CexE Is A Virulence Factor of *Citrobacter rodentium* And Present In Enteric Pathogens Of Humans

Running title (54 chars including spaces): CexE Is A Virulence Factor of *C. rodentium*

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Abstract (250 words)

CexE is a 12 kDa protein that was originally reported to be present in just three strains of enterotoxigenic *Escherichia coli* (ETEC); a frequent causes of diarrheal illnesses worldwide. However, an examination of recently sequenced genomes has revealed that CexE is actually present in a majority of ETEC strains. Homologs of CexE are also present in enteroaggregative *E. coli* (EAEC) and other enteric pathogens including *Yersinia enterocolitica*, *Providencia alcalifaciens*, and *Citrobacter rodentium*. CexE and its homologs are expressed within virulence regulons of ETEC, EAEC, and *C. rodentium*. This, along with its distribution across several species of enteric pathogens, suggest that CexE confers a selective advantage to these pathogens. However, this hypothesis has yet to be tested *in vivo*. Here we demonstrate that CexE is conditionally secreted to the external leaf of the outer membrane of ETEC. Although CexE does not appear to play a role in adherence *in vitro*, it does facilitate colonization of murine intestinal tissues by *C. rodentium* *in vivo*. In adult mice wild-type bacteria reached significantly higher loads and were shed in higher numbers than a *cexE::kan* mutant. A similar trend was observed in neonatal mice. In addition, all of the neonates infected with the wild-type strain succumbed to infection within 16 days of inoculation. In contrast, 45% of the neonates infected with the *cexE::kan* strain survived for the 30 day

duration of the experiment. These finding indicate that CexE is a conditionally secreted virulence factor that increases the colonization of hosts by enteric pathogens.

Introduction

Approximately half a million infants and children perish from diarrheal diseases every year (1). For citizens of low-income countries diarrheal diseases are projected to remain among the ten leading causes of death through 2030 (2). Of the many viral and bacterial pathogens that cause diarrhea enterotoxigenic *Escherichia coli* (ETEC) is one of the most frequent causes with at least 280 million people sickened annually; most in low-income nations (3). ETEC causes diarrhea by elaborating enterotoxins that disrupt the normal flow of ions across the apical membranes of enterocytes. This disruption leads to a net loss of water into the lumen of the intestinal tract resulting in profuse watery diarrhea (4). In some instances ETEC induced diarrhea results in severe dehydration that can result in death if not adequately addressed.

Although enterotoxins are the penultimate cause of diarrhea, ETEC must also colonize the gastrointestinal tract to cause disease. Colonization is itself dependent upon the expression of pili that function as adherence factors facilitating the direct attachment of ETEC to enterocytes (5, 6). Although ETEC are heterogeneous and there are numerous pilus serotypes, many are positively regulated at the level of transcription by the ETEC virulence regulator Rns (CfaD) (7, 8). In vitro DNase I footprinting studies have shown that each Rns activated pilin promoter contains a Rns DNA binding site immediately upstream of the promoter's -35 hexamer (9–11). In most cases the promoter proximal binding site is usually accompanied by one or more additional Rns binding sites further

upstream. However site directed mutagenesis studies have shown that occupancy of the promoter proximal site has the greatest contribution to promoter activation (9, 11). An in silico analysis of the genome of ETEC strain H10407 -a strain that has been used in several human challenge studies- led to the identification of two Rns binding sites upstream of *cexE* (12–14). As with pilin promoters, one of the binding sites was located immediately upstream of the -35 hexamer of *cexE*_p and the other further upstream. As expected from the positions of its binding sites, Rns was found to activate the expression of *cexE*. Although *cexE* does not encode a pilin nor is it required for the expression of pili, its regulation by the virulence regulator Rns implies that *cexE* encodes a virulence factor. However options to test this hypothesis are limited by the fact that H10407 is strictly a human pathogen that does not naturally infect mice nor other laboratory animals. Fortunately a homolog of *cexE* is present in the genome of *Citrobacter rodentium* (15). Because this natural murine pathogen is a well-developed model for enteric infections, we used murine *C. rodentium* challenge studies to interrogate the function of CexE *in vivo* (16).

RESULTS

CexE is broadly distributed across ETEC strains. CexE was initially identified as a member of the Rns regulon by an in silico search of the genome of ETEC strain H10407 for Rns binding sites (14). Since no other ETEC genomes were publicly available at that time, it was unknown if CexE was restricted to a few isolates or more broadly distributed amongst ETEC strains. However in the intervening years over 200 ETEC genomes have been sequenced and deposited in NCBI's databases; most as whole genome shotgun (WGS) sequences. Therefore to gauge the distribution of CexE across ETEC

strains we ran iterative BLASTN searches of NCBI's databases to identify genes with
homology to *cexE*. After each search the most distal member was used as the query for
a subsequent search until no additional members were found. BLASTP searches were
not effective because automated annotation pipelines apparently have a high failure
rate with regards to the identification of *cexE* alleles. The reasons for this are unclear as
these genes are open reading frames of ca. 360 bp; most with recognizable Shine-
Dalgarno sequences upstream of ATG start codons.

Of the 250 strains that were identified as ETEC –either explicitly in the record definition
lines or by the presence of genes encoding the heat-labile (LT) and/or heat-stable (ST)
enterotoxins– 76% were found to harbor *cexE* alleles. The prevalence of *cexE* alleles is
similar to the genes encoding LT and ST which are present in 64% and 78% of strains
identified as ETEC respectively. As with the enterotoxin genes, the prevalence of *cexE*
alleles suggest a selective advantage for their distribution and maintenance amongst
heterogeneous ETEC strains. The 190 alleles of *cexE* encode eleven distinct
polypeptides (Fig. 1). Hereafter these will be distinguished from each other by Greek
letter subscripts starting with CexE_α that was first identified by Pilonieta et al. in 2007
(14). Sequences of each are presented in S. Fig. 1. The amino terminus of each
polypeptide contains a putative signal peptide which suggest each polypeptide is
transported to the periplasm (17, 18). Subsequent cleavage of the signal peptide from
CexE_α by a periplasmic signal peptidase has been confirmed experimentally (14).

FIG 1

CexE homologs are prevalent amongst ETEC and a subset of other enteric pathogens.

Proteins from ETEC strains are designated with a single Greek letter. Dispersin is from EAEC strain 042. The abbreviations Cr, Pa, and Ye indicate sequences found in *C. rodentium*, *P. alcalifaciens*, and *Y. enterocolitica* respectively. Ec NCTC86 is the strain of *E. coli* that was originally isolated by Theodor Escherich in 1885. The phylogram was constructed using the maximum likelihood method on a multiple sequence alignment generated by the MUSCLE algorithm; both of which were run within the MEGA7 software package (19).

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94 With the lone exception of CexE_ψ all variants of CexE from ETEC strains clustered

95 within the same clade. Within the CexE_ψ clade are two homologs that were found in

60% of the 162 *Yersinia enterocolitica* strains in NCBI's WGS databases. In contrast to the situation in ETEC there is little variation between *Y. enterocolitica* strains; 93% of the polypeptides are identical to Ye_α found in a strain of *Y. enterocolitica* (ERL04757) that was isolated from human blood (20). Other species of *Yersinia* apparently lack CexE homologs. A CexE homolog is also present in the two complete genomes of *Citrobacter rodentium* as well as in one isolate of *Providencia alcalifaciens*. The former is a murine enteric pathogen while the latter has been described as an emerging enteric pathogen of humans (21–23). Notably the *P. alcalifaciens* homolog resides within a clade of ETEC proteins while the *Y. enterocolitica* and *C. rodentium* homologs are more closely related to dispersin of enteroaggregative *E.coli* (EAEC) (Fig. 1). Also within the dispersin clade is CexE_ψ from ETEC as well as a polypeptide from *E. coli* strain NCTC86. The latter was isolated by Theodor Escherich in 1885 as he sought to identify the cause of neonatal dysentery (24, 25).

CexE_α is a conditional coat protein in ETEC. Dispersin of EAEC has an amino terminal signal peptide and enters the periplasm through the general secretory pathway (18, 26). In a second translocation event dispersin crosses the outer membrane but remains associated with the outer membrane as a coat protein through electrostatic interactions (26). To determine if CexE_α and CexE_{Cr} are also coat proteins proteinase K was used as a membrane impermeable probe of their subcellular distribution. Robust expression of the target proteins dictated the culture media that were used. When ETEC was cultured in CFA CexE_α was well expressed but was not digested by proteinase K unless the outer membrane was permeabilized by Triton/EDTA (Fig. 2A). Quantification of multiple immunoblots relative to DnaK –a cytoplasmic protein that was not degraded

even when the outer membrane was permeabilized– revealed that there was no significant degradation of CexE α in the absence of Triton/EDTA (S. Fig. 2A). Thus, CexE α is localized in the periplasm when ETEC is cultured in CFA medium. Strikingly, proteinase K digested > 98% of CexE α when ETEC was grown in IMDM even in the absence of outer membrane permeabilization (Fig. 2A, S. Fig. 2A). A western blot of TCA precipitated IMDM supernatants found no detectable CexE α in the extracellular milieu (S. Fig. 3). These results demonstrate that translocation of CexE α across the outer membrane is influenced by culture conditions and that when it is secreted it remains predominately associated with the outer membrane. In contrast, CexE $_{Cr}$ -FLAG was not translocated across the outer membrane when *C. rodentium* was cultured in IMDM nor LB (Fig. 2B). It is unlikely that the < 3 kDa FLAG epitope tag prevented the translocation of CexE $_{Cr}$ because fusion of the 30 kDa TEM-1 β -lactamase enzyme to the carboxy terminus of CexE α did not prevent its translocation across the outer membrane of ETEC (S. Fig. 4). Given the conditional secretion of CexE α a more plausible explanation for the absence of CexE $_{Cr}$ translocation is that the in vitro conditions that were tested were not conducive to its translocation.

FIG 2

Culture medium influences the subcellular distribution of CexE α . Western blots of whole cell lysates of ETEC (H10407) or *C. rodentium* (GPM1830) in which the outer membrane was either left intact or permeabilized with Triton X-100 and EDTA prior to addition of proteinase K. Permeabilization was required for the digestion of CexE α when ETEC was grown in CFA but not IMDM. This indicates that CexE α is exposed on the surface of ETEC when the pathogen is cultured in the latter medium but remains within the bacterial envelope when ETEC is cultured in CFA. Likewise FLAG tagged CexE_{Cr} was retained within the envelope of *C. rodentium* when the murine pathogen was cultured in LB. Unlike with ETEC, IMDM did not result in translocation of CexE_{Cr} across the outer membrane. Lane numbering corresponds to quantification presented in S. Fig. 2. Abbreviations: Tri., Triton X-100; Prot. K, proteinase K; MW, molecular weight ladder; Ab, antibody specificity controls. Antibody specificity control lanes were loaded with whole cell lysates of ETEC strain GPM1163

(*cexE_α::kan*) or *C. rodentium* strain DBS100 which do not produce CexE_α or CexE_{Cr}-FLAG respectively.

CexE_α and CexE_{Cr} do not significantly modulate adherence. After finding that CexE_α can associate with the outer face of the outer membrane we next sought to determine if it has an effect on adherence. Although we observed that a *cexE_α::kan* mutant was slightly more adherent to HCT-8 cells than WT ETEC, the difference was not statistically significant (Fig. 3A). Likewise there was no significant difference between WT *C. rodentium* and a *cexE_{Cr}* mutant (Fig. 3B). However, the latter result was expected because CexE_{Cr} is not a coat protein under these conditions (Fig. 2B). In contrast, an insertion in *rns*—which abolishes expression of both CexE_α and the adhesive CFA/I pilus—significantly decreased adherence of the mutant compared to both the WT and *cexE_α::kan* strains of ETEC. These results suggest that CexE_α does not significantly modulate the adherence of ETEC even when the pathogen is cultured in IMDM; a condition in which CexE_α coats the outer membrane.

FIG 3

CexE_α and CexE_{cr} do not significantly modulate adherence to mammalian cells.

Adherent bacteria were enumerated after a 1 hr infection of HCT-8 cells in IMDM 10% FBS. **(A)** The difference between WT ETEC (GPM1168) and a *cexE_α::kan* (GPM1163) mutant was not statistically significant. In contrast, a mutant (GPM1236) lacking the Rns virulence regulator was significantly impaired in its ability to adhere to HCT-8 cells. **(B)** As with ETEC strains, the difference between WT *C. rodentium* (GPM1831) and a *cexE_{cr}::kan* mutant (GPM1827) was not statistically significant. **P* < 0.0001 by Tukey's multiple comparisons test.

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149 **CexE_{cr} is a virulence factor *in vivo*.** Although we did not observe secretion of CexE_{cr}

150 *in vitro* we considered it plausible that CexE_{cr} is a virulence factor because its

151 expression is regulated by the virulence regulator RegA (27). Its presence in a natural

murine pathogen is also fortuitous because there is no small animal model to evaluate the function of dispersin in EAEC. In addition the murine ETEC model has several limitations due to the fact that ETEC does not normally colonize mice (28). Thus we chose *C. rodentium* for our *in vivo* studies because it is the most appropriate of the three pathogens for murine infection studies. To determine whether or not CexE_{Cr} is a virulence factor we orogastrically inoculated C57BL/6 mice with *C. rodentium* strain GPM1831a or GPM1827 (*cexE_{Cr}::kan*). GPM1831a carries a kanamycin cassette in an intergenic region downstream of *cexE* and was considered our WT strain. On each of the three selected days after inoculation the infected animals shed fewer *cexE_{Cr}::kan* than WT bacteria (Fig. 4). Although the difference was not statistically significant on day three it was significant on days six and ten post inoculation. In addition mice shed greater numbers of both strains as the infection progressed. For WT *C. rodentium* this was expected based on previous *in vivo* studies (16). These results were not restricted to C57BL/6 mice as similar results were observed when 129X1/SvJ mice were used (S. Fig. 5). Not surprisingly, the differences in fecal shedding reflected the differences between the two strains within the intestines (Fig. 5).

FIG 4

Fecal shedding of WT *C. rodentium* exceeds that of a *cexE_{cr}* mutant. C57BL/6 mice were orogastrically inoculated with 10^8 CFUs with either WT (GPM1831a) or a *cexE_{cr}::kan* (GPM1827) mutant. Fecal pellets were collected on days 3, 6, and 10 post inoculation and CFUs were normalized to the mass of each pellet. Circles represent fecal pellets of separate animals; $n \geq 11$ mice per group. * $P < 0.05$ by Mann-Whitney U test.

FIG 5

Intestinal loads of mice infected with a *cexE_{cr}* mutant are lower than those infected with WT *C. rodentium*. C57BL/6 mice were orogastrically inoculated with 10⁸ CFU of either WT *C. rodentium* (GPM1831a) or a *cexE_{cr}::kan* mutant (GPM1827). Tissues were harvested 10 days after inoculation. CFUs were normalized to sample mass. The differences between the two strains of bacteria were significant in both the **(A-B)** small intestine and its contents as well as in the **(C-D)** large intestine and its contents. **P* < 0.005 by Mann-Whitney U test, n ≥ 11 mice per group.

170 To determine if the effect of CexE_{Cr} was age-dependent or -independent we also
 171 inoculated infant C57Bl/6 mice with both strains of *C. rodentium*. As with adult animals
 172 the loads of both strains increased as the infection progressed and significantly more
 173 WT than mutant bacteria were recovered from the large intestines of infant mice; for
 174 technical reasons the small intestines were not analyzed (Fig. 6). Although
 175 immunocompetent adult mice are able to clear *C. rodentium* infections, all infant mice
 176 infected with the WT strain perished (Fig. 6)(29). In contrast a significant number of the
 177 mice infected with the *cexE_{Cr}::kan* strain survived for the duration of the experiment. In
 178 aggregate these results demonstrate that *C. rodentium* uses CexE_{Cr} to achieve higher
 179 intestinal burdens and increase its virulence in both adult and infant mice.

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FIG 6

Infant mice infected with mutant *C. rodentium* had reduced colonization of their large intestines and enhanced survival compared to those infected with WT *C. rodentium*. Fifteen-day old infant C57Bl/6 mice were infected with 10^7 CFU of WT *C. rodentium* (GPM1831a) or a *cexE_{Cr}::kan* mutant (GPM1827). **(A)** *C. rodentium* colon titers, normalized to tissue mass, were determined at 2, 4, and 10 days post inoculation. * $P < 0.05$ ** $P < 0.005$ by Mann-Whitney U test. **(B)** In a parallel group of animals survival was monitored out to 30 days post inoculation. ≥ 10 animals were analyzed in two separate experiments. *** $P = 0.0005$ by log rank (Mantel-Cox) test.

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Discussion

A given isolate of *E. coli* that causes diarrheagenic disease in humans is often categorized as one of five pathovars; enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroinvasive *E. coli* (EIEC). A number of characteristics are used to distinguish these pathovars from one another including disease etiology, patterns of adherence, and repertoires of virulence genes. However this classification system belies the fact that isolates within each category may display considerable heterogeneity and originate from different phylogenetic lineages (30–32). Heterogeneity is particularly prevalent amongst ETEC isolates which is often considered an evolving pathogen. Thus, it is not surprising that although a majority of ETEC strains carry *cexE* alleles a minority do not. However, the fact that 76% of ETEC genomes harbor a *cexE* allele suggest a selective advantage to the gene's acquisition and maintenance. Moreover, *cexE* homologs have been acquired by another *E. coli* pathovar –EAEC– as well as three other species of enteric pathogens; *P. alcalifaciens*, *Y. enterocolitica* and *C. rodentium*. Overall these observations suggest that CexE and its homologs contribute to the pathogenicity of some gram-negative enteric pathogens.

ETEC, EAEC, and *P. alcalifaciens* do not naturally infect mice; therefore, we considered them unsuitable to evaluate the role of CexE and its homologs in vivo. In contrast both *Y. enterocolitica* and *C. rodentium* are capable of infecting mice including when administered orogastrically; arguably the most relevant route for enteric pathogens. Of the latter two pathogens we chose *C. rodentium* for our in vivo studies for the simple reason that we were unable to obtain an isolate of *Y. enterocolitica* that harbors a *cexE*

homolog. We found that mice inoculated with WT *C. rodentium* shed significantly more bacteria on days 6 and 10 post inoculation than mice inoculated with the *cexE_{Cr}* mutant. The observed CexE_{Cr}-dependent enhanced fecal shedding would seem to be consistent with the proposed function of the CexE homolog dispersin in EAEC. *In vitro* dispersin has been shown to decrease the adherence of EAEC to HEp-2 cells as well as human intestinal explants (26). Thus, as its name implies dispersin has been proposed to contribute to the pathogenicity of EAEC by increasing the dispersal of the pathogen throughout the intestinal tract. Decreased adherence would also be expected to result in greater fecal shedding of WT bacteria than *cexE* mutants. However, this model is at odds with the loads of *C. rodentium* within the intestinal tissues. In both the small and large intestines of adult mice, we found significantly higher loads of WT *C. rodentium* than the *cexE_{Cr}* mutant. A similar pattern was also observed in infant mice. Although we did observe that WT ETEC are less adherent to HCT-8 cells than a *cexE* mutant, the difference was not significant. Therefore, greater shedding of the WT strain compared to the *cexE_{Cr}* mutant is likely a consequence of higher loads of the WT strain within the intestines rather than an increased tendency of the WT strain to detach and disperse. Although the mechanism is currently unknown, our *in vivo* studies clearly demonstrate that CexE promotes colonization and/or survival of an enteric pathogen within the intestinal tract.

All CexE variants and dispersin have predicted amino terminal signal peptides that would result in their transport to the periplasm via the general secretory pathway (17). Such signal peptides are cleaved upon entry into the periplasm and cleavage of CexE_α's signal peptide has been experimentally verified (14). This is apparently

followed by a second translocation event that transports CexE_α to the external face of the outer membrane that is dependent upon by environmental conditions such as growth medium. For dispersin of EAEC this second translocation event requires five genes, *aatPABCD* (33). AatA likely forms a conduit through the outer membrane because it is a homolog of TolC; a protein that has been shown to form β-barrel pores in the outer membrane of *E. coli* and other gram-negative bacteria (34, 35). The other *aat* genes encode essential accessory proteins because deletion of any of the five genes abolishes translocation of dispersin across the outer membrane (33). Homologs of all five genes are also present in ETEC strains harboring *cexE* as well as *C. rodentium*, *Y. enterocolitica*, and *P. alcalifaciens* (unpublished observation). This suggest that the mechanism of CexE/dispersin translocation across the outer membrane is conserved across pathovars and species. Although we did not observe translocation of CexE_{Cr} in vitro, this is probably due to an inability to replicate the appropriate conditions. Conditional translocation of CexE_{Cr} is a valid possibility because we observed that CexE_α was translocated across the outer membrane when ETEC was cultured in IMDM but not CFA medium. The mechanism of this conditional translocation is currently under investigation.

Dispersin has been shown to decrease the adherence of EAEC to enterocytes in vitro by coating the outer membrane of the pathogen (26). Although we observed that CexE_α also coats the outer membrane of ETEC, WT ETEC was no less adherent to HCT-8 cells than a *cexE*_α mutant. Thus, CexE of ETEC does not appear to act in a dispersin-like manner which raises the question as to whether or not it acts locally at the outer membrane. The reported association of CexE_α with outer membrane vesicles (OMVs)

presents an alternative hypothesis in which CexE_α acts distally upon host cells OMVs are bacteria-derived nanostructures that can deliver virulence factors to host cells and other targets (36, 37). For example, OMVs have been shown to contain and also be coated with the LT enterotoxin of ETEC (38). Such LT laden OMVs facilitate the delivery of LT to the cytosol of mammalian cells (38). Since CexE_α colocalizes with LT in OMVs and OMVs deliver LT to host cells it is plausible that CexE also reaches host cells. Therefore, unraveling the mechanism of CexE's contribution to pathogenicity will require consideration that it may function locally at the bacterial outer membrane or alternatively at distal sites upon or within host cells.

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SUPPLEMENTARY MATERIAL

FIG S1 Sequences of CexE homologs

>CexE_α (ETEC)

MKKYILGVILAMGSLSAIAGGGNSERPPSVAAGECVTFNSKLGEIGGYSWKYSNDACNETVAK
GYAIGVAMHRTVNYEGGYSIQSSGIVKPGSDFIMKGGKTYKGHKKVSAGGDTPYWYK

274 >CexE_β (ETEC)
 275 MKKYILGVILAMGSLSAIAGGGNSERQPSVAAGEGVTFNSKLGEIGGYSWKYSNDACNETVAK
 276 GYAIGVAMHRTVNYEGGYSIQSSGIVKPGSDFIMKGGKTYKGHHKVSTGGETPYWYK
 277
 278 >CexE_γ (ETEC)
 279 MKKYILGIILTMGSFSALAGGAWQPSVGPGECSISFQSGLEIGGYRWWSSNGCNEVINRGYAL
 280 GVALERRVNYANGDSVSSAGVISPSRDYVMKGSKFSNGSPKVSTGGSSPYWYK
 281
 282 >CexE_δ (ETEC)
 283 MKNYILGIILTMGSFSALAGGVWQPSVGPGECSISFQPLGEIGGYRWWSSNGCNEVINRGFAL
 284 GVALERRVNYANGDSVSSAGVISPNSDYVIKGSKFSNGSPKVSTGGSSPYWYK
 285
 286 >CexE_ε (ETEC)
 287 MKKIILALPFLTSCFSAFAGGSGPEWQPQISPGQCIQYTEIGETGGYKWHNIDACNEVVHRGYA
 288 SGAFVSGKVVEGGETIEYTGIVKPDAPYTIQAPSTHNGKKKVGHGGAYTYWAR
 289
 290 >CexE_η (ETEC)
 291 MKKIILALPFLTSCFSAFAGSSGPEWQPQISAGQCIQYTEIGEAGGYKWNNIDYCNEVVRRGYA
 292 SGVFASGKVVEGGGVVEYTGLVKPDTPYAIQAPSTYNGKKKVGHGSAYTYWAR
 293
 294 >CexE_θ (ETEC)
 295 MKKLFFAFPLVISSFSTFAGGGSEWQPTVSPGQCIEYTEIGETGGYKWNDTDCNEVVRRGYA
 296 SGVMVSGKVFYDGAPSISYTGHVAPNKPYPARQAPLYNDGKKKKGHGDSYTYWAR
 297
 298

299 >CexE_l (ETEC)
300 MKKIILSLSVIVFSHSVSAGSTNWQPSVGPQGQCIVYAEIGETGGYKWNNQDDCNEVVRRGYAS
301 GVGVSGRVIYEGNTPGTNGDSIGYTGIVTPNKPYPYERQAPATYKGKKKVGHGDSYTYWAK
302
303 >CexE_k (ETEC)
304 MKRIILSLSVIFCSHSALAGSTDWQPSVGPQGQRIVYAEIGETGGYKWNNQNECNEVVRRGYAIG
305 VGVSGKVIYEGNKPGYNGDSISYSGIVTPYRDYKRQAPAVYNGKKKVAHGDSYTYWAK
306
307 >CexE_λ (ETEC)
308 MKRIILSLSVIFCSHSALAGSTDWQPSVGPQGQCIVYAEIGETGGYKWNNQNECNEVVRRGYAIG
309 VGVSGKVIYEGNKPGYNGDSISYSGIVTPDRDYKRQAPAVYNGKKKVAHGDSYTYWAK
310
311 >CexE_ψ (ETEC)
312 MNKIKFVIFSGILGLSLNAFAGGSGWNADNVDPSQCIKLSGVQYTYNSSASVCMQGLNEGKVR
313 GVSVSGEFYNDGTTSSFKGVVTPSTPVNTNQDINKTNKVG VQKYRALTEWVK
314
315 >Dispersin (EAEC)
316 MKKIKFVIFSGILGISLNAFAGGSGWNADNVDPSQCIKLSGVQYTYNSGVPVCMQGLNEGKVR
317 GVSVSGVFYYKDGTTSNFKGVVTPSTPVNTNQDINKTNKVG VQKYSALTEWVK
318
319 >Cr (*C. rodentium*)
320 MKLIGKFIGFAIMTISFYSFAGGGASSWIPNVAPSACVNIDESRISFTWNNNPECEKAISSGYAS
321 GVRIMGSASYVPDTTIAQFNKVLKRNMSLTIIDLDIYGSVNGYPAKLATMPIFRWES
322
323

324 >Pa (*P. alcalifaciens*)
325 MKKYILGIILTMGSFSALAGGAWQPSVGPGECSISFQSGLGEGGYKWWSSDGCNEVINRGHAL
326 GVGLERRVNYANGDYVSSAGVISPNRDYVIKGSTSSNGSPKVSTGGSSPYWYK
327
328 >Ec NCTC86 (*E. coli*)
329 MKKLNVSIFSIAACFSLNVFAGGGGWNSDLVDPQQCVKLSGAQYTYNSSSNKCMQGINEGKV
330 HGVSLFGTFYYGDGSQGTGKGRVSPGTTLNTNQDMNKTNKYGVKYKVITEWVR
331
332 >Ye_α (*Y. enterocolitica*)
333 MKLTGKFIGFVIMTISFSSFAGGGSSSWKPSVDPSACVNINEQRTPSTWYDNPVWFTWNANPE
334 CEKAISSGYASGIRIMGTVSYPVESTISQFNKVLKPNMSLTIVDLDIYGAVNGHPPRFASRPEFR
335 WES
336
337 >Ye_β (*Y. enterocolitica*)
338 MKLTGKFIGFVIMTISFSSFAGGGSSSWKPSVDPSACVNINEQRTPSTWYDNPVWFTWNANPE
339 CEKAISSGYASGIRIMGTVSYPVESTISHFNKVLKPNMSLTIVDLDIYGAVNGHPPRFASRPEFR
340 WES

FIG S2

Quantification of proteinase K digestion of antigens relative to DnaK.

(A) Quantification of CexE_α digestion from ETEC cultured in CFA or IMDM. **(B)**

Quantification of CexE_{cr} digestion from *C. rodentium* cultured in LB or IMDM. Column numbering corresponds to Western blot lanes in Fig 2. $n = 3$, $*P < 0.0001$ by Student's t -test. Black columns correspond to untreated cells (PBS). Gray columns correspond to cells treated with PBS, Triton (1%), and EDTA (10mM).

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FIG S3

CexE_α is not detectable in the culture

supernatants of ETEC. TCA precipitation of the supernatant of H10407 grown in either CFA or IMDM. Purified CexE_α was TCA precipitated as a positive control at approximately 10 ng of TCA precipitated protein. The limit of detection is estimated to be 10 ng of CexE_α Abbreviations: S, culture supernatant; P, cell pellet.

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FIG S4

Translocation of CexE_α-TEM-1 to the surface of ETEC cultured in IMDM. (A)

Western blot of whole cell lysates of ETEC strain GPM1163 / pGPM1034bla probed with anti-CexE and DnaK antibodies. Membrane permeabilization is not required for enzymatic digestion of a 43 kDa CexE_α-TEM-1 fusion protein indicating that the fusion protein is exposed on the surface of ETEC. (B) Quantification of CexE_α-TEM-1 digestion relative to DnaK. Column numbers corresponds to (A) Western blot lane numbers. $n = 3$, $*P < 0.0001$ by Student's t -test. Abbreviations: Prot. K, proteinase K; MW, molecular weight ladder; Ab, antibody specificity control with whole cell lysates of GPM1163 lacking the CexE_α-TEM-1 fusion protein.

FIG S5

A *CexE_{cr}* mutant has the same phenotype in 129X1/SJV and C57BL/6 mice.

129X1/SJV mice were orogastrically inoculated with 10¹⁰ CFUs with either WT *C. rodentium* (GPM1831a) or a *cexE_{cr}::kan* (GPM1827) mutant. Fecal pellets were collected on days 2 and 15 post inoculation and CFUs were normalized to the mass of each pellet. Although the difference between fecal shedding of the two strains was not significant at the early (A) time point it was statistically significant (B) 15 days after inoculation. Circles represent fecal pellets of separate animals; $n \geq 10$ mice per group.

* $P < 0.05$ by Mann-Whitney U test

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MATERIALS AND METHODS

Bacterial growth conditions.

Bacteria were cultured aerobically at 37°C in CFA medium (39), Lysogeny Broth (LB; HIMEDIA), or Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific). Growth media were supplemented with 50 µg/ml kanamycin or 100 µg/ml ampicillin as appropriate.

Bacterial strains and plasmids. Plasmid pGPM1034bla expresses a CexE-TEM-1 fusion protein from *cexEp* or T7p. It was constructed by amplification of the β -lactamase gene from pUC19 with oligonucleotide primer pair 1126/1127. The PCR product was digested with XhoI then ligated into the same site of pGPM1034. λ RED mediated recombineering was used for the construction of the following strains as previously described (40, 41). Kanamycin resistance cassettes targeting *cfaD* and *cexE_α* were amplified from pKD4 with primer pairs 608/625 and 622/623 respectively. A cassette for the disruption of the *lac* operon was amplified from JF876 with primers 108/518. Electroporation of the cassettes into H10407 / pSIM6 resulted in recombinants GPM1236, GPM1163, and GPM1168 respectively. Cassettes for epitope tagging, disruption, or insertion downstream of *cexE_{Cr}* were amplified from pSUB11 with primer pairs 1178/1179, 1177/1179, and 1193/1179 respectively. Electroporation of the cassettes into DBS100 / pSIM6 resulted in recombinants GPM1830, GPM1827, and GPM1831a respectively. Recombinants were cured of λ RED expression plasmids by passage at 42°C. Insertions were verified by PCR with primers flanking the insertion sites.

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Table 1

Strains used in this study

Strain	Description	Reference or source
H10407	ETEC O78:H11; CFA/I	(12)
GPM1168	H10407 $\Delta lacZYA514::kan$	this study
GPM1236	H10407 <i>rns::kan</i>	this study
GPM1163	H10407 <i>cexE_α::kan</i>	this study
JF876	H10407 $\Delta lacZYA::kan$	(42)
DBS100	<i>Citrobacter rodentium</i> (ATCC 51459)	(43)
GPM1831a	DBS100 <i>cexE_{Cr} Ω(66bp)::kan</i>	this study
GPM1827	DBS100 <i>cexE_{Cr}::kan</i>	this study
GPM1830	DBS100 <i>cexE_{Cr}-FLAG kan</i>	this study

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Table 2

Plasmids used in this study

Name	Description	Marker	Reference or source
pNEB193	cloning vector	Amp	New England Biolabs
pUC19	cloning vector	Amp	(44)
pGPM1034bla	CexE-TEM-1 expressed from T7p or <i>cexEp</i>	Amp, Kan	this study
pGPM1034	CexE expressed from T7p or <i>cexEp</i>	Kan	(14)
pKD4	PCR template for <i>kan</i> cassettes	Amp, Kan	(40)
pSIM6	λRED expression plasmid	Amp	(41)
pSUB11	PCR template for 3XFLAG epitope tagging by λRED	Kan	(45)

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Table 3

Oligonucleotide primers used in this study

Name	Sequence
108	AGCGGAACGGGAAGGCGA
518	GGCTGATGATCATAACCCTGCGTTTTGCA

608	<i>GATTCATAAATACACTGTATTATATACATCAAATTGTATTGTGTAGGCTGG</i> <i>AGCTGCTTC</i>
622	<i>CAATAACCGCCCCGAGCAAACAGCAAGGGCGATTTTGTTATCTTGTGTAGGC</i> <i>TGGAGCTGCTTCG</i>
623	<i>GGTAATTCTGAACGACCGCCTTCCGTTGCAGCAGGGGAGTGCCATATGAA</i> <i>TATCCTCCTTA</i>
625	<i>TCAAGTAGTCAAACACTCTAGATAACAACAGTATTGGCGCCCATATGAATAT</i> <i>CCTCCTTA</i>
1126	<i>GCAGATCTCGAGCACCCAGAAACGCTGGTGAAAG</i>
1127	<i>CTAGTACTCGAGTTACCAATGCTTAATCAGTGAG</i>
1177	<i>GAGGTTGCATATATGAACTTATAGGAAAATTCATTGGTTTTGCGATTATG</i> <i>ACAATTAGCGACTACAAAGACCATGACGG</i>
1178	<i>GGCTCAGTTAACGGATATCCAGCAAAGCTTGCCACTATGCCAATTTTTAGGTGG</i> <i>GAATCAGACTACAAAGACCATGACGG</i>
1179	<i>CATATTGGCGCAACAATATTAACCTACGCATAATGTGAAGTAGA</i> <i>AAATATTTTCATATGAATATCCTCCTTAG</i>
1193	<i>GCGAAAGCATATTGCTGTACAAAAATCAAAGTGCTCGTCATATGTTAACA</i> <i>TGACGAGCACGACTACAAAGACCATGACGG</i>

* Italics denotes sequences homologous to genomic targets for λ RED mediated recombineering.

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382 **Quantitative bacterial adherence assays.** HCT-8 cells, a human ileocecal epithelial
383 cell line, was purchased from the American Type Culture Collection (ATCC CCL-244).
384 HCT-8 cells were grown in IMDM supplemented with 10% fetal bovine serum (FBS) at
385 37°C in 5% CO₂. Approximately 24 hours before infection HCT-8 cells were seeded in

24-well tissue culture plates at ca. 10^5 cells per well. Bacteria were cultured in CFA medium to an OD₆₀₀ of 0.7-0.8. Bacteria were collected from 500 µl of culture by centrifugation then resuspended in 1 ml IMDM, 10% FBS. The tissue culture wells were aspirated, washed once with PBS, then 200 µl IMDM, 10% FBS was added to each well followed by 50 µl of the bacterial suspension for an MOI of ca. 50. After a one hour attachment period culture medium was aspirated and wells were washed four times with PBS to remove nonadherent bacteria. Adherent bacteria were collected in PBS, 0.1% (vol/vol) Triton X-100 and enumerated by serial dilution and plating. To control for differences between inocula adherent CFUs were normalized to inocula.

Trichloroacetic acid (TCA) precipitation of culture supernatants. H10407 /

pNEB193 was cultured in either CFA or IMDM to stationary phase. Bacteria were removed from the culture medium by centrifugation and successive filtration through 0.45 and 0.20 micron syringe tip filters. TCA was added to the clarified supernatants at a final concentration of 10% (vol/vol). Proteins were precipitated overnight at 4°C then pelleted for 8 min. at 21,000 x g. Protein pellets were washed twice with 500 µl ice-cold acetone to remove residual TCA, air-dried for 15 min. at room temperature, then resuspended in 60 µl of 1x SDS-PAGE loading buffer with 100 mM β-mercaptoethanol.

Proteinase K digests. Bacteria were collected from 400 µl of stationary phase cultures by centrifugation at 21,000 g for 3 min. and resuspended in 75 µl of either PBS or PBS, 10 mM EDTA, 1% (vol/vol) Triton X-100. Suspensions were equilibrated at 37°C for 1 hour prior to addition of proteinase K to a final concentration of 200 µg/ml. Some samples received 11 mM PMSF prior to enzyme addition. All samples were incubated for 1 hour at 37°C with continuous gentle rocking. Enzyme activity was terminated by

addition of PMSF followed by 25 μ l of 4x SDS-PAGE loading buffer with β -mercaptoethanol.

Immunoblots. Whole-cell lysates were subject to SDS-PAGE, transferred to PVDF membranes, and blocked in TBS-Blotto (25 mM TrisCl pH 7.6, 150 mM NaCl, 5% (wt/vol) powdered nonfat milk. Antibodies against CexE $_{\alpha}$ were produced by immunization of rabbits (Proteintech Group, Inc.) with purified CexE $_{\alpha}$ -His6 and used at a dilution of 1:10000 in TBS-Blotto with 0.05% vol./vol. Tween20 (14). Anti-DnaK (ab69617) and anti- β -lactamase (ab12251) antibodies were purchased from AbCam and used at dilutions of 1:10000 and 1:10000 respectively. Anti-FLAG (F1804) was purchased from Sigma-Aldrich and used at a dilution of 1:10000 HRP conjugated goat anti-rabbit (sc-2030) and goat anti-mouse (115-036-062) antibodies were purchased from Santa Cruz Biotechnology and Jackson ImmunoResearch Laboratories respectively. Secondary antibodies were used at a dilutions of 1:10000. Chemiluminescence was detected with a Odyssey FC Imaging System (LI-COR Biosciences). Densitometry analysis was performed utilizing ImageJ software.

In Vivo studies. All mice were bred and housed under barrier conditions in the Division of Veterinary Resources of the University of Miami Miller School of Medicine. Mice were regularly screened for specific common pathogens. C57Bl/6 mice were inoculated orogastrically with *C. rodentium* strains in PBS. Adults were inoculated with a 22-gauge, round-tipped feeding needle. Infant mice were used at 15 days of age and inoculated with PE-10 tubing (polyethylene tubing with an outside diameter of 0.61 mm) attached to a 30-gauge needle. Administered CFUs were determined by serial dilution and plating of inocula. Infant mice were weaned at 3 ½ weeks of age. On select days fecal

pellets were collected from adult mice and homogenized in sterile ddH₂O, diluted, and plated on MacConkey agar with kanamycin. Organs of adult mice were homogenized in PBS using a OMNI International Tissue Homogenizer (Kennesaw, GA) for 2 min at medium speed. Organs from infant mice were homogenized in HBSS using a Seward Biomaster 80 Stomacher (Brinkman, Westbury, NY) for 4 min. at high speed. Homogenates were diluted and plated on MacConkey agar plates with kanamycin. CFUs were normalized to the weight of fecal pellets and organs.

Statistical analysis. One-way ANOVA with Tukey's multiple comparisons test, Student's *t*-test, Mann-Whitney U Test, and Mantel-Cox logrank test. (GraphPad Prism Version 7.3 was utilized for statistical analysis).

Ethics statement. All animal experiments were approved by and performed in accordance with the University of Miami Institutional Animal Care and Use Committee guidelines.

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