Effects of hydroxylsine on the growth and morphology of Rhizobium leguminosarum bv. phaseoli

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Received February 4, 1998

It was found that both bacteroids and free-living cells of Rhizobium leguminosarum bv. phaseoli were highly susceptible to hydroxylsine (Hyl) and the inhibition of RNA and/or protein synthesis caused by the hydroxyl residue of Hyl appeared to be responsible for the growth inhibition. The size of free-living cells was enlarged by the addition of Hyl and some cells reached around 5 μm, which were close to the length of bacteroids. Under the same condition, the polyhydroxybutyrate (PHB) content in the cells was conspicuously increased. These results suggest that Hyl is not only a notable growth inhibitor of Rhizobium bacteria but also plays a role in a differentiation to bacteroids.

Key words: hydroxylsine (Hyl); Rhizobium; root nodule bacteria

Introduction

In symbiosis between rhizobia and leguminous plants, the ability for growth and nodulation of the rhizobia is important for both successful nitrogen fixation and competitive nodule formation, and is controlled by several factors derived from host plants. In the rhizosphere, leguminous plants are known to secrete some chemical compounds such as amino acids, saccharides, or vitamins. For example, it is known that Phaseolus vulgaris secretes mainly amino acids into the rhizosphere. It was reported that an average generation time of Bradyrhizobium bacteria was 10 to 15 d in the culture, while it took only 5 to 12 h in the host plant rhizosphere, indicating that some compounds in the root exudates were responsible for the stimulation of growth. Van Egbert reported that the increased number of the cells might be related to homoserine secreted from the root. On the other hand, Strijdom and Allen reported that the addition of D-amino acids such as D-Ala, D-His, or L-amino acids such as L-Cys suppressed the growth of R. meliloti, although little is known about the mechanism by which such a growth inhibition is brought about.

It was believed that the rhizobia lose the ability to grow concomitantly with differentiation of free-living cells into bacteroids and that the multiplication of bacteroids in the mature nodules of soybean is stopped. Sutton et al. reported that in the first 13 to 19 d of nodule development, only 2 to 4% of the bacteroids were dividing, although from 10 to 12 d, cell division was around 25 to 45%. However, Ozawa and Tsuji reported that when mannitol-yeast extract medium (YEM) was diluted 10 times, the colony formation was improved considerably. This suggests that the bacteroids do not lack the ability to multiply and indicates the presence of some compounds that inhibit the growth of bacteroids. It was reported that polyanamines such as spermidine which were present in nodules as well as in YEM, inhibited the growth of Bradyrhizobium japonicum and the concentration of this compound in soybean nodules was markedly increased with the cessation of the growth of bacteroids. In our previous study, we reported that hydroxylsine (Hyl), which was present in nodules and the roots of host plants, was a conspicuous growth inhibitor of free-living cells of Rhizobium bacteria. In this report, our objectives were to discover the mechanism of the growth inhibition by Hyl and the role of Hyl on the differentiation from free-living cells into bacteroids.

Materials and Methods

Bacterial strain and medium. Rhizobium leguminosarum bv. phaseoli USDA2676 was used throughout these experiments. This strain was kindly supplied by Dr. Van Berkum (Agricultural Research Service, USDA, Beltsville, MD). Free-living cells or bacteroids of this strain were grown in minimal medium (MM) at 30°C aerobically and the growth was monitored by measuring the turbidity of the culture at 660 nm.

Plant growth and inoculation. Phaseolus vulgaris L. cv. Taishoukintoki was kindly provided by the Tokachi Federation of Agricultural Cooperatives, Obihiro, Japan. The seeds were surface sterilized with 80% ethanol for 2 min, then 10% sodium hypochlorite for 1 min, and then sufficiently washed with sterilized distilled water. An overnight culture of R. leguminosarum bv. phaseoli USDA2676 was prepared in YEM-HM medium at 30°C and the cell density was adjusted to 10⁶ cells per ml by 0.01 M phosphate buffer, pH 7.2, with 0.15 M NaCl. The seeds were inoculated with 10⁶ cells per seed and grown in vermiculite, which was moistened with Norris and Datta solution in glass pots in a growth chamber controlled at 22°C for

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13 h and 16°C for 11 h at day time and night time, respectively.

**Preparation and growth of bacteroids.** Nodules (around 0.1 g) detached from 50-d-old plants were surface-sterilized with 70% (v/v) ethanol for 2 min followed by 10% (v/v) sodium hypochlorite for 30 s. After rinsing with sterilized distilled water, the nodules were crushed in 2 ml of 1% NaCl and one-tenth ml of the suspension was transferred to 5 ml of the liquid MM with or without hydroxylsine (Hyl) and incubated at 30°C aerobically.

**Pulse labeling of DNA, RNA and protein in vivo.** When *R. leguminosarum* bv. *phaseoli* USDA2676 cells were in the early logarithmic phase (OD_{600}=0.1) in MM at 30°C, Hyl (final conc. 1 mm) was added to the culture. One ml of the culture in the presence or absence of Hyl was withdrawn at zero, 10, 20, 30, and 60 min after the addition of Hyl and incubated with [methyl-^3H]-thymine (0.0185 MBq/ml), [2-^14C]-uracil (0.0037 MBq/ml), or [^14C]-leucine (0.00185 MBq/ml) for 5 min exactly at 30°C. One ml of 10% TCA (trichloroacetic acid) was added to the labeled sample and the mixture was left for 30 min on ice.[^18,19] Half of the mixture was then filtered through a membrane filter (Millipore Co.; pore size 0.45 μm) and washed with 0.85% NaCl (1 ml×15 times), then the radioactivity on the filter was counted in a scintillator (Aquasol 2) using a liquid scintillation counter (Packard Instrument Co. Inc. Trycarbud4640).

**Light micrographs.** One tenth ml of overnight culture of free-living cells or bacteroids suspension of *R. leguminosarum* bv. *phaseoli* USDA2676 was transferred to 5 ml of the liquid MM with or without Hyl (1 mm). The cells were withdrawn at an appropriate period and after staining with Fuchsin, observed by light microscope (Olympus BH-2, Tokyo, Japan) at a magnification of 1,000× under oil immersion.

**Measurement of polyhydroxybutyrate (PHB).** Overnight culture of *R. leguminosarum* bv. *phaseoli* USDA2676 cells (4 ml) were transferred to 200 ml of MM with or without Hyl (1 mm) and incubated at 30°C aerobically. At desired periods, the culture was centrifuged at 8,000 g for 10 min at 4°C and after removal of the supernatant, the pellets were washed with 50 mm phosphate buffer, pH 7.6, containing 0.15 m NaCl, several times. The pellets were then dried on the dry bath and PHB in the cells was depolymerized to crotonic acid by the addition of 1 ml conc. H_2SO_4 for 30 min.[^20] Crotonic acid in the sample was measured by HPLC (Jasco UVIDC-100-V, Hitachi Custom Ion-Exchange Resin #2618 column) after an appropriate dilution of the sample with 0.05% H_3PO_4.

**Chemicals:** 6-Hyl (mixture of DL- and DL-allo) was purchased from Sigma Chem. Co., L- and DL-Lys were from Wako Pure Chem. Industries, Ltd., N\(^2\)-methyl-L-Lys was from Aldrich Chem. Co., DL-ethionine was from Nakarai Chem. Co., Ltd., and standard crotonic acid was from Kishida Chem. Co., Ltd.

**Results and Discussion**

A previous study showed that among the major amino acids and other amino compounds, Hyl present in the nodules and roots was 0.6 to 0.7 μmol/g wet weight (which corresponds to around 0.6 to 0.7 mm) and about 1 μmol/g wet weight (which corresponds to around 1 mm), respectively, and the growth of *Rhizobium* bacteria was severely inhibited only in the presence of Hyl.[^13] Hyl was also found in both nodules (formed with *R. leguminosarum* bv. *phaseoli* USDA2676) and roots of the host plant (*Phaseolus vulgaris* L. cv. Taishoukintoki) at a level of 0.4 to 0.7 μmol/g wet weight (which corresponds to around 0.4 to 0.7 mm). Figure 1 shows the effects of (D)L-Lys and its analog, Hyl, and methyl-L-Lys on the growth of *R. leguminosarum* bv. *phaseoli* USDA2676. Without the additives, the cells started to grow without a lag and reached early stationary phase 96 h after the start of incubation. However, in the presence of 0.1 mg ml, the growth of

![Fig. 1. Effects of Lys and Its Analogs on the Growth of R. leguminosarum bv. phaseoli USDA2676](image1.png)

Forty-eight hour old cultures (0.1 ml) were transferred to 5 ml of minimal medium containing (A) Hyl (△; 0.1 mm, ○; 0.5 mm, ●; 1 mm), (B) L-Lys (△; 1 mm), DL-Lys (●; 1 mm) or methyl-L-Lys (●; 1 mm) at zero time and incubated at 30°C aerobically. Growth of the control is shown as open circles.

![Fig. 2. Comparison of the Effects between Hyl and Ethionine on the Growth of R. leguminosarum bv. phaseoli USDA2676](image2.png)

Forty-eight hour old cultures (0.1 ml) were transferred to 5 ml of minimal medium at zero time and (A) Hyl (1 mm) or (B) ethionine (1 mm) was added at the point indicated by the arrows (●; zero h, △ 18 h and/or ○; 25 h). Growth of the control is shown as open circles.
the cells was almost halted for 20 h and then the cells started to grow. The addition of 0.5 mM or 1 mM Hyl extended the lag time to 70 h (Fig. 1A). Hyl was incorporated into the cells after the addition, but the level decreased concomitantly with the recovery of the growth. The level of Hyl in the medium also tended to decrease gradually, suggesting that it was metabolized (data not shown). On the other hand, when 1 mM (D)L-Lys or methyl-L-Lys was added to the culture, the cells could grow without a lag and there were no significant inhibitory effects on the growth (Fig. 1B). These results indicate that the hydroxyl residue of Hyl could be responsible for the growth repression.

Next, the effect of the growth repression by Hyl was compared with that by ethionine (Fig. 2). Ethionine is an analog of methionine, and it is believed that this compound is incorporated into protein in place of methionine, thus inhibiting the growth of cells. When Hyl (1 mM) was added to the culture at zero, 18, or 25 h after the incubation, the growth of the cells was halted immediately after the addition and the cells could not grow during the 60 h of the experiments (Fig. 2A). On the other hand, when ethionine was added to the culture at zero or 18 h after the incubation, the cells could still continue to grow and reached more than 85% of the control by 60 h after the incubation (Fig. 2B). These results indicate that the mechanism of the growth repression by Hyl is quite different from that caused by an amino acid analog like ethionine.

Figure 3 shows the effects of Hyl on the DNA, RNA, and protein synthesis in R. leguminosarum bv. phaseoli USDA2676 cells. The incorporation of radioactive precursors into the cells in the presence of Hyl was shown as the ratio (%) to that without the compound (control). When Hyl (1 mM) was added to the culture at zero time, the rate of both RNA and protein synthesis was promptly reduced to 31% (53.5 kdpn/mg dry weight of cell) and 40% (816.3 kdpn/mg dry weight of cell) of the control, respectively. Particularly the rate of protein synthesis dropped to 3% (62.1 kdpn/mg dry weight of cell) of the control 10 min after the addition of Hyl and was maintained at a low level for the duration of the experiment. The results of 2-dimensional polyacrylamide gel electrophoresis of cell-free extracts at 24 h after the addition of Hyl showed that many protein spots disappeared, indicating that protein synthesis was repressed under this condition (data not shown). On the other hand, the rate of RNA synthesis was maintained at a higher level as compared to that of both RNA and protein synthesizes and it was more than 60% of the control during the periods tested. These results seem to show that Hyl decreased the RNA and/or protein synthesis, followed by the growth inhibition of the cells.

*Rhizobium leguminosarum* bv. *phaseoli* USDA2676 bacteroids were isolated as described in Materials and Methods and the growth was compared with that of the free-living cells in the presence of Hyl (Fig. 4). The growth of free-living cells reached the early stationary phase at 3 to 4 d after the incubation, while in the presence of Hyl, the growth of the cells was halted for 3 d and then grew were slowly than the control. The growth reached around 70% of the control at 10 d after the addition of Hyl. On the other hand, the bacteroids started to grow with the lag time of 3 d after the incubation, while in the presence of Hyl, the growth was severely inhibited and the cells did not grow during 10 d of the experiment. These results seem to show the possibilities that Hyl inhibited the growth of bacteroids as well as of free-living cells or functioned to maintain the condition of bacteroids, and that free-living cells and bacteroids differ from each other in response to Hyl. However, the details are obscure.

Figure 5 shows the effects of Hyl on the morphology of the root nodules.
of *R. leguminosarum* bv. *phaseoli* USDA2676 free-living cells and bacteroids. Without the addition of Hyl, the free-living cells maintained almost the same size (at most 2 μm) during the incubation (Fig. 5A, B). However, when Hyl was added to the culture, the length of the cells was increased and some cells reached about 5 μm at 8 d, which was close to the length of bacteroids (Fig. 5C, D). Under this condition the cells similar to the size of free-living cells in the absence of Hyl were also mixed with them. These results seem to suggest that there might be a possibility that the enlarged cells were dividing. Zurkowski reported that during the incubation at the elevated temperature the bacterial cell size was increased, and after 5 d of incubation, about 60% of the cells were normal sized because of the cell division. On the other hand, when the bacteroids were incubated without the addition of Hyl, the length of the cells was decreased gradually and became almost like that of free-living cells at 9 d (Fig. 5A, E). In the presence of Hyl, the distinct decrease of the cell length did not occur and some cells still maintained the length of around 4 to 6 μm (Fig. 5F).

The level of polyhydroxybutyrate (PHB) in free-living cells, which is one of the marker products of bacteroids, was measured (Fig. 6). In the absence of Hyl, the PHB content in the cells increased up to about 0.2 (mg crotonic acid/mg dry weight of cell) until 2 d after the incubation, and then decreased for the duration of this experiment. However, in the presence of Hyl, the PHB content in the cells started to increase gradually after 2 d of incubation, followed by an increase of the turbidity of the culture at 660 nm (Figs. 4 and 6). At 10 d after the incubation, the PHB level reached about 0.38 (mg crotonic acid/mg dry weight of cell) (Fig. 6). At that time, the length of some cells was around 5 μm and the cells similar to the size of free-living cells in the absence of Hyl were also mixed with them with nearly the same results as seen at 8 d (Fig. 5C) (data not shown). Fast- and slow-growing rhizobia such as *R. leguminosarum* bv. *phaseoli*, *R. leguminosarum* bv. *trifolii*, *R. meliloti*, and *R. japonicum* are known to synthesize PHB as an intracellular carbon and energy storage polymer. Karr et al. reported that the PHB level in *R. japonicum* bacteroids isolated from the mature nodules was 40 to 45% of dry weight of the cells (0.4 to 0.45 mg crotonic acid/mg dry weight of cells).

Previous studies report on the enlargement of cell size in the presence of organic acids such as succinate. Urban and Dazzo reported that bacteroid-like morphology of *R. trifolii* was induced in the presence of succinate. Alicia et al. also showed that the growth of *R. meliloti* free-living cells was inhibited by the addition of succinic acid, concomitantly with the enlargement of cell size. However, there are no mention of the increase of the marker product of bacteroids with the enlargement of cell size. This study shows that the PHB content in the free-living cells was increased distinctly in the presence of Hyl concomitantly with the enlargement of cell size, as with bacteroids. These results seem to show that Hyl is not only a notable growth inhibitor of free-living cells of *Rhizobium* bacteria but also plays a role in the dynamic change between free-living cells and bacteroids such as differentiation to bacteroids and their main-
References


