論文の内容の要旨

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論文題目

Study on the spatial localization and function of *N*-acetylmuramoyl-L-alanine amidases during filamentous growth and sporulation in *Streptomyces griseus*
（放線菌の菌糸成長と胞子形成における*N*-アセチルムラモイル-L-アラニンアミダーゼの局在と機能に関する研究）

Introduction

The gram-positive, soil-inhabiting, filamentous bacterial genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites such as antibiotics, parasiticides, herbicides, and pharmacologically active substances. Another characteristic feature of the genus is its complex multicellular development. On an agar medium, spores germinate to form a branched, multinucleoid substrate mycelium, which then produces an aerial mycelium. After septa have been formed at regular intervals along the aerial hyphae, long chains of uninucleoid spores are formed. On the other hand, some *Streptomyces* species, including the streptomycin producer *Streptomyces griseus*, can make submerged spores in liquid medium. In *S. griseus*, sporogenic hyphae, which seem to be equivalent to aerial hyphae on solid culture, are formed from vegetative hyphae and subsequently undergo septation to form chains of unicellular submerged spores.

Peptideglycan (PG), which comprises alternating copolymer of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) with pentapeptide side-chains branching from the MurNAc residues, is a primary constituent of the Gram-positive bacterial cell wall. It functions in maintaining cell shape and cytoplasmic turgor pressure. PG is a dynamic macromolecule that is actively remodeled to enable cell growth and morphological differentiation. For the remodeling of cell wall, synthesis and cleavage of PG must be tightly regulated, with the activities of biosynthetic

and hydrolytic enzymes coordinated in both space and time. Cell wall hydrolases are diverse enzymes that are typically grouped on the basis of substrate specificity and resulting cleavage products. *N*-acetylmuramoyl-L-alanine amidase, which cleaves between MurNAc and the first residue (L-Ala) of the peptide side chain, is one of the major cell wall hydrolases. Although significant remodeling of the cell wall must accompany the filamentous growth and morphological changes associated with the different stages of the *Streptomyces* life cycle, little is known about cell wall remodeling in *Streptomyces*. In this study, to investigate cell wall remodeling in *S. griseus*, the spatial localization and function of the five *N*-acetylmuramoyl-L-alanine amidases were examined. The spatial localization and function of a novel component of the bacterial core morphogenic apparatus, RodZ, were also examined.

1. The spatial localization of five *N*-acetylmuramoyl-L-alanine amidases during filamentous growth and sporulation in *S. griseus*

Although *N*-acetylmuramoyl-L-alanine amidase has an important role in cleaving the septum to release daughter cells after cell division in *Escherichia coli*, the function of it in a filamentous bacterial genus *Streptomyces* was unknown. In silico analysis of the *S. griseus* genome revealed that *S. griseus* has five secreted *N*-acetylmuramoyl-L-alanine amidases, designated as AmiA to AmiE (Fig. 1). Each protein was produced as a fusion protein carrying a 3 x FLAG tag at its C-terminus in *S. griseus* and its localization in hyphae cultured in a liquid medium was examined by immunofluorescence microscopy.

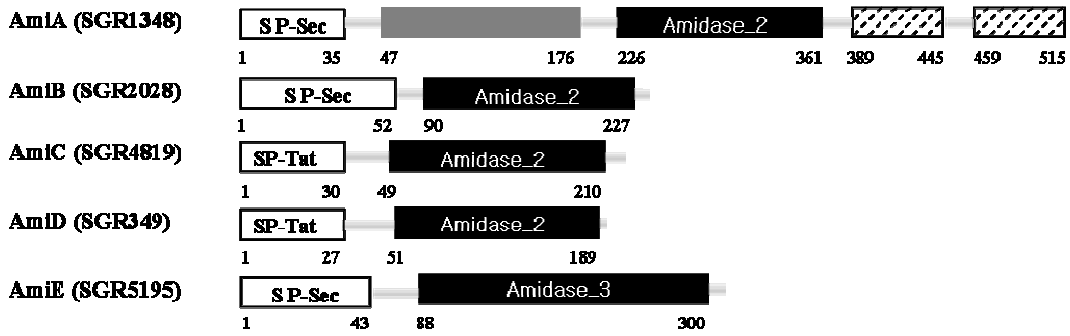

Figure 1. Schematic representation of the domain structure of five *N*-acetylmuramoyl-L-alanine amidases in *S. griseus*. *N*-acetylmuramoyl-L-alanine amidase has an amidase_2 or amidase_3 domain (indicated by a black box). AmiE has an amidase_3 domain, while others have an amidase_2 domain. AmiA, AmiB, and AmiE have signal peptides (SP-Sec) for secretion by the Sec pathway, while AmiC and AmiD have signal peptides (SP-Tat) for secretion by the Tat system. AmiA has a transglycosylase-like domain (grey box) and two PG-binging domains (hatched box), as well as an amidase_2 domain.

All of the five *N*-acetylmuramoyl-L-alanine amidases showed an interesting localization. The foci were observed at (i) tips of sporogenic hyphae and (ii) several spots arranged spirally with almost regular intervals along the sporogenic hyphae (Fig. 2). The foci appeared to be dispersed after septa were formed. Because AmiC and AmiD have a Twin-arginine translocation (Tat)-dependent signal peptide, a fusion protein carrying GFP at the C-terminus (AmiC-GFP or AmiD-GFP) was also produced in *S. griseus* and localization of them in living hyphae cultured on an agar medium was examined by fluorescence microscopy. The pattern of localization of AmiC-GFP and AmiD-GFP in aerial hyphae was very similar to that of AmiC-FLAG and AmiD-FLAG in sporogenic hyphae. This result suggested that AmiC and AmiD were transported across the cell membrane by the Tat system, which is dedicated to the translocation of folded proteins, and localized at their destination even as GFP fusion proteins. Then, AmiD was fused with RFP in a similar manner and localization of AmiC-GFP and AmiD-RFP was examined in a same recombinant strain. AmiC-GFP and AmiD-RFP were almost colocalized. This result suggested that not only AmiC and AmiD but also other three *N*-acetylmuramoyl-L-alanine amidases were colocalized all together at specific positions in the surface of sporogenic or aerial hyphae before septation.

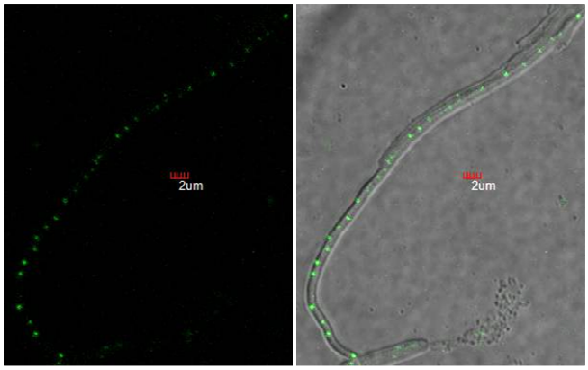

Figure 2. Localization of AmiA-FLAG in the surface of sporogenic hyphae in *S. griseus* as determined by immunofluorescence microscopy.

2. Functional analysis of five *N*-acetylmuramoyl-L-alanine amidases in *S. griseus*

The course of transcription of the five *N*-acetylmuramoyl-L-alanine amidase genes in liquid culture was examined by semi-quantitative RT-PCR. Transcription of *amiA*, *amiC*, and *amiE* was greatly increased in the late growth phase, while transcription of *amiB* and *amiD* was almost constant during growth. This result suggested that AmiA, AmiC, and AmiE should be involved in morphological development. To reveal the function of the *N*-acetylmuramoyl-L-alanine amidases, each *ami* gene was deleted from the chromosome. Among five amidase-mutants, only the Δ*amiA* mutant showed a distinct phenotype, as determined by scanning electron microscope. In the Δ*amiA* mutant, approximately 40% of aerial hyphae produced spores with abnormal length because of septaion with irregular intervals. The other mutants showed a similar disturbance in the position of septation with a very low frequency (approximately 1%). Then, a Δ*amiAC* double mutant and a Δ*amiACE* triple mutant were also constructed. In the Δ*amiAC* and Δ*amiACE* mutants, approximately

70% and 90% of aerial hyphae, respectively, produced abnormal spores. This result indicated that *N*-acetylmuramoyl-L-alanine amidases, especially AmiA, AmiC, and AmiE, had an important role in the formation of septa. Localization of the amidases at spots with almost regular intervals along sporogenic hyphae also supported this notion. Because the aerial hyphae of the Δ*amiACE* mutant had a rough appearance and sometimes swelled in the middle part, the amidases may have some influence on biogenesis of cell wall. Localization of the amidases at hyphal tips, where cell walls are generated and the hyphae extend, may support this idea.

3. The spatial localization and function of RodZ in *S. griseus*

Recently, a new common player, RodZ, in bacterial cell morphogenesis was discovered. RodZ is a membrane protein with bitopic topology such that the N-terminal region including a helix-turn-helix motif is in the cytoplasm, whereas the C-terminal region is exposed to periplasm. In *E. coli*, RodZ forms helical structures associated with the cell membrane, similar to the MreB bacterial actin. The N-terminal cytoplasmic domain containing a helix-turn-helix motif may mediate important interactions between RodZ and MreB. In contrast, the C-terminal region of RodZ probably interacts with enzymes that contribute to PG synthesis in the periplasm. Thus, RodZ probably mediates spatial information from cytoskeletal proteins in the cytoplasm to a PG synthesis machinery in the periplasm. By a Blast search, only one RodZ homolog (SGR1770) was found in *S. griseus*. To investigate the function of the RodZ homolog, the *rodZ* homolog gene (named *rodZ* hereafter) was deleted from the *S. griseus* chromosome. Interestingly, the Δ*rodZ* mutant showed similar phenotypes as the Δ*amiACE* triple mutant; the aerial hyphae of the Δ*rodZ* mutant produced spores with abnormal length, had a rough appearance, and sometimes swelled in the middle part. On the other hand, RodZ was produced as a fusion protein carrying GFP at its C-terminus in *S. griseus* and its localization in living hyphae cultured on an agar medium was examined by fluorescence microscopy. The pattern of localization of RodZ-GFP was very similar to that of the *N*-acetylmuramoyl-L-alanine amidases; the foci were observed at both tips and several spots arranged spirally with almost regular intervals along the aerial hyphae.

4. Colocalization of RodZ and AmiC in *S. griseus*

Because functional and spatial relationships between RodZ and the *N*-acetylmuramoyl-L-alanine amidases were suggested as described above, RodZ-GFP and AmiC-RFP were simultaneously produced in a same recombinant strain. Expectedly, exact colocalization of RodZ-GFP and AmiC-RFP was observed. However, RodZ seemed to be unnecessary for the localization of *N*-acetylmuramoyl-L-alanine amidases, because AmiC and AmiD could localize at specific positions in the surface of aerial hyphae even in the Δ*rodZ* mutant. Further work is needed to reveal the molecular mechanism for localization of *N*-acetylmuramoyl-L-alanine amidases.