, and its role was confirmed in phage binding assays with and without anti-YaeT antiserum. Heterologous cloning of *E. coli yaeT* to enable Stx phage adsorption to *Erwinia carotovora* and the phage adsorption patterns of bacterial species possessing natural *yaeT* variants further supported this conclusion. The use of an essential and highly conserved protein by the majority of Stx phages is a strategy that has enabled and promoted the rapid spread of shigatoxigenic potential throughout multiple *E. coli* serogroups and related bacterial species. Infection of commensal bacteria in the mammalian gut has been shown to amplify Shiga toxin production in vivo, and the data from this study provide a platform for the development of a therapeutic strategy to limit this YaeT-mediated infection of the commensal flora.**

Shiga toxin-producing *Escherichia coli* (STEC) strains, the most notorious serotype of which is O157:H7, possess a variety of factors that contribute to their pathogenic profiles. The emergence of these strains as serious food-borne pathogens of humans has been multifaceted (55), but the pivotal event in their emergence was undoubtedly acquisition of the ability to produce Shiga toxin, which is also referred to as Shiga-like toxin or verotoxin (19), following infection by a bacteriophage carrying the *stx* genes (36). Stx phages are lambdoid phages that carry two small genes comprising the Shiga toxin operon that encodes a typical AB<sub>5</sub> toxin. Essentially, the only trait that all Stx phages share is carriage of an *stx* operon, which can encode one of two distinct toxin types, Stx1 or Stx2; each type is comprised of multiple variants (3). Stx phages have different morphologies (4, 34, 38, 39), and the exact identities of the genetic modules that make up their mosaic genomes vary, although the general organization of these modules is con-

served. Recombination events within bacterial lysogens are thought to promote bacteriophage mosaicism, which is found among all lambdoid phages (3). Determination of the means by which Stx phages recognize and infect their bacterial hosts has been confounded by this vast heterogeneity.

STEC strains can possess more than one *stx* operon and can be infected with multiple Stx phages (4, 39). Even a single *E. coli* cell can be infected more than once by the same Stx phage or related Stx phages (4). However, no phage that carries more than one *stx* operon has ever been identified. Thus, a multiply Stx phage-infected *E. coli* strain can produce more than one type of Shiga toxin and/or high levels of Shiga toxin, and both of these properties are associated with a greater potential to cause serious life-threatening disease (4). A multiply infected host cell also serves as a reservoir for recombination events in situ, potentiating the creation of novel Stx phages, which may ultimately expand their host range and thus result in the emergence of new toxigenic bacterial strains (4). This could partially explain the heterogeneity of Stx phages and the rapid evolution and spread of STEC and STEC-like disease that has been remarkable since the first discovery in the early 1980s (40).

It was initially proposed that only rough strains of *E. coli*, lacking an intact cell envelope and unable to colonize the gastrointestinal tract, could be infected by Stx phages (50). For this reason, the crucial role of Stx phages in the continuing evolution and spread of STEC and STEC-like disease was largely ignored. However, it has been established more recently that smooth, wild-type strains of *E. coli* can be infected by Stx phages (22, 44) and that ab initio Stx phage infection of *E. coli* can actually take place in the animal gut (14, 15).

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<sup>∇</sup> Published ahead of print on 10 August 2007.

TABLE 1. Strains, plasmids, and bacteriophages used in this study

Strain(s), plasmid, or bacteriophage	Relevant characteristics	Reference(s) or source
<i>E. coli</i> strains		
MC1061		8
DM1187 <sup>Rif</sup>	<i>recA441 lexA51 sfiA</i> , rifampin resistant	22
MC1061-MRL1	Phage resistant	This study
MC1061-MRL2	Phage resistant	This study
MC1061-MRL3	Phage resistant	This study
SG13009		17
M15		QIAGEN
<i>S. flexneri</i> PT2a and PT6		
		22
<i>S. sonnei</i> PT36		
		22
<i>P. luminescens</i> subsp. <i>laumondii</i> TTO1		
	Genome sequencing completed at the Institut Pasteur	Institute Pasteur, Paris France
<i>S. enterica</i> serovar Choleraesuis SC-B67		
	Genome sequencing completed at the Chang Gung Genomic Medical Center, Chang Gung Memorial Hospital	Cheng-Hsun Chiu, Chang Gung Children's Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan
<i>E. carotovora</i> subsp. <i>atroseptica</i> SCRI1043		
	Genome sequencing completed at the Sanger Institute	ATCC BAA-672
<i>C. rodentium</i> ATCC 51459		
		ATCC 51459
Plasmids		
pUC19	Cloning vector, Amp <sup>r</sup>	57
pUC18	Cloning vector, Amp <sup>r</sup>	57
pUCΦR1	pUC19 with a 4.5-kb BamHI chromosome fragment	This study
pUCΦR1A	pUC18 with a 2.5-kb BamHI-KpnI fragment	This study
pUCΦR1B	pUC19 with a 2.0-kb KpnI-BamHI fragment	This study
pUCΦR1C	pUC18 with a 1.9-kb XhoI fragment	This study
pUCΦR1D	pUC19 with a 2.8-kb BamHI-PstI fragment	This study
pQE32	Cloning vector, places six His residues at the amino terminus of a recombinant protein, Amp <sup>r</sup>	QIAGEN
pSCHyaeT	pQE32 possessing <i>yaeT</i> minus the leader peptide coding region	This study
pREP4	Constitutively produces the <i>lac</i> repressor protein encoded by <i>lacI</i>	QIAGEN
Bacteriophages		
φE86654-VT1	Wild-type Stx1-encoding phage	4
φE86654-VT2	Wild-type Stx2-encoding phage	4
φE85539-VT2a	Wild-type Stx2-encoding phage	4
φE83819-VT1	Wild-type Stx1-encoding phage	4
φD155-VT1	Wild-type Stx1-encoding phage	4
φ24 <sub>B</sub> ::kan	Mutant φE86654-VT2, VT operon interrupted with <i>aph-3</i> , lysogens are Kan <sup>r</sup>	22, 42
φ24 <sub>B</sub> ::cat	Mutant φE86654-VT2, VT operon interrupted with <i>cat</i> , lysogens are Cat <sup>r</sup>	4

Originally, it was hypothesized that Stx phages recognized some epitope on the lipopolysaccharide (LPS) of rough *E. coli* strains that was masked in smooth, wild-type strains possessing an intact cell envelope, a concept for which there is a precedent (50). Since it has been shown that the epitope recognized by Stx phages is not masked in smooth strains (22, 44), there must be some other target for adsorption to the host surface. Stx phages are heterogeneous, and many different bacterial species, even members of different bacterial genera belonging to and not belonging to the *Enterobacteriaceae*, have been reported to produce Shiga toxin (4, 18, 22). Therefore, either multiple host proteins are recognized by different Stx phages or the phages must adsorb to a highly conserved gene product on

the bacterial surface. Alternatively, both mechanisms might control recognition of potential host cells.

Here, the host recognition marker that φ24<sub>B</sub>, an Stx phage studied in some detail previously (4, 22, 23, 42), uses to initiate infection is described. We chose to measure phage-host cell recognition directly through adsorption assays rather than relying upon the ability of the Stx phage to produce plaques on indicator cell lawns. This is important when the ability of isogenic mutant host cells or cells of other bacterial strains to support phage binding is examined because the metabolic state of the host cell, the presence of restriction and modification systems, and other factors can significantly affect the ability to detect plaques on bacterial lawns (12, 24, 28, 47, 49).

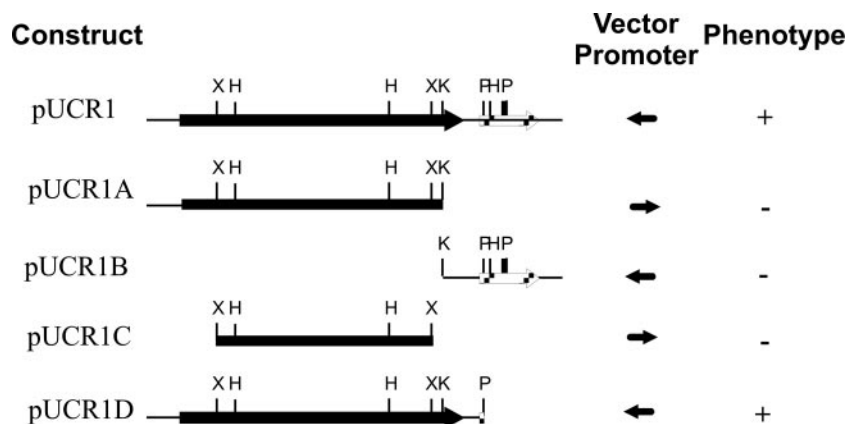


FIG. 1. Subcloning of the open reading frame associated with adsorption of  $\phi$ 24<sub>B</sub> to MRL1. Construct pUCR1 was one of the four original clones that complemented the  $\phi$ 24<sub>B</sub> phage adsorption defect. All four clones shared the two open reading frames present in pUCR1; the solid box represents *yaeT*, and the striped box represents *skp*. When fragments from pUCR1 were subcloned into pUC19 or pUC18 (indicated by the vector promoter direction: leftward, pUC19; rightward, pUC18), only the complete *yaeT* gene found in pUCR1 and pUCR1D complemented the MRL1 adsorption defect. Restriction sites are as follows: X, XhoI; H, HincII; K, KpnI; P, PstI.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and bacteriophages.** *E. coli* K-12 strains MC1061 and DM1187 are susceptible to a variety of Stx phages (4, 22) and were used as hosts for all phage infections, as indicated below. Other bacterial strains, plasmids, and bacteriophages used in this study are described in Table 1. Unless indicated otherwise, cultures were grown in Luria-Bertani Miller broth (1% tryptone, 0.5% yeast extract, 1% NaCl [VWR]) or on plates prepared by addition of 1.5% (wt/vol) agar (Difco). When bacteriophages were used, the growth medium was supplemented with 0.01 M CaCl<sub>2</sub> (LB-Ca). The antibiotics used were kanamycin (50  $\mu$ g ml<sup>-1</sup>; Merck), ampicillin (100  $\mu$ g ml<sup>-1</sup>; Sigma), and chloramphenicol (30  $\mu$ g ml<sup>-1</sup>; Sigma).

**Plaque assay.** *E. coli* MC1061 was cultured to an optical density at 600 nm (OD<sub>600</sub>) of 0.5 in LB-Ca at 37°C with shaking. Aliquots (200  $\mu$ l) were then incubated at 37°C with 200  $\mu$ l of serially diluted phage suspensions. After 30 min, 5 ml of VT top agar (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl, 0.4% [wt/vol] Difco agar, 0.01 M CaCl<sub>2</sub>) was added to each infection mixture, which was then mixed and poured onto LB plates. The plastic petri plates were vented and incubated overnight at 37°C to allow plaques to develop.

**Identification of an Stx phage-resistant mutant.** We used a strategy similar to that of Kilno and Rothman-Denes (26) in which proteins involved in infection with bacteriophage N4 were identified. A 100-ml culture of *E. coli* strain MC1061 was infected at a multiplicity of infection of 0.1 with phage  $\phi$ E86654-VT2 in LB-Ca. The infection was allowed to proceed overnight at 37°C, and then the culture was serially diluted before plating onto LB agar. The majority of cells were lysed during this initial infection step, and while most of the survivors were lysogens, a few cells were found to have lost their susceptibility to Stx phages. This was confirmed experimentally using a phage cocktail consisting of  $\phi$ E86654-VT1,  $\phi$ E86654-VT2,  $\phi$ E85539-VT2a,  $\phi$ E83819-VT1, and  $\phi$ D155-VT1 (4) and parameters identical to those used for the initial infection step. Any colonies were examined for their ability to adsorb phage in order to identify potential phage-resistant mutants.

**Phage adsorption assay.** All bacterial strains were grown to an OD<sub>600</sub> of 0.5 in LB-Ca with shaking at 37°C. Approximately 10<sup>7</sup> PFU ml<sup>-1</sup> phage was added to each culture or LB-Ca alone. The phage were allowed to adsorb to the cell surfaces for 15 min at 37°C. Subsequently, the infection mixtures were centrifuged in a microcentrifuge for 5 min at 5,000 rpm to remove cells and adherent phage. The supernatant was harvested and titrated in a classical plaque assay using the wild-type MC1061 strain as the host. The data from the wild-type strain infection served as the control. The level of phage adsorption was determined as follows: percentage of phage adsorption = [(PFU from LB-Ca control - PFU from sample infection)/PFU from wild-type MC1061 infection]  $\times$  100.

**Identification of *yaeT*.** A genetic library from *E. coli* MC1061 was created following the ligation of partially Sau3AI-digested chromosomal DNA (2- to 5-kb fragments) into BamHI-restricted, dephosphorylated expression vectors pUC18 and pUC19 (Amersham-Pharmacia). This library was transformed into MC1061-MRL1. Transformants were isolated on LB agar containing 100  $\mu$ g ml<sup>-1</sup> ampicillin and then used to inoculate LB-Ca. A phage,  $\phi$ 24<sub>B</sub>::kan (22), was

added to this culture at a concentration of  $2.5 \times 10^9$  PFU ml<sup>-1</sup>, and the infection mixture was incubated at 37°C with shaking (200 rpm). After 5 h, 1-ml portions of the infection mixture were plated onto LB agar containing 50  $\mu$ g ml<sup>-1</sup> kanamycin and 100  $\mu$ g ml<sup>-1</sup> ampicillin. No colonies were isolated. However, when 1 ml of the infection mixture was subcultured into 10 ml LB-Ca and further incubated overnight, 30 colonies ml<sup>-1</sup> were obtained on the selective medium.

**DNA preparations.** Plasmid DNA preparations were made routinely using a QIAprep Spin miniprep kit according to the manufacturer's recommendations (QIAGEN). Chromosomal preparations were made using 2 ml of an overnight culture, the cells of which were harvested by centrifugation and suspended in 600  $\mu$ l of lysing buffer (0.5% [wt/vol] sodium dodecyl sulfate [SDS], 0.1 mg proteinase K [Sigma], 10 mM Tris, 0.5 mM EDTA; pH 8.0) for 1 h at 37°C. The DNA was further purified by cetyltrimethylammonium bromide treatment (100  $\mu$ l of 5 M NaCl, 80  $\mu$ l of 10% [wt/vol] cetyltrimethylammonium bromide in 0.7 M NaCl) followed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1) and a second extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated with 0.6 volume of isopropanol, and RNA was removed by treatment with 50 ng of RNase (Sigma) followed by a second phenol-chloroform-isoamyl extraction and ethanol precipitation (5).

**SDS-PAGE and Western blot analysis.** Three identical SDS-polyacrylamide gel electrophoresis (PAGE) gels were prepared by the method of Laemmli (27), using a 10% separating gel and a 5% stacking gel. One gel was stained with Coomassie blue R250, and the other two were transferred to nitrocellulose membranes (Bio-Rad) using a semidry transfer apparatus according to the manufacturer's (Bio-Rad) instructions. The blots were incubated in blocking buffer consisting of 3.0% (wt/vol) bovine serum albumin in Tris-buffered saline (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) for 1 h at room temperature, which was followed by incubation with the primary antibody, either anti-RGS-His (1:1,000; Clontech) or anti-YaeT (1:50,000; this study) in the same buffer for 1 h at room temperature. The blots were washed twice for 10 min in 20 mM Tris-HCl (pH 7.5)-500 mM NaCl-0.05% Tween 20-0.2% Triton X-100; then one of the blots was incubated in blocking buffer containing anti-mouse alkaline phosphatase conjugate (1:10,000; Sigma), and the other blot was incubated in blocking buffer containing anti-rabbit alkaline phosphatase conjugate (1:10,000; Sigma). The blots were developed using Sigma Fast 5-bromo-4-chloro-3-indolylphosphate (BCIP)—nitroblue tetrazolium tablets according to the manufacturer's instructions.

**Creation of H-YaeT.** The *yaeT* gene from pUC $\phi$ R1D was amplified using primers BamH I YaeT5' (5'-GTA TAC GGT GCT GAA GGG ATC CTA GTG AAA G-3') and yaeT 3'PstI (5'-CIT AGC TTG CAT GCC TGC-3'). These primers were used to amplify the portion of the *yaeT* open reading frame that corresponded to the mature, processed protein product. This was achieved by using Platinum Pfx DNA polymerase (Invitrogen Life Technologies) according to the manufacturer's instructions and the following parameters: denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 68°C for 1.5 min. Primer BamH I YaeT5' has nucleotide substitutions at residues 21 and 24, which result in addition of a BamHI restriction endonuclease site. Cleavage of the amplification product with

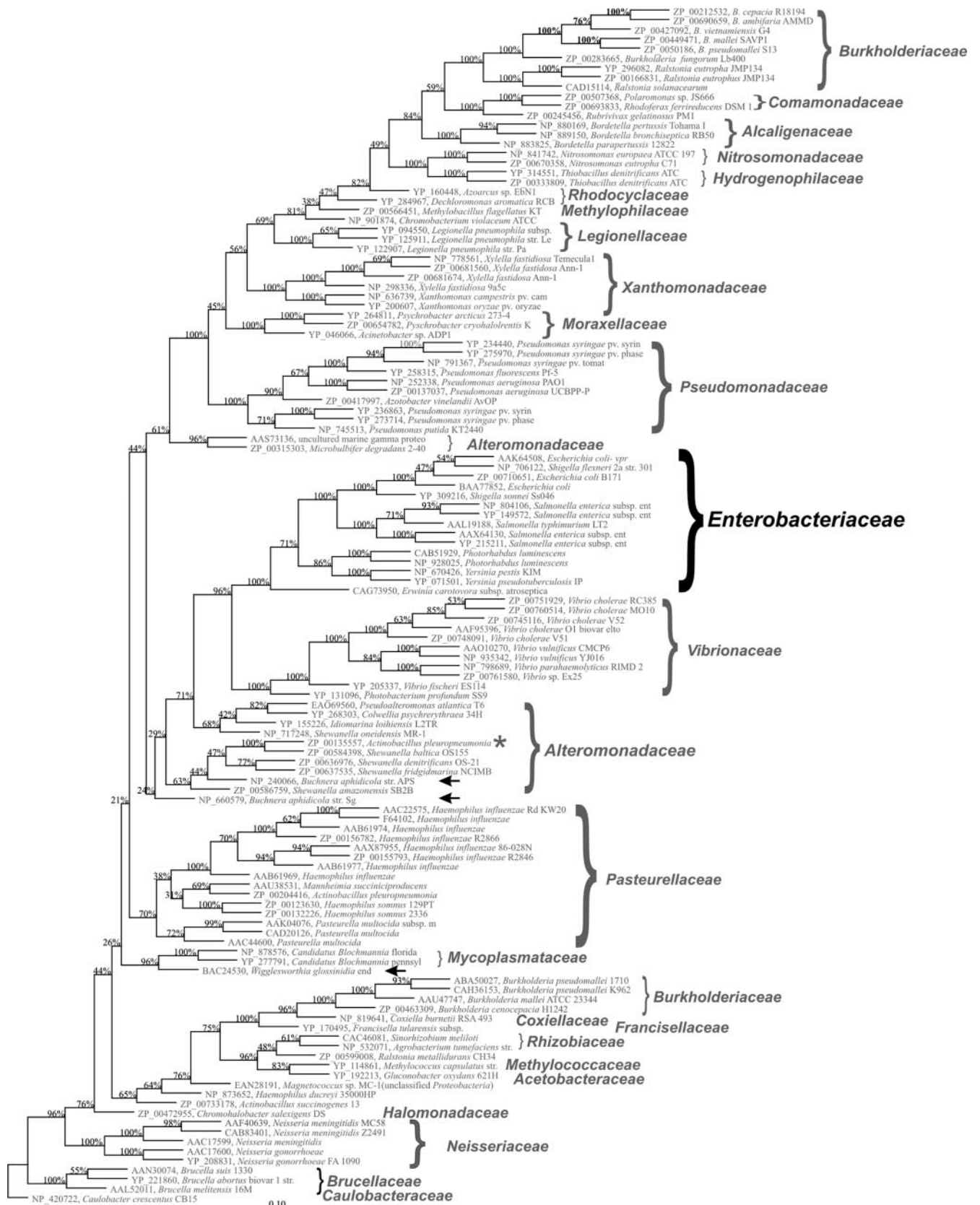


FIG. 2. Phylogenetic tree of YaeT orthologues for most of the gram-negative bacteria for which there are complete genome sequences. Homologues of YaeT were identified by BLASTP analyses and used to identify all homologues of YaeT possessing at least 30% amino acid identity. The sequences were aligned and then subjected to a maximum parsimony analysis using ARB (29). Branches were validated through 100 bootstrap analyses. Scale bar = 1% amino acid changes. The *Enterobacteriaceae* is indicated by larger type, and a few unusual members of this family are indicated by arrows. The asterisk indicates a bacterial species that clusters with an unrelated bacterial family.

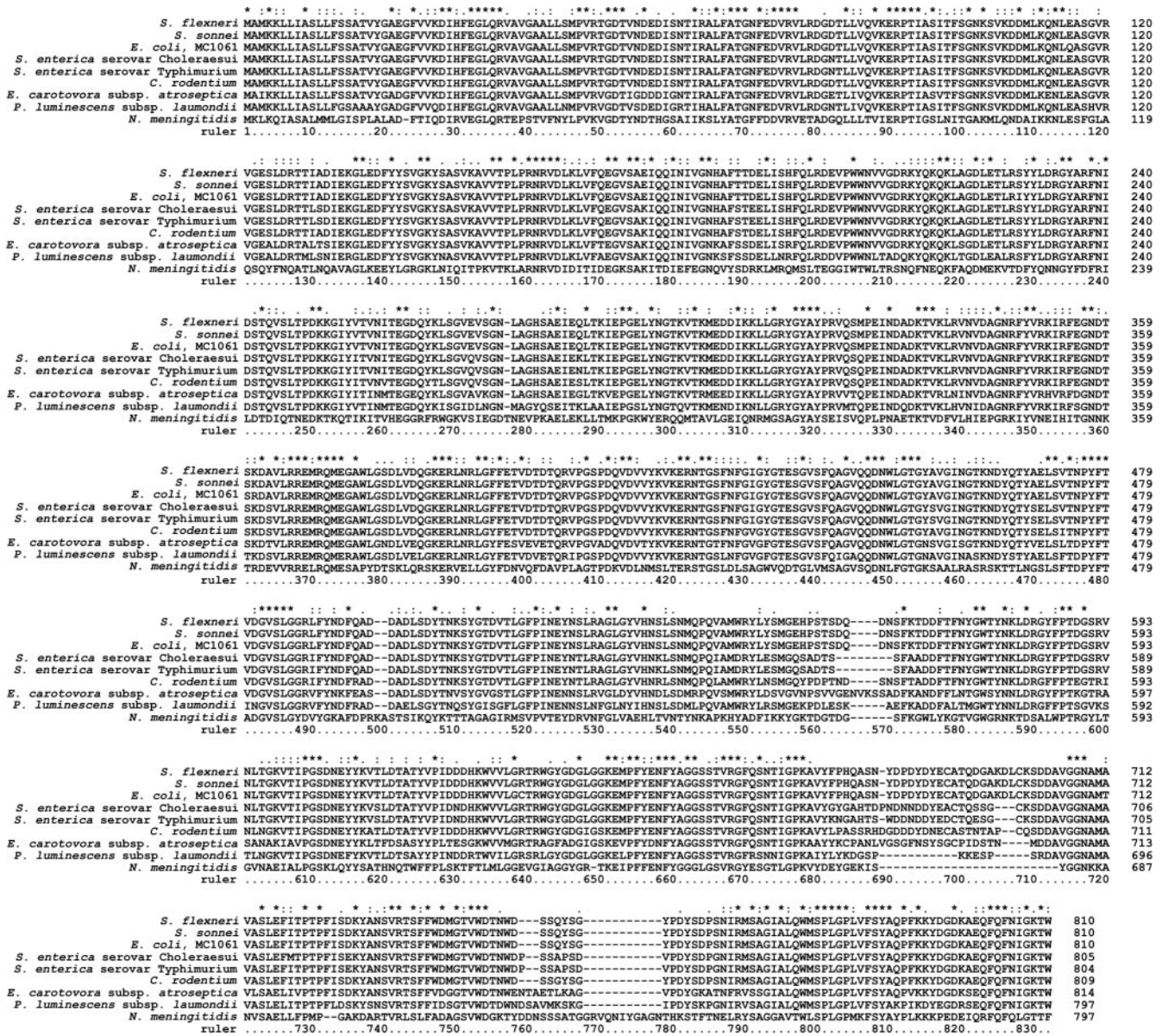


FIG. 3. Multiple alignment of YaeT from members of the *Enterobacteriaceae*. Clustal X (46) was used to align YaeT sequences from eight members of the *Enterobacteriaceae* against the orthologue from *Neisseria meningitidis*, for which a structure has been proposed (51). The alignments were used to identify regions possessing the greatest similarities and disparities; the latter are mostly associated with the carboxyl terminus and correspond to predicted extracellular loops. The conservation of residues is indicated above the alignments as follows: asterisk, complete identity; colon, conservation of a strong group (46); period, conservation of a weak group (46).

BamHI endonuclease removed all sequences encoding the leader peptide of YaeT, and subsequent cleavage with the restriction endonuclease PstI removed all traces of the pUC19 vector. The BamHI-PstI fragment was then cloned into similarly digested pQE32 (QIAGEN) to create plasmid pSCHyaeT, which codes for expression of histidine-tagged recombinant YaeT (H-YaeT). This clone was identified in *E. coli* strain SG13009 carrying plasmid pREP4, which prevents expression from the *lacZ* promoter in the absence of an inducer molecule, by growth on LB agar plates containing ampicillin and kanamycin. Its identity was confirmed by Western blot analysis using the primary antibody RGS-His (QIAGEN) to identify a ~90-kDa histidine-tagged protein. H-YaeT differs from the wild-type processed YaeT in that the sequence of the former begins with MRGSHHHHHHGLVKDIHFE, while the sequence of the latter begins with AEGFVVKDIHFE.

**Purification of H-YaeT protein.** H-YaeT was purified using affinity Ni-nitrilotriacetic acid chromatography (QIAGEN) under denaturing conditions, as spec-

ified by the manufacturer. Briefly, *E. coli* strain SG13009 carrying both pSCHyaeT and pRep4 was grown in 200 ml LB containing both ampicillin and kanamycin to an OD<sub>600</sub> of 0.6. Production of H-YaeT was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were incubated for a further 4 h. The cells were harvested by centrifugation at 4,000 × g for 15 min and suspended in 20 ml lysing buffer (100 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Tris-HCl, 8 M urea; pH 8). Cells were then lysed using a probe sonicator set to an amplitude of 22 μm using four 15-s bursts with intervening 30-s intervals at 0°C. The resultant lysates were centrifuged at 4,000 × g for 25 min, and the supernatant was collected. The lysates were then incubated with 5 ml of the Ni-nitrilotriacetic acid matrix for 1 h at room temperature with shaking. The resulting slurry was then poured into a chromatography column, and the flowthrough was collected for analysis. The column was washed with 8 ml of wash buffer (100 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Tris-HCl, 8 M urea; pH 6.5). His-YaeT was eluted with elution buffer (100 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Tris-HCl, 8 M urea; pH 5.9), and fractions were collected

at 2-min intervals. Samples were subsequently analyzed by SDS-PAGE and Western blotting.

**Protein assay.** Protein concentrations were determined using the bicinchoninic acid assay. Briefly, twofold dilutions of fraction V purified bovine serum albumin (Sigma Chemical Co.) were prepared as standards. These samples, along with dilutions of the Ni<sup>2+</sup>-purified H-YaeT, were incubated with a bicinchoninic acid-CuSO<sub>4</sub> solution (50:1, vol/vol; Sigma Chemical Co.) and incubated for 1 h at 37°C. Concentrations of protein were determined spectrophotometrically at 562 nm.

**Antibody production.** Approximately 5 mg of H-YaeT was purified by nickel affinity chromatography in the denaturing elution buffer. The resulting preparation was sent to Biogenesis (Poole, England), where it was used to inoculate two New Zealand White rabbits using the custom antibody production service of this company.

## RESULTS

In order to investigate the initial steps in the infection of *E. coli* by Stx phage  $\phi$ 24<sub>B</sub>, a strategy similar to that used to identify the host ligand for bacteriophage N4 was adopted (26). Spontaneous Stx phage-resistant mutants were identified following infection of an *E. coli* K-12 strain with a cocktail of Stx phages induced from clinical isolates (4). Each Stx phage was independently capable of infecting the host strain. Three resistant mutants that failed to produce phage particles following UV induction were obtained, and one strain (MRL1), which was later determined to be incapable of supporting the adsorption of  $\phi$ 24<sub>B</sub>, was used to screen an expression library created from the Stx phage-susceptible *E. coli* K-12 strain MC1061. Four clones that conferred phage adsorption potential as well as phage susceptibility to MRL1 were identified, and all four shared a region of identity containing two open reading frames (Fig. 1). Following independent subcloning of the two open reading frames, it was determined that the gene conferring the ability to support phage adsorption, as well as the phage-susceptible phenotype, was *yaeT* (11, 16, 30, 54), also referred to as *yzzN* (2) and *ecfK* (10), while the second gene, *skp*, encoding a periplasmic molecular chaperone, did not confer phage susceptibility. The *yaeT* gene has recently been identified elsewhere as an essential gene in *E. coli* (16) involved in populating the LPS outer membrane with proteins (11, 43, 54, 56).

BLASTP analyses of YaeT identified a homologue in all gram-negative bacterial genome sequences (Fig. 2), predictably in view of its proposed role in biogenesis of the LPS membrane. YaeT has an especially high level of sequence conservation among most members of the *Enterobacteriaceae* (Fig. 3), yet the orthologue in *Erwinia carotovora* subsp. *atroseptica* fell outside the main clade (Fig. 2). The degree of conservation among members of the *Enterobacteriaceae* is particularly apposite considering that many of its members have been reported to produce Shiga toxin, implying that at some time they were infected by an Stx phage (22, 37, 45, 48). It would have been informative to have sequenced the *yaeT* gene possessed by the MRL1 strain, but unfortunately this strain was lost. However, complementation of MRL mutants identified only YaeT, a predicted outer membrane protein, as the potential phage adsorption target. A series of experiments designed to ablate expression of *yaeT* in the wild-type *E. coli* K-12 strain MC1061 were initiated, but these experiments did not produce knockout mutants and predated the discovery that *yaeT* was an essential gene (16). Although a *yaeT* deletion mutant has been described previously (10), it was created only

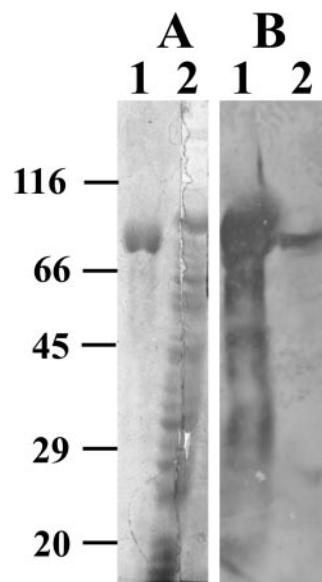


FIG. 4. SDS-PAGE and Western blot identification of purified histidine-tagged recombinant YaeT. (A) Coomassie blue-stained SDS-PAGE gel. (B) Western blot analysis using rabbit anti-H-YaeT (1:50,000). Lane 1, nickel affinity-purified H-YaeT; lane 2, whole-cell lysates of *E. coli* strain MC1061.

after the gene was first duplicated in cells by complementation (D. L. Smith and D. Missiakas, personal communication). Conditional mutants or complementation strategies used to study sigma E-regulated genes (10) were also unsuitable as even short-term expression of *yaeT* would be sufficient to populate the cell surface with YaeT, which would subsequently be able to support phage adsorption. Consequently, the initial mutation and complementation experiments with the MRL mutants served to identify YaeT as a potential receptor for the short-tailed Stx phage, and the experiments described below were designed to provide the formal proof. These experiments centered on the use of an *in vivo* binding assay based on measurement of phage adsorption in the presence and absence of specific YaeT antiserum. This approach was supported by experiments in which the relationship between phage binding and *yaeT* expression and copy number were studied, together with heterologous cloning of *E. coli yaeT* to confer phage adsorption ability on *E. carotovora*. The data were correlated with predicted phage adsorption profiles for other species with natural variations in the *yaeT* sequence.

The amino terminus of YaeT was fused to a histidine tag (H-YaeT) to enable easy purification of recombinant H-YaeT, and monospecific rabbit antiserum was raised against H-YaeT (Fig. 4). This antiserum was capable of recognizing both recombinant and native YaeT in Western blots at a 1:50,000 dilution (Fig. 3B), and it could also be used to block phage adsorption in a dose-dependent fashion (Fig. 5). Preimmune sera from the same rabbit, which recognized many *E. coli* proteins in Western blots at a lower dilution (1:10,000) (data not shown), had no effect in the adsorption assay. Additionally, *yaeT* was fused to a promoterless *lacZ* gene in order to confirm that growth conditions shown to affect *yaeT* expression (10) affected its expression in our strain; phage adsorption

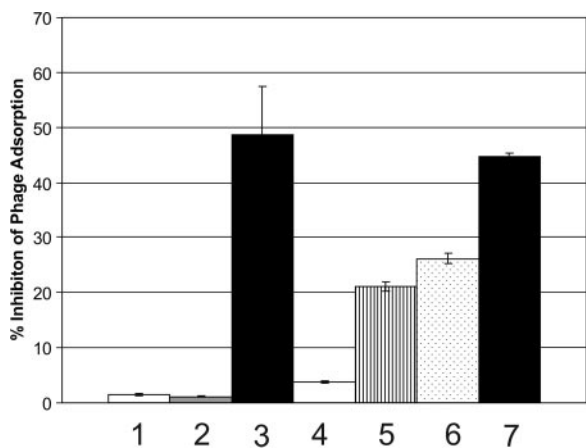


FIG. 5. Inhibition of  $\phi 24_B$  adsorption using rabbit anti-H-YaeT antiserum. A total of  $5 \times 10^7$  MC1061 cells were incubated with no serum (bar 1), rabbit preimmune serum (1:5 dilution) (bar 2), or rabbit anti-H-YaeT serum (1:5 dilution) (bar 3) prior to infection with  $\phi 24_B$  ( $5 \times 10^8$  PFU). The error bars indicate the standard errors of the means ( $n = 15$ ). In a separate experiment, using the same numbers of cells and phage, various dilutions of the anti-H-YaeT serum were added to the cells prior to infection with  $\phi 24_B$  (bar 4, none; bar 5, 1:60 dilution; bar 6, 1:60 dilution; bar 7, 1:6 dilution) The error bars indicate the standard errors of the means ( $n = 5$ ).

levels correlated to the expression levels of  $y_{aeT}$ , and factors such as growth at 42°C caused a twofold increase in expression from  $y_{aeT}$  and also doubled the number of phages that could become adsorbed to a single cell (data not shown). *E. coli* clones carrying *yaeT* on a high-copy-number vector (pUC19 or TOPO vectors [Invitrogen]) also exhibited the ability to adsorb significantly increased numbers of phage (data not shown). All of this evidence indicated that YaeT was acting as the cell surface ligand for phage adsorption, but independent confirmation was still required.

There have been several previous reports of the functional expression of outer membrane proteins that have been exchanged between various members of the *Enterobacteriaceae*,

including *E. coli* and *E. carotovora* (1, 9, 25). The latter species is an unusual member of the *Enterobacteriaceae* in that it is a plant pathogen and is not known to colonize animals. Use of this species as a recombinant host for studying Stx phage adsorption in the laboratory also guards against the potential creation of a new human or animal pathogen. Our adsorption assays demonstrated that phage  $\phi 24_B$  was not capable of adsorption to the surface of *E. carotovora* strain SCRI1043. So, in essence, *E. carotovora* was a potential adsorption-deficient mutant that still possessed a functional orthologue of YaeT. The *E. coli yaeT* gene was introduced into *E. carotovora* carried on the low-copy-number vector pKT230 (6) possessing the RSF1010 broad-host-range replicon, which is known to function in *E. carotovora* (52). Western blot analysis using the antiserum raised against recombinant *E. coli* H-YaeT indicated that the *E. coli* YaeT was expressed in the *E. carotovora* clone carrying pKT230 *yaeT* (Fig. 6). Although the anti-H-YaeT serum was able to recognize the endogenous *Erwinia* YaeT orthologue, its reactivity with *E. coli* YaeT was much greater (Fig. 6B). The ability of  $\phi 24_B$  to adsorb to *E. carotovora* containing pKT230 with or without the cloned *yaeT* gene was determined (Fig. 6C). Adsorption could be observed in a dose-dependent fashion only in the *E. carotovora* strain carrying the *E. coli yaeT* gene, and this adsorption was specifically inhibited with the anti-H-YaeT sera, thus providing formal proof that YaeT serves as the adsorption target for  $\phi 24_B$  infection of *E. coli*.

Mutational studies of essential gene products present challenges, especially if the only available measure of their activity is the ability to support the binding of a ligand once the essential gene product has been appropriately folded and inserted into the LPS outer membrane. In order to better understand the adsorption of  $\phi 24_B$  to YaeT, the natural sequence variation in *yaeT* orthologues across members of the *Enterobacteriaceae* was utilized. Most of the sequence diversity can be found in the carboxy-terminal portion of the YaeT protein (Fig. 3). The model produced by Voulhoux et al. (51) predicted the conformation of Omp85 (a YaeT orthologue) in the *Neis-*

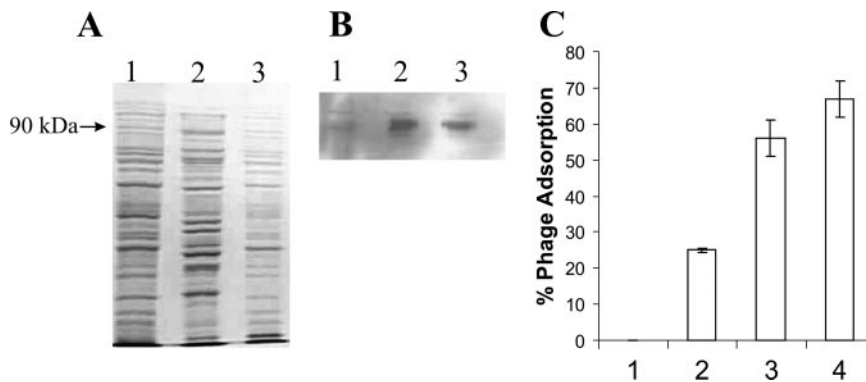


FIG. 6. Complementation of a natural *yaeT* mutant confers the ability to support  $\phi 24_B$  adsorption. (A) SDS-PAGE analysis of total cell protein from *E. carotovora* subsp. *atroseptica* (lane 2), *E. coli* strain MC1061 (lane 2), and *E. carotovora* subsp. *atroseptica* carrying the construct pKT230*yaeT* (lane 3). The region corresponding to 90 kDa is indicated on the left. (B) Western blot analysis of total cell proteins from panel A. (C) Phage binding to YaeT on the surface of *E. carotovora* carrying pKT230*yaeT*. Data were calculated for plasmid-free and pKT230-containing strains challenged with  $3.4 \times 10^7$  phage particles. The x axis indicates different total numbers of *E. carotovora* cells, as follows: bar 1,  $1.5 \times 10^8$  cells bearing pKT230; and bars 2, 3, and 4,  $1.5 \times 10^8$ ,  $3 \times 10^8$ , and  $5 \times 10^8$  cells carrying pKT230*yaeT*, respectively. The error bars indicate the standard errors of the means ( $n = 5$ ).

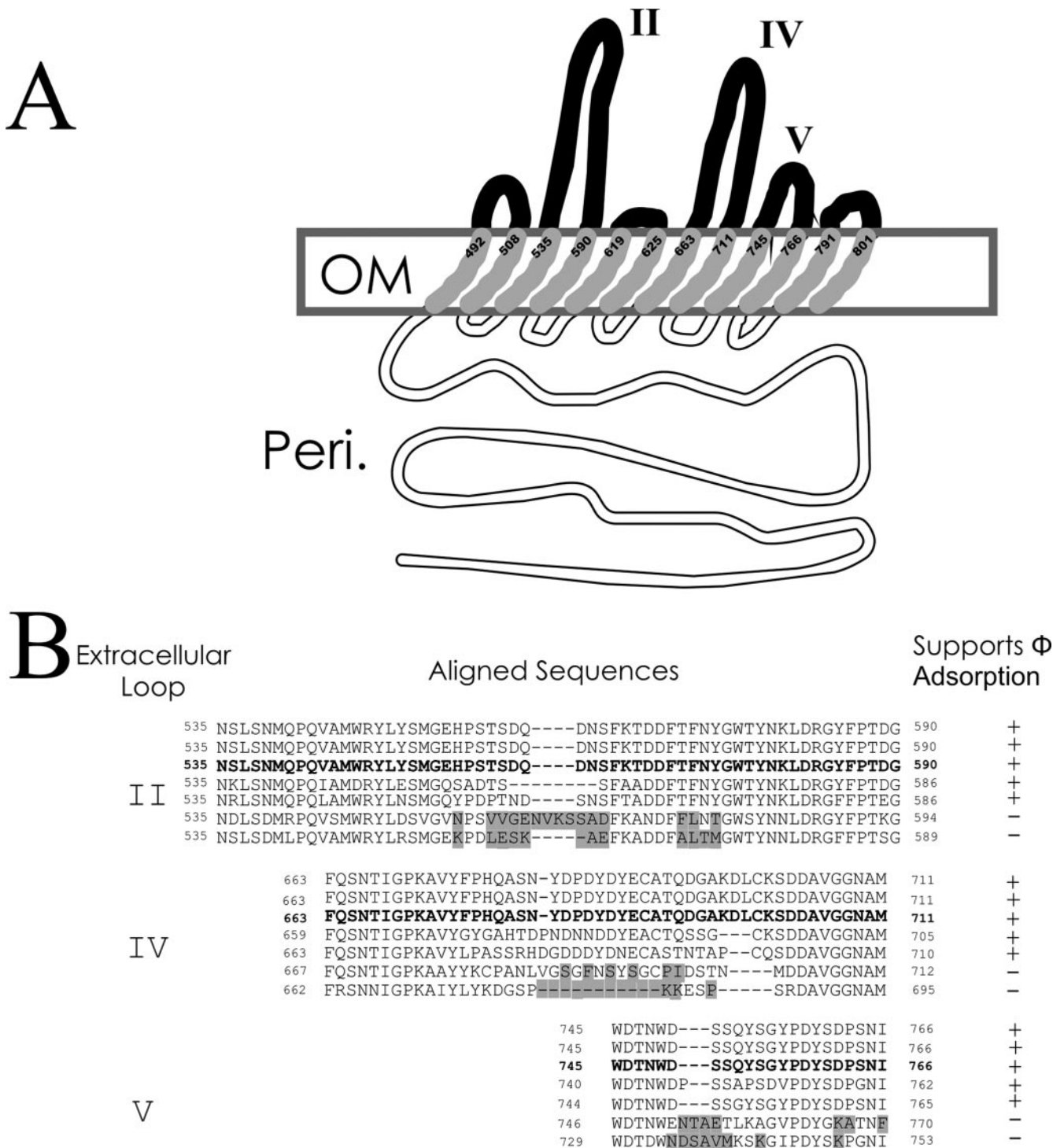


FIG. 7. Relationship between  $\phi$ 24<sub>B</sub> phage adsorption and *yaeT* sequence variation with the predicted protein conformation in *Enterobacteriaceae* strains. (A) Proposed configuration of YaeT in the *E. coli* LPS membrane. The black loops are the predicted extracellular loops; the gray segments are predicted outer membrane (OM)-spanning regions; and the open segments are predicted periplasmic (Peri.) regions of YaeT. The extracellular loops are numbered from the amino terminus. (B) Identification of amino acid substitutions that correlate with the loss of Stx phage adsorption ability. The highlighted sequences are the changes predicted to be responsible for the loss of the ability to support phage adsorption. The sequences are sequences of, from top to bottom, *S. flexneri*, *S. sonnei*, *E. coli* strain MC1061, *S. enterica* serovar Choleraesuis strain SC-B67, *C. rodentium* strain ICC168, *E. carotovora* subsp. *atroseptica* strain SCR11043, and *P. luminescens* subsp. *laumondii* strain TTO1.



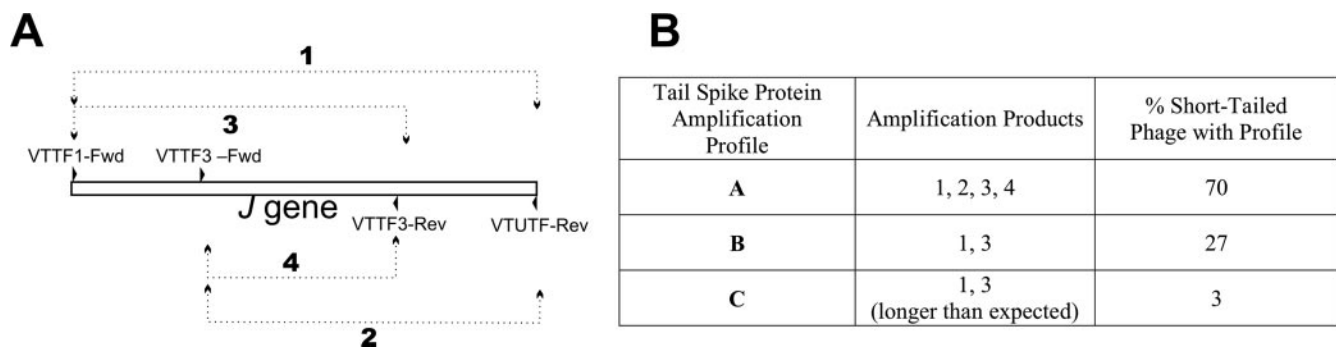


FIG. 8. Variations in tail spike proteins that recognize YaeT as an adsorption target. Amplification primers were designed against the gene sequence encoding the tail spike protein (the *J* gene orthologue) of  $\phi$ 24<sub>B</sub> and 933W (VTTF1-Fwd, GTGTTGTTTCGGGGACG; VTUTF-Rev, TCATTCTCCTGTCTGCC; VTTF3-Fwd, TGCAGAGGAAAGCTCGAC; VTTF3-Rev, GCAGCCTCTTCTGCCTT). The primers were used in combinations of VTTF1-Fwd with VTUTF-Rev or VTTF3-Rev and of VTTF3-Fwd with VTUTF-Rev or VTTF3-Rev (combinations 1, 3, 2, and 4, respectively). All variations of tail spike proteins identified by this means were capable of adsorption to *E. coli* MC1061, and this adsorption could be blocked with the anti-YaeT sera. Three different amplification profiles were found for the short-tailed phages listed in the table in panel B. The majority (70%) of phages possessed amplification profile A, which results from the production of all four amplification products of the anticipated size. Profile C was found only rarely and is characterized by the failure of the VTUTF-Rev primer to produce any products in combination with either 5' primer, while the products of VTTF3-Rev were ~800 bp larger than expected.

seria LPS membrane and is used here to propose a model for the *E. coli* YaeT molecule (Fig. 7). The majority of unconserved residues in the YaeT orthologues across the *Enterobacteriaceae* are associated with regions that are predicted to be extracellular loops and specifically the middle sequences of those loops (Fig. 7). In order to investigate which orthologues of YaeT could support phage adsorption and thus identify important sequences for  $\phi$ 24<sub>B</sub> adsorption, assays were carried out with *Shigella flexneri* strains PT2a and PT6, *Shigella sonnei* strain PT36, *E. coli* strain MC1061, *Salmonella enterica* serovar Choleraesuis strain SC-B67, *Citrobacter rodentium* strain ICC168, *E. carotovora* subsp. *atroseptica* strain SCRI1043, and *Photobacterium luminescens* subsp. *laumondii* strain TTO1 (Fig. 6). Only *E. carotovora* subsp. *atroseptica* and *P. luminescens* subsp. *laumondii* were unable to support  $\phi$ 24<sub>B</sub> adsorption. The greatest degree of sequence diversity is found in the central sections of extracellular loops II, IV, and V, and thus predictions concerning the important residues in  $\phi$ 24<sub>B</sub> adsorption can be made (Fig. 7), although additional experiments are necessary to prove these predictions. The anti-H-YaeT serum was capable of blocking  $\phi$ 24<sub>B</sub> adsorption to all of the susceptible strains (data not shown).

DISCUSSION

It is widely accepted that lambdoid bacteriophages are genetic mosaics, and the Stx phages are no exception. In fact, the only single attribute known to be shared by all Stx phages is the carriage of the genes encoding some variant of Shiga toxin (3). Thus, the impact of the identification of a ubiquitous host receptor for the short-tailed Stx phage  $\phi$ 24<sub>B</sub> is dependent on the relative frequency of this phage type among circulating populations of Stx phages. A total of 76 Stx phages were induced from a collection of 460 *E. coli* O157:H7 strains that were obtained from a large farming area over a 5-year period in Cheshire, United Kingdom (41). Most (70%) of the induced Stx phages are *Podoviridae* (short tail, icosahedral head), like 933W (35) and  $\phi$ 24<sub>B</sub> (4). The predominance of short-tailed

*Podoviridae* among Stx phages isolated directly from the environment has also been reported previously (13, 33). Examination of the tail spike sequences from the induced Stx phages, as well as 933W,  $\phi$ 24<sub>B</sub>, and short-tailed phages in the DNA sequence databases (GenBank accession numbers BAB87868, NP\_612899, NP\_050557, NP\_286993), NP\_309255, and NP\_859099), confirmed that all of these phages possess tail spike proteins with identical or highly conserved sequences (Fig. 8). Through examination of the short-tailed Stx phage genomes that are available, it is clear that they possess only the tail spike protein for host recognition, as does bacteriophage lambda for recognition of its potential host cells (7, 31). The ability of all of the short-tailed phages in our collection to adsorb to the surface of *E. coli* K-12 strain MC1061 was demonstrated, as was inhibition of binding in competition assays with anti-H-YaeT (data not shown). Thus, YaeT is likely to be an Stx phage recognition site of considerable importance to the epidemiology of Shiga toxin dispersal among populations of *E. coli* and possibly gram-negative bacteria in general.

There have been previous attempts to identify the host ligand recognized by Stx phages (21, 32, 53), using long-tailed or short-tailed phages. It is clear that long-tailed Stx phages, such as H19-B (35), PP01 (32), and P27 (38), recognize other host-encoded surface molecules, and there are data that support the use of surface ligands, such as OmpC (52). The data on short-tailed phage adsorption sites have been more difficult to interpret (35). The approach reported here directly determines adsorption of the phage to the host surface, in contrast to other studies that have examined the ability of the phage to infect a host cell, propagate, and infect surrounding cells to a level sufficient to produce plaques on bacterial lawns. Many contributing factors can affect the overall ability of a phage to elicit a productive infection and form plaques, and measuring phage adsorption is the most direct and appropriate technique for identifying phage-host binding ligands.

We propose that the recognition of YaeT and its orthologues among the members of the *Enterobacteriaceae* has contributed to the rapid evolution of STEC and STEC-like patho-

gens. Although there is considerable genetic heterogeneity among Stx phages, our data suggest that the large majority share an identical tail spike protein and thus the ability to recognize susceptible host cells in the same fashion, via YaeT. The use of a highly conserved essential gene product as the receptor for adsorption by a phage is likely to ensure that host bacterial cells will always be available in the gut or other environments that support *Enterobacteriaceae* populations. The function of YaeT and its conserved structure ensure that it will always be present on the surface of potential host cells and that susceptible bacterial populations will always be present in the gut environment. This differs fundamentally from the other phage adsorption targets, which do not provide an essential function to the cell. Mutations or conditions that abrogate production of the phage adsorption target are very unlikely to occur, so spread of the bacteriophage should be enhanced according to our current understanding of herd immunity concepts (20). Together then, the ability of Stx phage to convert its host cell into a deadly human pathogen and its ability to infect related strains or species by utilizing a conserved surface receptor underlie the rapid evolution, emergence, and expansion of STEC and STEC-like pathogens, which have echoed throughout the scientific literature since the first *E. coli* O157:H7 outbreak in 1982. These abilities also support the view that STEC and STEC-like pathogens will continue to evolve as the short-tailed Stx phages direct conversion of additional susceptible bacterial strains. For example, we have determined that pathogenic strains of *S. enterica* can support the adsorption of the short-tailed Stx phages (Fig. 7), with corresponding inhibition by anti-H-YaeT (data not shown), and this was predicted by the conservation of the *yaeT* allele with that of *E. coli* (Fig. 3 and 7). Salmonellae have yet to be linked to Stx phage-related disease, but there has been a report of a member of the *Moraxellaceae* (*Acinetobacter haemolyticus*) carrying an inducible Stx phage, producing Stx2, and associated with a case of bloody diarrhea in an infant (18). Understanding how Stx phages recognize commensal bacteria in the human gut should provide a platform for a therapeutic strategy aimed at limiting the potentiation of Stx production via phage conversions in the infected intestine, and the Stx phage recognition site, YaeT, provides a possible target. Expression levels of *yaeT* have been previously shown to fluctuate with environmental conditions (10), especially those that affect the integrity of the outer membrane. As *yaeT* expression is controlled by the sigma E stress regulon (10), it would not be unreasonable to expect the expression of this gene to be up-regulated in the mammalian gut environment, and this would facilitate the infection of susceptible commensal gut bacteria (14, 15) and possibly explain why infection with an Stx2-encoding phage was more successful in a porcine ileal loop model than under in vitro laboratory conditions (47).

In addition to elucidating a mechanism for Stx phage recognition of potential host cells, the antisera, phages, and YaeT variants described in this study constitute a set of tools that should be useful for studying the essential role that YaeT plays in populating the outer membrane with proteins, as well as for identifying the exact epitope(s) recognized by the phage tail-spike protein.

## ACKNOWLEDGMENTS

We thank Sarah S. Cookson for her contribution to the production of the anti-YaeT sera. We also thank the Institute Pasteur and Cheng-Hsun Chiu at the Chang Gung Children's Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan, for the kind provision of sequenced bacterial strains.

This research was supported by the Biotechnology and Biological Sciences Research Council (United Kingdom), the Department of Environment Food and Rural Affairs (United Kingdom), and the Higher Education Funding Council for England (grants to H.E.A., J.R.S., and A.J.M.). The contributions of the Natural Environment Research Council to M.J.S. and of the former Centre for Applied Microbiology Research Porton Down to studentships for M.J.S. and C.E.J. are gratefully acknowledged.

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