OmpT Outer Membrane Proteases of Enterohemorrhagic and Enteropathogenic *Escherichia coli* Contribute Differently to the Degradation of Human LL-37

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Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are food-borne pathogens that cause serious diarrheal diseases. To colonize the human intestine, these pathogens must overcome innate immune defenses such as antimicrobial peptides (AMPs). Bacterial pathogens have evolved various mechanisms to resist killing by AMPs, including proteolytic degradation of AMPs. To examine the ability of the EHEC and EPEC OmpT outer membrane (OM) proteases to degrade α -helical AMPs, *ompT* deletion mutants were generated. Determination of MICs of various AMPs revealed that both mutant strains are more susceptible than their wild-type counterparts to α -helical AMPs, although to different extents. Time course assays monitoring the degradation of LL-37 and C18G showed that EHEC cells degraded both AMPs faster than EPEC cells in an OmpT-dependent manner. Mass spectrometry analyses of proteolytic fragments showed that EHEC OmpT cleaves LL-37 at dibasic sites. The superior protection provided by EHEC OmpT compared to EPEC OmpT against α -helical AMPs was due to higher expression of the *ompT* gene and, in turn, higher levels of the OmpT protein in EHEC. Fusion of the EPEC *ompT* promoter to the EHEC *ompT* open reading frame resulted in decreased OmpT expression, indicating that transcriptional regulation of *ompT* is different in EHEC and EPEC. We hypothesize that the different contributions of EHEC and EPEC OmpT to the degradation and inactivation of LL-37 may be due to their adaptation to their respective niches within the host, the colon and small intestine, respectively, where the environmental cues and abundance of AMPs are different.

Antimicrobial peptides (AMPs) are small cationic peptides secreted into the extracellular environment by epithelial cells. AMPs have both bactericidal and immunomodulatory properties, making them key players in the innate immune response to infection. AMPs are known to bind to the anionic cell membrane and lyse bacterial cells by forming pores. They also bridge innate and adaptive immunity by recruiting immune cells to the site of infection. On the basis of their three-dimensional structures and disulfide bridge patterns, AMPs are divided into distinct families, the cathelicidins and the α - and β -defensins (18). LL-37 is the sole human AMP of the cathelicidin family. It consists of 37 amino acids, including 11 positive residues, and forms an amphipathic α helix when bound to membranes (2). LL-37 is synthesized as the precursor human cationic antimicrobial protein 18 (hCAP18) that is processed into the biologically active peptide by the serine protease proteinase 3. LL-37 is expressed by different cell types, including neutrophils, bone marrow cells, and epithelial cells of the lung and intestine. The distribution of LL-37 expression along the gastrointestinal tract is uneven, being limited to surface epithelial cells of the stomach and colon (14, 15). In addition to its antimicrobial activity, LL-37 has a broad range of immunomodulatory functions (30).

Bacterial pathogens have evolved different mechanisms to resist the killing action of AMPs (32). Gram-negative and Gram-positive bacteria modify their surface lipopolysaccharides (LPSs) and lipoteichoic acids, respectively, by adding positively charged moieties that prevent the electrostatic interaction of AMPs with bacterial surfaces. Efflux pumps can export AMPs before they damage the cytoplasmic membrane. AMPs can also be proteolytically degraded and inactivated by surface or secreted proteases. OmpTins are proteases found at the outer membrane (OM) of various Gram-negative pathogens of the *Enterobacteriaceae* family

(13, 16, 21). OmpTins share high amino acid sequence identity (45 to 80%) and adopt a conserved β -barrel fold, with the active site facing the extracellular environment (41). OmpTins possess a unique active site that combines elements of both serine and aspartate proteases, and interaction with LPS is critical for activity (8). OmpTins impact bacterial virulence by degrading or processing a number of host proteins or peptides. For example, *Yersinia pestis* Pla and *Salmonella enterica* PgtE control human plasmin activity by processing the proenzyme plasminogen and inactivating the plasmin inhibitor α_2 -antiplasmin and plasminogen activator inhibitor 1 (PAI-1) (12, 22, 23). Pla and PgtE were also shown to cleave serum components and affect complement activity (33, 38). Several ompTins, including OmpT, Pla, and PgtE, have been associated with the degradation of AMPs. *Escherichia coli* K-12 OmpT was reported to efficiently degrade the AMP protamine (39). Other studies have shown that PgtE and Pla cleave α -helical AMPs such as human LL-37 and the synthetic α -helical peptide C18G, whose sequence has been optimized for maximal antibacterial activity (4, 10, 11).

Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are two genetically related bacteria

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
EHEC EDL933	Wild-type EHEC O157:H7	34
EHEC $\Delta ompT$	EDL933 $\Delta ompT$	This study
EHEC $\Delta ompT$ (pEHompT)	EDL933 $\Delta ompT$ mutant expressing <i>ompT</i> from pEHompT	This study
EHEC $\Delta ompT$ (pEPompT)	EDL933 $\Delta ompT$ mutant expressing <i>ompT</i> from pEPompT	This study
EHEC $\Delta ompT$ (pEHompT-FLAG)	EDL933 $\Delta ompT$ mutant expressing <i>ompT</i> -FLAG from pEHompT-FLAG	This study
EHEC $\Delta ompT$ (pEPompT-FLAG)	EDL933 $\Delta ompT$ mutant expressing <i>ompT</i> -FLAG from pEPompT-FLAG	This study
EHEC $\Delta ompT$ (pEPpromEHompT)	EDL933 $\Delta ompT$ mutant expressing <i>ompT</i> from pEPpromEHompT	This study
EHEC $\Delta ompT$ (pEHpromEPompT)	EDL933 $\Delta ompT$ mutant expressing <i>ompT</i> from pEHpromEPompT	This study
EPEC E2348/69	Wild-type EPEC O127:H6 Str ^r	25
EPEC $\Delta ompT$	E2348/69 $\Delta ompT$	This study
EPEC $\Delta ompT$ (pEPompT)	E2348/69 $\Delta ompT$ mutant expressing <i>ompT</i> from pEPompT mutant	This study
EPEC $\Delta ompT$ (pEHompT)	E2348/69 $\Delta ompT$ mutant expressing <i>ompT</i> from pEHompT	This study
EPEC $\Delta ompT$ (pEPompT-FLAG)	E2348/69 $\Delta ompT$ mutant expressing <i>ompT</i> -FLAG from pEPompT-FLAG	This study
EPEC $\Delta ompT$ (pEHompT-FLAG)	E2348/69 $\Delta ompT$ mutant expressing <i>ompT</i> -FLAG from pEHompT-FLAG	This study
EPEC $\Delta ompT$ (pEPpromEHompT)	E2348/69 $\Delta ompT$ mutant expressing <i>ompT</i> from pEPpromEHompT	This study
EPEC $\Delta ompT$ (pEHpromEPompT)	E2348/69 $\Delta ompT$ mutant expressing <i>ompT</i> from pEHpromEPompT	This study
Sm10 (λ Pir)	<i>thi thr leuB tonA lacY supE recA::RP4-2-Tc::Mu-Kan Kan^r</i>	37
Sm10 (λ Pir)(p Δ EPompT)	Sm10 (λ Pir) containing p Δ EPompT	This study
χ 7213	<i>thr-1 leuB6 flhA21 lacY1 glnV44 recA1 asdA4 thi-1 RP4-2-Tc::Mu [-pir] Kan^r</i>	35
χ 7213 (p Δ EHompT)	χ 7213 containing p Δ EHompT	This study
Plasmids		
pRE112	Sucrose-sensitive (<i>sacB1</i>) suicide vector, Cm ^r	7
p Δ EHompT	EHEC $\Delta ompT$ deletion construct in pRE112	This study
p Δ EPompT	EPEC $\Delta ompT$ deletion construct in pRE112	This study
pACYC184	Multicopy plasmid, Tet ^r Cm ^r	NEB
pEHompT	EHEC <i>ompT</i> cloned into pACYC184	This study
pEHompT-FLAG	C-terminally FLAG-tagged EHEC <i>ompT</i> cloned into pACYC184	This study
pEPompT	EPEC <i>ompT</i> cloned into pACYC184	This study
pEPompT-FLAG	C-terminally FLAG-tagged EPEC <i>ompT</i> cloned into pACYC184	This study
pEPpromEHompT	EHEC <i>ompT</i> under the control of the EPEC <i>ompT</i> promoter cloned into pACYC184	This study
pEHpromEPompT	EPEC <i>ompT</i> under the control of the EHEC <i>ompT</i> promoter cloned into pACYC184	This study

that cause severe diarrheal diseases in humans (28). Together with the mouse enteric pathogen *Citrobacter rodentium*, EHEC and EPEC belong to a group of pathogens that cause histopathological lesions known as attaching and effacing (A/E) lesions. A/E lesions are characterized by the localized effacement of intestinal microvilli, the intimate attachment of bacteria to the enterocyte plasma membrane, and the formation of pedestal-like structures at sites of bacterial attachment. All three pathogens carry a pathogenicity island known as the locus of enterocyte effacement (LEE) that is required for the formation of A/E lesions. We have recently shown that CroP, the omp⁺ of *Citrobacter rodentium*, degrades α -helical AMPs, including the mouse cathelicidin mCRAMP (24). CroP-mediated degradation of AMPs occurred before they reached the periplasmic space and triggered a PhoPQ-mediated adaptive response, resulting in AMP resistance. Since CroP is 74% identical to *E. coli* OmpT, we hypothesized that OmpT would confer on pathogenic *E. coli* (EHEC and EPEC) resistance against human LL-37 and other α -helical AMPs. In this study, we show that the EHEC and EPEC OmpT proteins contribute differently to the degradation of α -helical AMPs. EHEC OmpT readily degraded and inactivated AMPs to promote bacterial survival, whereas EPEC OmpT was found to have a more marginal role in AMP degradation.

MATERIALS AND METHODS

Media and reagents. Bacteria were grown at 37°C with aeration in Luria-Bertani (LB) broth or N-minimal medium (29) adjusted to pH 7.5 and supplemented with 0.2% glucose and 1 mM MgCl₂. When appropriate, media were supplemented with streptomycin (50 μ g/ml), chloramphenicol (30 μ g/ml), or DL-diaminopimelic acid (DAP; 50 μ g/ml). C18G, LL-37, and peptides corresponding to LL-37 cleavage products were synthesized with a purity of >85% (BioChemia). Polymyxin B (PMB) was purchased from Sigma. AMPs were reconstituted in sterile deionized water. Restriction enzymes and Phusion DNA polymerase were from New England BioLabs.

Construction of EHEC and EPEC *ompT* deletion mutants. The bacterial strains and plasmids used in this study are listed in Table 1. DNA purification, cloning, and transformation were performed according to standard procedures (36). The EHEC and EPEC $\Delta ompT$ deletion mutants were generated by *sacB* gene-based allelic exchange (6). Genomic DNA from EHEC or EPEC was used as a template to PCR amplify the upstream (primer pair EH1/EH2 or EP1/EP2; Table 2) and downstream (primer pair EH3/EH4 or EP3/EP4) sequences of the *ompT* genes. The resultant PCR products were treated with KpnI and ligated together. The ligation products were used as the DNA template in a PCR using primer pairs EH1/EH4 and EP1/EP4 for EHEC and EPEC, respectively. PCR fragments were gel purified, digested with the appropriate restriction enzymes (Table 2), and ligated into pRE112 cleaved with XbaI and SacI. Resultant plasmids p Δ EHompT and p Δ EPompT were verified by sequencing. The p Δ EHompT construct was conjugated into wild-type EHEC using *E. coli*

TABLE 2 Primers used in this study

Primer	Sequence ^a	Use ^b	Restriction site
EH1	GCTCTAGAAATTGCCGCCACTGTGAGTCAT	Generate pΔEHompT and screen for ΔompT	XbaI
EH2	GGGGTACCCATAAAAGGTCTCCATTCAATCG	Generate pΔEHompT	KpnI
EH3	GGGGTACCTAACGAAGTTAACGAGATTTTC	Generate pΔEHompT	KpnI
EH4	GCGAGCTCACGACTCCATCAAGAACGATAGA	Generate pΔEHompT and screen for ΔompT	SacI
EH5	GCTCTAGACGACCTGATTATGCCATTACATA	Generate pEHompT	XbaI
EH6	GCGAGCTCAAATCTGGTTAACTTCGTAA	Generate pEHompT	SacI
EH7	AAAAGGTCTCCATTCAATCGTTTAAATG	Generate pEPromEHompT	
EH8	ATGCGGGCGAAACTTCTGGGAATAG	Generate pEPromEHompT	
EH9	GCGGAGCTCCTATCATTACTTGTCGTCATCGTCCTTGTAGTCAAAG GTGTACTTAAGACCAGCAG	Generate pEHompT-FLAG and pEPompT-FLAG	SacI
EH10	GGACTATTCCCAGAAGTTTCG	Generate pEHpromEPompT	
EP1	GCTCTAGAGCGTGAACGTTATCTACAGG	Generate pΔEPompT and screen for ΔompT	XbaI
EP2	GGGGTACCGTCAGGAGTAAACGATAAAGT	Generate pΔEPompT	KpnI
EP3	GGGGTACCTAACACGTTAAATAGATTTTC	Generate pΔEPompT	KpnI
EP4	GCGAGCTCGAAACCTCGTTGCTGGAAGC	Generate pΔEPompT and screen for ΔompT	SacI
EP5	GCTCTAGACTTAGAACTCCAGGAACGACAT	Generate pEPompT	XbaI
EP6	GCGAGCTCAATCTATTTAACGTTGTAA	Generate pEPompT	SacI
EP7	TGACAACCCCTATTGCGATCAGC	Generate pEHpromEPompT	
qEH810	TCGGCTCCTTCCCGAATGGAG	qPCR EHEC ompT F	
qEH811	GATGCTTCCACCCAGCCGC	qPCR EHEC ompT R	
qEH812	GTGCTGCATGGCTGTCGTCA	qPCR EHEC 16S RNA F	
qEH813	AGCACGTGTGTAGCCCTGGT	qPCR EHEC 16S RNA R	
qEP808	CAGCGGCTGGGTGGAAGCAT	qPCR EPEC ompT F	
qEP809	ACCCGATTCCATGCGCCTTCA	qPCR EPEC ompT R	
qEP814	AACGCGTTAAGTCGACCGCC	qPCR EPEC 16S RNA F	
qEP815	CGGCTCCCGAAGGCACATTC	qPCR EPEC 16S RNA R	

^a Restriction sites are underlined.^b F, forward; R, reverse.

χ7213 as the donor strain. Integration of the plasmid into the chromosome was selected for by plating bacteria on LB agar supplemented with chloramphenicol. The pΔEPompT construct was introduced into wild-type EPEC by conjugation using *E. coli* Sm10 (λ Pir) as the donor strain; integration of the plasmid into the chromosome was selected for by plating bacteria on LB agar supplemented with chloramphenicol and streptomycin. Chloramphenicol-resistant transformants of EPEC and EHEC were then cultured on peptone agar containing 5% sucrose to isolate colonies that were sucrose resistant. The resultant colonies were also tested for chloramphenicol sensitivity. Gene deletions were verified by PCR using primer pair EH1/EH4 or EP1/EP4. Plasmids used for complementation were constructed by PCR amplifying the genes of interest with their promoters from the appropriate genomic DNA using primer pair EH5/EH6 or EP5/EP6. The resultant PCR products were cloned into the XbaI and EcoRV restriction sites of plasmid pACYC184, generating plasmids pEHompT and pEPompT. Plasmids containing FLAG-tagged EHEC and EPEC *ompT* were generated by PCR amplifying the genes of interest with their promoters from genomic DNA using primer pair EH5/EH9 or EP5/EH7. The PCR products were then treated with XbaI and ligated into pACYC184, as described above. Promoter-swapping constructs were generated by amplifying the promoter of EHEC or EPEC *ompT* using primer pair EH5/EH10 or EP5/EH7. The resultant PCR products were digested with XbaI, treated with T4 polynucleotide kinase, and ligated to the PCR products of the open reading frame of EPEC *ompT* (primer pair EP7/EP6) or EHEC *ompT* (primer pair EH8/EH6). The ligation products were then used as the DNA template in a PCR using primer pair EH5/EP6 or EP5/EH6. The PCR products were then digested with XbaI and ligated into pACYC184 that had been treated with XbaI and EcoRV, generating pEHpromEPompT and pEPpromEHompT.

MIC determination. MICs were determined in 96-well microtiter plates using the broth microdilution method (42). Briefly, bacterial cells were grown to an optical density at 600 nm (OD₆₀₀) of 0.5 in N-minimal

medium, diluted to 5×10^5 CFU/ml in the same medium, and aliquoted into rows of wells. Twofold serial dilutions of the tested AMP were added to each row of wells. The plates were incubated at 37°C for 24 h. The lowest concentration of AMP that did not permit any visible growth, determined by absence of turbidity, was the MIC. Determination of MIC values was repeated at least three times.

Proteolytic cleavage of AMPs, tandem MS, peptide separation, and N-terminal sequencing. Bacterial cells were grown to an OD₆₀₀ of 0.5 in N-minimal medium supplemented with 0.2% glucose and 1 mM MgCl₂. Culture aliquots (10⁷ cells) were incubated with 10 μg of AMP, to facilitate visualization of the degradation products, for various times at 37°C in a total volume of 25 μl. Bacterial cells were pelleted by centrifugation. The supernatant was removed, and Tris-Tricine sample buffer (Bio-Rad) was added. Aliquots (5 μl) were heated at 100°C for 5 min and resolved by Tris-Tricine SDS-PAGE (10 to 20% acrylamide; Bio-Rad). After fixation for 30 min in 5% glutaraldehyde, the peptides were stained with Coomassie blue G-250. For mass spectrometry (MS) analysis, supernatants were filtered using a 0.2-μm-pore-size polyvinylidene difluoride (PVDF) filter and stored at -20°C. Samples (5 μg) were injected on a Zorbax octyldecyl silane (ODS) column installed on an Agilent 1100 series liquid chromatograph connected to a Sciex-Applied Biosystems QTRAP 4000 mass spectrometer. Peptides were eluted using a gradient with solvent A (0.1% trifluoroacetic acid [TFA] in water) and solvent B (0.1% TFA in acetonitrile). Enhanced MS scans are acquired between 350 and 2,000 *m/z* with a source voltage of 2,075, scan speed at 4,000 atomic mass units (amu)/s, and active dynamic fill time. Information-dependent MS/MS analysis was performed on the six most intense multiply charged ions; a dynamic exclusion of 120 s was used to limit resampling of previously selected ions. MS/MS scans were acquired at between 70 and 2,000 amu/s, and the fixed fill time was set at 25 ms with quadrupole Q0 trapping and rolling collision energy of ±3 eV. LL-37 cleavage products were separated on an ODS

TABLE 3 MICs of AMPs for EHEC and EPEC strains

Strain	MIC ($\mu\text{g/ml}$)					
	LL-37	LL-37 ₁₋₇	LL-37 ₈₋₁₈	LL-37 ₁₉₋₃₇	C18G	PMB
EHEC wild type	16	>1,024	>1,024	512	32	2
EHEC $\Delta ompT$	8	>1,024	>1,024	512	4	2
EHEC $\Delta ompT$ (pEHompT)	64	>1,024	>1,024	512	128	2
EHEC $\Delta ompT$ (pEPompT)	32	ND ^a	ND	ND	32	2
EPEC wild type	16	>1,024	>1,024	1,024	16	2
EPEC $\Delta ompT$	16	>1,024	>1,024	1,024	8	2
EPEC $\Delta ompT$ (pEPompT)	32	>1,024	>1,024	1,024	64	2
EPEC $\Delta ompT$ (pEHompT)	64	ND	ND	ND	64	2

^a ND, not determined.

column, placed into different tubes, and submitted to N-terminal sequencing on an Applied Biosystems 494cLC automated sequence system.

FRET activity assay. The synthetic fluorescence resonance energy transfer (FRET) peptide substrate containing ortho-aminobenzoic acid (Abz) as the fluorescent and group 2,4 nitrophenyl (Dnp) as the quencher [2Abz-SLGRKIQI-K(Dnp)-NH₂] was purchased from AnaSpec. To perform the assay, bacterial cells were grown to an OD₆₀₀ of 0.5 in N-minimal medium supplemented with 0.2% glucose and 1 mM MgCl₂. Cells were pelleted by centrifugation and resuspended in phosphate-buffered saline (PBS; pH 7.4). The FRET substrate (final concentration, 3 μM) was transferred into a quartz cuvette equipped with a stir bar. After 30 s, 1.5×10^9 cells were added. For normalization, PBS was added instead of cells. The fluorescence emission was monitored over 60 min at 22°C with an excitation wavelength at 325 nm and emission wavelength at 430 nm. Both excitation and emission slits were set at 5 nm.

qPCR. Quantitative PCR (qPCR) was performed as previously described (24). Briefly, bacterial strains were grown to an OD₆₀₀ of 0.5 in N-minimal medium supplemented with 0.2% glucose and 1 mM MgCl₂. Total RNA was isolated using TRIzol reagents (Invitrogen) and treated with a DNA-free kit (Ambion) to remove any remaining DNA. The absence of contaminating DNA was confirmed by qPCR using primers qEP814 and qEP815 (Table 2). RNA (1 μg) was reverse transcribed using Superscript II (Invitrogen) with 0.5 μg of random hexamer primers (Sigma). As a negative control, a reaction mixture without Superscript II was also included (NRT). qPCRs were performed in a Rotor-Gene 3000 thermal cycler (Corbett Research) by using a QuantiTect SYBR green PCR kit (Qiagen), according to the manufacturer's instructions. Primers used are listed in Table 2. The level of *ompT* transcript was normalized to 16S RNA and analyzed using the $2^{-\Delta CT}$ (where C_T is threshold cycle) method (26). Reverse transcription (RT) was performed three times independently, and the NRT sample was used as a negative control.

OM protein extraction and Western blotting. Bacterial strains were grown in N-minimal medium supplemented with 0.2% glucose and 1 mM MgCl₂ to an OD₆₀₀ of 0.5. To generate whole-cell lysates, cells were harvested by centrifugation and the pellet was resuspended in Laemmli sample buffer. Total membrane fractions were isolated by osmotic lysis as described elsewhere (43). Briefly, cells were pelleted by centrifugation, washed with PBS, resuspended in osmotic shock buffer (0.5 M sucrose, 40 mM Tris-HCl [pH 7.4], 5 mM EDTA, 100 $\mu\text{g/ml}$ lysozyme), and incubated at room temperature for 30 min with gentle rocking. An equal volume of MgCl₂ (20 mM) was added to the mixture, and cells were pelleted by centrifugation (10,000 rpm, 20 min). The pellet was resuspended in hypotonic solution (20 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1 mM protease inhibitor cocktail [Sigma], 25 $\mu\text{g/ml}$ DNase I) and incubated at room temperature for 20 min. Total membranes were collected by centrifugation (15,000 rpm, 30 min, 4°C) and solubilized with sarcosyl (10 mM Tris-HCl [pH 8.3], 2% sarcosyl, 5 mM MgCl₂) at 10°C for 30 min. Sarcosyl-soluble and -insoluble fractions were separated by ultracentrifugation at 45,000 rpm for 1 h. Sarcosyl-soluble fractions, corresponding to the inner membrane fractions, were collected. Sarcosyl-insoluble frac-

tions, corresponding to the OM fractions, were solubilized with Triton X-100 (50 mM Tris-HCl [pH 8.3], 10 mM EDTA, 1% Triton X-100) at 25°C for 30 min. Protein samples were boiled for 5 min, loaded on 10% SDS-polyacrylamide gels, and transferred to a PVDF membrane (Millipore). OmpT-FLAG was visualized using a polyclonal anti-FLAG antibody (Sigma). The rabbit polyclonal antibody raised against CroP was produced at the Comparative Medicine Animal Resources Centre (McGill University).

RESULTS

OmpT contributes to AMP resistance. The chromosomes of both EPEC and EHEC contain the *ompT* gene coding for the OmpT OM protease. Both open reading frames share 98% identity at the amino acid level. To assess the contribution of OmpT to the degradation of α -helical AMPs, *ompT* deletion mutants were generated in both EHEC and EPEC. These *ompT* deletion mutants were then compared to the wild-type strains by determining MIC values of the AMPs LL-37 and C18G. As shown in Table 3, the deletion of EHEC *ompT* resulted in 2-fold and 8-fold decreases in the MICs of LL-37 and C18G, respectively. Complementation of the EHEC $\Delta ompT$ mutant with a pACYC184-derived plasmid encoding EHEC OmpT under the control of its native promoter (pEHompT) restored resistance to both AMPs above wild-type levels. Compared to the MICs for the EHEC $\Delta ompT$ mutant, 8- and 32-fold increases in the MICs of LL-37 and C18G, respectively, were obtained. Interestingly, complementation of the EHEC $\Delta ompT$ mutant with the pEPompT plasmid, which encodes EPEC OmpT under the control of its native promoter, led to more modest increases in MICs. In EPEC, the deletion of *ompT* resulted in only a 2-fold decrease in the MIC of C18G and did not affect the MIC of LL-37 (Table 3). Complementation of the EPEC $\Delta ompT$ mutant with pEPompT resulted in a 2-fold increase in the MIC of LL-37 and an 8-fold increase in the MIC of C18G compared to the MICs for the EPEC $\Delta ompT$ mutant. Complementation of the EPEC $\Delta ompT$ mutant with pEHompT resulted in 4- and 8-fold increases in the MICs of LL-37 and C18G, respectively. For both EHEC and EPEC, no differences in the MICs of PMB, a cyclic lipopeptide that was previously shown to resist proteolytic degradation (24), were observed (Table 3). Taken together, these data indicate that OmpT is likely to contribute to AMP resistance in both EHEC and EPEC. The MIC values of C18G determined for the EHEC strains are very similar to those previously obtained for *C. rodentium* under similar experimental conditions (24). In contrast, the smaller differences in MICs obtained for the EPEC strains may suggest that EPEC OmpT plays a more marginal role in AMP resistance than EHEC OmpT.

FIG 1 Proteolytic degradation of LL-37 and C18G. OmpT-mediated degradation of LL-37 (A) and C18G (B). LL-37 or C18G (10 μ g) was incubated with the indicated strains for the indicated times. The resulting AMP cleavage products were separated by Tris-Tricine SDS-PAGE and visualized by Coomassie staining. Asterisks, migration of the dye front; pound sign, an aberrantly migrating band that is observed only after complete C18G cleavage and may correspond to cleavage product aggregates. Data shown are representative of at least three independent experiments.

EHEC and EPEC cleave AMP substrates at different rates. To confirm the proteolytic degradation of α -helical AMPs by OmpT, time course experiments monitoring the cleavage of LL-37 and C18G were conducted. AMPs were incubated with the various bacterial strains, and degradation products were analyzed by Tris-Tricine SDS-PAGE. As shown in Fig. 1A, cleavage of LL-37 by EHEC wild type was observed within the first 5 min of incubation and was complete by 60 min. As expected, no cleavage was observed for the EHEC $\Delta ompT$ mutant, but complementation of the deletion mutant with pEHompT resulted in complete cleavage of LL-37 within 30 min. In EPEC, evidence of LL-37 cleavage by the wild-type strain appeared only after 30 min, and cleavage was incomplete by 60 min. The EPEC $\Delta ompT$ mutant did not cleave LL-37, and complementation of this strain with pEPompT resulted in almost complete cleavage by 60 min. Both EHEC and EPEC wild-type strains cleaved C18G more rapidly than LL-37 (Fig. 1B). Cleavage of C18G by EHEC wild type was complete by 5 min. The EHEC $\Delta ompT$ mutant did not cleave C18G for the duration of the assay. Complementation of the EHEC $\Delta ompT$ mutant with pEHompT resulted in complete cleavage of C18G by 2 min. In the case of EPEC wild type, some cleavage products were observed after 5 min, but degradation remained incomplete at 15 min. The EPEC $\Delta ompT$ mutant did not cleave C18G, and complementation of this mutant with pEPompT resulted in the complete cleavage of C18G by 5 min. Altogether, these data show that

FIG 2 Mass spectrometry analysis of LL-37 degradation products. OmpT-dependent LL-37 cleavage products were detected by liquid chromatography and analyzed by MS/MS. Shown is a schematic of the LL-37 amino acid sequence. Dibasic sequences are shown in black. Vertical filled arrows, OmpT cleavage sites; horizontal open arrows, fragments detected by MS/MS analysis.

both EHEC OmpT and EPEC OmpT mediate degradation of LL-37 and C18G. They also indicate that both AMPs are cleaved more rapidly by EHEC than EPEC.

EHEC OmpT cleaves LL-37 at dibasic sites. *E. coli* K-12 OmpT was previously reported to preferentially cleave substrates between two consecutive basic amino acids (40). LL-37 contains 11 basic residues, including the 2 dibasic sequences RK and KR (Fig. 2). LL-37 was incubated with EHEC $\Delta ompT$ (pEHompT) cells, and at various time points, cleavage products were separated from bacterial cells by filtration and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Under these conditions, LL-37 was observed as a single peak at m/z 1,124.5, corresponding to the quadruply protonated molecular ion of LL-37 with a molecular mass of 4,494 Da that is consistent with the calculated mass of 4,493.3 Da. Following 5 min of incubation with EHEC $\Delta ompT$ (pEHompT) cells, additional peaks corresponding to molecular species of 2,188, 2,341, and 3,644 Da were observed (Fig. 2). Further incubation with bacterial cells for up to 1 h did not lead to the appearance of additional peaks, indicating that OmpT did not further degrade LL-37. N-terminal sequencing by Edman degradation of the LL-37 cleavage products from the 5- and 15-min time points further confirmed the cleavage sites obtained by LC-MS/MS (data not shown). In addition, peptides corresponding to the LL-37 cleavage products were synthesized, and MICs were determined. Large increases in MIC values were obtained for EHEC and EPEC strains (Table 3), indicating that OmpT-mediated cleavage of LL-37 is protective. These data clearly show that EHEC OmpT cleaves LL-37 at two dibasic sites and inactivates its bactericidal activity.

Cleavage of a synthetic FRET substrate by EHEC and EPEC OmpT. To further compare the catalytic activities of the EHEC OmpT and EPEC OmpT, we measured the cleavage of the synthetic FRET substrate containing the dibasic sequence RK in its center [2Abz-SLGRKIQR-K(Dnp)-NH₂]. This substrate is derived from the protein C2 of the classical complement pathway (20). Cleavage of the substrate was monitored by measuring fluorescence emission over time. Incubation of the substrate with EHEC wild type resulted in a rapid increase in fluorescence, reaching a maximum value of 175 arbitrary units (AU) after 1 h (Fig. 3A). Incubation with the EHEC $\Delta ompT$ strain resulted in minimal fluorescence emission that remained below 20 AU for the duration of the assay, indicating that substrate cleavage was due to OmpT. Complementation of the EHEC $\Delta ompT$ strain with pEHompT resulted in an even more rapid increase in fluorescence, followed by a plateau at the value of 200 AU after 15 min. As shown in Fig. 3B, EPEC wild type exhibited a lower rate of cleavage than EHEC wild type. The fluorescence level increased only to about 80 AU

FIG 3 Proteolytic cleavage of a synthetic FRET peptide. The synthetic FRET peptide containing the dibasic sequence RK was incubated with various EHEC and EPEC strains. Peptide cleavage, indicated by the increase in fluorescence, was measured over time. (A) Fluorescence of the FRET peptide incubated with EHEC wild type (black), EHEC $\Delta ompT$ mutant (red), and EHEC $\Delta ompT$ (pEHompT) mutant (blue); (B) fluorescence of the FRET peptide incubated with EPEC wild type (black), EPEC $\Delta ompT$ mutant (red), and EPEC $\Delta ompT$ (pEPompT) mutant (blue). All samples were normalized against a PBS blank; data shown are representative of three independent experiments.

during the course of the assay. The EPEC $\Delta ompT$ mutant showed a slow increase in fluorescence that remained below a value of 25 AU, indicating that substrate cleavage was primarily OmpT dependent. Complementation of the EPEC $\Delta ompT$ mutant with pEPompT resulted in an increased rate in substrate cleavage, with fluorescence reaching a maximum value of 190 AU. These data indicate that EHEC OmpT cleaved the FRET substrate more rapidly than EPEC OmpT. Altogether, these data suggest that EHEC OmpT is more efficient than EPEC OmpT at protecting cells from AMPs (Table 3) and at cleaving LL-37, C18G, and the FRET substrate (Fig. 1 and 3). Several explanations may account for these apparent differences in activity. First, the *ompT* genes may be differentially expressed in EHEC and EPEC. Second, the OmpT proteins may be differentially targeted to the OM of EHEC and EPEC.

FIG 4 Expression of the *ompT* gene. (A) Transcription of *ompT* in the EHEC and EPEC wild-type and $\Delta ompT$ strains; (B) transcription of *ompT* in the EPEC wild-type, $\Delta ompT$, $\Delta ompT$ (pEPompT), and $\Delta ompT$ (pEHompT) strains. Expression of *ompT* was quantified by RT-qPCR. Relative mRNA expression is representative of *ompT* expression normalized against 16S RNA. Results are expressed as means $\pm$ SDs. Statistical significance was assessed using a one-way analysis of variance and Tukey's *post hoc* comparison test. Unless otherwise indicated, asterisks indicate statistical significance versus wild type (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Third, the catalytic activities of the OmpT proteins may be differentially regulated by unknown factors.

Expression of the *ompT* gene is higher in EHEC than in EPEC. To examine the expression of the *ompT* gene in EHEC and EPEC, relative transcript levels were analyzed by quantitative RT-PCR. The level of *ompT* transcript was normalized to the 16S RNA level and analyzed using the $2^{-\Delta CT}$ method (26). Expression of the *ompT* gene was 32-fold higher in EHEC wild type than in EPEC wild type (Fig. 4A). As expected, the *ompT* transcript was absent in the EHEC and EPEC $\Delta ompT$ strains. As shown in Fig. 4B, complementation of the EPEC $\Delta ompT$ strain with either pEPompT or pEHompT resulted in the overexpression of the *ompT* gene. Compared to the levels in wild-type EPEC, the levels of the *ompT* transcript were 50- and 100-fold higher in the EPEC $\Delta ompT$ mutant complemented with pEPompT or pEHompT, respectively. Interestingly, complementation of the EPEC $\Delta ompT$ mutant with

pEHompT resulted in a significant increase in the level of *ompT* transcript compared to that for the same strain complemented with pEPompT. These qPCR results indicate that the faster degradation of OmpT substrates by EHEC may be due to a higher expression of the *ompT* gene in EHEC than EPEC under our experimental conditions.

Presence of OmpT at OM of EHEC and EPEC. To correlate the transcription levels of *ompT* with the presence of OmpT at the OM of EHEC and EPEC, FLAG-tagged *ompT* plasmids were generated and protein levels were examined by immunoblotting. First, the presence of the OmpT protein at the OM was investigated by fractionating whole-cell lysates of the EPEC $\Delta ompT$ mutant complemented with pEPompT-FLAG. The band corresponding to OmpT-FLAG was detectable in the whole-cell lysate and in the OM fraction but undetectable in the cytoplasmic and inner membrane fractions (Fig. 5A). Similar results were obtained for the EHEC $\Delta ompT$ mutant complemented with pEHompT-FLAG (data not shown). Western blot analysis of whole-cell lysates from EHEC and EPEC $\Delta ompT$ strains complemented with either pEHompT-FLAG or pEPompT-FLAG revealed that EPEC OmpT-FLAG was expressed at lower levels than EHEC OmpT-FLAG, regardless of the host strain (Fig. 5B). To confirm these results and investigate the expression of OmpT in the wild-type strains, whole-cell lysates were analyzed using an antiserum developed against the CroP OM protease of *C. rodentium* (74% identical to OmpT). As shown in Fig. 5C, a band corresponding to wild-type OmpT was easily detected in EHEC lysates, but this band was undetectable in EPEC lysates. In addition, Western blot analysis of the EHEC and EPEC strains complemented with their respective genes confirmed that EHEC OmpT is expressed at higher levels than EPEC OmpT (Fig. 5C), confirming the results obtained with the OmpT-FLAG proteins. Altogether, these data are in good agreement with the qPCR data. They show that both OmpT proteins are properly targeted to the OM. They also indicate that the OmpT protein is present at higher levels at the OM membrane of EHEC than that of EPEC and that the differences in expression levels are conferred in *cis* by the *ompT* gene or its promoter rather than by *trans*-acting factors differing between the two bacterial strains.

OmpT promoter swapping. To test whether the lower expression of EPEC *ompT* is caused by differences in the promoter regions, we swapped the EHEC *ompT* promoter with that of EPEC *ompT* to generate plasmid pEPpromEHompT that contains the EPEC *ompT* promoter in front of the EHEC open reading frame. Similarly, the EPEC *ompT* promoter was swapped with that of EHEC to generate plasmid pEHpromEPompT that contains the EHEC *ompT* promoter in front of the EPEC open reading frame. The amounts of OmpT produced by the various EPEC and EHEC strains were analyzed by Western blotting. As shown in Fig. 6A, the EPEC $\Delta ompT$ mutant complemented with pEHompT produced larger amounts of OmpT than the same strain complemented with pEPompT, confirming the results shown in Fig. 5B. When the EHEC *ompT* gene was under the control of the EPEC *ompT* promoter (pEPpromEHompT), the level of OmpT produced was similar to that produced by the EPEC $\Delta ompT$ mutant complemented with pEPompT, indicating that the EPEC *ompT* promoter is responsible for the decreased OmpT expression observed in EPEC (Fig. 6A). Conversely, when the EPEC *ompT* gene was under the control of the EHEC *ompT* promoter (pEHpromEPompT), the level of OmpT produced was similar to that pro-

FIG 5 Detection of the OmpT protein by Western blotting. (A and B) OmpT-FLAG was detected using a polyclonal anti-FLAG antibody; filled arrows, OmpT-FLAG protein species. (A) Whole-cell lysates (WCL), soluble fractions (CYT), inner membrane fractions (IM), and OM fractions (OM) from the EPEC $\Delta ompT$ mutant with the empty vector ($\Delta ompT$) or pEPompT-FLAG (pEPompT); (B) whole-cell lysates of the EHEC $\Delta ompT$ mutant with the empty vector ($\Delta ompT$), pEHompT-FLAG (pEHompT), or pEPompT-FLAG (pEPompT) and of the EPEC $\Delta ompT$ mutant with the empty vector ($\Delta ompT$), pEPompT-FLAG (pEPompT), or pEHompT-FLAG (pEHompT); (C) OmpT was detected using a polyclonal anti-CroP antibody. Whole-cell lysates of EHEC wild type, $\Delta ompT$ mutant, and $\Delta ompT$ (pEHompT) mutant and of EPEC wild type, $\Delta ompT$ mutant, and $\Delta ompT$ (pEPompT) mutant. All samples were normalized (by OD₆₀₀) to ensure that the same number of cells was used. Data shown are representative of three independent experiments. Filled arrows, OmpT protein species; asterisks, cross-reactive bands.

duced by the EPEC $\Delta ompT$ gene complemented with pEHompT (Fig. 6A). A similar trend was observed when the EHEC $\Delta ompT$ gene was complemented with these various plasmids (Fig. 6B). Densitometry analyses were performed on 3 independent Western blots to quantify OmpT amounts (data not shown). Statistically significant differences in the amount of OmpT produced by pEPompT and pEHompT were observed ($P < 0.05$), regardless of genetic background. By analyzing OmpT bands corresponding to the promoter swap constructs, we found that the amount of

FIG 6 The EPEC *ompT* promoter lowers the expression of EHEC OmpT. (A and B) OmpT from various EPEC (A) and EHEC (B) strains was detected by Western blotting of whole-cell lysates using a polyclonal anti-CroP antibody. Asterisks, cross-reactive bands. Data shown are representative of three independent experiments. (C and D) Cleavage of the FRET peptide was measured over time. (C) Fluorescence of the FRET peptide incubated with EPEC wild type and $\Delta ompT$, $\Delta ompT$ (pEPompT), $\Delta ompT$ (pEHompT), $\Delta ompT$ (pEPpromEHompT), and $\Delta ompT$ (pEHpromEPompT) mutants; (D) fluorescence of the FRET peptide incubated with EHEC wild type and $\Delta ompT$, $\Delta ompT$ (pEHompT), $\Delta ompT$ (pEPompT), $\Delta ompT$ (pEPpromEHompT), and $\Delta ompT$ (pEHpromEPompT) mutants. All samples were normalized against a PBS blank; data shown are representative of two independent experiments.

OmpT was dependent on the promoter used ($P < 0.05$ for EPEC and $P < 0.01$ for EHEC). To correlate OmpT expression with enzymatic activity, cleavage of the FRET substrate was measured. As shown in Fig. 6C, the EPEC $\Delta ompT$ mutant complemented with pEHompT cleaved the FRET substrate faster than the other EPEC strains, as indicated by the rapid increase in fluorescence with a plateau at about 200 AU. When the EPEC $\Delta ompT$ mutant was complemented with pEPpromEHompT, the increase in fluorescence was very similar to that of the same strain complemented with pEPompT (Fig. 6C). When the EPEC $\Delta ompT$ mutant was complemented with pEHpromEPompT, the increase in fluorescence was very similar to that of the same strain complemented with pEHompT. A similar trend was observed when the EHEC $\Delta ompT$ gene was complemented with these various plasmids (Fig. 6D). Taken together, these data clearly show that the differential *ompT* expression, protein level, and OmpT activity in EHEC and EPEC are due to differences in the promoters.

DISCUSSION

During colonization at mucosal surfaces, EHEC and EPEC must overcome innate host defenses such as the secretion of various AMPs by epithelial cells, including the human cathelicidin LL-37. Proteolytic degradation of α -helical AMPs by OM proteases of the omptin family has been shown to be one resistance mechanism used by Gram-negative pathogens. The *S. enterica* PgtE, *Y. pestis* Pla, and *C. rodentium* CroP OM proteases were reported to degrade α -helical AMPs (10, 11, 24). To investigate the involvement of EHEC and EPEC OmpT in the degradation of α -helical AMPs, we generated and characterized *ompT* deletion mutants. When

tested under the same experimental conditions, consistent differences in the rates of α -helical AMP degradation and subsequent contributions to resistance were observed between EHEC OmpT and EPEC OmpT. These differences in AMP resistance were directly associated with decreased EPEC *ompT* expression and OmpT protein levels compared to EHEC. Promoter-swapping experiments showed that the decreased expression of EPEC *ompT* was promoter dependent.

Striking differences in the proteolytic degradation of α -helical AMPs were observed between EHEC and EPEC cells expressing OmpT at wild-type levels (Fig. 1). In contrast to what was observed for EHEC cells, EPEC cells poorly degraded both LL-37 and C18G. The presence of OmpT at the OM of wild-type EPEC cells is supported by several lines of evidence. First, peptide bands corresponding to small amounts of cleavage products were observed at later time points for EPEC wild type but not for the EPEC $\Delta ompT$ mutant. Second, the intensity of these bands increased dramatically upon complementation and, thus, overexpression of EPEC OmpT. The peptide degradation patterns obtained for the EPEC $\Delta ompT$ (pEPompT) mutant were somewhat similar to those obtained for EHEC wild-type cells. Third, wild-type EPEC cells readily cleaved the FRET substrate in an OmpT-dependent manner (Fig. 3B). The OmpT protease from *E. coli* K-12 was previously reported to preferentially cleave substrates between two consecutive basic amino acids (5, 40). Our results are in agreement with these previous reports, since EHEC OmpT rapidly cleaves LL-37 at dibasic sequences (Fig. 2). We did not observe any evidence of preferential cleavage at the RK or KR site of LL-37. In addition, no further cleavage of the LL-37 fragments by OmpT was observed at

the later time points, confirming that OmpT exhibits narrow cleavage specificity against LL-37. The substrate specificity of omptins depends on the sequence variability of the five outer loops (22). Because there are no amino acid changes in the EHEC and EPEC outer loop sequences, it is most likely that both EHEC and EPEC OmpT cleave LL-37 with the same specificity. Our results clearly indicate that the *ompT* gene is differentially regulated in EHEC and EPEC in a promoter-dependent manner. Such differential gene regulation between EHEC and EPEC is not unprecedented. The LEE, the major pathogenicity island of A/E pathogens, is known to exhibit subtle differences in gene regulation between EHEC and EPEC (27).

Although our results showed that EPEC OmpT poorly degrades LL-37 and C18G (Fig. 1), the MIC values, determined under similar growth conditions, indicate that the EPEC Δ *ompT* mutant is slightly more resistant to α -helical AMPs than the EHEC Δ *ompT* mutant (Table 3). In contrast to what was observed in EHEC, deletion of *ompT* in EPEC resulted in MIC values that were unchanged for LL-37 and minimally different for C18G. Despite this apparent minor role for OmpT in EPEC, we noted that the MIC values of LL-37 and C18G for EPEC were not strikingly lower than those obtained for EHEC wild type. Although we cannot rule out the possibility that expression of EPEC *ompT* is up-regulated during infection by stimuli that were not present in the growth medium used, these observations may suggest that EPEC relies, at least partly, on other mechanisms to resist AMP killing. Several possible mechanisms may account for this. For example, capsules, curli fibers, and efflux pumps have all been associated with AMP resistance by other pathogens (3, 9, 19, 31). Whether these previously described resistance mechanisms play a role in EHEC and/or EPEC AMP resistance remains to be determined. Additionally, we cannot completely rule out the possibility of an uncharacterized resistance mechanism in EPEC.

We previously identified and characterized CroP, the omptin from the mouse A/E pathogen *C. rodentium*, and showed that it can proteolytically degrade α -helical AMPs (24). By comparing the omptin mutants from the three A/E pathogens, striking similarities are noticed between EHEC and *C. rodentium*. Identical MIC values of C18G were obtained for the various strains of EHEC and *C. rodentium* under similar growth conditions. In addition, both wild-type EHEC and *C. rodentium* readily degraded both C18G and their native host cathelicidins, LL-37 and mCRAMP, respectively. In contrast, the contribution of EPEC OmpT to α -helical AMP resistance appears to be more marginal. Notably, the differential contribution of omptins to AMP resistance in these three A/E pathogens appears to correlate with their niches in their respective hosts. Both EHEC and *C. rodentium* infect the distal part of the large intestine, whereas EPEC infects the small intestine (28). Large quantities of LL-37 were detected in the epithelial cells of the human colon, whereas little or no expression was seen within epithelial cells of the small intestine (14). Likewise, mCRAMP was produced in larger amounts by the mouse colon epithelium than by epithelial cells that line the small intestinal villi and crypts (17). Therefore, high levels of omptin expression by A/E pathogens correlate with colonization at intestinal sites where the largest amounts of cathelicidins are produced. The smaller quantity of LL-37 present in the small intestine is probably compensated for by the secretion of large amounts of α -defensins from Paneth cells (1). It remains unclear whether EPEC OmpT and other omptins play a role in the proteolytic

degradation of defensins. Ongoing work in our laboratories is exploring this possibility.

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