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The C-terminus of the ESAT6-like secretion system virulence factor EsxC mediates divalent cation-dependent homodimerization

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ABSTRACT

The human pathogen *Staphylococcus aureus* encodes the ESAT6-like Secretion System (ESS). The ESS pathway secretes pathogenic substrates such as EsxA, EsxB, EsxC, EsxD and EssD that mediate staphylococcal establishment in persistent abscess lesions. The biochemical behavior of these substrates is not fully understood. EsxC is species-specific lysine-rich homodimer that lacks recognizable topogenic sequence. Studies have shown that EsxC is required for the secretion of other substrates, thereby revealing its biomedical importance. Here, EsxC self-association was investigated in the presence of several metal ion chelators. Results show that EsxC homodimerization is abolished in the presence of EDTA and EGTA, suggesting a role for calcium in mediating EsxC self-association. Complementation experiments confirm that EsxC homodimerization is calcium-dependent. N- and C-terminal truncations of EsxC were constructed, followed by bacterial two-hybrid screening. Results show that EsxC self-association is mediated by its C-terminal domain. Affinity purification of recombinant EsxC to apparent homogeneity, followed by chemical crosslinking and SDS-PAGE led to the detection of the monomeric and dimeric forms of the protein. In contrast and when a purified EsxC variant lacking the C-terminus was subjected to similar conditions, only the monomeric form was observed. These *in vivo* and *in vitro* data highlight the contribution of the C-terminus of the virulence factor EsxC to self-association, and document a previously unreported role for calcium in mediating protein-protein interactions in this pathogenic secretion system.

1. Introduction

Microbial infections are often accompanied by the recruitment of secretion systems encoded on the genomes of various pathogens (Stoop et al., 2012; Green and Mecsas, 2016; Palmer, 2017). At least nine secretion systems have been described to date, many of which contribute to microbial virulence (Lasica et al., 2017). A common theme of these systems is the assembly of a membrane-spanning complex in the microbial envelope, followed by the secretion of a number of substrates into the host side (Houben et al., 2012; Aly et al., 2017). In *Staphylococcus aureus*, the ESAT6-like Secretion System (ESS) is a non-canonical pathway that shares genetic features of the prototypical *Mycobacterium tuberculosis* Type VII Secretion System (T7SS) (Abdallah et al., 2007; Warne et al., 2016).

The staphylococcal ESS system is a cluster of genes that code for a collection of soluble and membrane proteins. Some of the soluble components of the ESS pathway, such as EsxA, EsxB, EsxC and EsxD, are secreted effectors (Fig. 1A). In addition, a fifth effector, EssD, has been found to exhibit broad nuclease activity towards double stranded DNA (Ohr et al., 2017). Unlike secretion systems that secrete a subset of proteins harboring classical leader peptides, substrates secreted by the staphylococcal ESS pathway lack Sec-dependent or commonly known topogenic sequences (Burts et al., 2005; Anderson et al., 2013). They are rather secreted as full-length proteins and contribute to staphylococcal pathogenesis (Burts et al., 2005; Korea et al., 2014).

EsxA and EsxB have the salient feature of a tryptophan-X-glycine (WXG) motif found in the middle of the protein sequence (Sundaramoorthy et al., 2008; Fan et al., 2015). Both proteins belong to the WXG superfamily of substrates the members of which are broadly existent in several firmicutes and other bacterial species (Pallen, 2002; Poulsen et al., 2014; Warne et al., 2016). Previous

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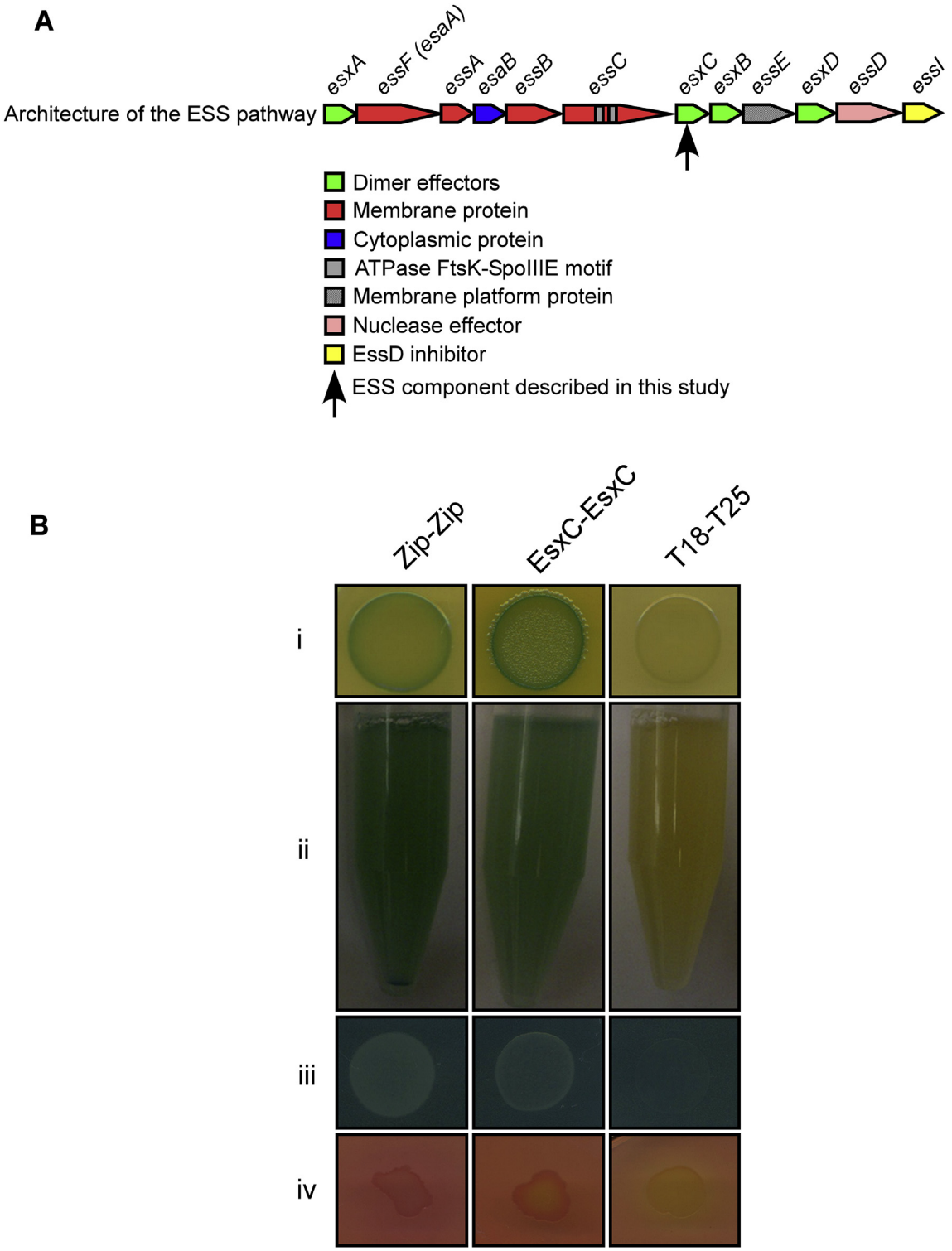

Fig. 1. ***In vivo*** **examination of EsxC self-association using bacterial two-hybrid approach.** (A) Schematic representation of the ESS pathway composition in *S. aureus*, outlining the potential location/role of gene products and the location of *esxC* within the cluster. (B) i- *E. coli* DHM1 cells harboring the T18-*leucine zipper*/T25-*leucine zipper* (Zip-Zip) as positive control, T18-empty vector/T25-empty vector (T18-T25) as negative control or T18-*esxC*/T25-*esxC* (EsxC-EsxC) were spotted on LB plates supplemented with the chromogenic substrate X-Gal, ampicillin and kanamycin for plasmid propagations and IPTG for fusion gene inductions. Blue color indicates positive interaction and white color indicates negative interaction. ii- For further validation on a liquid medium, *E. coli* DHM1 cells harboring Zip-Zip as positive control, T18-T25 as negative control or EsxC-EsxC were inoculated into LB liquid medium supplemented with X-Gal, ampicillin, kanamycin and IPTG, similar to the previous panel. Blue color indicates positive interaction and white color indicates negative interaction. iii- *E. coli* DHM1 cells harboring Zip-Zip, T18-T25 or EsxC-EsxC were spotted on M63 minimal medium plates supplemented with 0.2% maltose, ampicillin, kanamycin as well as IPTG. Bacterial cell survival indicates positive interaction, whereas cell death is considered negative interaction. iv- As a last method to confirm EsxC-EsxC interaction, *E. coli* DHM1 cells harboring Zip-Zip, T18-T25 or EsxC-EsxC were further analyzed on MacConkey agar plates supplemented with 1% maltose, ampicillin and kanamycin for plasmid propagations and IPTG for fusion gene inductions. Red color indicates positive interaction and white color indicates negative interaction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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