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The c-di-GMP phosphodiesterase VmpA absent in *Escherichia coli* K12 strains affects motility and biofilm formation in the enterohemorrhagic O157:H7 serotype

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a foodborne pathogen that resists the acidic gastric environment, colonizes the gut epithelium, and causes hemorrhagic colitis and hemolytic–uremic syndrome, especially in children. The genomic island OI-47 of *E. coli* O157:H7 contains a gene, *z1528*, encoding an EAL-domain protein potentially involved in c-di-GMP hydrolysis that is absent in non-pathogenic *E. coli*. This gene, designated *vmpA*, is co-transcribed with *ycdT*, which is present in non pathogenic *E. coli* and encodes a diguanylate cyclase involved in c-di-GMP synthesis. To test for *vmpA* function, we constructed a *vmpA* knockout mutant. We also overexpressed *vmpA*, purified the VmpA protein and assayed for its activity *in vitro*. We found that VmpA possesses c-di-GMP phosphodiesterase activity and that the *vmpA* mutation results in increased biofilm formation, and reduced swimming motility, which is consistent with the function determined *in vitro*. Unexpectedly, suppressor mutations arise frequently in the *vmpA* background suggesting that VmpA plays an important regulatory role in *E. coli* O157:H7. These findings represent an example of remarkable flexibility in the organization of c-di-GMP signaling pathways in closely related species.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), particularly the O157:H7 serotype, is a foodborne pathogen that causes diseases in humans ranging from mild diarrhea to life-threatening complications such as hemolytic–uremic syndrome (Paton and Paton, 1998). The main reservoir of EHEC strains is healthy ruminants (Caprioli et al., 2005). Outbreaks have been associated with exposure to the farm environment, and with the consumption of undercooked meat, raw milk and dairy products, water, and fruits or vegetables contaminated with ruminant manure (Cieslak et al., 1993; O'Brien et al., 2001; Yatsuyanagi et al., 2002).

Cyclic dimeric GMP (c-di-GMP) is a cytoplasmic second messenger involved in regulation of various cellular functions including biofilm formation, motility and virulence,

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in response to environmental factors (reviewed in Tamayo et al., 2007; Hengge, 2009). Intracellular concentration of c-di-GMP is controlled by the opposite activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) that synthesize and degrade c-di-GMP, respectively. Protein domains associated with DGC and PDE activities have been designated GGDEF and EAL, based on the conserved amino acid motifs present in these domains. Yet another protein domain, HD-GYP, also possesses c-di-GMP PDE activity (Ryan et al., 2006). While c-di-GMP has been shown to regulate virulence in various bacterial species including *Vibrio cholerae*, *Salmonella enterica* Typhimurium, *Pseudomonas aeruginosa*, *Yersinia pestis*, *Borrelia burgdorferi*, *Brucella melitensis* and others (Tischler et al., 2002; Tischler and Camilli, 2004, 2005; Hisert et al., 2005; Kulasakara et al., 2006; Bobrov et al., 2011; He et al., 2011; Petersen et al., 2011), surprisingly few studies have explored the role of c-di-GMP in *E. coli* pathogenesis. Claret et al. (2007) showed that in the Crohn disease-associated adherent-invasive *E. coli*, c-di-GMP signaling pathways affect the fine tuning of flagellar and type-1 pili synthesis. Sjostrom et al. (2008) showed that in a newborn meningitis *E. coli* isolate, a putative PDE-encoding gene is present in the main S fimbrial operon. In non-pathogenic *E. coli*, c-di-GMP signaling has been shown to play a role in synthesis of curli, an important pathogenesis factor (Weber et al., 2006; Pesavento et al., 2008), however the role of c-di-GMP in curli synthesis in pathogenic *E. coli* has not been elucidated. The molecular mechanisms of c-di-GMP involvement in all of these processes remain unknown.

It has been generally accepted that reduced intracellular c-di-GMP levels are associated with increased motility, reduced biofilm formation and increased virulence in acute infections. We found that gene *z1528*, hereby designated *vmpA* gene, from the most virulent EHEC serotype O157:H7 predicted to encode an EAL domain PDE is absent in non-pathogenic *E. coli*. In this study, we explored *vmpA* function and its involvement in biology of O157:H7.

2. Material and methods

2.1. Bacterial strains and genetic manipulations

E. coli O157:H7 strain EDL933 was used throughout the study. Mutant strains and plasmids are described in Table 1. *E. coli* strains screened for the presence of *vmpA* are listed in Table 2 and were previously described (Girardeau et al., 2005). In-frame substitution of the *vmpA* gene by a gene conferring resistance to chloramphenicol in *E. coli* EDL933 was done by using the one-step PCR-based method of Datsenko and Wanner (2000). Trans-complementation of the mutant strain was done by cloning the coding sequence of *vmpA* under the P_{ara} promoter in pBAD30, a low copy vector, generating p(vmpA). Induction of *vmpA* expression was achieved with 0.5 mM arabinose. For cis-complementation, the *vmpA* coding sequence flanked by 1.1 kb upstream to the start codon was PCR amplified using high fidelity Pfx DNA polymerase (Invitrogen, Saint-Aubin, France) adding the BamHI restriction sites at the 5′- and 3′-end, and inserted in the suicide vector pKO3 (Link et al., 1997). The insert in the recombinant plasmid was entirely sequenced to check that no mutation was introduced during PCR. Then the gene encoding kanamycin resistance was cloned downstream to *vmpA* into the SmaI site of the recombinant pKO3::vmpA plasmid. The vmpA-kan DNA fragment flanked by 1 kb upstream and 1 kb downstream sequences was PCR amplified using high fidelity Pfx polymerase, treated with DpnI to eliminate the plasmid DNA, and used to replace the vmpA::Cam mutation of the *vmpA* mutant by the one step PCR method. Allelic

Table 1
EHEC strains, plasmids and oligonucleotides used in this study.

Strains and plasmids	Relevant characteristics	Reference
Strains		
EDL933	Wild type EHEC O157:H7	O'Brien et al. (1983)
ΔvmpA	EDL933 Δ*vmpA* Cam^r	This work
ΔvmpA::vmpA	*ΔvmpA cis* complementation Cam^r Kan^r	This work
Plasmids		
pBAD30	Low copy P_{ara} Amp^r	Guzman et al. (1995)
pvmpA	pBAD30::vmpA	This work
pKO3		Link et al. (1997)
pKO3::vmpA::Kan		This work

Oligonucleotides use	Sequence(5′ → 3′)[a]	
RT-PCR 1	TATTGGCGCTGTTGTGACTC	This work
RT-PCR 2	CCCAGGTGTCGTTGACTTTT	This work
RT-PCR 3	TATTGGCGCTGTTGTGACTC	This work
RT-PCR 4	CAGTAACACCCCCAGAATGC	This work
RT-PCR 5	CCGGAGAATTTTCTGTGCAT	This work
RT-PCR 6	GCGTTCGGTAATCTCAAGGA	This work
vmpA in situ hybridization	CGACTGGCTGATAACGCACT (F)	This work
	TCTCTACAGGCAGGACACCA (R)	This work
vmpA mRNA quantification	CGATGTTTACGGTACGAGCT (F)	This work
	GGCATAAGGAACACTATGGC (R)	This work
rpoA mRNA quantification	GGTGAGAGTTCAGGGCAAAG (F)	This work
	ACCGCGCTGAACTTTGATAC (R)	This work

[a] F: forward primer; R: reverse primer.