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STRUCTURE NOTE

Crystal structure analysis reveals a novel forkhead-associated domain of ESAT-6 secretion system C protein in *Staphylococcus aureus*

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Key words: ***FHA domain; Esx secretion;*** **Staphylococcus aureus.**

INTRODUCTION

Pathogenic bacteria secrete many types of virulence factor or exotoxin to infect host cells and cause disease. They are usually secreted by the Sec translocon across the membrane by a mechanism that requires N-terminal signal peptides for recognition. However, Sec-independent systems for secretion of virulence factors, including Gram-negative type III and IV protein secretion systems, have been identified in Group A streptococci and other Gram-positive species.[1–4] The Esx secretion system in *Staphylococcus aureus* has recently been proposed to be one such Sec-independent secretion system.[3,5] Esx is a small secreted protein belonging to the *e*arly *s*ecreted *a*ntigen *t*arget 6 kDa (ESAT-6)-like proteins, which have a common WXG motif at the center of the sequence.[6] Esx homologs have also been identified in other bacteria, including *Mycobacterium tuberculosis*, *Clostridium acetobutylicum*, *Bacillus subtilis*, and *Listeria monocytogenes*.[5,6] The present study was performed in the pathogenic bacterium *S. aureus*, which has two Esx proteins, EsxA and EsxB, the molecular masses of which are 6 and 10 kDa, respectively, within a gene cluster comprised of eight ORFs [Fig. 1(A)].[7] Based on the deletion of the *esxa* gene in all versions of the attenuated *Mycobacterium bovis* Bacille Calmette–Guérin vaccine strain,[8] the observation that EsxA induces a strong T-cell response during infection,[9] and that mutants that fail to secrete EsxA and EsxB show defects in the pathogenesis of *S. aureus* murine abscesses,[5] these proteins in pathogenic bacteria are thought to be among the most important virulence factors in pathogenesis.[5] Interestingly, in every species in which *esx* genes are found, there is an ORF with two or three FtsK/SpoIIIE domains, which is *E*SAT-6 *s*ecretion system C protein (EssC) in the case of *S. aureus*, in the gene cluster that includes the *esx* genes [Fig. 1(A,B)].[6] The FtsK/SpoIIIE domain—PFAM entry PF01580—was found in the FtsK cell division protein from *Escherichia coli* and in the SpoIIIE sporulation protein that regulates prespore-specific gene expression in *B. subtilis*.[10,11] This domain contains a putative ATP-binding P-loop motif, and is categorized as an AAA+ domain observed in

Grant sponsors: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Ministry of Education, Science, Sports, and Culture of Japan.

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Received 19 September 2006; Accepted 18 October 2006
Published online 6 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/prot.21302

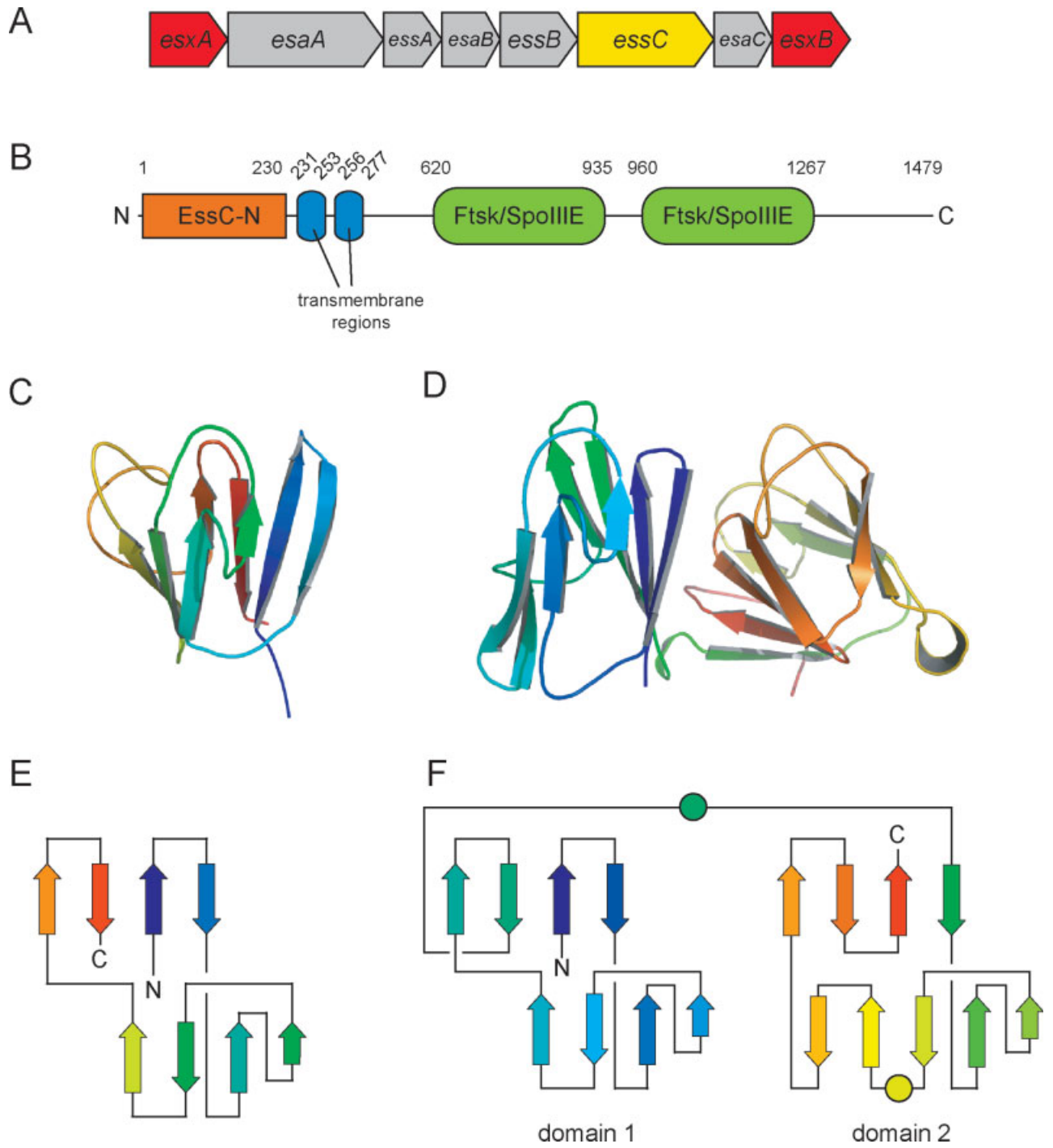

Figure 1

*(**A**) The gene cluster encoding* essCC. esx *genes and the* essC *gene are shown in red and yellow, respectively. (**B**) The domain structure of EssC. The N-terminal region is represented as an orange box. Transmembrane regions and FtsK/SpoIIIE domains are shown as blue and green ellipses, respectively. The numbers indicate the residue numbers at the start and end of each domain. (**C**) Ribbon diagram of the FHA domain in Ki67 from human (PDB ID: 1r21). The model is colored according to the sequence from blue at the N-terminus to red at the C-terminus, and is oriented in the direction of the domain1 in EssC-N described in Figure 1(D). (**D**) Ribbon diagram of EssC-N. The model is colored according to the sequence from blue at the N-terminus to red at the C-terminus. (**E**) Topology diagrams of FHA domain in human Ki67, the ribbon diagram of which is shown in Figure 1(C). The arrows and circles represent β-strands and α-helices, respectively. The model is colored according to the sequence from blue at the N-terminus to red at the C-terminus. (**F**) Topology diagrams of EssC-N. The model is colored according to the sequence from blue at the N-terminus to red at the C-terminus.*

DOI 10.1002/prot

many ATPases involved in macromolecular secretion systems, including those from Gram-negative type IV protein secretion systems.[12] Thus, the FtsK/SpoIIIE domain has been suggested to be involved in generating energy for the secretion of Esx proteins,[6] and this was corroborated by experiments using an *essC* mutant strain of *S. aureus*, where deletion of the FtsK/SpoIIIE domain in EssC resulted in abolition of production and secretion of EsxA and EsxB.[5] Although extensive studies have focused on the mechanisms of Esx secretion, the details remain unclear. To obtain insight into the Esx secretion mechanism, we investigated EssC from *S. aureus* from a structural viewpoint. EssC from *S. aureus* is a protein of 1479 amino acids and is thought to be a membrane-associated protein as it contains two transmembrane regions from positions 231 to 253 and 256 to 277 [Fig. 1(B)]. Both N- and C-terminal regions separated by transmembrane regions are thought to be located in the cytoplasm. FtsK/SpoIIIE domains are iterated twice in the C-terminal region [Fig. 1(B)]. In the present study, we determined the crystal structure of the N-terminal region of EssC from *S. aureus* at a resolution of 1.75 Å by the SAD method. Structural analysis indicated that this region is composed of two iterations of forkhead-associated (FHA) domain-like structures. We inferred the function of EssC based on the structural information.

MATERIALS AND METHODS

Construction of expression vectors for the N-terminal domain of EssC

The gene encoding the N-terminal region of EssC was amplified using KOD-Plus DNA polymerase (Toyobo) with *Staphylococcus aureus strain Mu50* genomic DNA as a template and the following degenerate primers. NdeI and XhoI sites were incorporated into the EssC-N-S (5′-GGAATTC*CATATG*CATAAATTGATTATAAAATATAACAA ACAATTG-3′) and EssC-N-AS (5′-CCG*CTCGAG*TGTA TTGTTCTTCTGTATTGGCTG-3′) sequences, respectively (restriction enzyme recognition sites are underlined). The PCR products were inserted into the NdeI and XhoI sites of the pET26b vector (Novagen), resulting in attachment of a His6-tag at the C-termini of the expressed N-terminal region of EssC. The accuracy of the DNA sequence was confirmed using an ABI 310 Genetic Analyzer (Applied Biosystems).

Expression and purification of N-terminal region of EssC

E. coli strain BL21 (DE3) transformed with the expression vector encoding the gene corresponding to the N-terminal region of EssC was grown at 37°C in LB medium supplemented with 100 μg mL^{-1} kanamycin until early stationary phase. To induce expression of the desired protein, IPTG was added to a final concentration of 0.5 m*M*, and the culture was grown for 16 h at 25°C. The selenomethionine derivative of the N-terminal region of EssC was expressed in a methionine auxotroph, *E. coli* strain B834 (DE3), grown in M9 medium supplemented with 1 m*M* selenomethionine. Cells were harvested by centrifugation at 4°C and 5000*g* for 10 min, washed with a buffer comprised of 50 m*M* phosphate (pH 8.0) and 300 m*M* NaCl, and then resuspended in the same buffer. The suspension was sonicated, followed by centrifugation at 40,000*g* for 30 min at 4°C. The supernatant was loaded onto a HisTrap column (GE Healthcare Bio-Sciences AB), preequilibrated with a buffer containing 50 m*M* phosphate buffer (pH 8.0) and 300 m*M* NaCl. After the column had been washed with 20 mL of the same buffer, the adsorbed protein was eluted with a buffer containing 50 m*M* phosphate (pH 8.0), 300 m*M* NaCl, and 500 m*M* imidazole. Fractions containing the N-terminal region of EssC were loaded onto a HiPrep desalting column (GE Healthcare Bio-Sciences AB) preequilibrated with a buffer containing 20 m*M* Tris–HCl (pH 8.0), 500 m*M* NaCl, and 1 m*M* EDTA to change the buffer. Fractions containing the desired protein were further purified on a HiLoad 26/60 Superdex 200-pg column (GE Healthcare Bio-Sciences AB) equilibrated with 20 m*M* Tris–HCl (pH 8.0) containing 300 m*M* NaCl and 1 m*M* EDTA.

Crystallization of N-terminal region of EssC

The purified protein was dialyzed against 20 m*M* Tris–HCl (pH 8.0) and concentrated to 20 mg/mL. Initial crystallization conditions were screened by the sparse matrix method at 20°C using Crystal Screen and Crystal Screen 2 kits (Hampton Research). Crystals of the N-terminal region of EssC most suitable for further analyses were grown by the hanging-drop vapor diffusion method from 100 m*M* Bis–Tris (pH 5.5), 20% polyethylene glycol 3350, and 0.2*M* magnesium acetate.

X-ray diffraction

X-ray diffraction of selenomethionine (Se-Met)-substituted N-terminal region of EssC was performed on the beamline BL5A at Photon Factory (Tsukuba, Japan) under cryogenic conditions (100 K). For single-wavelength anomalous diffraction phasing, a wavelength was chosen based on the fluorescence spectrum of the Se K absorption edge, corresponding to the maximum f'' (peak, 0.97938 Å). The diffraction data set was collected to a resolution of 1.75 Å with a Quantum 315 detector (ADSC). The crystal of the Se-Met-substituted N-terminal region of EssC belongs to space group $P2_12_12_1$ with unit cell dimensions of $a = 45.37$ Å, $b = 66.01$ Å, and $c = 88.29$ Å. One molecule of the protein was in the asymmetric unit with a V_M value of 2.39 $Å^3$ Da^{-1}. The

diffraction data were indexed, integrated, scaled, and merged using the HKL2000 program package.[13]

Structure solution and refinement

The structure of the Se-Met-substituted N-terminal region of EssC was solved by the single-wavelength anomalous diffraction method. The program SOLVE/RESOLVE[14,15] was used to calculate the initial phases, to improve the phases, and to perform automatic model building. The program RESOLVE successfully placed 156 of 238 residues. The complete atomic model with a total of 186 residues, including side chains, was rebuilt automatically using the program LAFIRE.[16]

Positional and individual *B* factor refinements were carried out with the program CNS,[17] using reflections ranging from 40 to 1.75 Å. A random of 5% of all observed reflections were set aside for crossvalidation analysis and used to monitor the refinement by calculating the free *R* value (R_{free}). The final model consisted of 186 residues, one chloride ion, and 196 water molecules with crystallographic *R* and R_{free} values of 19.5 and 22.2%, respectively. The stereochemical quality of the final refined model was analyzed with the program PROCHECK.[18] The crystallographic parameters and refinement statistics are summarized in Table I.

Table I
X-ray Data Collection and Refinement Statistics

	Se-Met substituted structure (SAD)
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	$a = 45.37$
	$b = 66.01$
	$c = 88.29$
Beamline	Photon Factory BL5
Resolution[a] (Å)	40–1.75 (1.81–1.75)
Wavelength (Å)	0.97938
R_{sym}[a,b] (%)	8.3 (31.3)
Completeness[a] (%)	100 (100)
Observed reflections	213,164
Unique reflections	26,172
Multiplicity	8.1
Refinement and model quality	
Resolution range (Å)	40–1.75
No. of reflections	27,431
R-factor[c]	0.195
R_{free}-factor[d]	0.222
Total protein atoms	1501
Total ligand atoms	1
Total water atoms	196
Average *B*-factor (Å^2)	22.14
r.m.s.d. from ideal	
Bond lengths (Å)	0.007
Bond angles (°)	1.44
Improper angles (°)	0.79
Dihedral angles (°)	27.04
Ramachandran plot	
Residues in most favored regions (%)	91.9
Residues in additional allowed regions (%)	7.5
Residues in generously allowed regions (%)	0.6

[a]The values in parentheses refer to data in the highest resolution shell.
[b]$R_{sym} = \Sigma_h \Sigma_i |I_{h,i} - \langle I_h \rangle| / \Sigma_h \Sigma_i |I_{h,i}|$, where $\langle I_h \rangle$ is the mean intensity of a set of equivalent reflections.
[c]*R*-factor $= \Sigma |F_{obs} - F_{calc}| / \Sigma F_{obs}$, where F_{obs} and F_{calc} are observed and calculated structure factor amplitudes, respectively.
[d]R_{free}-factor was calculated for *R*-factor, with a random 5% subset from all reflections.

RESULTS AND DISCUSSION

The crystal structure of the N-terminal region of EssC (EssC-N) was determined by the SAD method at a resolution of 1.75 Å. The final model contained 186 of the 238 residues of EssC-N, one chloride ion, and 196 water molecules. The C-terminal region (residues 187–238) could not be built because of the poor electron density map. The revealed structure consists of two domains, N-terminal (residues 1–77; domain1) and C-terminal (residues 82–186; Domain 2) domains, connected by a short linker with a short helix [Fig. 1(D)]. Despite the dissimilarity of their amino acid sequences, both domains construct similar β-sandwich structures, which superimposed well with a low root mean square difference (r.m.s.d.) of 1.71 Å for 77 Cα atoms. Domain1 is comprised of eight β-strands, and domain2 is comprised of nine β-strands and one helix. Some differences were observed between the two domains, for example, the number and order of the β-strands in the β-sandwich structure [Fig. 1(F)].

A structural alignment of each domain against all of the proteins in the Protein Data Bank [the atomic coordinates for the N-terminal domain of EssC from *Staphylococcus aureus* have been deposited in the Protein Data Bank (PDB # 1WV3)] by SSM at the European Bioinformatics Institute showed that the structures of domain1 and domain2 were similar to those of FHA domains; in the case of domain1, the FHA domain of the function unknown protein from *Arabidopsis thaliana* (1uht) with r.m.s.d. of 2.15 Å, that of Ki67 from human (1r21) with r.m.s.d. of 2.62 Å [Fig. 1(C,E)], that of kinase-associated phosphatase from *Arabidopsis* (1mzk) with r.m.s.d. of 2.27 Å, and that of CHK2 kinase from human (1gxc) with r.m.s.d of 2.51 Å; in the case of domain2, FHA domain of CHK2 kinase from human (1gxc) with r.m.s.d. of 2.36 Å and that of RAD53 from *yeast* (1k2n) with r.m.s.d of 2.40 Å. The FHA domain is a small protein module that recognizes phosphothreonine epitopes on proteins.[18,19] It has been identified in more than 200 proteins in species ranging from prokaryotes to higher eukaryotes with a great deal of functional diversity, including roles in intracellular signal transduction, transcription, protein transport, DNA repair, and protein degradation.[19] These findings suggest that EssC-N has a role in interaction with other proteins in the Esx secretion system.

DOI 10.1002/prot

Although FHA domains were found in many bacterial genomes by iterative PSI-BLAST searches, they were conserved in three restricted groups of bacteria—the mycobacteria and their relatives, the cyanobacteria, and selected Gram-negative proteobacteria.[20] The FHA domains identified previously have several common motifs in amino acid sequence: GR at the end of strand three, SXXH just before strand five, and NG just before strand seven.[20] EssC-N from *S. aureus*, however, does not have these motifs, and the iteration of FHA domain-like structures in this region could not be predicted from the amino acid sequence. The structures of several FHA domains and their complexes with short phosphopeptides have been reported previously,[21–27] where R31, S45, K46, and S65 (residue numbers are those of Ki67 FHA domain from human) located in a region where connecting loops are centered interacted with the phosphate group.[21] These phosphate binding residues are not conserved in the structures of domain1 or domain2 in EssC-N from *S. aureus*. Serine/threonine protein kinases and phosphatases are present among the neighboring genes of many FHA domain-containing proteins.[20] However, no genes for such enzymes were found around the EssC gene in the *S. aureus* genome. These observations suggest that EssC-N may not recognize phosphorylated proteins and may act as a distinct protein module from FHA domains investigated to date. EssC-N may have acquired novel mechanism of recognition to other proteins while retaining the FHA domain-like structure.

EssC from *S. aureus* has two iterations of FtsK/SpoIIIE domains in the C-terminal region, as described earlier [Fig. 1(B)]. The *esx* gene cluster, which contains an ORF with three iterations of the FtsK/SpoIIIE domains, that is, CAC0039, was also identified in the genome of *C. acetobutylicum*.[6,20] CAC0039 also has a transmembrane region in the interior of the ORF, where FtsK/SpoIIIE domains are located in the C-terminal region. Interestingly, PSI-BLAST search showed that the N-terminal region of CAC0039 also has an FHA domain, which possesses common sequential features to this domain, resulting in the identical domain composition to EssC from *S. aureus*, that is, FHA domains in the N-terminal region, transmembrane region in the center of ORF, and FtsK/SpoIIIE domains in the C-terminal region. FHA has been identified not only in the Esx secretion system but also in a Type III secretion system, which is another Sec-independent secretion system observed in Gram-negative bacteria involved in secretion and injection of pathogenicity proteins into the cytosol of host cells.[20] These entities of FHA domains in the Sec-independent secretion systems of virulence proteins suggest the universal importance of the FHA domain in the virulence secretion systems in bacteria.

EssC from *S. aureus* is equipped with two pairs of FHA-like domains and FtsK/SpoIIIE-like domains. Each domain may act independently in binding to its target protein, which has not been identified. Two secreted Esx proteins, that is, EsxA and EsxB, are involved in the esx gene cluster of *S. aureus* [Fig. 1(A)]. EssC is an essential protein for Esx secretion where mutations in the *essc* gene abolished production and secretion of EsxA and EsxB.[5] Thus, it is likely that EssC directly recognizes and binds Esx proteins. The most likely candidate for the target of FHA-like domains in Essc-N could be EsxA and EsxB. We are now attempting to observe the interaction of EssC with these molecules. Elucidating their binding will provide critical insights into the mechanism of the Sec-independent secretion mechanisms of virulence proteins.

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 DOI 10.1002/prot