The role of pyridoxal phosphate in the function of EspB, a protein secreted by enteropathogenic *Escherichia coli*

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Abstract The sequence of EspB, a secreted protein required for virulence of enteropathogenic *Escherichia coli* (EPEC), reveals a motif common to enzymes that bind pyridoxal phosphate. Pyridoxal phosphate was not found by fluorometry in concentrated supernatants of EPEC cultures that contain EspB. Plasmids containing cloned *espB*, in which the lysine residue conserved in the motif was substituted with either an arginine or methionine residue, remained capable of complementing an *espB* deletion mutant to restore EspB function. The results of these studies do not support a role for pyridoxal phosphate in EspB function.

Key words: Protein secretion; Pyridoxal phosphate; Bacterial pathogenesis; Fluorometry; Enteropathogenic *Escherichia coli*

1. Introduction

Enteropathogenic *Escherichia coli* (EPEC), a leading cause of infantile diarrhea in the developing world, disrupts host cell microvilli, induces rearrangements of actin, and initiates the formation of a cup-like pedestal to which the bacteria intimately adhere in a process known as the attaching and effacing effect [1]. A chromosomal pathogenicity island encoding a type III secretion pathway is necessary and sufficient for this attaching and effacing effect [2,3] and is used by EPEC to secrete several proteins [4] including the translocated intimin receptor (Tir). Tir is inserted into the host cell membrane, where it serves as the receptor for the bacterial outer membrane protein intimin [5], which is required for intimate attachment. EspB, another protein secreted via the type III system, is required for EPEC virulence in an adult volunteer model [6] and for numerous in vitro phenotypes attributed to EPEC including attaching and effacing activity [7], activation of NF-κB [8], and in polarized monolayers changes in short-circuit current [9], transepithelial electrical resistance [10], and transmigration of leukocytes [11]. The precise function of EspB is not known, but since it is required for translocation of Tir into the host cell membrane [5], it may be considered part of the EPEC translocation apparatus. On the other hand, EspB itself is inserted via the type III system into the host cell [12,13] and expression of EspB within host cells leads to dramatic changes in the cytoskeleton [14], suggesting that EspB may itself be a bacterial effector protein. Analysis of the predicted amino acid sequence of EspB from EPEC strain E2348/69 revealed the presence at residues 136–145 (TSAKSIGTVS) of a near perfect consensus sequence common to the pyridoxal 5′-phosphate-binding motif of several type II aminotransferase enzymes [7]. The consensus pattern for this type of enzyme is T-[LIVMFYW]-[STAG]-K-[SAG]-[LIVMFYWR]-[SAG]-X(2)-[SAG], where X is any amino acid and the indicated alternative amino acids are allowed at bracketed positions. Of particular note is the presence, at position four, of a critical lysine residue, which forms a covalent bond with pyridoxal 5′-phosphate [15]. In the EspB protein from EPEC strain E2348/69, there is a single amino acid difference at position two, placing a serine at this position rather than one of the seven amino acids previously recognized in the consensus pattern. Since substitution of a single amino acid, other than at the site of the critical lysine residue, does not necessarily preclude interaction of pyridoxal phosphate with binding domains in other transaminases [16], we sought to determine whether EspB incorporates pyridoxal phosphate at this site. To test the hypothesis that EspB uses pyridoxal phosphate as a cofactor for its function, we sought fluorometric evidence of pyridoxal phosphate in EPEC supernatants containing EspB and performed site-directed mutagenesis of the putative pyridoxal phosphate-binding motif.

2. Materials and methods

2.1. Bacterial strains and plasmids

The prototypic EPEC serotype O127:H6 strain E2348/69 and isogenic strain UMD864 containing an in-frame deletion of the *espB* gene have been described [7]. Plasmids and oligonucleotides used are indicated in Table 1.

2.2. Preparation of culture supernatants

Protein samples were prepared from supernatants concentrated using a stirred-cell filtration device as previously described [17]. The protein concentration for each sample was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). The percent of EspB present in each supernatant preparation was estimated by Coomassie blue staining and the concentration of EspB was calculated on this basis.

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Table 1
Plasmids and oligonucleotides used in this study

Designation	Sequence or source	Description or purpose[a]
pMSD3	[7]	Plasmid carrying a 2.3 kb *Bgl*II fragment of EPEC DNA that includes the *espB* gene
pACYC184	[22]	Cloning vector containing chloramphenicol and tetracycline resistance
pKT.K139R	This study	Plasmid carrying the *espB* gene with the K to R mutation in the PLP motif
pCO.K139M	This study	Plasmid carrying the *espB* gene with the K to M mutation in the PLP motif
Donne-3	5′-GGG TCG ACG ATC TAC GCG GAT GGA CA-3′	Upstream end of the 2.3 kb *Bgl*II fragment of pMSD3, containing *espB*, with the *Sal*I site incorporated
Donne-4	5′-GGG CAT GCG ATC TTT TAT CGT TTT CA-3′	Downstream end of the 2.3 kb *Bgl*II fragment with the *Sph*I site incorporated
Donne-19	5′-GTT GAA CGC GAA ATC C-3′	Nucleotides 698–683 of *espB*; used to sequence the K to R mutation
Donne-132	5′-GCC GCT TCT ACT TCT GCG AGG TCT ACT GGT ACA GTC-3′	Nucleotides 508–530 of *espB*; with Donne-133 introduces a change at nucleotide 417 to generate the K to R mutation
Donne-133	5′-CTC GCA GAA GTA GAA GCG GCT TGC-3′	Nucleotides 527–503 of *espB*; with Donne-132 introduces the K to R mutation
Donne-201	5′-GCC GCT TCT ACT TCT GCC ATG TCT ATC GGT ACA GTC-3′	Nucleotides 507–543 of *espB*; with Donne-202 introduces a change at nucleotide 416 to generate the K to M mutation
Donne-202	5′-ATG GCA GAA GTA GAA GCG GCT TGC-3′	Nucleotides 550–526 of *espB*; with Donne-201 introduces the K to M mutation

[a]Nucleotide positions according to GenBank–EMBL accession number Z21555.

2.3. Fluorometric detection of pyridoxal phosphate

Samples of concentrated secreted proteins were assayed for pyridoxal phosphate in two independent experiments using the method of Adams [18], which takes advantage of the high fluorescence yield of pyridoxal phosphate after reaction with cyanide. Briefly, each protein sample was suspended in potassium phosphate buffer, acidified by the addition of an equal volume of 11% TCA, incubated with KCN and adjusted to a final pH of 3.8. Samples were excited at 325 nm in a Shimadzu RF 5301 PC fluorometer and the emission spectra from 370 to 520 nm were recorded.

2.4. Site-directed mutagenesis

A two-step overlap PCR technique was used to engineer mutations of the pyridoxal phosphate-binding domain. The plasmid pMSD3 was used as the template for each PCR reaction. Oligonucleotides Donne-3 and Donne-4 bracket the 2.3 kb region of DNA in which the *espB* gene lies and incorporate a *Sal*I and *Sph*I site, respectively. Two additional oligonucleotides, Donne-132 (or Donne-201) and Donne-133 (or Donne-202), are complementary and correspond to the sequence of the pyridoxal phosphate-binding site with a single nucleotide change at position 526 or 527 of the *espB* gene which, when translated, changes the amino acid 139 from a lysine to arginine or methionine, respectively. Donne-3 and Donne-133 (or Donne-202) and Donne-4 and Donne-132 (or Donne-201) were used in separate reactions to amplify the 2.3 kb template containing the *espB* gene. The result is two half products with a complementary 18 bp overhang corresponding to the pyridoxal phosphate-binding sequence with the introduced mutation. These products were then combined and subjected to amplification using Donne-3 and Donne-4 to regenerate the recombinant *espB* gene, containing the desired point mutation. The second amplification products were then cloned into the pACYC184 vector via the *Sal*I and *Sph*I sites. The resulting plasmids containing the K139R and the K139M mutations were confirmed by sequence analysis and designated pKT.K139R and pCO.K139M, respectively. In each case, PCR reactions were carried out for 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. DNA sequencing of plasmid templates was performed at the University of Maryland Biopolymer Laboratory using a model 373A sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Fluorescence actin staining test

The fluorescence actin staining test, which measures the accumulation of filamentous actin beneath areas of attaching and effacing bacteria [19], was performed in chamber slides as previously described [20].

3. Results and discussion

We used a fluorometric method to determine whether EPEC culture supernatants containing EspB also contain pyridoxal phosphate. Fig. 1 shows the absorption spectra, after excitation at 325 nm, for the supernatant proteins containing 1 μM of secreted EspB from the wild type strain, E2348/69, and an equal concentration of protein from the *espB* deletion mutant, UMD864, respectively. By comparison to the spectra for the purified pyridoxal 5′-phosphate samples, and phosphorylase A, a pyridoxal phosphate containing enzyme, it is apparent that there is little, if any, pyridoxal phosphate present in the supernatant samples assayed. To insure that pyridoxal phosphate, if present, could be detected in EPEC supernatants using this assay, we also analyzed supernatant proteins from the *espB* deletion mutant UMD864 after the addition of 1 μM pyridoxal phosphate. If EspB binds pyridoxal phosphate on an equimolar basis as predicted, then the addition of 1 μM of pyridoxal phosphate to the deletion mutant sample should have the effect of bringing the intensity of the emission spectra for UMD864 up to the level of the wild type strain. As seen in Fig. 1, the absorption spectrum for this sample was intermediate between the 0.2 and 2.0 μM pyridoxal phosphate controls, with a much greater peak at 420 nm than that of the wild type sample. These results demonstrate that pyridoxal phosphate is not present as cofactor for EspB upon secretion of the protein from EPEC.

To address the possibility that the pyridoxal phosphate motif is important to the function of EspB during infection, we generated two *espB* constructs with defined mutations in the pyridoxal phosphate-binding site (Table 1). Plasmids containing these mutant *espB* alleles were then introduced into the *espB* deletion strain, UMD864. The *espB* mutant constructs were tested for their ability to complement UMD864 to restore the attaching and effacing phenotype. This process may be monitored by fluorescence microscopy using fluorescein isothiocyanate-conjugated phalloidin, which binds to ac-

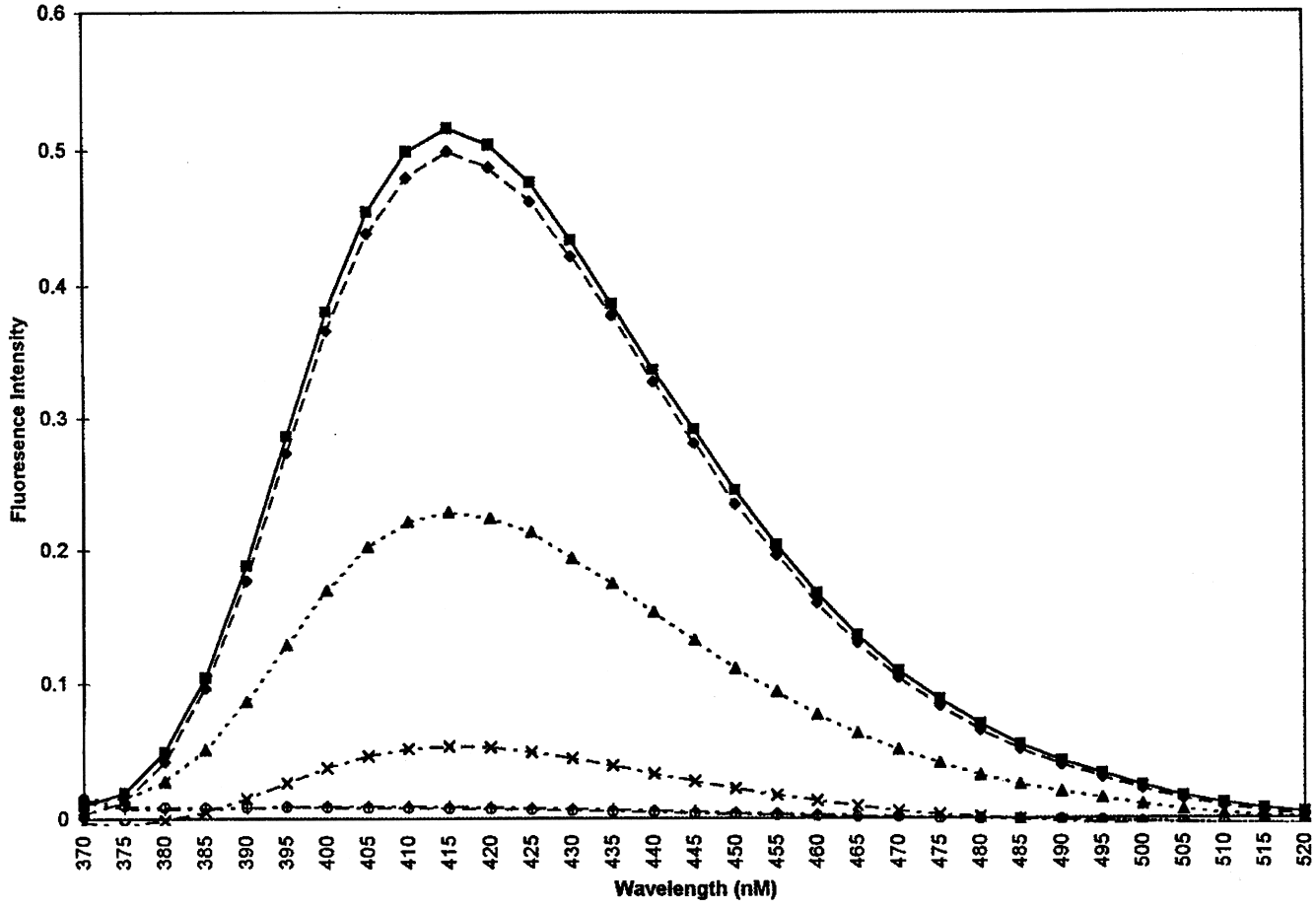

Fig. 1. Emission spectra of bacterial supernatants after excitation at 325 nm. Proteins secreted by either the wild type EPEC strain, E2348/69, or the *espB* deletion strain, UMD864, were assayed for the presence of pyridoxal phosphate by fluorometry as described in Section 2. For wild type supernatant samples 1 μM EspB was tested (○). For UMD864, an equivalent amount of protein was assayed (+). An equivalent amount of secreted proteins from strain UMD864 to which 1 μM pyridoxal phosphate was added (▲), 2 μM phosphorylase A (■), 0.2 μM pyridoxal phosphate (×) and 2 μM pyridoxal phosphate (♦) were also tested as controls.

cumulated filamentous actin underneath adherent bacteria [19]. If EspB requires the pyridoxal phosphate motif to perform its function, then substitution of the critical lysine residue in the pyridoxal phosphate-binding site with either arginine or methionine would be expected to abolish the ability of EspB to recruit filamentous actin in host cells. We found that recombinant strains containing either of the pyridoxal phosphate point mutation constructs were able to cause the condensation of actin under their sites of adherence, as was wild type (data not shown). In contrast, the *espB* mutant lacking a complementing plasmid was unable to recruit actin. Thus, substitutions at lysine 139 in the putative EspB pyridoxal phosphate-binding site had no effect on the function of the EspB protein.

The results of this study fail to support the earlier suggestion that EspB is a pyridoxal phosphate-dependent, type II transaminase. That we could not detect pyridoxal phosphate in samples containing ample quantities of EspB, while we were able to detect the cofactor when it was added exogenously, clearly indicates that it is not associated with EspB in significant quantities. To more definitively exclude a role of pyridoxal phosphate in EspB function, we replaced the critical

Table 2
The pyridoxal phosphate motif of EspB from *E. coli* strain E2348/69, comparable regions of the EspB sequence from other attaching and effacing strains, and the consensus pyridoxal phosphate motif

Strain	Accession number	Sequence						
E2348/69	Q05129	T	**S**	A	K S	I	G	T V S
C. rodentium	AAD51754	**A**	**S**	A	K S	V	N	T A S
EDL933	AAC31499	**A**	**S**	S	K A	**A**	G	A A S
413.89-1	CAA67984	T	**S**	S	K A	I	**D**	A A S
E65/56	AAD34584	T	**S**	S	K A	I	**D**	A A S
4221	AAD12778	T	**S**	S	K A	I	**D**	A A S
RDEC-1	AAB69980	T	**S**	S	K A	I	**D**	A A S
B10	AAC82360	T	**S**	S	K A	I	**D**	A A S
Pyridoxal phosphate consensus		T	[LIVMFYW]	[STAG]	K [SAG]	[LIVMFYWR]	[SAG]	X X [SAG]

Residues in bold differ from the consensus sequence.

lysine residue that would represent the site of covalent binding to pyridoxal phosphate with either arginine, a relatively conservative replacement, or methionine, which is similar in size, but lacks the amine residue necessary for forming a Schiff's base with the cofactor. Even though this lysine residue is absolutely conserved in all EspB sequences reported to date (Table 2), neither replacement had any detectable effect on function, clearly excluding an important role of pyridoxal phosphate in EspB function. That pyridoxal phosphate is not involved in EspB function is surprising, but not unprecedented. There are several proteins that have been reported to possess the consensus pattern for pyridoxal phosphate-binding but do not bind or require this cofactor for their function [21]. In addition, as more EspB sequences have accumulated, it has become apparent that EspB proteins from other attaching and effacing strains have even more variation from the consensus pyridoxal phosphate-binding motif than does the original sequence (Table 2). We therefore conclude that EspB does not require the cofactor pyridoxal phosphate for its essential function in EPEC infection.

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