Short Communication

Identification of the Hydrogen Uptake Gene Cluster for Chemolithoautotrophic Growth and Symbiosis Hydrogen Uptake in *Bradyrhizobium diazoefficiens*

SACHIKO MASUDA1,*, MASAKI SAITO1, CHIKA SUGAWARA1, MANABU ITAKURA1, SHIMA EDA1, and KIWAMU MINAMISAWA1

1Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai, Miyagi 980–8577, Japan

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The hydrogen uptake (Hup) system of *Bradyrhizobium diazoefficiens* recycles the H2 released by nitrogenase in soybean nodule symbiosis, and is responsible for H2-dependent chemolithoautotrophic growth. The strain USDA110 has two *hup* gene clusters located outside (locus I) and inside (locus II) a symbiosis island. Bacterial growth under H2-dependent chemolithoautotrophic conditions was markedly weaker and H2 production by soybean nodules was markedly stronger for the mutant of locus I (*ΔhupS1L1*) than for the mutant of *hup* locus II (*ΔhupS2L2*). These results indicate that locus I is primarily responsible for Hup activity.

Key words: *Bradyrhizobium diazoefficiens*, chemolithoautotrophic growth, hydrogenase, symbiosis

Soybean bradyrhizobia have two lifestyles: as symbiotic bacteroids that fix atmospheric nitrogen in host plants or as free-living soil bacteria. As a symbiont, *B. diazoefficiens* synthesizes a hydrogen uptake (Hup) system that recycles the H2 formed as a byproduct of nitrogenase activity (4). This symbiotic hydrogen oxidation increases nitrogen fixation efficiency, thereby enhancing the productivity of the legume host (3, 6). As free-living cells, *Bradyrhizobium diazoefficiens* Hup+ strains have the ability to grow chemolithoautotrophically by using H2 as an electron donor (7).

Two sets of *hup* genes have been identified in *B. diazoefficiens*: a large cluster outside the symbiosis island (*hup* locus I, genome position 7,620,025–7,645,755) and a small cluster inside the symbiosis island (*hup* locus II, genome position 1,888,916–1,902,575) (6, 11). A transcriptome analysis previously showed that several *hup* genes located outside the symbiosis island were up-regulated during the chemolithoautotrophic growth, whereas several *hup* genes located inside the symbiosis island were up-regulated in symbiotic bacteroids (1, 6). These findings imply that *hup* locus I plays an important role in chemolithoautotrophic growth, while symbiotic *hup* locus II may contribute to Hup activity in the nodules. In the present study, we constructed *hupSL* deletion mutants in order to clarify the contribution of each *hup* gene cluster during the chemolithoautotrophic growth and nodulation of *B. diazoefficiens* USDA110.

Materials and Methods

The strains and plasmids used in this study are listed in Table 1. The HM salt medium for the preculture and Hup medium for chemolithoautotrophic growth were described previously (14). Antibiotics were added to the media for *B. diazoefficiens* USDA110 and *Escherichia coli* strains as described previously (13).

We generated *hupS1L1* and *hupS2L2* deletion mutants as follows. A DNA fragment containing *hupFDCL1S1V* was isolated from plasmid pUC18 clone library of *B. diazoefficiens* USDA110 sequences (11), and inserted into the HindIII site of pK18mob. The resultant plasmid, pK18mob-*hupS1*, was digested with *Apa*I and ligated with the *Apa*IEcoRI adaptor, yielding pK18mob-*hupS1*.

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Results

Wild-type USDA110 and the *ΔhupS1L1* mutant grew on Hup agar medium under chemolithoautotrophic growth conditions (Fig. 2A). However, the *ΔhupS2L1* mutant showed markedly weaker growth than that of the wild type on this medium (Fig. 2A). The height of plants inoculated with *ΔhupS1L1* appeared to be lower than those inoculated with the wild type and *ΔhupS2L2* mutant (Fig. 2B); however, no significant differences were observed in plant dry weights or fresh nodule weights (Table 2). H2 was not produced from the nodulating roots of the wild type or *ΔhupS1L1* mutant (Fig. 2C), indicating that Hup activity compensated for the production of H2 via

* Corresponding author. E-mail: smasuda@cc.tuat.ac.jp; Tel/Fax: +81–42–367–5847.
† Present address: Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Saiwaicho, Fuchu, Tokyo 183–8509, Japan
Two hydrogenases of Bradyrhizobium

Table 1. Strains and plasmids used in the present study.

| Strain or plasmid       | Relevant characteristicsa | Reference or source |
|-------------------------|---------------------------|---------------------|
| **Bradyrhizobium diazoefficiens** |                           |                     |
| USDA110                 | Soybean bradyrhizobia, wild type | 11                  |
| AhupS1L1                | USDA110 hupS1L1::aadA; Sm r Sp r | This study          |
| AhupS2L2                | USDA110 hupS2L2::ter, Te r | This study          |
| *Escherichia coli*      |                           |                     |
| DH5α                    | cloning strain            | Toyobo Inc.         |
| **Plasmids**            |                           |                     |
| p34S-Tc                 | Plasmid carrying a 2.1-kb Tc cassette; Te r | 2                   |
| pHP45I                  | Plasmid carrying a 2.1-kb Ω cassette; Sp r Ap r | 15                 |
| pK18mob-hup1            | pK18mob carrying a 5.9-kb hupFDCL1S1VU HindIII fragment of brp15657; Km r | This study          |
| pK18mob-hup2            | pK18mob carrying an ApaI/EcoRII adaptor; Km r | This study          |
| pK18mob-hupomega        | pK18mob carrying hupFDCL1S1VU::aadA; Sp r Km r | This study          |
| pK18mob-hupII           | pK18mob carrying a 7.6-kb hupS1L1::CDFH1Xhol fragment of brp07428; Te r | This study          |
| pK18mob-hupIII          | pK18mob carrying an EcoO109I/BamHI1 adaptor; Km r | This study          |
| pK18mob-hupITc          | pK18mob carrying hupS1L1::ter, Te r Km r | This study          |
| pK18mob                 | cloning vector; pMB1ori oriT; Km r | 16                  |
| prK2013                 | ColE1 replicon carrying RK2 transfer genes; Km r | 5                   |
| brp15657                | pUC18 carrying hupUVS1L1,CDFG | 11                  |
| brp07423                | pUC18 carrying hupKHFDCLS2 | 11                  |

Ap r, ampicillin-resistant; Te r, tetracycline-resistant; Km r, kanamycin-resistant; Sm r, streptomycin-resistant; Sp r, spectinomycin-resistant.

a Osaka, Japan.

Fig. 1. Gene maps of *hup* gene clusters in *B. diazoefficiens*. (A) Gene map of *hup* locus I showing the genome position of *hup* locus I and brp15657. pK18mob-hup1 carries a 5.9-kb DNA fragment containing *hupFDCL1S1V* genes. pK18mob-hup1 was ligated with an ApaI/EcoRII adaptor. pK18mob-hupomega had an omega cassette, which was inserted at the EcoRI site of pK18mob-hup1. (B) Gene map of *hup* locus II showing the genome position of *hup* locus II and brp07428. The strategy used to construct the *hupS1L1* deletion mutant is shown as the *hupS1L1* deletion mutant. H, HindIII; A, ApaI; E, EcoRI; X, Xhol; Ec, EcoO109I; B, BamHI; Te r, tetracycline-resistant.

Nitrogenase. In contrast, H₂ was produced by the *AhupS1L1* nodule genes in USDA110 (6.4 μmol h⁻¹ g fresh nodule weight⁻¹) (Fig. 2C), indicating that Hup activity was lower than the production of H₂ via nitrogenase. These results suggest that *hup* genes outside the symbiosis island are the primary cluster involved in chemolithoautotrophic growth and Hup activity in the nodules.

Discussion

In the present study, the mutation of *hupS1L2* did not change nodule H₂ production from that by wild-type USDA110 (Fig. 2C), even though some genes on *hup* locus II were up-regulated in symbiotic bacteroids (1, 6). *Hup* locus I contains a complete set of the *hup-hyp-hox* gene cluster, and is missing from *hup* locus II (11). Thus, *hupS1L2* in locus II may not be fully induced without the *hup* gene assemblage, resulting in no or weak Hup activity by the *hupS1L2* genes. On the other hand, the *hup* gene cluster outside the symbiosis island, which we identified as the primary *hup* gene cluster contributing to Hup activity in free-living and symbiotic cells, is located on a typical genomic island (trnM element) of *B. diazoefficiens* USDA110. The trnM element is likely acquired in the USDA110 lineage after the divergence of strains USDA110 and USDA67 because *B. japonicum* USDA67 completely lacks this element (10, 11, 12).

Hup genes were also found in the symbiosis island of the USDA67 genome even though USDA67 was previously reported to exhibit no Hup activity (10, 11, 12). The *hup* genes in USDA67 on the symbiosis island had 99–100% amino acid sequence identity to the corresponding genes in *hup* locus II of USDA110. In contrast, USDA67 *hup* genes had only 43–83% amino acid sequence identity to genes in *hup* locus I of USDA110, which is similar to the homology (45–83%) between *hup* genes in loci I and II of USDA110. These results suggest that *hup* genes on locus II of USDA110 and *hup* genes in USDA67 do not contribute to the Hup activities of these strains and appear to be derived from the acquisition of symbiosis islands. Therefore, our results imply the horizontal gene transfer of the primary *hup* cluster via the genomic
Fig. 2. Comparison of chemolithoautotrophic growth (A) and symbiotic phenotypes (BC) between hup mutants and the wild-type strain of B. diazoefficiens USDA110. (A) Growth phenotype on Hup agar medium under an atmosphere of 84% N₂, 10% H₂, 5% CO₂, and 1% O₂ at 25°C. (B) Plant growth 30 d after inoculation. (C) Concentration of H₂ produced by the root nodules. White squares, wild type; black squares, ΔhupS₁L₁ mutant; grey squares, ΔhupS₂L₂ mutant.

Table 2. Plant dry weights and fresh nodule weights of inoculated wild-type USDA110 and mutants.

| Strains | Plant dry weight (g) | Fresh nodule weight (g) |
|---------|---------------------|------------------------|
| USDA110 | 6.9 ± 0.9ᵃ          | 1.36 ± 0.20ᵃ           |
| ΔhupS₁L₁| 6.8 ± 1.1ᵃ          | 1.38 ± 0.20ᵃ           |
| ΔhupS₂L₂| 6.9 ± 0.6ᵃ          | 1.29 ± 0.13ᵃ           |

Plants were harvested 30 d after inoculation. Values are presented as an average and standard deviation (n=5). Tukey’s multiple comparison test was used for statistical analyses (p<0.05).

island to the lineage of B. diazoefficiens rather than symbiosis island transfer.

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References

1. Chang, W.S., W.L. Franck, E. Cytryn, S. Jeong, T. Joshi, D.W. Emerich, M.J. Sadowsky, D. Xu, and G. Stacey. 2007. An oligonucleotide microarray resource for transcriptional profiling of Bradyrhizobium japonicum. Mol. Plant-Microbe Interact. 20:1298–1307.
2. Dennis, J.J., and G.J. Zylstra. 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. Appl. Environ. Microbiol. 64:2710–2715.
3. Drevon, J., V.C. Kalia, M. Heckmann, and I. Salsac. 1987. Influence of the Bradyrhizobium hydrogenase on the growth of Glycine and Vigna species. Appl. Environ. Microbiol. 53:610–612.
4. Evans, H.J., A.R. Harker, H. Papen, S.A. Russell, F.J. Hanus, and M. Zuber. 1987. Physiology, biochemistry, and genetics of the uptake hydrogenase in rhizobia. Annu. Rev. Microbiol. 41:335–361.
5. Figruski, D.H., and D.R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. U.S.A. 76:1648–1652.
6. Franck, W.L., W.S. Chang, J. Qiu, M. Sugawara, M.J. Sadowsky, S.A. Smith, and G. Stacey. 2008. Whole-genome transcriptional profiling of Bradyrhizobium japonicum during chemoaotrophic growth. J. Bacteriol. 190:6697–6705.
7. Hanus, F.J., R.J. Maier, and H.J. Evans. 1979. Autotrophic growth of H₂-uptake-positive strains of Rhizobium japonicum in an atmosphere supplied with hydrogen gas. Proc. Natl. Acad. Sci. U.S.A. 76:1788–1792.
8. Inaba, S., F. Ikenishi, M. Itakura, M. Kikuchi, S. Eda, N. Chiba, C. Katayama, Y. Suwa, H. Mitsu, and K. Minamisawa. 2012. N₂O emission from degraded soybean nodules depends on denitrification by Bradyrhizobium japonicum and other microbes in the rhizosphere. Microbes Environ. 27:470–476.
9. Itakura, M., K. Tabata, S. Eda, H. Mitsu, K. Murakami, J. Yasuda, and K. Minamisawa. 2008. Generation of Bradyrhizobium japonicum mutants with increased N₂O reductase activity by selection after inoculation of a mutated dnaQ gene. Appl. Environ. Microbiol. 74:7258–7264.
10. Itakura, M., K. Saecki, H. Omori, et al. 2008. Genomic comparison of Bradyrhizobium japonicum strains with different symbiotic nitrogen-fixing capabilities and other Bradyrhizobiaceae members. ISME J. 3:326–339.
11. Kaneko, T., Y. Nakamura, S. Sato, et al. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium Bradyrhizobium japonicum USDA110. DNA Res. 9:189–197.
12. Kaneko, T., H. Maita, H. Hirakawa, N. Uchiike, K. Minamisawa, A. Watanabe, and S. Sato. 2011. Complete genome sequence of the soybean symbiont Bradyrhizobium japonicum strain USDA45. Gene. 2:763–787.
13. Masuda, S., S. Eda, S. Ikeda, H. Mitsu, and K. Minamisawa. 2010. Thiosulfate-dependent chemolithoautotrophic growth in Bradyrhizobium japonicum. Appl. Environ. Microbiol. 76:2402–2409.
14. Masuda, S., S. Eda, C. Sugawara, H. Mitsu, and K. Minamisawa. 2010. The cbbL gene is required for thiosulfate-dependent autotrophic growth of Bradyrhizobium japonicum. Microbes Environ. 25:220–223.
15. Prentki, P., and H.M. Krisch. 1984. In vitro insertional mutagenesis with a selective DNA fragment. Gene. 29:303–313.
16. Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene 145:69–73.