Short Communication
Characterization of Vesicle Differentiation Mutants of Frankia casuarinae

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The nitrogen-fixing actinobacterium Frankia develops unique multicellular structures called vesicles, which are the site of nitrogen fixation. These vesicles are surrounded by a thick hopanoid lipid envelope that protects nitrogenase against oxygen inactivation. The phenotypes of five mutants that form smaller numbers of vesicles were investigated. The vesicles of these mutants were smaller than those of the wild type and had a phase dark appearance. They induced the expression of a glutamine synthetase gene in hyphal cells in response to ammonium starvation. These results suggest that genes impaired in the mutants do not function in global nitrogen regulation, but specifically function in vesicle differentiation.

Key words: glutamine synthetase, multicellular bacteria, nitrogen fixation, vesicle

Nitrogen is an essential element for all living organisms. Most organisms cannot utilize dinitrogen gas (N₂) because of its stable triple bound. Nitrogen-fixing (N₂-fixing) bacteria have the ability to reduce N₂ to ammonia (NH₃), and assimilate it to organic compounds such as amino acids. Fixed nitrogen flows into ecology, and, thus, N₂-fixing bacteria play an important role in global nitrogen cycles.

N₂ fixation is catalyzed by nitrogenase, which is a complex metalloenzyme composed of dinitrogenase (NifDK) and dinitrogenase reductase (NiFH) (Dixon and Kahn, 2004). Since nitrogenase is highly oxygen-labile, N₂-fixing bacteria adopt diverse strategies (behavioral, physiological, and structural) to protect nitrogenase against oxygen inactivation (Gallon, 1992).

Frankia spp. are N₂-fixing multicellular actinobacteria. Under NH₃-depleted and aerobic conditions, Frankia develop spherical multicellular structures called vesicles (Fig. S1), which are the site for N₂ fixation (Huss-danell, 1997). These vesicles are surrounded by a thick envelope composed of dozens of hopanoid lipid layers (Berry et al., 1993). Since the envelope functions as a barrier to oxygen penetration, nitrogenase, which is expressed inside vesicles, retains its activity (Benson and Silvester, 1993). Vesicles are not formed under anaerobic conditions and N₂ fixation occurs in hyphal cells (Murry et al., 1985). Genes related to vesicle differentiation have not yet been identified, except for those related to hopanoid lipid synthesis, which are ubiquitous in the microbial world (Kannenberg and Poralla, 1999).

We previously isolated five N₂-fixation mutants of Frankia casuarinae (G21E10, G23C4, G23D3, N7C9, and N10E6), which had smaller numbers of vesicles (<15% of the wild type) (Kucho et al., 2017) (Table S1). These mutants are considered to have defects in the generation of vesicle primordia. In the present study, we characterized the phenotypes of these mutants in more detail.

We used F. casuarinae strain CcI3 as the wild type (WT) (Nouiou et al., 2016). Frankia strains were grown in NH₃-repleted (N+) BAP-TN+ liquid medium (Kucho et al., 2009) at 28°C with stirring until the mid-logarithmic phase, and cells were then transferred to NH₃-depleted (N−) BAP-TN− medium (Kucho et al., 2009). Vesicles were observed 7 d after being transferred to N− conditions using phase-contrast (for size measurements) and dark-field (for envelope evaluations) optical systems with the microscope MT5310L (Meiji Techno). The vesicle sizes of G21E10, G23D3, N7C9, and N10E6 were markedly smaller than those of WT (~60%), while those of G23C4 were slightly smaller (80% of WT) (Fig. 1 and Table S1). When observed under the dark-field microscope, the thickness of the envelope was proportional to its brightness (Parsons et al., 1987) because the light effect was attributed to birefringence produced by structural layering of the vesicle envelope. In WT, approximately 40% of vesicles showed a bright appearance, indicative of a well-developed envelope (Fig. 2 asterisk and Fig. S2). Approximately 60% of WT vesicles also had a thick-walled stalk (Fig. 2 arrowhead and Fig. S2). In all mutants, the frequency of vesicles with a well-developed envelope was significantly less than that in WT and was markedly lower in G23C4 (5%) and N10E6 (0%) (Fig. 2 and S2). Furthermore, G23D3 and N10E6 produced fewer vesicles with a thick-walled stalk (Fig. 2 and S2). These results indicate that genes impaired in these mutants are important not only for the generation of primordia, but also for the maturation of vesicles (size expansion and envelope development).

The genes impaired in these mutants may be directly involved in the vesicle differentiation process. Alternatively, these genes may function in the perception or signaling of a NH₃-starvation status and their mutations indirectly disabled downstream vesicle differentiation (Fig. 3). To clarify the site of function, we investigated the expression of a NH₃-responsive gene in hyphal cells. If the latter is the case, these mutants will not be able to induce gene expression in hyphal cells or induce vesicle formation (Fig. 3). Therefore, we focused on a glutamine synthetase (GS) gene, which converts NH₃ and glutamate to glutamine. Frankia has two types of GS enzyme—GSI and GSII—that show distinct...
biochemical and regulatory properties, and the expression of the GSII gene was previously shown to be up-regulated in hyphal cells (and in vesicles) in response to NH₃-starvation (Schultz and Benson, 1990; Ghodhbane-Gtari et al., 2014). We also investigated the expression of a gene involved in NH₃-responsive regulation (ntrB, see below).

Frankia cells were acclimated to N− conditions as described above. Cells were collected by centrifugation 4 d after being transferred to N− conditions, and total RNA was purified by the cetyltrimethylammonium bromide (CTAB) method (Kucho et al., 2009). Contaminating DNA was removed by the TURBO DNA-free kit (Thermo Fisher Scientific). The cDNAs of the GSII (francci3 3143), ntrB (francci3 3178), and 16S rRNA (francci3 R0040, internal standard) genes were synthesized using PrimeScript reverse transcriptase (Takara Bio) in a 20-μL reaction mixture containing 1.5 μg of total RNA and 2 pmol of gene-specific reverse primers (GSII, Ghodhbane-Gtari et al., 2014; 16S rRNA, Kucho et al., 2017; ntrB, 5′-cccacatctcgggcagtt-3′) at 42°C for 30 min and then at 50°C for 15 min. Regarding GSII and 16S rRNA, real-time PCR was performed using the Probe qPCR mix (Takara Bio) in a 20-μL reaction mixture containing 4 pmol of forward and reverse primers (GSII, 5′-acgccatcgtcgcctgct-3′; 16S rRNA, Kucho et al., 2017), 4 pmol of the TaqMan probe (GSII, 5′- aecccatctcgggcagtt-3′; 16S rRNA, Kucho et al., 2017), and cDNA derived from 100 ng (GSII) or 1 ng (16S rRNA) of

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**Fig. 1.** Size of vesicles. Each dot represents an average calculated from between 20 and 36 independent vesicles from a biological replicate. The bar represents the standard error. Medians calculated from two biological replicates are shown by open boxes.

**Fig. 2.** Dark-field microscopic images of vesicles. A vesicle and stalk with a well-developed envelope are shown by an asterisk and arrowhead, respectively. The bar represents 3 μm.

**Fig. 3.** Schematic diagram of sequential events predicted to occur in Frankia in response to NH₃ starvation under aerobic conditions. This is a working hypothesis that requires further evidence.
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system (Merrick and Edwards, 1995), and homologs of its components (glnBD and ntrBC) have been found in Frankia genomes. Semi-quantitative reverse transcription PCR showed that an ntrB homolog (francci3_3178) was expressed in WT and all the mutant strains (Fig. 5 and Table S1). Genome analyses revealed that three of the mutants (G23C4, N7C9, and N10E6) did not carry mutations in these homologs (Kuco et al., 2017). Collectively, these results indicate that the genes responsible for the phenotypes of the three mutants are not related to global nitrogen regulation, but specifically function in the vesicle differentiation process (Fig. 3). The mutants G21E10 (Kuco et al., 2017) and G23D3 (K. Kucho, unpublished) carried an identical amino acid substitution in the homolog of ntrB (francci3_3178), whereas the same mutation was found in a revertant strain that formed vesicles and fixed N₂ (K. Kucho, unpublished). Therefore, the mutation in the ntrB homolog did not appear to cause the mutant phenotypes and G21E10 and G23D3 may be impaired in other genes that specifically function in the vesicle differentiation process.

Vesicle differentiation-specific genes are considered to be unique for Frankia and have not yet been identified. Two laboratories recently reported the successful transformation of Frankia spp. (Gifford et al., 2019; Pesce et al., 2019). Using these methods, we will be able to identify the genes responsible for the mutant phenotypes using complementation experiments with a genomic library of the WT strain.

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