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ORIGINAL ARTICLE

Regions involved in fengycin synthetases enzyme complex formation

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Abstract *Background:* Fengycin is a lipopeptide antibiotic synthesized nonribosomally by five fengycin synthetases. These enzymes are linked in a specific order to form the complex. This study investigates how these enzymes interact in the complex and analyzes the regions in the enzymes that are critical to the interactions.
Methods: Deletions were generated in the fengycin synthetases. The interaction of these mutant proteins with their partner enzymes in the complex was analyzed *in vitro* by a glutathione S-transferase (GST) or nickel pulldown assay.
Results: The communication-mediating donor (COM-D) domains of the fengycin synthetases, when fused to GST, specifically pulled down their downstream partner enzymes in the GST-pulldown assays. The communication-mediating acceptor (COM-A) domains were required for binding between two partner enzymes, although the domains alone did not confer specificity of the binding to their upstream partner enzymes. This study found that the COM-A domain, the condensation domain, and a portion of the adenylation domain in fengycin synthetase B (FenB) were required for specific binding to fengycin synthetase A (FenA).
Conclusion: The interaction between the COM-D and COM-A domains in two partner enzymes is critical for nonribosomal peptide synthesis. The COM-A domain alone is insufficient for interacting with its upstream partner enzyme in the enzyme complex with specificity; a region that contains COM-A, condensation, and a portion of adenylation domains in the downstream partner enzyme is required.

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Introduction

Fengycin, an antifungal antibiotic synthesized by *Bacillus subtilis*,[1–3] is a cyclic lipopeptide.[4,5] This peptide is synthesized nonribosomally by five fengycin synthetases,[6–11] which interlock in the order of FenC-FenD-FenE-FenA-FenB to form a complex.[12] The formation of the enzyme complex allows the elongating peptide to be transferred efficiently and correctly between the synthetases, and is therefore crucial for fengycin synthesis.[12–16] Our previous study showed that two partner fengycin synthetases in the enzyme complex interact *via* the C-terminal region of an upstream enzyme and the N-terminal region of a downstream enzyme.[12] In addition, peptide synthetases contain a communication-mediating donor (COM-D) domain of approximately 20 amino acids at the C-terminus, and a communication-mediating acceptor (COM-A) domain of approximately 15 amino acids at the N-terminus.[17,18] The COM-D and COM-A domains between two partner enzymes are paired, and are crucial to peptide synthesis.[17–19] Based on the crystal structure of surfactin synthetases A–C (SrfA-C), it may be that a COM-hand motif located downstream of the COM-A domain in the condensation domain may be involved in the interaction between the COM-A domain and the COM-D domain in the upstream partner enzyme.[20]

In this study, we found that the COM-A domain alone is insufficient for two fengycin synthetases to interact with specificity; the N-terminal region of the synthetase, which includes the condensation domain and a portion of the adenylation domain, is required for specific recognition of the upstream partner enzyme. This study sheds light on the mechanisms involved in the regulation of the complex assembly.

Methods

Bacterial strains and culturing conditions

Escherichia coli was cultured in Luria–Bertani broth or on Luria–Bertani agar. *E. coli* BL21(DE3) and *E. coli* JM109 were used for protein expression. Recombinant protein expression was induced using isopropyl β-D-1-thiogalactopyranoside.[21] *Bacillus subtilis* F29-3, a fengycin-producing strain, was cultured in soybean-mannitol-nitrate media for 16 hours to express fengycin synthetases.[4]

Plasmids

Plasmids pFA230F, pFA230H, pFB200, pFB210F, pFB220, pFD210, and pFE210 have been reported.[10,12] Different DNA segments in the 3′ region in *fenA*, *fenC*, *fenD*, and *fenE* genes were amplified by polymerase chain reaction and inserted at the EcoRI-XhoI sites in pGEX4T-1 (GE Health Science, Little Chalfont, UK) to generate plasmids that encoded glutathione S-transferase (GST)-proteins with deletions in the C-terminal region of FenA (Figure 1A), FenC, FenD, and FenE. In the 5′ region in *fenB*, DNA was similarly amplified and inserted into the KpnI-XhoI sites in pET30b (Merck Millipore, Darmstadt, Germany) to create pFB210H, pFB230, and pFB240 (Figure 2A). Plasmid pFB210H was digested by NdeI and self-ligated to form pFB211 (Figure 2A). Plasmid pFC310, which encodes CC730, was constructed by inserting a polymerase chain reaction-amplified DNA fragment at the BamHI-HindIII sites in pETGEXT. An EcoRI-HindIII fragment containing the *gfp* gene from pEGFP-c1 (Clontech, Mountain View, CA) was inserted into pET28a (Merck Millipore, Darmstadt, Germany) to generate pET-eGFP. DNA fragments encoding the N-terminal 15- and 102-amino acid regions in FenB were inserted at the NcoI-BamHI sites in pET-eGFP to generate pFB270 and pFB260, respectively (Figure 2A). Plasmids expressing AC1194dE, AC1194 (COM-D_E), BN657 (COM-A_A), and His-FenB (COM-A_A) were generated by an inverse polymerase chain reaction method.[22]

Pulldown studies

E. coli cells from a 100-mL culture were homogenized three times with an Amicon French Press (Thermo Spectronic, Rochester, NY) at 1,400 p.s.i. Lysates were centrifuged at 16,000 × *g* for 20 minutes at 4°C. The GST-pulldown assay was conducted in accordance with a method described elsewhere.[23] His-tagged proteins in bacterial lysates were bound to nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen, Hilden, Germany) for 1 hour at 4°C in 50 mM monosodium phosphate, pH 8.0, 300 mM sodium chloride, and 10 mM imidazole. The beads were thereafter mixed with a bacterial lysate at 4°C for 1 hour to pull down proteins interacting with the His-proteins. Proteins pulled down by the bead were finally subjected to immunoblot analysis.

Immunoblotting

Immunoblot analysis was conducted using anti-FenB[12], anti-histidine (LTK, Taiwan), anti-GST (Santa Cruz Biotechnology, Dallas, TX), anti-green fluorescent protein (GFP) (Sigma, Kawasaki, Japan), anti-Flag (Sigma, Kawasaki, Japan), anti-rabbit HRP (Santa Cruz Biotechnology, Dallas, TX) or anti-mouse HRP (Santa Cruz Biotechnology, Dallas, TX) antibodies, based on methods described elsewhere.[23]

Results

Identification of regions in FenA that interact with FenB

In this study, we examined how FenA interacts with its partner enzyme, FenB. We first expressed a GST-fusion protein, AC1194, which contains 1194 amino acids at the C-terminal region in FenA from pFA310 (Figure 1A). The GST-pulldown assay revealed that AC1194-glutathione-Sepharose beads pulled down His-FenB from the *E. coli* JM109(pFB200) lysate (Figure 1B, Lane 3). We found that deletion derivatives of AC1194, including AC883, AC313, and AC148, which respectively contain the 883-amino acid, 313-amino acid, and 148-amino acid C-terminal regions in FenA fused to GST (Figure 1A), also pulled down His-FenB from the *E. coli* JM109(pFB200) lysate (Figure 1B, Lanes

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