Isolation of a pink-pigmented facultative methylotrophic bacteria and its growth-promoting effect on pepper plants

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Microorganisms offer a low-cost, eco-friendly alternative to chemical fertilizers. A pink-pigmented facultative methylotrophic bacterium Gen-B2 was isolated from sugarcane rhizosphere and its growth-promoting activity was detected using *in vitro* experiments. Based on morphological, biochemical and molecular characteristics, Gen-B2 was identified as *Methylobacterium populi*. Pepper seedling inoculation significantly benefitted shoot/root fresh/dry weight, plant height, chlorophyll content and average true leaf number. Determination of activities related to growth promotion indicated that Gen-B2 promoted plant growth through nitrogen fixation and indole acetic acid synthesis. The present study suggests that this bacterium has great potential to be used as a bioinoculant for sustainable cultivation.

Keywords: Growth promotion, indole-3-acetic acid, *Methylobacterium populi*, nitrogen fixation, pepper plants, sugarcane rhizosphere.

CHEMICAL fertilizers are used on a large scale in modern agriculture because they can provide plants with essential nutrients such as nitrogen, phosphorus and potassium[1]. However, inadequate management of such fertilizers, such as their excessive use, has caused a global problem of soil deterioration, which adversely affects crop production[2]. Therefore, there is an urgent need for a more environment-friendly alternative.

Living plants are constantly involved in interactions with a wide range of microbes. In particular, plants are associated with microbes that colonize the rhizosphere, a narrow soil zone influenced by root secretion[3,4]. They can stimulate plant growth through the availability of nutrients originating from genetic processes, such as biological nitrogen fixation and phosphate solubilization, and through the production of phytohormones and siderophores[1].

Pink-pigmented facultative methylotrophic bacteria (PPFM), which belong to the genus *Methylobacterium*, are commonly found in the rhizosphere, as well as on the leaf and seed surfaces of a wide variety of plants[5,6]. PPFM can use one-carbon compounds (C1), such as methanol and methylamine, as energy and carbon sources, in addition to various C2, C3 and C4 compounds[7,8]. The associations between *Methylobacterium* spp. and host plants can be strong or symbiotic to weak or epiphytic and intermediate or endophytic[9–11]. PPFM can promote plant growth and seed germination by fixing nitrogen and producing ACC (1-aminocyclopropane 1-carboxylate) deaminase, indole acetic acid (IAA), cytokinins and siderophores[12–15]. Based on their importance in the rhizosphere and their potential for commercial applications, methylotrophic bacteria have received significant attention as bioinoculant substitutes for chemical fertilizers[8].

During a screening of PPFM strains in Southeast China (e.g. sugarcane and pepper), the Gen-B2 strain isolated from the rhizosphere of sugarcane showed a strong ability to promote cabbage seed germination. In this study, Gen-B2 has been identified and its ability to promote plant growth and the associated activities was evaluated. The results show that the methylotrophic strain Gen-B2 has potential as a bioinoculant.

Materials and methods

PPFM strains isolated from the rhizosphere

PPFM Gen-B2 was isolated from the rhizosphere of healthy sugarcane obtained from the Nansha District of Guangzhou, China. Rhizosphere soil samples were collected according to Bulgarelli *et al.*[16]. The rhizosphere suspensions were diluted in gradient and spread on ammonium mineral salt (AMS) plates[17], supplemented with 0.5% (v/v) methanol as the sole carbon source at a dilution of 10^{-5}–10^{-7}. The plates were incubated upside down at 28°C for 48 h, and single colonies were purified and stored in a 30% glycerol solution at –80°C.

Primary screening with cabbage seeds

To determine whether the isolated strains could promote plant growth, we tested the strains on cabbage seed germination

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in vitro. Isolated bacterial strains were incubated in liquid AMS medium and cultured on a rotary shaker at 180 rpm for 48 h at 30°C. The cabbage seeds were sterilized and washed before being sparsely placed (50 seeds/plate) in a petri dish containing a thin layer of sterilized gauze soaked with 10 ml liquid culture containing one of the bacterial strains. Each treatment had three replicates and the plates with fresh liquid AMS medium were used as control. Petri dishes were incubated in the dark at 25°C for five days. On the third and fifth days, germinated seeds were counted and germination rates were calculated.

Identification of Gen-B2

The Gen-B2 bacteria that exhibited germination promotion activity were stored in the laboratory on agar slants with AMS. Phenotypic characteristics such as colony morphology, pigment production and Gram-staining were tested, as previously described[18]. Biochemical characteristics such as carbon resource utilization, enzyme activity and antibiotic tolerance were detected (VITEK® 2 System; bioMérieux, France) with a Gram-negative bacteria identification card. The total DNA was extracted and the *mxaF* genes were amplified using the primers *mxa* f1003 (5′-GCGGCACCAACTGGGGCTGGT-3′) and *mxa* r1561 (5′-GGGCAGCATGAAGGGCTCCC-3′) to confirm the genus[19]. The 16S rRNA was amplified using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′)[20]. To identify the species of Gen-B2, phylogenetic analysis based on maximum parsimony (MP) and Bayesian analyses (BA) were conducted with 16S rRNA and sequences of other related species obtained from GenBank (NCBI, MD, USA). Sequence of *Escherichia coli* (GenBank No: J01859) was used as outgroup.

Growth-promoting effect of Gen-B2 on pepper in the greenhouse

The soil for the greenhouse experiment was a mixture of peat and vermiculite in the ratio 5 : 1 (v/v). The soil was autoclaved four times at 15 psi for 30 min. Sterilized plastic pots (0.5 l) were each filled with 200 g of autoclaved soil and moistened to 80% field capacity. Pepper seedlings (3–4 weeks old) with similar growth were transplanted into the pots, with two plants per pot. The liquid inoculum was prepared by inoculating the liquid AMS medium (with 0.5% (v/v) methanol) with a single, fresh Gen-B2 colony and incubating it at 28°C and 180 rpm for seven days.

The treatments were as follows: (i) using root irrigation, each pot was inoculated with 30 ml of Gen-B2 fermentation broth (FB) one day after transplantation. (ii) using foliar spraying, each pot was inoculated with 10 ml of Gen-B2 FB on the first, eighth and fifteenth day after seedling transplantation. (iii + iv) Controls for root irrigation and foliar spraying, where FB was replaced with liquid AMS medium. Each treatment had five replications cultivated at 28°C under a 16 h/8 h light/dark regime for three weeks. Plants were watered every 1–2 days until the end of the experiment. After three weeks, the plants were collected and plant height, shoot/root fresh/dry weight, leaf chlorophyll content and true leaf number were recorded. The greenhouse experiment was repeated twice.

Gen-B2 enzyme activity determination

Nitrogen-fixing activity: The Gen-B2 strain was inoculated on an AMS medium without a nitrogen source. The strain inoculated on a normal AMS medium with a nitrogen source (NH_4Cl) was used as control[21]. Each treatment had three replicates, which were incubated at 28°C. Strain growth was observed after seven days.

Cellulase activity: This was assayed according to the method proposed by Kim and Wimpenny[22] using the KW medium. After incubation for three days at 30°C, the agar medium was flooded with an aqueous solution of 1% Congo red for 30 min, followed by immersion in 1 M NaCl for 10 min. The appearance of clear zones around the colonies was considered as evidence of cellulolytic activity.

Siderophore detection: Siderophores were detected using the assay of Louden Brian and Haarmann Daniel[23]. Chrome azurol S (CAS) agar plates were used to identify siderophores in Gram-negative bacteria. After incubation for three days at 30°C, a yellow halo was observed.

IAA assay: The production of IAA was quantified using a Salkowski colorimetric assay according to Ventorino *et al.*[24], with minor modifications. Briefly, 1 ml of Gen-B2 (10^7 cfu/ml) was inoculated in liquid nutrient broth with L-tryptophan (2 mg/l) and incubated at 26°C at 160 rpm for seven days. The broth was centrifuged and filtered using a 0.22 μm microporous membrane to collect the sterile filtrate. An equal volume of Salkowski's reagent (2 ml of 0.5 M $FeCl_3$ with 49 ml of 35% (v/v) $HClO_4$) was added to the sterile filtrate (2 ml) and incubated at room temperature for 30–60 min. Liquid nutrient broth with L-tryptophan but without inoculation was used as the control. The standard curve was constructed based on the absorbance value of the standard IAA substance at 530 nm with concentrations of 0, 0.2, 1.0, 2.0, 3.0, 6.0, 11.0, 20.0 and 45.0 μg/ml. IAA concentration was determined using spectroscopic absorbance measurements at 530 nm, according to the standard curve.

ACC deaminase activity assay: The ACC deaminase activity of Gen-B2 was assayed according to the method of Penrose and Glick[25]. The negative control contained Dworkin and Foster salts minimal medium without ACC,

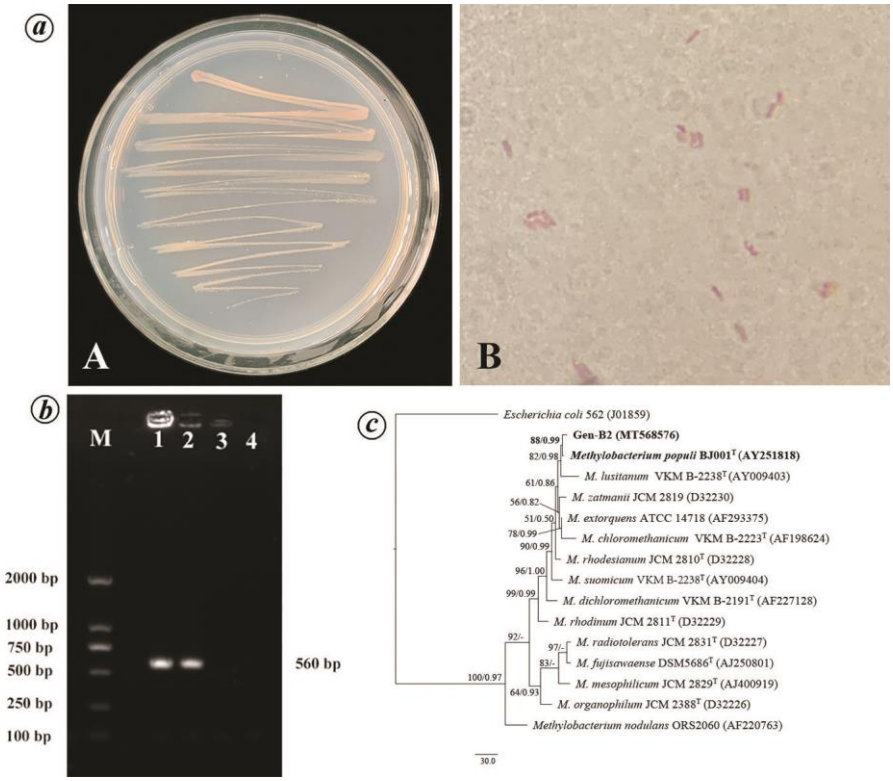

Figure 1. Identification of *Methylobacterium populi* Gen-B2. ***a***, Cultural characteristics (**A**, Colonies on ammonium mineral salt (AMS). **B**, Gram-staining). ***b***, Specific amplification of *mxaF* genes (1 and 2 are two replicates of Gen-B2 and 3 and 4 are negative controls). ***c***, Phylogenetic analysis with 16S rRNA sequences, T, Sequence of type strain.

and the positive control contained $(NH_4)_2SO_4$ (0.2% w/v) instead of ACC. The plates were incubated at 28°C for 72 h, after which the growth of Gen-B2 on the ACC plates was compared with the positive and negative controls.

Statistical analysis

Experimental data were statistically analysed using a single factor analysis of variance (Microsoft Excel 2010 and SPSS 19.0; IBM, Armonk, NY, USA). Differences among treatments were identified using Fisher's least significant difference test ($P = 0.05$).

Results

Screening of PPFM strains for high seed germination ability

Pink colonies on the AMS medium were selected for purification on new AMS plates. To determine whether the isolated bacteria could stimulate seed germination, we tested the selected strains on cabbage seeds. Strain Gen-B2 demonstrated a significant ability to promote cabbage seed germination. When treated with Gen-B2 FB, the seeds germinated on the second day and the germination rate reached 59.04% on the third day. In contrast, seed germination in the control group began on the third day with a germination rate of 39.39%. On the fifth day after inoculation, the average germination rate of the control was 47.74% and the average seedling length was 20.03 ± 0.02 mm, whereas the average germination rate of the Gen-B2 treatment group was 70.16% and the average seedling length was 25.29 ± 0.03 mm. The average values of both germination rate and seedling length were significantly greater than those of the control group (Supplementary Figure 1).

Methylobacterium populi (Gen-B2) and its characteristics

The bacterial strain Gen-B2 was Gram-negative. It was an obligate aerobe and the optimum growth temperature was 26°–30°C. When cultured on AMS solid medium, the colonies had a diameter of 1–3 mm, were opaque and light pink, protruded upwards, had neat edges and were dry (Figure 1 *a*). Carbon source utilization and other biochemical characteristics of the colonies are listed in the Supplementary Table 1 and largely aligned with those of *Methylobacterium* spp. An expected amplification product

Table 1. Growth indices of pepper seedlings under different treatments

Treatment	Fresh weight (g)		Dry weight (g)		Plant height (mm)	Chlorophyll content	Leaf number
	Shoot	Root	Shoot	Root			
A	2.86 ± 1.21a	0.79 ± 0.27a	0.34 ± 0.18a	0.09 ± 0.03ab	116.88 ± 4.67b	24.89 ± 0.69a	17
B	2.84 ± 1.00a	1.22 ± 0.36a	0.42 ± 0.17a	0.15 ± 0.04a	146.63 ± 6.35a	25.79 ± 0.95a	19
C	1.10 ± 0.83b	0.14 ± 0.05b	0.09 ± 0.06b	0.10 ± 0.05c	98.50 ± 3.50bc	17.03 ± 0.43b	10
D	0.56 ± 0.18b	0.22 ± 0.14b	0.07 ± 0.02b	0.04 ± 0.01bc	90.50 ± 12.87c	15.05 ± 1.98b	9

Means ± standard error, $n = 5$, different letters in the same column indicate significant differences ($P < 0.05$) between treatments according to Fisher's least multiple comparison.

Figure 2. Effect of Gen-B2 fermentation broth (FB) on pepper growth three weeks after inoculation. ***a***, Irrigation with 30 ml liquid AMS. ***b***, Spraying with 30 ml liquid AMS three times. ***c***, Irrigation with 30 ml Gen-B2 FB. ***d***, Spraying with 30 ml Gen-B2 FB three times.

of 560 bp was obtained using the specific primers for the *mxaF* genes (Figure 1 *b*), which further confirmed Gen-B2 as a *Methylobacterium* bacterium. In an optimal phylogenetic analysis tree based on the complete sequence of 16S rRNA with *Escherichia coli* (J01859) as an outgroup, Gen-B2 was clustered with the sequence of the type strain of *M. populi* (AY251818) with high support (Figure 1 *c*). Based on its morphology, phylogeny and biochemical properties, Gen-B2 was identified as *M. populi*. The complete 16S rRNA sequence has been deposited in GenBank (NCBI, USA) with accession number MT568576. The patent strain was deposited in the Guangdong Microbial Culture Collection Center (GDMCC No: 61052), China.

Effect of Gen-B2 on pepper growth in the greenhouse

Measured three weeks after transplanting, the shoot/root fresh/dry weight, plant height, chlorophyll content and mean number of true leaves were significantly higher for pepper seedlings treated with Gen-B2 FB by root irrigation or foliar spraying than those treated with AMS medium. Pepper seedlings treated three times with foliar spraying exhibited greater root dry weight, plant height and number of true leaves than those treated once with root irrigation. There was no significant difference in growth indices between the two control treatments (Table 1 and Figure 2).

Enzyme activity related to growth promotion

Activities related to growth promotion revealed that Gen-B2 showed similar growth on AMS plates without a nitrogen source and on those with a nitrogen source (NH_4Cl; Supplementary Figure 2), indicating that Gen-B2 can fix nitrogen. Based on the absorbance value of the standard IAA substance at 530 nm, the standard curve was obtained as $y = 44.716x + 0.812$ ($R^2 = 0.9985$; Supplementary Figure 3 *a*). After incubation for seven days, the average absorbance value of the treated group at 530 nm was 0.126 ± 0.009, indicating that Gen-B2 can secrete IAA. On the seventh day, the concentration reached 6.461 ± 0.401 μg/ml according to the standard curve (Supplementary Figure 3 *b*). Strain Gen-B2 did not grow on plates with ACC as the unique nitrogen source. Similarly, neither cellulase nor siderophore activity was observed by plate determination.

Discussion and conclusion

Long-term use of chemical fertilizers has led to poor soil physio-chemical properties and nutrient balance, resulting in increased plant diseases and a reduction in crop productivity. As commercial alternatives, bioinoculants can reduce environmental hazards and diseases, and promote plant growth. Methylotrophic bacteria associated with the plant rhizosphere are important bioinoculants due to cytokinin production[26], N_2 fixation[27], IAA production[24] and siderophore activity[21]. In this study, we have identified and demonstrated the growth-promoting activity of PPFM strain Gen-B2 on pepper plants. This strain exhibits good potential as a bioinoculant.

Gen-B2 was screened from the sugarcane rhizosphere based on its significant activity during cabbage seed germination. Seeds treated with Gen-B2 FB improved the germination rate and germination time of the cabbage. The average seedling length in the treated group was significantly more than the control, indicating the positive role that Gen-B2 plays in plant growth. This corroborates the results obtained in other studies for sugarcane and tomato when seeds were inoculated with PPFM strains[5,21,28].

To better understand the Gen-B2 bacterial strain, it was identified to the species level using a polyphasic approach with phenotypic, molecular and biochemical characteristics as *M. populi*[29,30]. In previous studies, this has shown multiple plant growth-promotion activities[24].

Previous studies have reported that some *Methylobacterium* species isolated from the phyllosphere show significant growth-promotion activity[21,26,31,32]. To explore the growth-promotion activity of Gen-B2 through leaves, foliar spraying treatment was added to the pot experiment. All Gen-B2 treatments resulted in a significant increase in pepper growth. Additionally, the foliar spraying assay was shown to be an easy and efficient method for using pink-pigmented methylobacteria to promote growth and increase plant yield[31]. This may reflect the ability of methylotrophs to efficiently survive on leaves using methanol released through the stomata via pectin demethylation[31]. In the pot experiment, the dry weight and plant height of pepper seedlings treated with three sprays were significantly higher than those treated with one-time irrigation. The average number of true leaves was also greater, indicating that for the same total amount of applied bacteria, multiple applications are better than a single one. However, further studies on the exact influence of fertilization methods and frequency are required.

The effect of *Methylobacterium* on promoting plant growth is generally attributable to the synthesis of biologically active compounds. Bacterial IAA plays a major role in host root system development and plant growth[33]. Ivanova *et al.*[34] were the first to report on the significant production of IAA by different methylotrophs. In the present study, *M. populi* (Gen-B2) produced 6.46 ±0.401 μg/ml IAA after seven days of *in vitro* incubation. This value is greater than that previously reported for *M. populi* VP2, wherein IAA production was 5.27 ± 0.05 μg/ml in the presence of L-tryptophan[24]. The preliminary detection of Gen-B2 under *in vitro* conditions also provides qualitative evidence for nitrogen fixation, which is consistent with previous studies on PPFM bacteria (e.g. *Methylobacterium nodulans*, *Methylobacterium radiotolerans*)[27,35]. Furthermore, previous studies have shown that PPFM strain *M. populi* VP2 can solubilize phosphate and produce low quantities of siderophores[24]; such abilities were not observed for strain Gen-B2 in this study.

The above results indicate that strain Gen-B2 (*M. populi*) isolated from sugarcane rhizosphere demonstrates multiple plant-growth promoting activities through IAA production and nitrogen fixation. However, field conditions are more complicated, and additional studies with varied bacterial formulations and environmental conditions will be needed to evaluate the growth-promotion properties of Gen-B2 as a bioinoculant under field conditions.

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