

NOTE

Contribution of a Novel Gene, *rpeA*, Encoding a Putative Autotransporter Adhesin to Intestinal Colonization by Rabbit-Specific Enteropathogenic *Escherichia coli*[∇]

Denisse L. Leyton,¹ Louise M. Adams,² Michelle Kelly,¹ Joan Sloan,¹ Marija Tauschek,² Roy M. Robins-Browne,^{2,3} and Elizabeth L. Hartland^{1,3*}

Australian Bacterial Pathogenesis Program, Department of Microbiology, Monash University, Victoria 3800,¹ Australian Bacterial Pathogenesis Program, Department of Microbiology and Immunology, University of Melbourne, Victoria 3010,² and Microbiological Research Unit, Murdoch Children's Research Institute, Royal Children's Hospital, Parkville 3052,³ Australia

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Rabbit-specific enteropathogenic *Escherichia coli* (REPEC) is an attaching and effacing pathogen of young rabbits. Using signature-tagged mutagenesis, we identified several known colonization factors of REPEC as well as a gene predicted to encode a novel autotransporter protein. This novel gene was termed *rpeA* for REPEC plasmid-encoded autotransporter.

Attaching and effacing (A/E) pathogens induce distinctive lesions on the host intestine that are characterized by localized destruction of intestinal microvilli, intimate attachment of the bacteria to the host cell surface, and rearrangement of cytoskeletal proteins beneath tightly adherent bacteria (5). Rabbit-specific enteropathogenic *Escherichia coli* (REPEC) is an A/E pathogen of weanling rabbits that induces A/E lesions indistinguishable from those induced by the human pathogens enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (1, 14, 17). REPEC-induced disease shows the same clinicopathological features and age and tissue specificity as human infection with EPEC, and as such, REPEC is a useful small animal model for studying the contribution of specific virulence factors to colonization of the host and development of diarrhea (17). In addition to the locus for enterocyte effacement (LEE), strains of REPEC also produce fimbriae that are necessary for colonization, although these vary among different strains and serotypes (4, 20). REPEC O15:H– strain 83/39 produces Ral (rabbit adherence locus) fimbriae, which are encoded by the 95-kb plasmid pRAP (1).

The aim of this study was to identify novel colonization factors in A/E pathogens, using REPEC as a model organism. We used signature-tagged mutagenesis (STM) to screen a library of REPEC O15:H– strain 83/39 transposon mutants simultaneously for loss of virulence in weanling rabbits (Microscience, Wokingham, Berkshire, United Kingdom) (10). Ten pools of 21 mutants were used to infect two 5- to 6-week-old New Zealand White rabbits per pool by oral gavage. Each rabbit received 2 ml of inoculum containing approximately $6 \times$

10^6 CFU each of the 21 different mutants pooled from individual cultures grown statically in Penassay broth at 37°C overnight. The optical density at 600 nm of each mutant was checked, and mutants displaying in vitro growth defects were excluded from the screen. The viable count of the inoculum strain was determined by retrospective plating of serial dilutions onto LB agar supplemented with kanamycin. DNA was isolated from the remaining inoculum for amplification of input pool DNA. Infected rabbits were examined daily for 7 days and monitored for body weight and evidence of diarrhea, such as loose stools and soiling of the hind limbs. Stool samples were taken from the rabbits daily using sterile rectal swabs inserted approximately 2 cm into the anus, and the rabbits were killed on day 7 after infection. Collected stools and duodenum, jejunum, ileum, cecum, and colon scrapings were emulsified in 0.5 ml phosphate-buffered saline and plated on MacConkey agar supplemented with rifampin and kanamycin. More than 10,000 colonies were used to extract output pool DNA at day 5 and day 7. Tags from input and output pools were amplified by PCR using primers P2 and STM-R2 (Table 1). Amplified input and output tags were each spotted in duplicate onto Hybond N⁺ membranes (Amersham Biosciences, Buckinghamshire, United Kingdom), and a digoxigenin-labeled 40-bp oligonucleotide homologous to one of the 21 signature tag sequences was then used to simultaneously probe input and output pools. Twenty-one different hybridizations were performed to detect the 21 tags. Tags that were present in the input pool but absent in the output pool denoted putative attenuated mutants. A derivative of REPEC 83/39, the REPEC *Δeae* intimin mutant (12), carrying tag H7 was included in each input pool as a negative control and was not recovered from any of the 10 output pools. Twenty-seven mutants that showed weak or absent hybridization signals in the output pool compared to those in the corresponding input pool

* Corresponding author. Mailing address: Department of Microbiology, Monash University, Victoria 3800, Australia. Phone: (61) 3 9905 4323. Fax: (61) 3 9905 4811. E-mail: Liz.Hartland@med.monash.edu.au.

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TABLE 1. Oligonucleotide primers used in this study

Primer	Nucleotide sequence ^a
P2	5'-TACCTACAACCTCAAGCT-3'
STM-R2	5'-CAAACCGTTATTCATTTCG-3'
<i>rpeA</i> -F	5'-CTTACACGTGGCACTAG-3'
<i>rpeA</i> -R	5'-CAGTGATCGCTGAAGGAG-3'
<i>ral</i> -F	5'-ATGGATAAGCGTACGCC-3'
<i>ral</i> -R	5'-AATACTAACTGGTAGGG-3'
Tn5F	5'-CAGGCATGCAAGCTTCG-3'
Tn5R	5'-GGTACCGAGCTCGAATT-3'
<i>prpeA</i> -F	5'-CGGGATCCCGATCTCGAACAACATTTA CACG-3'
<i>prpeA</i> -R	5'-CGGGATCCCGTTACTTGTCTCATCGT CTTTGTAGTCGAACATATTCAAAC CAAG-3'
pKD4 <i>rpeA</i> -F	5'-ATGAACAAGATTTTTTCACTTAAATTT AGCACCCTTCTGGTGGTTTAAATGCA TGTGTAGGCTGGAGCTGCTTCG-3'
pKD4 <i>rpeA</i> -R	5'-TCAGAACTCATATTTCAAACCAAGAAT TCCAACAGTATCGGTTTTGAAGTCACC ACCATATGAATATCTCCTTA-3'
<i>rall</i> -F	5'-CTGGTACTGGGAAATTAC-3'
<i>rpeA</i> -RT	5'-GAAGCAATATGGCGTCG-3'
<i>ralG</i> -F	5'-CAAATTCAGTTATTGGTAC-3'
<i>ralH</i> -R	5'-CCATTTGGAGCCTCATC-3'

^a Engineered restriction sites are underlined, and pKD4 sequences are italicized.

were identified (Table 2). Marker rescue of the transposon insertion sites of these mutants identified six known LEE-encoded virulence determinants, including *escV*, *escD*, *rorf8*, *map*, *espB*, and *tir*. In addition, we identified three insertions into the *ral* operon and one insertion into another fimbrial gene, designated z2203, in the genome of EHEC O157:H7 EDL933. Other insertions were found in genes encoding putative cell wall components, regulators, and several hypothetical proteins (Table 2). One novel gene identified by this screen was predicted to encode a member of the serine protease autotransporters of *Enterobacteriaceae* (SPATE) family of autotransporters, and we termed this gene *rpeA* for REPEC plasmid-encoded autotransporter.

A fosmid gene library was constructed from REPEC 83/39 to obtain nucleotide sequence information about *rpeA* and flanking DNA (Copy Control, Epicenter, Madison, WI). *rpeA* comprised a 3,684-bp open reading frame flanked by genes that code for hypothetical proteins (Fig. 1). Sequencing of fosmid DNA also revealed that *rpeA* is located downstream of the *ral* operon and is therefore carried by the virulence plasmid pRAP (1). *rpeA* was predicted to encode a 1,228-amino-acid precursor protein with a molecular mass of 135 kDa. Although slightly smaller than other reported SPATE proteins, RpeA exhibited a significant degree of amino acid sequence identity with several SPATE proteins, including Hbp, Tsh, Pic, SepA, EpeA, and the serine protease adhesins App from *Neisseria meningitidis* and Hap from *Haemophilus influenzae* (Table 3) (2, 7, 9, 13, 16, 18). Amino acid sequence analysis showed that, similar to other SPATE proteins, RpeA comprised an extended N-terminal leader peptide signal sequence that possessed a conserved extension beginning with an MNKI(F/Y)S LK(F/W/Y)S motif followed closely by a second motif, GLIA VSELAR, comprising conserved aromatic and hydrophobic residues. The C-terminal β domain of the predicted *rpeA* prod-

TABLE 2. Transposon insertion sites of REPEC 83/39 mutants attenuated for colonization

Group and strain(s)	Disrupted gene or homologue	Putative function ^a
LEE		
83E7	<i>escV</i>	T3SS
81F9	<i>escD</i>	T3SS
54F5	<i>rorf8</i>	T3SS
81E6	<i>map</i>	T3SS effector
28A7	<i>espB</i>	T3SS translocator/effector
91A6	<i>tir</i>	T3SS effector
Fimbriae		
81F5	<i>ralD</i>	Fimbrial usher
91H5	<i>ralH</i>	Minor fimbrial subunit
81H9	<i>rall</i>	Minor fimbrial subunit
28F5	z2203	Fimbrial usher
Cell wall		
28E1	<i>yaiP</i>	Glycosyltransferase
91F9	<i>waaD</i>	Lipopolysaccharide synthesis
91H9	z1190	Glycosyltransferase
54F9	<i>nlpA</i>	Predicted lipoprotein
67F12	<i>nlpC</i>	Predicted lipoprotein
Regulation		
81A8	<i>afrR</i>	Transcriptional activator
91E1	z3632	Sensor histidine kinase similar to EvgA
Metabolism		
80E1	<i>ydiF</i>	Acetoacetyl-coenzyme A transferase
67A6	<i>cadB</i>	Lysine/cadaverine antiporter
91A10	<i>lysP</i>	Lysine specific permease
54A6	<i>gltB</i>	Glutamate synthase
Other		
54H9	z0853	Hypothetical protein
67F5	<i>ydfQ</i>	Hypothetical protein
91F7, 28A3	z1419, z1420	Hypothetical proteins
28H2	b1168	Putative membrane protein
81H5	<i>rpeA</i>	Putative autotransporter

^a T3SS, type III secretion system.

uct also exhibited a conserved 3-amino-acid motif, (Y/V/I/F/W)-X(E)-(F/W), which is essential for outer membrane localization of Hap (8). RpeA differed from other SPATE proteins, however, in that it did not have a characteristic C-terminal domain cleavage site located between two asparagine residues. This may indicate that the RpeA passenger domain is not cleaved from the outer membrane and instead remains attached to the bacterial surface, similar to the autotransporter Hia from *Haemophilus influenzae* (19). Alternatively, RpeA may employ a mode of cleavage different from those of other SPATE proteins. Although we made several attempts to express epitope-tagged RpeA consistently in REPEC 83/39, this was unsuccessful (data not shown). In addition, we produced several forms of recombinant RpeA, but all were insoluble, and antibodies generated to the insoluble protein did not recognize native RpeA from REPEC strains. Therefore, we were unable to confirm localization of the protein in REPEC or

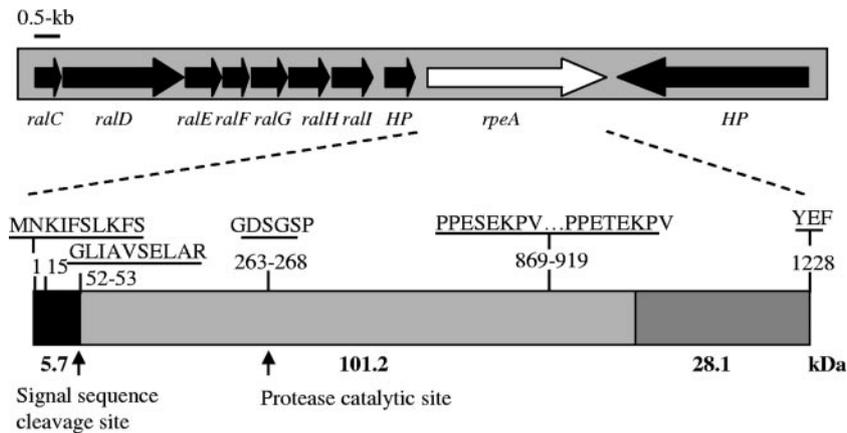


FIG. 1. Schematic representation of a segment of pRAP containing *rpeA*. The white arrow represents *rpeA* (3,684 bp), and the dark arrows represent other open reading frames carried by this fragment. HP is hypothetical protein. The domain organization of RpeA is indicated below the fragment and shows the N-terminal cleavage site predicted by the SignalP method and two N-terminal conserved motifs as well as the serine protease motif, the proline-rich tandem repeat in the passenger domain, and the conserved YEF motif at the C terminus. The predicted molecular mass of each domain is indicated in kDa.

perform functional assays. Nevertheless, the putative passenger domain of RpeA possessed a potential serine protease motif, GDSGSP, which was conserved in sequence and location in comparison to other SPATE proteins, suggesting that the protein may have protease activity (Fig. 1) (6).

We used primers specific for *rpeA* (*rpeA*-F and *rpeA*-R) to determine the distribution of this gene among REPEC strains and other *E. coli* pathogens by PCR. We found that *rpeA* was present in 7 out of 10 REPEC strains tested, and 5 of these 10 strains also carried *ral* (Table 4). The *ral* operon was detected using the primer pair *ral*-F/*ral*-R (Table 1). All REPEC strains that carried *ral* also carried *rpeA*. As *rpeA* is a novel gene, we also tested its prevalence among other *E. coli* pathogens, including 25 strains of EPEC, 25 strains of EHEC, and 5 strains each of enteroaggregative *E. coli*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, and *Shigella flexneri*, by PCR; however, results for all were negative (data not shown). Therefore, *rpeA* appears to be present predominantly in strains of REPEC and could not be detected in the other pathotypes of *E. coli* tested here.

Nucleotide sequence analysis suggested that *rpeA* may be transcriptionally linked to the *ral* operon (Fig. 1). To determine if *rpeA* was expressed in the same operon as *ral*, we extracted whole-cell RNA from two different serotypes of REPEC, namely, wild-type REPEC O15:H– strain 83/39 and

wild-type REPEC O103:H2 strain E22. Bacteria were grown to mid-log phase, preparations were treated with DNase, and RNA was purified using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. This was followed by a second treatment with DNase. *rpeA* and *ral* expression was then analyzed by reverse transcription (RT)-PCR. Random hexamers and Superscript II RNase H– reverse transcriptase (Invitrogen) were used to generate cDNA from purified RNA. PCR of cDNA and a negative control lacking reverse transcriptase was performed with primer pairs *rpeA*-F/*rpeA*-R, *ralG*-F/*ralH*-R, *rall*-F/*rpeA*-R, and *rall*-F/*rpeA*-RT (Table 1). Primers overlapping *ralG* and *ralH* as well as *rall* and the start of *rpeA* were used to determine whether *ral* and *rpeA* expression was transcriptionally coupled. In both REPEC E22 and REPEC 83/39, *ralG* and *ralH* were expressed from the same mRNA whereas *rpeA* expression was independent of the most distal *ral* gene, *rall* (Fig. 2).

Loss of the *rpeA* transposon mutant 81H5 (REPEC *rpeA*::mini-Tn5 Km2) from output pools during our STM screen implied that this derivative of REPEC 83/39 was defective for intestinal colonization. To determine whether *rpeA* contributed to adherence in vitro, we constructed a defined nonpolar deletion mutant of *rpeA*, using the λ Red recombi-

TABLE 3. Shared amino acid sequence similarity between RpeA and its closest relatives

Protein	GenBank accession no.	Major reported function	Length (amino acids)	% Amino acid identity for:			
				Entire protein	Leader sequence	Passenger domain	β domain
Hbp/Tsh	CAA11505	Hemoglobin binding protein/hemagglutinin	1,377	37	48	39	24
Pic	AAK00464	Mucinase	1,371	34	42	37	22
SepA	CAC05786	Invasion	1,364	33	39	35	23
EpeA	AY258503	Mucinase	1,359	32	48	33	22
App	CAC1467	Adhesin	1,449	28	20	31	20
Hap	AAX87307	Adhesin	1,436	28	16	31	20

TABLE 4. Prevalence of *ral* and *rpeA* among different strains of REPEC

Strain	Serogroup	Presence (+) or absence (-) of:		
		<i>eae</i> ^a	<i>rpeA</i>	<i>ral</i>
REPEC 83/39	O15:H-	+	+	+
REPEC RDEC-1	O15:H-	+	+	-
REPEC 83/146	O153:H7	+	+	+
REPEC 84/110/1	O103:H2	+	+	-
REPEC B10	O103:H2	+	-	-
REPEC 82/123	O109:H2	+	+	+
REPEC 82/90	O132:H2	+	+	+
REPEC 82/260	O20:H7	+	-	-
REPEC 82/183	O128:H2	+	-	-
REPEC E22	O103:H2	+	+	+

^a Marker for the LEE pathogenicity island.

nase system and primers pKD4*rpeA*-F and pKD4*rpeA*-R (Table 1) (3). Fluorescence actin staining of HeLa cells infected with the REPEC $\Delta rpeA$ mutant showed that the mutant still carried a functional LEE pathogenicity island capable of inducing actin polymerization (data not shown) (11). Quantitative adherence assays comparing wild-type REPEC 83/39 and REPEC $\Delta rpeA$ were performed as described previously (15) but revealed no significant difference in the abilities of the two strains to adhere to HeLa cells (data not shown). Since REPEC 83/39 produces several adhesins, the expression of one or all of these may mask the activity of RpeA in vitro (11, 14). Therefore, to determine if RpeA possessed host cell binding activity in the absence of other adhesins, we tested the ability of poorly adherent *E. coli* strain XL1-Blue to adhere to HeLa

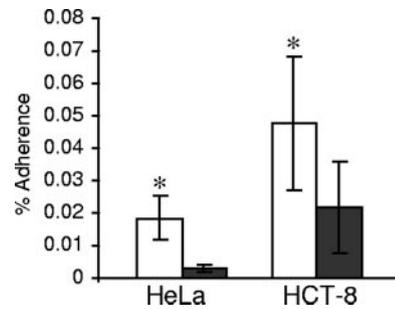


FIG. 3. Quantitative adherence of *E. coli* XL1-Blue carrying pRpeA (white bars) and *E. coli* XL1-Blue carrying pUC18 (black bars) to HeLa and HCT-8 cells. Results are expressed as percentages of the original inoculum recovered from quadruplicate wells and are the means for at least four independent experiments. *, adherence significantly greater than that of *E. coli* XL1-Blue carrying pUC18 ($P < 0.05$; unpaired, two-tailed *t* test).

cells and HCT-8 cells when expressing RpeA from a plasmid. To construct pRpeA, *rpeA* from REPEC 83/39 was amplified using the primers *rpeA*-F and *rpeA*-R (Table 1). The final product was cloned into the BamHI site of pUC18 to generate pRpeA, which was subsequently introduced into *E. coli* XL1-Blue. The results showed that *E. coli* XL1-Blue carrying pRpeA was significantly more adherent for both cell types than *E. coli* XL1-Blue carrying pUC18 alone (Fig. 3), indirectly suggesting by genetic studies that RpeA possessed some host cell binding activity.

To assess further the role of *rpeA* in adherence and colonization of rabbits, we next tested the ability of the defined nonpolar $\Delta rpeA$ mutant of REPEC 83/39 to colonize weanling

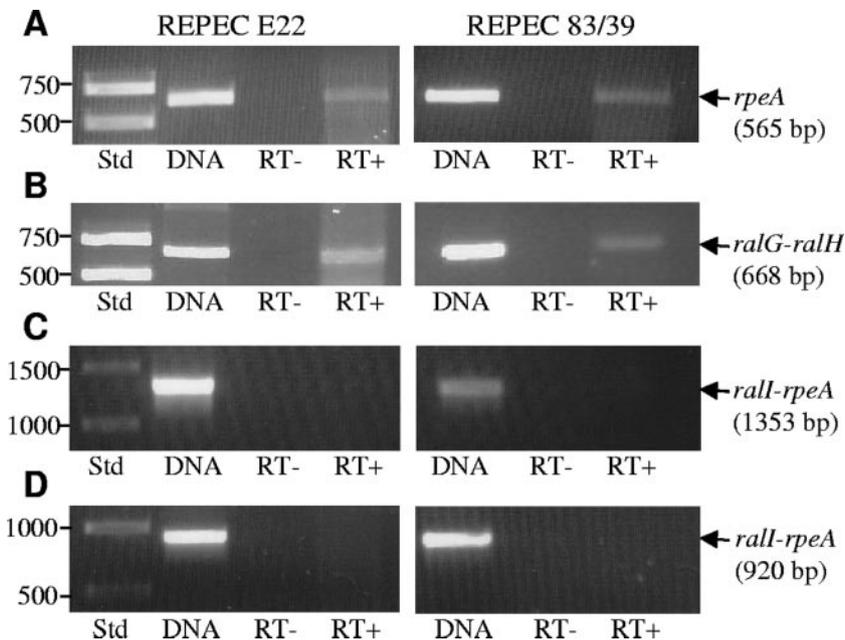


FIG. 2. Analysis of *ral* and *rpeA* expression by RT-PCR in wild-type REPEC strains E22 (left panels) and 83/39 (right panels). (A) Expression analysis of *rpeA* using primers *rpeA*-F and *rpeA*-R, internal to *rpeA*. (B) Expression of *ralG-ralH* using primers *ralG*-F and *ralH*-R. (C) Expression analysis of *rall-rpeA* using primers *rall*-F and *rpeA*-R, yielding a 1,353-bp product. (D) Expression analysis of *rall-rpeA* using primers *rall*-F and *rpeA*-RT, yielding a 920-bp product. Std indicates DNA size markers shown in base pairs; DNA indicates the PCR control; RT- indicates RT-PCR in the absence of reverse transcriptase; RT+ indicates PCR from cDNA.

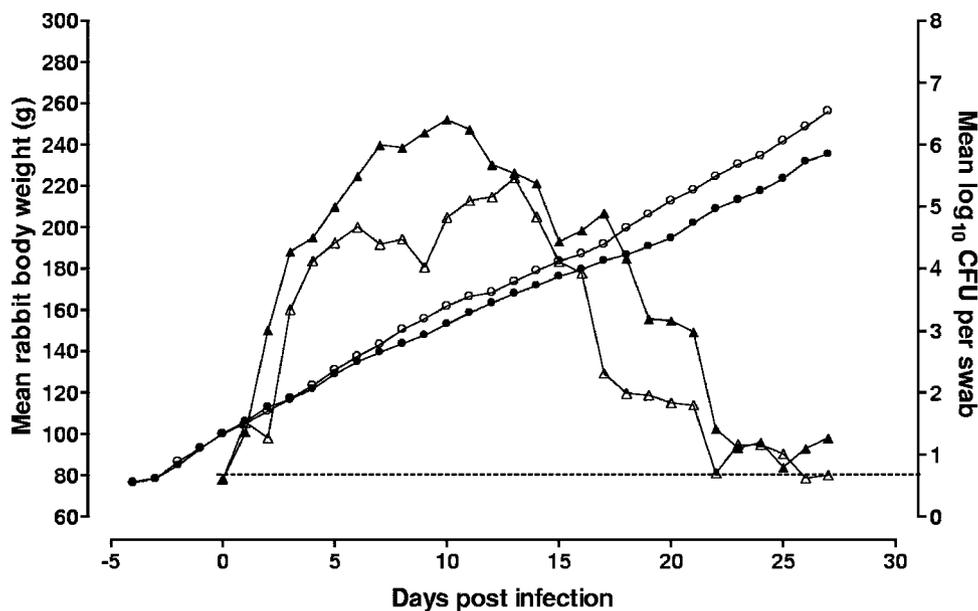


FIG. 4. Mean weights (circles) and mean \log_{10} numbers of CFU per swab (triangles) of rabbits infected with wild-type REPEC 83/39 (solid shapes) and the REPEC $\Delta rpeA$ mutant (open shapes). Animals were weighed daily, and fecal swabs were taken daily for 27 days after inoculation. The dotted line indicates the CFU detection limit.

rabbits in a single infection. Two groups of six rabbits received 6×10^6 CFU each of wild-type REPEC 83/39 and REPEC $\Delta rpeA$. Rectal swabs from animals inoculated with wild-type REPEC 83/39 and the REPEC $rpeA$ mutant were taken each day over a 27-day period to assess levels of colonization, and animals were weighed daily to monitor changes in body weight, which indicates the severity of REPEC-induced disease. The $\Delta rpeA$ mutant was recovered in lower numbers on 21 of 27 days, took longer to colonize the rabbits, and was cleared more quickly than wild-type REPEC 83/39 (Fig. 4). This lower bacterial burden translated into slightly higher weight gain for rabbits infected with the $\Delta rpeA$ mutant than for those infected with wild-type REPEC 83/39 (Fig. 4). Overall, rabbits were colonized to significantly higher levels with wild type REPEC 83/39 ($\geq 10^6$ CFU on 28 of 162 group days) than with the $\Delta rpeA$ mutant ($\geq 10^6$ CFU on 7 of 162 group days; $P = 0.0002$; Fisher's exact test, two-tailed). These results indicated that when tested with a single infection, $rpeA$ was found to be essential for full colonization of weanling rabbits by REPEC 83/39. In addition, these findings corroborated results from the STM screen where the $rpeA$ transposon mutant 81H5 was not detected in output pools by day 7 when samples were taken for the STM screen.

Concluding remarks. We have shown previously that REPEC 83/39 requires both *ral* and *eae* to colonize the rabbit intestine (12). In this study, the construction of a defined non-polar mutant of $rpeA$ showed that this novel gene also plays a role in intestinal colonization, albeit a more minor one than either *eae* or *ral*. The $rpeA$ mutant took longer to colonize rabbits, was cleared more quickly than wild-type REPEC 83/39, and did not achieve the high bacterial numbers associated with REPEC 83/39 infection. These results explain the identification of $rpeA$ through our STM screen, in which colonization-defective mutants present in lower numbers 7 days after infec-

tion were identified by weak or absent hybridization signals in output pool DNA.

The predicted product of $rpeA$ is the latest member of the autotransporter family of proteins and appears most closely related to serine protease adhesins from gram-negative pathogens. The presence of $rpeA$ in a pathogen of rabbits provides an opportunity to study the role of autotransporter adhesins in a small animal model of intestinal colonization. In particular, it will now be possible to assess the contributions of specific domains of autotransporter adhesins, such as the proline-rich region and the serine protease motif, to virulence in a natural infection model.

Nucleotide sequence accession number. The $rpeA$ nucleotide sequence presented in this study was assigned GenBank accession number AY552473.

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