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Colonization of Maize and Rice Plants by Strain *Bacillus megaterium* C4

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Abstract. *Bacillus megaterium* C4, a nitrogen-fixing bacterium, was marked with the *gfp* gene. Maize and rice seedlings were inoculated with the, GFP-labeled *B. megaterium* C4 and then grown in gnotobiotic condition. Observation by confocal laser scanning microscope showed that the GFP-labeled bacterial cells infected the maize roots through the cracks formed at the lateral root junctions and then penetrated into cortex, xylem, and pith, and that the bacteria migrated slowly from roots to stems and leaves. The bacteria were mainly located in the intercellular spaces, although a few bacterial cells were also present within the xylem vessels, root hair cells, epidermis, cortical parenchyma, and pith cells. In addition, microscopic observation also revealed clearly that the root tip in the zone of elongation and differentiation and the junction between the primary and the lateral roots were the two sites for the bacteria entry into rice root. Therefore, we conclude that this Gram-positive nitrogen-fixer has a colonization pattern similar to those of many Gram-negative diazotrophs, such as *Azospirillun brasilense* Yu62 and *Azoarcus* sp. As far as we know, this is the first detailed report of the colonization pattern for Gram-positive diazotrophic *Bacillus.*

The green fluorescent protein (GFP) is widely used as a reporter in studies of gene expression and protein localization in diverse organisms. One interesting application is the colonization analysis of diazotrophs [15] and plant pathogens [13]. Plant colonization patterns of many Gram-negative diazotrophs have been elucidated clearly through the use of GFP reporter as well as the reporters of β-galactosidase (LacZ) and β-glucuronidase (GUS). Endophytic diazotrophs, such as *Azoarcus* sp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, and some strains of *Azpspirillum brasilense* [2, 18], can penetrate deeply into plants, being found in the outer cortex layers, stele, and xylem vessels. Once inside a plant, these bacteria can spread systemically and reach aerial parts [8, 15]. In contrast, most nonendophytic diazotrophs, e.g., some strains of *Azospirilium*, live either on the surface of the roots, particularly in the root hair and elongation zones, or within disrupted epidermises/outer cortices [8, 14, 17].

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Genus *Bacillus* has great potential uses in agriculture. Its members are able to produce antimicrobial metabolites to control plant pathogens; to fix nitrogen; to form endospores to resist desiccation, heat, and UV irradiation, and survive in adverse conditions. Plant colonization studies revealed that *Bacillus* has various colonization patterns on plants. *Bacillus pumilus* SE34, a plant-growth-promoting rhizobacterium (PGPR), colonized in tomato roots, stems, and leaves at 6 weeks after inoculation [22]. *Bacillus mojavensis* AB1 colonized in leaves and twigs of the coffee plants [12]. However, these experiments were performed by isolations of *Bacillus* strains marked with rifampicin (Rif) resistance gene, and isolation procedures are not sufficient to prove that *Bacillus* can colonize inner tissues of plants. As far as is known, it has not been described using GFP reporter or other reporters to study the colonization patterns of Gram-positive diazotrophic *Bacillus*.

B. megaterium C4 is a nitrogen fixer, which was originally isolated from the maize rhizosphere by our laboratory [3]. It has been demonstrated that the bacterium has nitrogenase acitivity (11.60 nmole ethylene/

OD_{600} with reference to [3]) and *nifH* gene amplified by polymerase chain reaction [3]. However, whether *B. megaterium* C4 colonizes root surface or resides in the interiors of roots, stems, and leaves has not been determined. In this study, a GFP-labeled *B. megaterium* C4 was constructed, and maize and rice seedlings were inoculated with the GFP-labeled bacterium. At certain days after inoculation, the different portions of the seedlings were observed under confocal laser scanning microscope. Our experimental results indicated that the bacterium could infect the maize roots through the cracks formed at the lateral root junctions and then penetrate to the vascular tissues and migrate to stems and leaves. Rice colonization studies here revealed clearly that the two sites for the bacteria entry into rice root are the root tip in the zone of elongation and differentiation and the junctions between the primary and lateral roots.

Materials and Methods

Bacterial strains and plasmids. *B. megaterium* C4 was isolated from maize rhizosphere by our laboratory [3]. pGFP4412 is an *Escherichia coli–Bacillus cereus* shuttle vector containing *gfp* (mut3a), neomycin (7 μg/mL), and ampicillin (100 μg/mL) resistance genes [4, 20].

Constrution of GFP-labeled *B. megaterium* C4. The competent cells of *B. megaterium* C4 were prepared as described [10]. The preparation procedure was as follows. First, incubate a single colony of *B. megateriuni* C4 into 5 mL of Luria-Bertani broth (LB) medium [16] and grow overnight at 30°C with shaking at 200 rpm. Second, incubate 5 mL of the culture into 400 mL of LB medium in a 2-L Erlenmeyer flask and grow at 30°C with shaking at 160 rpm to an OD_{650} of 0.2. Third, aliquot the culture into several 50-mL prechilled, sterile polypropylene tubes and leave the tubes on ice for 5 to 10 min. Centrifuge cells for 10 min at 8000 rpm at 4°C. Then resuspend each pellet in 10 mL of ice-cold SG buffer containing 272 mM sucrose and 15% glycerol [9], centrifuge as above, and repeat this process four times. Finally, resuspend each pellet with 1 mL SG buffer and aliquot 50 μL competent cells to each enpendorf. Store the competent cells at −70 °C for future use.

GFP-labeled *B. megaterium* C4 was constructed by transferring pGFP4412 into competent *B. megaterium* C4. For transformation, 1 μL of plasmid pGFP4412 was added to 50 μL of competent cells in a 2-mm electroperation cuvette. The plasmids were electroporated into the cells by using an electroporation system (Bio-Rad) set at 1.6 kV/cm, 25 μF, 200 Ω, and 416 ms. The cells were immediately transferred to 1 mL of LB medium and incubated for 1 h at 30°C with shaking at 80 rpm, and then they were plated on selective medium (LB medium containing 7 μg/mL neomycin). Transformants, which emitted green fluorescence, were screened with confocal laser scanning microscope (excitation wavelength was 488 nm).

Plant growth and inoculation. Maize (*Zea mays* L. ev, Nongda 108) seeds were surface-sterilized by soaking in 75% ethanol for 10 min and in 1% sodium hypochlorite solution for 5 min, and then washed with sterile water. Then they were germinated in sterile plates containing sterile water for 4 days at 26°C until the root seedlings were approximately 1 cm in length. After germination, the seeds were transferred into sterile flasks (6 cm in diameter and 10 cm in height) with N6 semisolid agar medium [11] and cultured at 25°C and 14 h light per day in growth chamber. Each plant was inoculated with 1×10^8 bacterial cells of GFP-labeled *B. megaterium* C4. At 3 days after culture in a growth chamber, each plant was inoculated with 1×10^8 bacterial cells of GFP-labeled *B. megaterium* C4 at the root by using a micropipet. Each flask was enveloped with a sterile ventilate film in order to keep sterile. Plants with no inoculation were used as negative control.

Rice (*Oryza sativa* L. cv. Yuefu) seeds were planted and inoculated using the same methods as described above for maize.

Laser confocal microscopic observation of the inoculated seedlings. The plants, grown in the gnotobiotic condition were taken out and their root surfaces were rinsed clean with sterile water. The tissues of roots, stems, and leaves were optically sectioned in transverse and longitudinal directions. The sections were examined under the Bio-Rad MRC1024 laser confocal microscope [1].

Results

Expression of the *gfp* gene in *B. megaterium* C4. GFP-labeled *B. megaterium* C4 was constructed by transferring pGFP4412, an *E.coli-B. cereus* shuttle vector containing *gfp* (mut3a) gene, into *B. megaterium* C4, GFP-labeled *B. megaterium* C4 was detected by laser confocal microscope [Fig. 1(1)], indicating that the *gfp* gene was well expressed in *B. megaterium* C4.

Colonization of maize roots by GFP-labeled *B. megaterium* C4. The maize roots at 1, 3, 5, 7, 9, and 11 days after inoculation were optically sectioned, and the sections were examined under laser confocal microscope.

One day after inoculation, the bacterial cells were found to colonize the surfaces of the primary and lateral roots in the root hair zone [Fig. 1(2) and (3)], and the lateral root junction [Fig. 1(2)]. The result that the bacteria were concentrated at the lateral root junction suggested that the crack formed at the lateral root junction is probably a site of entry into maize roots for this Gram-positive nitrogen-fixer, as reported in many microorganisms [5]. However, in this study bacterial cells were not found at the root tip, which is another major route of entry into roots for many microorganisms [5].

The longitudinal and transverse sections of the primary root [Fig. 1(4) and (5)] demonstrated that majority of the bacteria lived in the intercellular spaces of cortical parenchyma and a few bacterial cells were located within epidermises, root hair, and cortex cells at 3 days after inoculation.

Five days after inoculation, the bacteria had progressed towards the inner cortex of the primary root [Fig. 1(6)].

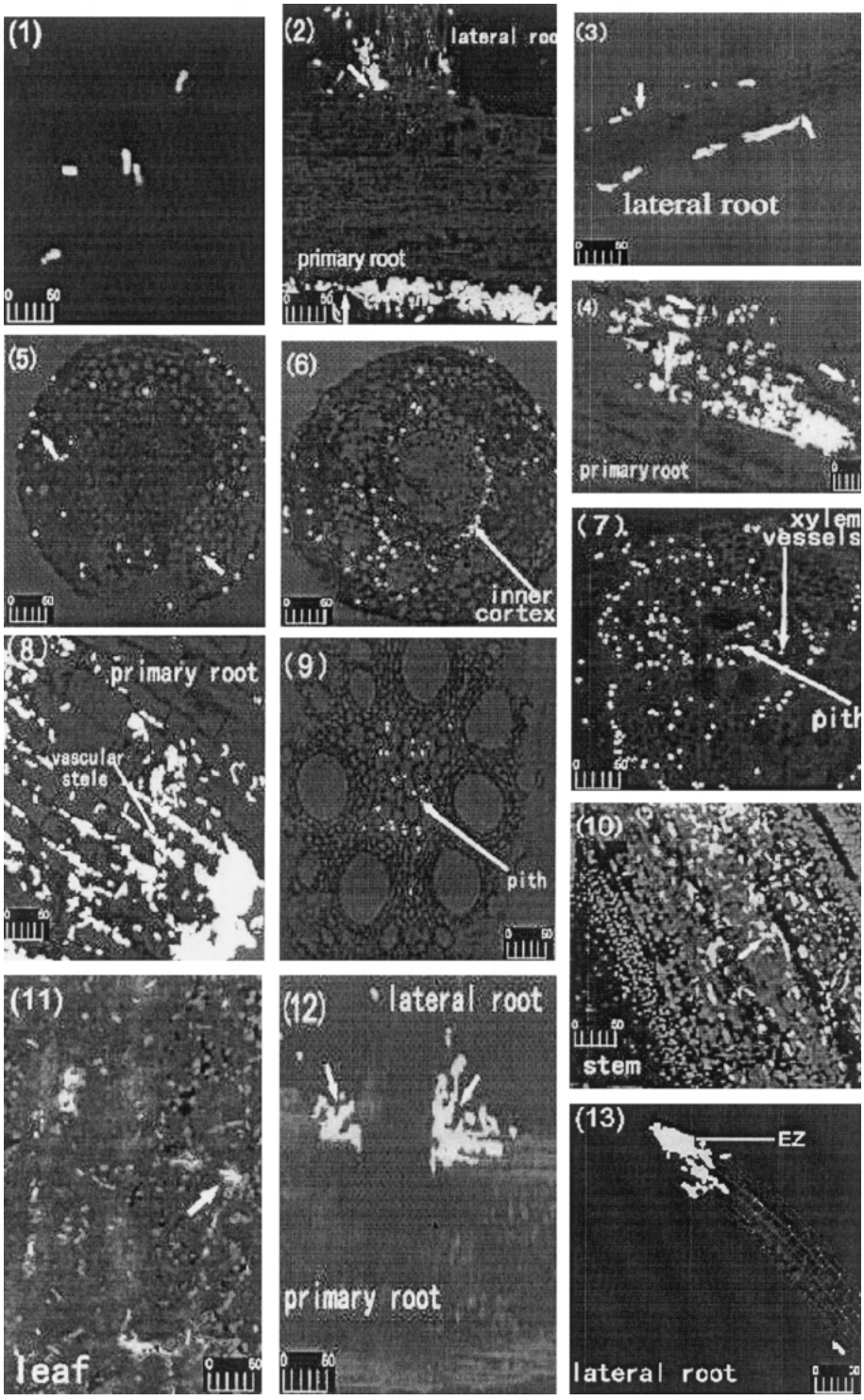

Fig. 1. Confocal image of GFP-labeled *Bacillus megaterium* C4 (**1**) and colonization of this GFP-labeled bacterium in maize roots, stems, and leaves (**2**)–(**11**) and in rice roots (**12**)–(**13**). (**1**) Confocal image of GFP-labeled *B. megaterium* C4 grown in LB medium. (**2**) and (**3**) Longitudinal sections of the primary and lateral roots showing the bacterial cells (arrows) assembled on the surfaces of primary and lateral roots and the lateral root junction at 1 day after inoculation. (**4**) and (**5**) Longitudinal and transverse sections of primary roots showing the majority of the bacteria distributed in the intercellular space and some located within epidermis, root hair cells [arrows in (**4**)], cortex [arrows in (**5**)] at 3 days after inoculation. (**6**) Bacteria penetrated into inner cortex of the primary root at 5 days after inoculation. (**7**) and (**8**) Seven days after inoculation, bacteria penetrated into stele of the primary root, including xylem vessels. The asterisk in (**8**) indicates that the bacterial cells were clumped. (**9**) Representative example of colonized pith. (**10**) and (**11**) Longitudinal sections showing the bacteria distributed within maize stem (**10**) and leaf (**11**) at 30 days after inoculation. Arrows show the GFP-labeled bacteria. (**12**) Longitudinal section of rice root showing that the bacteria colonized the junctions (arrows) between the primary and lateral roots and penetrated into the outer cell layers of cortex. (**13**) Longitudinal section of the infected root tip in the zone of differentiation and elongation (EZ) of the rice lateral root.

Seven days after inoculation, the bacteria reached the stele and penetrated into xylem vessels and pith of the primary root [Fig. 1(7)] and the bacteria were obviously clumped [Fig. 1(8)]. Fig. 1(9) is a representative example of colonized pith at 7 days after inoculation.

Nine and 11 days after inoculation, the colonization pattern of the root issues by the bacteria was similar to

that observed at 7 days after inoculation. The results indicated that the bacterial cells finished the infection process of maize roots within 7 days.

Colonization of maize stems and leaves. The maize stems and leaves were observed under the confocal laser scanning microscope at 30 and 40 days after inoculation. In the inoculated maize, the bacteria were found in stems and leaves at 30 and 40 days after inoculation [Fig. 1(10)–(11)], indicating that the bacteria migrated slowly from roots to stems and leaves. In contrast, no GFP-labeled *B. megaterium* C4 cells were found in negative control plants.

Infection process of rice roots. The rice roots at 1, 3, and 5 days after inoculation were optically sectioned, and the sections were observed under a confocal laser scanning microscope.

One day after inoculation, the bacteria were found to colonize mainly the surface of the primary roots in the zones of differentiation, elongation, and root hair (figure not shown) and the lateral root junction [Fig. 1(12)], Fig. 1(12) also revealed that the bacterial cells had invaded into epidermises and the outer cell layers of cortex, probably via the lateral root junctions. The longitudinal sections of lateral root revealed that 3 days after inoculation, the bacteria colonized the root tip in the zone of differentiation and elongation, which was swollen, and the bacteria were in large cell aggregates [Fig. 1(13)]. The results indicated that the zone of differentiation and elongation was another major site for the bacteria entry into rice. At this time, some bacteria also invaded rapidly into the cortex of the primary root (figure not shown). Five days after inoculation, the bacteria reached the stele (figure not shown).

Discussion

B. megaterium C4 is a Gram-positive nitrogen fixer, which was originally isolated from maize rhizosphere by our laboratory. In this study, we constructed GFP-labeled *B. megaterium* C4 and used a confocal laser scanning microscope to study the colonization patterns of *B. megaterium* C4 on maize and rice.

Our results here showed that the bacteria shared the similar three stages in invading the maize roots with many microorganisms, such as *Pseudomonas solanacearum* (now called *Ralstonia splanacearum*) [21]. The first stage was that the bacteria colonized mainly the surfaces of primary and lateral roots and the junctions between the primary and lateral roots. Surface colonization was followed by cortical infection, the second stage of the root infection. The third stage was characterized by stele infection and penetration into xylem vessels. The GFP-labeled *B. megaterium* C4 cells were found to live mainly in the intercellular spaces, although they were also present within the epidermises, xylem vessels, and the cells of the root hair, cortex, and pith. The data are consistent with the report of Sevilla et al. [19] that the bacterial cells of *G. diazotrovhicus*, an endophytic bacterium, were located mostly in the intercellular spaces of the roots and stems, but in some cases, they were also present in the xylem vessels. Our results also showed that *B. megaterium* C4 could spread systemically and reach maize stems and leaves from roots. James [8] reported that endophytic diazotrophs had the ability to colonize the root cortex, and might even penetrate the endodermis to colonize the stele, from which they might be subsequently translocated to the aerial parts, According to the definition of James, *B. megaterium* C4 might be referred to as a diazotrophic endophyte.

It has been reported that many microorganisms enter into plant roots by the following three putative pathways [8, 15]. One site of primary colonization is the root tip in the zone of elongation and differentiation, where the bacteria can invade inter- and intracellularly and can penetrate central tissues that will later differentiate into the stele. Another route of entry is the points of emergence of lateral root. The third route is the axils of emerging or developed lateral roots. Our study on the rice root infection revealed clearly that the root tip in the zone of elongation and differentiation and the junctions between the primary and the lateral roots were the two sites for the bacteria entry into rice root. However, in maize roots the bacterial cells were found concentrated at the lateral root junctions, but not found at the maize root tip, indicating that entry of the bacteria into maize roots could have occurred via these cracks formed at the lateral root junction. The image of Fig. 1(4) appears to suggest that the bacteria might directly enter the cortex through epidermis in the root hair zone of the primary root, just as reported *Klebsiella oxytoca* SA2, which penetrated into cortex of the maize root in the maturity zone [1]. We also observed that the GFP-labeled *B. megaterium* C4 directly penetrated into cortex of wheat root through epidermis in the root hair zone (figure not shown). These data suggested that *B. megaterium* C4 might use similar mechanisms for invading the different plants.

In summary, our report here showed that *B. megaterium* C4, a Gram-positive nitrogen-fixer, has a similar colonization pattern to those of many Gram-negative endophytic diazotrophs, such as *A. brasilense* Yu62, *G. diazotrophicus, Azoarcus* sp. and *K. oxytoca* SA2. As

far as we know, it is the first detailed report of colonization pattern for Gram-positive diazotrophic *Bacillus*. Some studies showed that that endophytic nitrogen-fixing bacteria, like *G. diazotrophicus, Azocarus* sp. or *Klebsielle pneumoniae*, could fix nitrogen inside plants and provide ammonia to plants [6, 7, 19]. Thus, colonization studies of nitrogen-fixing bacillus will be great importance in agriculture.

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