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Article

Bacillus simplex—A Little Known PGPB with Anti-Fungal Activity—Alters Pea Legume Root Architecture and Nodule Morphology When Coinoculated with *Rhizobium leguminosarum* bv. *viciae*

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Abstract: Two strains, 30N-5 and 30VD-1, identified as *Bacillus simplex* and *B. subtilis*, were isolated from the rhizospheres of two different plants, a *Podocarpus* and a palm, respectively, growing in the University of California, Los Angeles (UCLA) Mildred E. Mathias Botanical Garden. *B. subtilis* is a well-known plant-growth promoting bacterial species, but *B. simplex* is not. *B. simplex* 30N-5 was initially isolated on a nitrogen-free medium, but no evidence for nitrogen fixation was found. Nevertheless, pea

plants inoculated with *B. simplex* showed a change in root architecture due to the emergence of more lateral roots. When *Pisum sativum* carrying a *DR5::GUSA* construct, an indicator for auxin response, was inoculated with either *B. simplex* 30N-5 or its symbiont *Rhizobium leguminosarum* bv. *viciae* 128C53, GUS expression in the roots was increased over the uninoculated controls. Moreover, when pea roots were coinoculated with either *B. simplex* 30N-5 or *B. subtilis* 30VD-1 and *R. leguminosarum* bv. *viciae* 128C53, the nodules were larger, clustered, and developed more highly branched vascular bundles. Besides producing siderophores and solubilizing phosphate, the two *Bacillus* spp., especially strain 30VD-1, exhibited anti-fungal activity towards *Fusarium*. Our data show that combining nodulating, nitrogen-fixing rhizobia with growth-promoting bacteria enhances plant development and strongly supports a coinoculation strategy to improve nitrogen fixation, increase biomass, and establish greater resistance to fungal disease.

Keywords: anti-fungal activity; lateral roots; nodulation; plant growth-promoting bacteria; phosphate solubilization; siderophores

1. Introduction

Soil bacteria that associate with plant roots and have a positive effect on growth are often called plant-growth promoting rhizobacteria (PGPR). The more general term plant-growth promoting bacteria (PGPB) [1], used in this report, has been utilized to describe microbes that colonize and positively impact the phytosphere, *i.e.*, the surfaces of stems, leaves, seeds, and roots.

PGPBs fix nitrogen, secrete plant hormones or antibiotics, solubilize phosphate, inhibit pathogenic microbes, or modify insoluble Fe^{3+} to utilizable forms of iron [2,3]. In addition, soil bacteria stimulate phytoremediation and by attaching to roots, improve plant health by increasing the numbers of biodegradative bacteria [4]. Many PGPB studies focus on Gram-negative bacteria such as the pseudomonads, which are simple to culture and suitable for genetic manipulation. However, a large number of *Bacillus* species, Gram-positive, rod-shaped bacteria, also exhibit plant growth-promoting (PGP) activity. Their ability to produce dormant, heat- and desiccation-tolerant spores unlike Gram-negative microbes not only enables them to survive severe stress in the field, but has also led to their utilization in commerce because of the easy dispersal and long shelf life of their spores [5]. *Bacillus* species reported as having PGP ability include *B. subtilis* [6–8], *B. amyloliquefaciens* [9], *B. pumilus* [10], *B. licheniformis* [11], *B. thuringiensis* [12], *B. cereus* [13], *B. megaterium* [14], and many others. Combinations of *Bacillus* spp. also enhance plant growth. Researchers describe the interactions of *Bacillus* species as well as other Gram-positive bacteria with plants [15], whereas others present overviews of the commercial products available for plant growth promotion, several of which include *Bacillus* spp. [16,17].

Bacillus spp. enhance plant growth in myriad ways: By sequestering or mobilizing heavy metals in contaminated or serpentine soils [18], by controlling pathogens via induced systemic resistance (ISR) [17], by synthesizing antibiotics or volatile compounds [19–21], by producing plant hormones such as indole-3-acetic acid (IAA), cytokinin, and gibberellic acid (GA) or by reducing ethylene

concentration via *acdS* (1-aminocyclopropane-1-carboxylic acid deaminase) activity [22], and/or by acting synergistically with nitrogen-fixing or phosphorous-acquiring symbiotic microbes [11,23–25]. PGP bacilli are commonly detected in the rhizosphere, phyllosphere, or as endophytes [10,26,27].

In a survey of soil bacteria isolated from the Mildred E. Mathias Botanical Garden (MEMBG), we discovered two *Bacillus* strains, one identified as *B. simplex* and the other as *B. subtilis*. Other than the studies reporting that certain *B. simplex* strains promote growth of kiwifruit [28], Arabidopsis [19], and tomato and wheat [29], few accounts describe this species as a PGPB [30–32]. On the other hand, *B. subtilis* strains are well known PGPBs.

Previous studies demonstrated that coinoculating legumes with rhizobia and various *Bacillus* species, including *B. subtilis*, resulted in altered root architecture and enhanced nodulation for bean [33,34], peanut [35], pigeon pea [36], and soybean [12,37]. In preliminary experiments, we found that two legume species used as models for determinate and indeterminate nodule formation, *Lotus japonicus* and *Medicago truncatula*, respectively, showed enhanced lateral root formation in response to *B. simplex* inoculation. Determinate and indeterminate nodules differ in the site of original cell divisions giving rise to the nodule, the length of the root hair that is infected, the presence of an ephemeral *versus* a persistent apical meristem, and the downstream stages of nitrogen assimilation [38].

In this report, we analyzed a *DR5::GUS*-containing *Pisum sativum* L. [39], an indeterminate nodule-forming legume, with the following goals: (1) To determine whether *Bacillus* inoculation alone would enhance the growth of pea; (2) To learn whether pea nodulation was changed upon coinoculation with *Rhizobium leguminosarum* bv. *viciae* 128C53 (Rlv) and *Bacillus*; and (3) To deduce likely mechanisms that could account for growth enhancement in response to *Bacillus* inoculation either alone or in combination with rhizobia. Pea was selected as a host plant because: (1) *DR5::GUS* expression has not yet been employed to monitor the effect of bacterial inoculation on auxin response in this legume; and (2) it is a large legume making it easier to detect potentially subtle differences in dry weight and shoot length measurements. We focused on the effects of *B. simplex* inoculation because this species has been understudied compared to *B. subtilis*. However, because *B. subtilis* species are commonly used for biocontrol and have other PGP effects, we included *B. subtilis* 30VD-1, which was isolated at the same time as the *B. simplex* strain, in our studies.

Based on our analysis, we propose that bacteria-derived or plant-produced auxin may be one of the major factors influencing pea root architecture and growth. Furthermore, bacterial traits such as phosphate solubilization, siderophore production, and anti-fungal activity are also likely to be involved in pea growth promotion, and thus contribute to the success of a multi-species strategy for inoculating crops in the field.

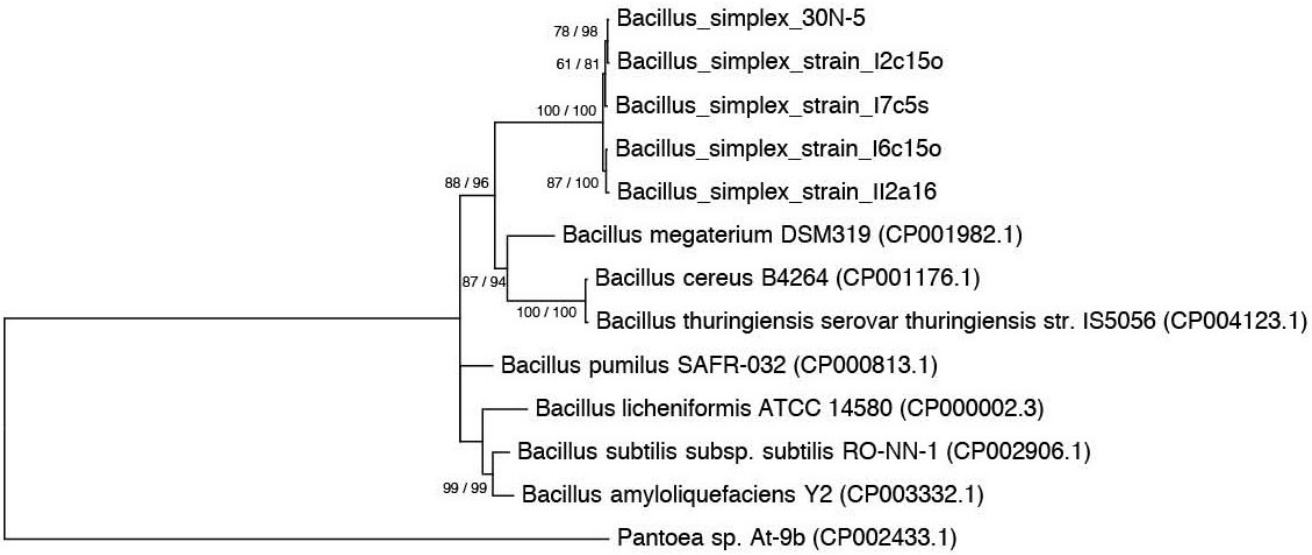
2. Results and Discussion

The 16S rRNA gene of strains 30N-5 and 30VD-1 (Table 1) was PCR-amplified using a colony PCR procedure (see Experimental Section). A preliminary phylogenetic analysis of the nucleotide sequence for a partial 16S rDNA gene showed that strain 30N-5 is nested within a well-supported clade (100% bootstrap) that includes five other *B. simplex* strains. We found that strain 30N-5 is 100% identical to *Bacillus simplex* NH25 and >99.4% identical to all other *B. simplex* strains. Two 16S PCR

products of 1465 and 1458 bp were sequenced and found to be 100% identical to *B. subtilis* subsp. *subtilis* strain 168.

To obtain even finer resolution, a phylogenetic tree was constructed with a concatenated set of four protein-encoding genes: glyceraldehyde-3-phosphate dehydrogenase (*gap*A), RNA polymerase beta subunit (*rpo*B), excision nuclease subunit A (*uvr*A), and phosphoglycerate kinase (*pgk*) [31,40]. As shown in Figure 1, strain 30N-5 is nested within the clade that contains five *B. simplex* strains, which are distinct from other *Bacillus* species previously reported to be PGPB.

Figure 1. Multilocus sequence analysis of closely related members of the genus *Bacillus* and position of *B. simplex* 30N-5 within the *B. simplex* clade based on a concatenated set of four protein-coding genes (*gap*A, *rpo*B, *uvr*A, *pgk*). The bootstraps reported are listed in the order Maximum Likelihood/Neighbor Joining. Also, the values are only reported if both methods support the clade with a >50% bootstrap value.



2.1. Nitrogen Fixation Tests

Because *B. simplex* 30N-5 was originally isolated on an N-free medium, primers that targeted *nifH* were used in a colony PCR using *Sinorhizobium* (*Ensifer*) *meliloti* strain 1021 as the positive control. We did not find any evidence for *nifH* in *B. simplex* 30N-5 despite the fact that we based our primer set on data from [41] and that several reports were published earlier regarding *nifH* presence in various *Bacillus* genomes [41–43]. We also found no evidence for *nif* genes in an analysis of a draft sequence of the *B. simplex* genome. Thus, we conclude, in agreement with [44] who reported that *Bacillaceae* lack *nifH*, that *B. simplex* is not a diazotroph even though 30N-5 was isolated on a solidified N-free medium.

One possible explanation for its growth on medium lacking nitrogen is that *B. simplex* is a very good N scavenger. When inoculated into an N-free liquid medium, *B. simplex* 30N-5 ceased growth unless the medium was supplemented with ACC, suggesting that *B. simplex* has AcdS (1-aminocyclopropane-1-carboxylate (ACC) deaminase) activity. AcdS catalyzes the breakdown of ACC to 2-oxobutanoate and ammonia. A number of rhizosphere bacteria use ACC, the precursor of the

plant-growth inhibitor ethylene, as a nitrogen source [45]. When *B. simplex* 30N-5 was cultured on an N-free medium with or without ACC, growth occurred in liquid medium only when ACC was present. However, in solid medium, growth occurred with or without ACC. This response took place whether Bacto-Agar or Phytagar was used as a solidifying agent, suggesting that a small amount of N may have been present in the agar.

Table 1. Strains used in this study.

Strain	Relevant Characteristics	Source/Reference
Bacillus simplex 30N-5	Isolated from the rhizosphere of a *Podocarpus nagi* tree growing in the MEMBG.	This work
Bacillus subtilis 30VD-1	Isolated from soil in the palm section of the MEMBG, adjacent to *Brahea edulis* (Aracaceae; Guadalupe Palm), a tree indigenous to Guadalupe Island in Mexico.	This work
Bacillus subtilis BAL218	JH642 *trpC2, pheA1*	Beth A. Lazazzera (University of California, Los Angeles)
Sinorhizobium (*Ensifer*) *meliloti* 1021	Wild-type *Sinorhizobium* (*Ensifer*) *meliloti*, Str^r	Laboratory strain
Rhizobium leguminosarum bv. viciae 128C53	Wild-type Rlv	Laboratory strain
Rhizobium leguminosarum bv. viciae 128C53/pHC60	Wild-type Rlv with GFP, Tet^r	This work
Fusarium oxysporum forma specialis conglutinans race 2 PHW 808	Isolated from cabbage.	[46]
Fusarium oxysporum forma specialis matthioli race 2 PHW 726	Isolated from *Mathiola incana*.	[46]
Fusarium verticillioides NRRL 13993	Isolated from maize ear.	[47]
Nectria hematococca 77-113-4	Pea pathogen, member of the *Fusarium solani* complex	Martha C. Hawes (University of Arizona)

We next utilized primers for *acdS* [48] to determine whether an ACC deaminase gene was present in the *B. simplex* genome. A band of the expected size was amplified and cloned into a TOPO® vector. Instead of an *acdS* sequence, the primers amplified products that were determined to encode histidyl-tRNA synthetase, acetyl CoA acetyltransferase, and D-fructose-6-phosphate amidotransferase based on NCBI BLAST. Previously, an endophyte identified as *B. simplex* XAS5-3 was shown to exhibit low levels of ACC deaminase activity [22]. We next checked the draft genome of *B. simplex* 30N-5 and found it contains a sequence with high identity to a gene encoding a pyridoxal phosphate-dependent enzyme. ACC deaminase is a member of this family of enzymes, and in *Bacillus* spp., these genes are often annotated as *acdS.* Nonetheless, experimental evidence will be needed to prove whether or not this gene actually encodes AcdS, but in any case, its putative presence

in the *B. simplex* draft genome suggests the reason for the initial selection of strain 30N-5 on an N-free medium.

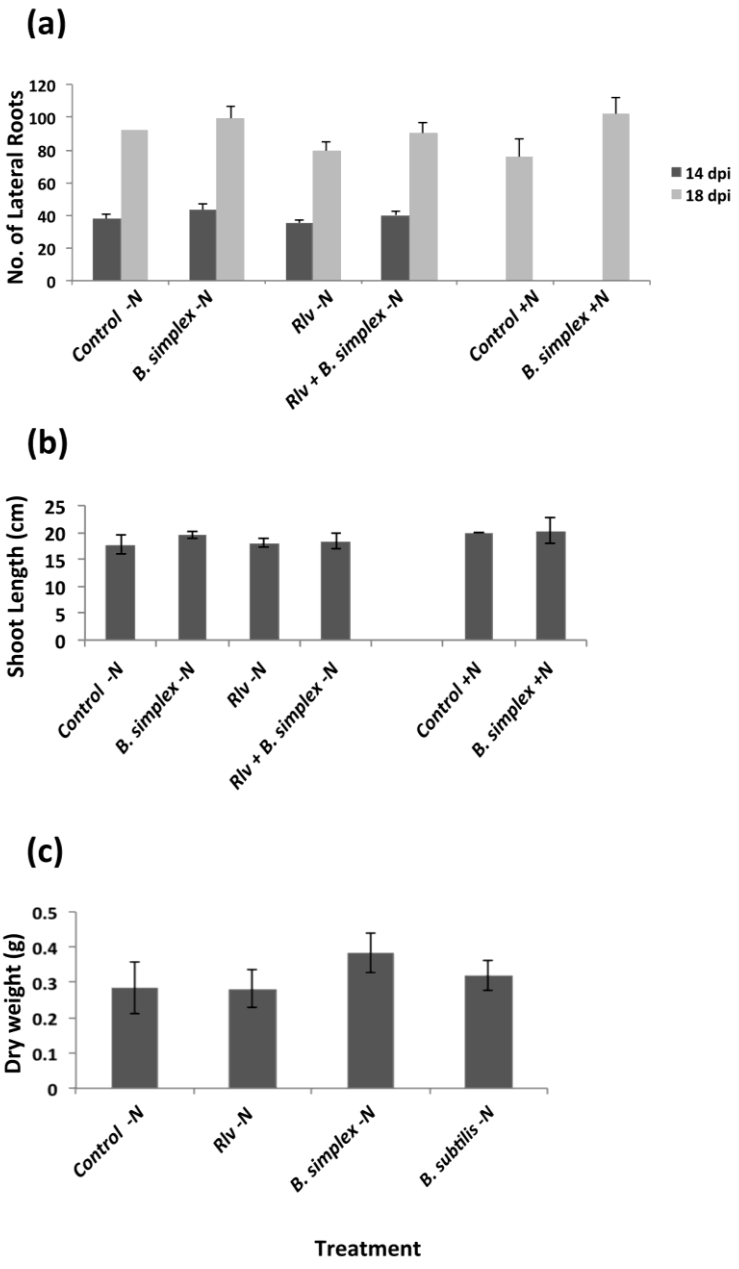
2.2. Inoculation with B. simplex 30N-5 Alters Root Architecture

Because several *B. simplex* strains are reported to be PGPBs, we choose to study strain 30N-5's plant growth-promoting capabilities, specifically as a helper for nodulation. We found that lateral root number increased in response to *B. simplex* inoculation compared to the control (uninoculated) when peas were grown in +N medium as early as 14 days following inoculation (dpi) (Figure 2) and that the difference from the control was statistically significant (Figure 3a). When pea plants grown in –N medium were either left uninoculated, inoculated with *B. simplex* or *B. subtilis* or Rlv singly, or mixed with either *Bacillus* species and Rlv in a 1:1 ratio, only a slight change in root architecture was observed for *B. simplex*-inoculated plants 14 and 18 dpi (Figure 3a). For Rlv-inoculated plants, the number of lateral roots decreased by 18 dpi. With respect to shoot length, no obvious statistical difference was observed 18 dpi (Figure 3b) although in some cases, greater root development led to taller plants (Figure 2).

Figure 2. Change in pea root architecture for either **(a)** uninoculated plants or (**b**) plants inoculated with *B. simplex* 30N-5 and grown in a +N medium for 2 weeks, Bar, 1 cm.

Plants were grown in pots for the dry weight measurements because root volume was likely to be less constrained than in Magenta jars. The largest increase in root dry weight occurred following *B. simplex* inoculation. By contrast, *B. subtilis* and Rlv-inoculated root dry weight increases did not statistically differ from that of the uninoculated control (Figure 3c) although a trend towards increased biomass was suggested. No difference was observed among the treatments with respect to shoot dry weight.

Figure 3. Growth measurements of uninoculated and inoculated pea plants grown in Magenta jars (**a**,**b**) or pots (**c**); **(a)** Average number of lateral roots 14 dpi and 18 dpi from either four or six different treatments including the uninoculated control (without or with N); *B. simplex* (without or with N) or Rlv alone (without N) and the two bacteria mixed together in a 1:1 ratio (without N); **(b)** Same treatment as above, but shoot length measurements were made 18 dpi; **(c)** Effect of *Bacillus* strains on pea root biomass. Peas inoculated with *B. simplex*, *B. subtilis*, or *R. leguminosarum* bv. *viciae*, or left uninoculated were grown in pots in a UCLA Plant Growth Center greenhouse (22.3 °C) under N-deficient conditions (−N). At 18 dpi, roots were isolated and dried at 55 °C for 48 h. For **a**–**c**, the number of plants measured per treatment was 8–10. All experiments were repeated at least 3 times. Significance between means was tested, after a variance analysis was performed, using Duncan's Multiple Range test at $p < 0.05$. Standard errors are illustrated.



Many *B. subtilis* strains synthesize auxin [49], but because so few *B. simplex* strains have been studied, it is not known whether they share this ability. IAA secreted into the growth medium of *B. simplex* cultures incubated with varying concentrations of L-tryptophan (an IAA precursor) was measured following published procedures (see Experimental Section). A positive linear relationship between the concentration of L-tryptophan and the Optical Density ratio ($OD_{535/600}$) was seen indicating the presence of auxin in the supernatant (Appendix Figure A1). No positive correlation was observed between the absorbance ratios and the varying L-tryptophan concentrations in uninoculated control experiments or in the medium alone (data not shown).

2.3. Effects on Nodulation in Response to Rhizobium leguminosarum *bv.* viciae

Because auxin has been implicated in both nodulation and lateral root formation and because *B. simplex* secretes IAA, we utilized pea plants with a *DR5*::*GUS* promoter. These stable transgenic lines were originally developed to monitor auxin activity and response in vegetative and reproductive tissues [39]. GUS expression was detected in embryos, root tips, developing flowers, procambial cells, and pollen—sites known to correlate with high auxin concentrations.

Upon inoculation with either *B. simplex* 30N-5 or Rlv singly or mixed together in a 1:1 ratio, an increase in GUS expression in pea root tips over the uninoculated controls was observed (Figure 4a–c). Because nodules developed only after Rlv inoculation, the blue color first became apparent within inner cortical cells in close association to the root vascular bundle (vb) where nodules originate (Figure 4d). Nodules of varying developmental stages that stained positively for GUS activity are illustrated in Figure 4e,f. Immature but emergent nodules exhibited terminal GUS staining (Figure 4e). With maturity, the staining of the nodule meristem lessened, but the GUS stain remained closely associated with the immature vascular bundles of the developing nodule (Figure 4f).

2.4. Nodulation in Response to Coinoculation

The most striking phenotype resulting from the mixed inoculation experiments was the increase in nodule size as well as the clustering of nodules (Figures 4c and 5). A similar response occurred with *B. subtilis* 30VD-1 coinoculated with Rlv for pea plants that were grown in pots in the greenhouse. Like the *B. simplex*-Rlv coinoculation, the nodules were clustered and redder than the nodules initiated by Rlv alone.

As described above for Rlv only, the earliest stages of nodule development took place in the inner cortex and the young, emergent nodules exhibited GUS staining in the nodule meristem and in the vascular bundles (Figure 5b). In some young nodules, the nodule meristem lost the blue color early in development (Figure 5c) whereas in others, the color was evident even in the later stages (Figure 5d). More mature nodules exhibited additional branching of the vascular bundles with the distal areas losing GUS staining as the vascular bundles matured (Figure 5e,f).

Figure 5g illustrates a near-median, sagittal [50] section of a nodule stained for GUS (arrow). The interior cells of the nodule are occupied by bacteroids. Examination at a higher magnification did not reveal any confirmable bacilli or spores in or between nodule cells. Attempts to determine whether bacilli were present using an antibody against *Bacillus* spore walls or a specific staining reaction to identify Gram-positive organisms in sectioned material [51] were unsuccessful.

Figure 4. GUS staining of pea roots and nodules 14 dpi; (**a**) Uninoculated control. GUS staining is mostly localized to the lateral root primordia and the youngest root tips; (**b**) Root inoculated with *R. leguminosarum* bv. *viciae*. The blue color is detected in the nodules (arrows), lateral root primordia, and in young root tips; (**c**) Root coinoculated with *B. simplex* and *Rlv*. The nodules (white arrowheads) are larger and clustered closer to the crown of the root. Bar, 1 cm; **(d)** Nodule primordium (black arrow) 10 dpi with *R. leguminosarum* bv. *viciae*. The white arrow points to a vascular bundle (vb); **(e)** Emergent nodule (ca. 14 dpi) with GUS staining in the nodule meristem (m) and young vascular bundles; **(f)** Staining is apparent in the nodule meristem (m) and most recently developed vascular bundles (vb) (arrow); 32 dpi; Bars, 200 µm.

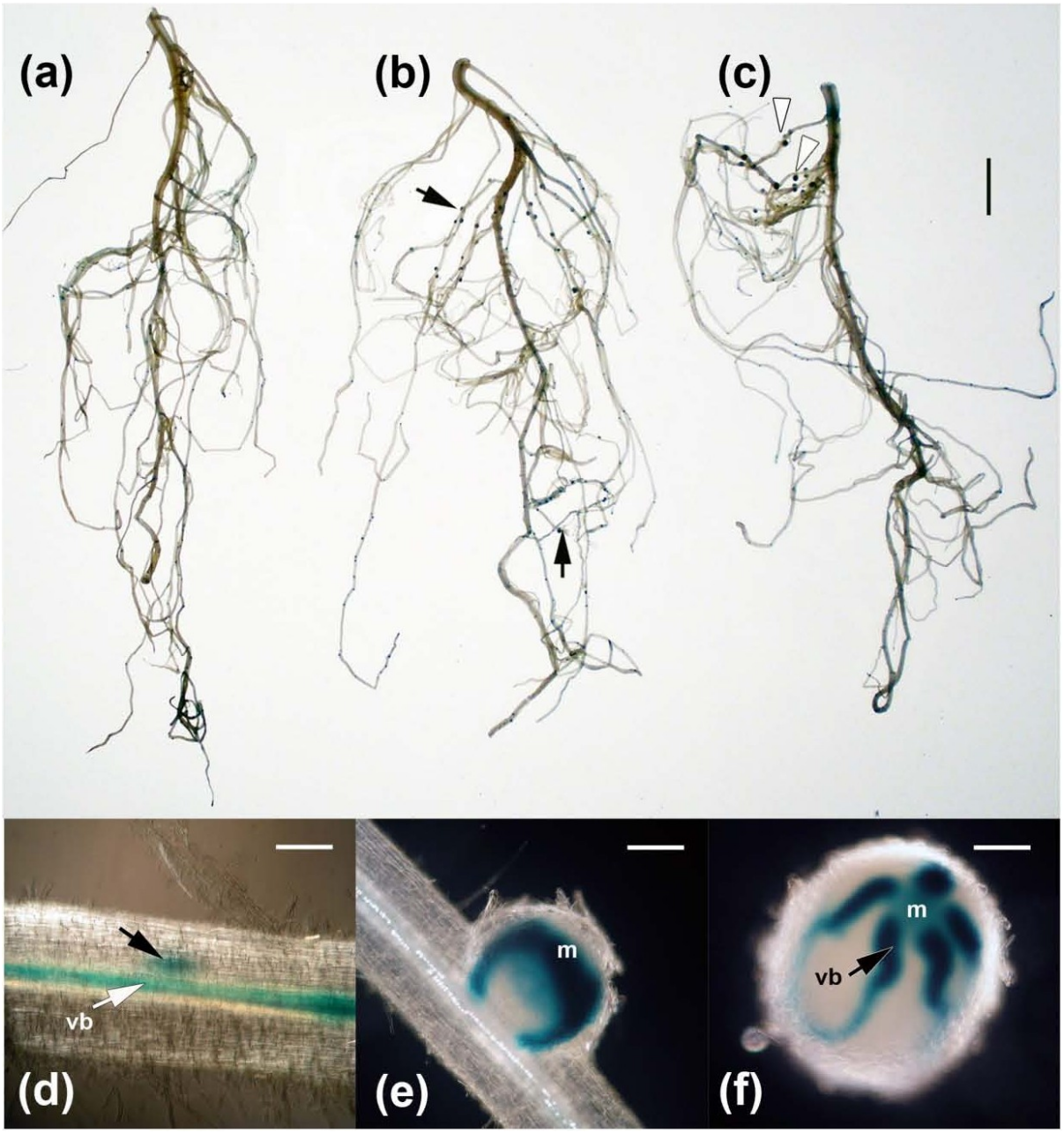
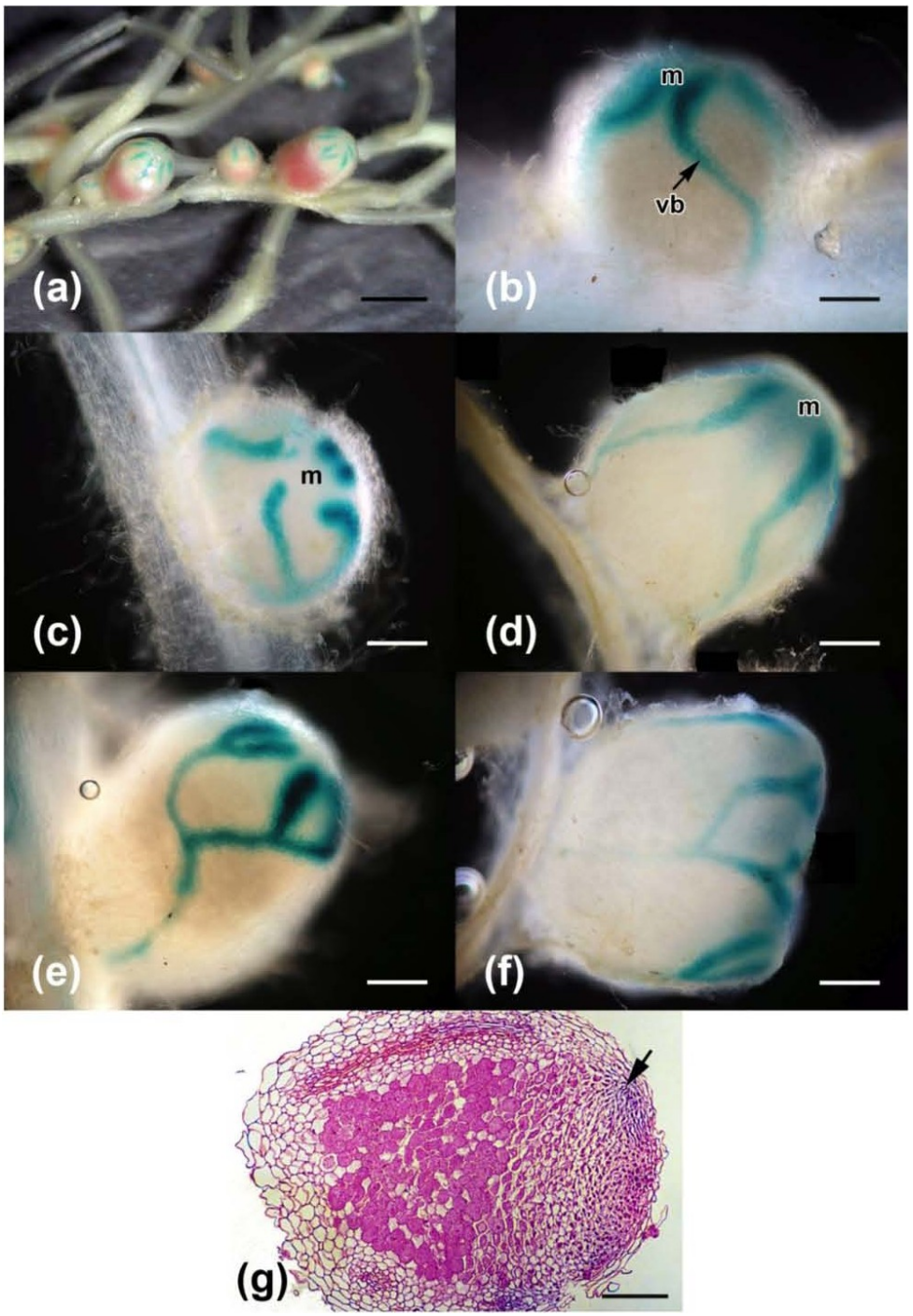


Figure 5. Effect of coinoculation; (**a**) A cluster of nodules of different ages showing GUS staining in the young vascular bundles; 32 dpi. The red color is due to the presence of leghemoglobin. Bar, 2 mm; (**b**) A young nodule (14 dpi) exhibits localization of GUS activity in the nodule meristem (m) and young vascular bundles (vb); (**c**) Loss of GUS staining in the nodule meristem (m); 14 dpi; (**d**) Retention of some GUS staining in the meristem (m); 32 dpi; (**e**) Branching of the vascular bundles in a more mature nodule; 32 dpi; (**f**) A nodule with extensive vascular development; 32 dpi; (**g**) Near-median plastic section of a 26 dpi nodule originally stained for GUS, which is localized towards the apical end near the vascular bundle (arrow). The section was stained with safranin O. Bars for (**b**) through (**g**), 200 µm.



To determine whether both bacterial species were found in the nodules from coinoculated plants, we squashed surface-sterilized nodules to look for Rlv/pHC60 *versus* bacteria of a different morphology or lacking GFP. Table 2 shows the numbers of bacteria recovered from suspensions obtained from one Rlv/pHC60-nodule and three nodules of a coinoculated plant. Three colonies, one from a nodule removed from an Rlv/pHC60-inoculated plant and two from nodules of coinoculated plants, were chosen for PCR analysis to verify that both strains were present in the nodule. The GFP-fluorescing colony derived from the Rlv/pHC60 nodule was identified as Rlv (99% identity over 329 bp). The non-fluorescent colonies isolated from nodules of coinoculated plants were identified as *Bacillus* sp. (97%, 329 bp) and *B. simplex* (99%, 890 bp). Thus, both bacterial species are housed within nodules although *B. simplex* is significantly outnumbered.

Table 2. Recovery of bacteria from nodules of *Rhizobium leguminosarum* bv. *viciae*-inoculated and coinoculated plants.

Dilution	**Rlv/pHC60-inoculated**		**Coinoculated 1**		**Coinoculated 2**		**Coinoculated 3**	
	Total # colonies	Non-GFP colonies	Total # colonies	Non-GFP colonies	Total # colonies	Non-GFP colonies	Total # colonies	Non-GFP colonies
1:100	247	0	n.d. *	n.d.	435	1	n.d.	n.d.
1:1000	n.d.	n.d.	88	3	126	2	136	1

* The 1:10 dilutions and the columns indicated by "not determined" (n.d.) contained either no or too many bacteria to count.

Nodule clustering following coinoculation with Rlv and either *Bacillus* strain might be a consequence of increased root hair development as reported by [34] for bean, or alternatively, caused by the branching of the nodule primordium. It is well known that lateral root development is positively influenced by auxin treatment and this might also affect nodule status. The clustering of nodules and their increased size is likely to be correlated with the increase in the number of vascular bundles, brought about by branching of the nodule primordia under coinoculation conditions. Auxin, when applied exogenously, increases root mass both by stimulating the elongation of the primary root, and by increasing the number of lateral roots [14]. Increased root mass due to more lateral roots enhances the plants' ability to anchor themselves in the soil, enables them to obtain increased amounts of water and mineral nutrients through increased root surface area, and promotes the extension of the primary roots into deeper soil levels sooner after seed germination [52].

In earlier studies, *M. truncatula* plants inoculated with *S. meliloti* 1021, either engineered to overproduce IAA (strain RD64) by introducing a plasmid encoding the indole-3-acetamide pathway or supplemented with 0.5 mM IAA, exhibited increased nitrogen fixation, which was linked to the improved phosphate solubilization activity of *S. meliloti* strain RD64 and IAA-supplemented strain 1021 [53]. Also, the RD64 and IAA-treated 1021 strains induced the formation of *M. truncatula* nodules that had a significantly larger nodule meristem, resulting in increased nodule size, nitrogen fixation, and stem dry weight [54]. These descriptions resemble the phenotypes observed for peas coinoculated with Rlv and *B. simplex*, although [54] did not report whether the nodule vasculature was more elaborated or whether the nodules were clustered together.

2.5. Mechanisms by Which B. Simplex *30N-5 and* B. Subtilis *30VD-1 Could Promote Plant Growth*

Because "direct mechanisms of plant growth promotion are difficult to differentiate from disease suppression" [16], we first investigated whether the *B. simplex* strain promoted plant growth by producing siderophores or solubilizing phosphate, two basic mechanisms of plant growth promotion. Moreover, the ability of the *Bacillus* strains to solubilize phosphate and produce siderophores might also be involved in enhancing nodule function. Bacterial siderophore production promotes growth by supplying iron directly to the plant or by depriving other microbes, potentially deleterious ones, of iron [55]. We found that strains 30N-5 and 30VD-1 were able to solubilize phosphate and synthesize siderophores (Appendix Figure A2). On the other hand, other known *B. simplex* strains, such as *B. simplex* KBSIF-3 [29] and *B. simplex* XAS35-3 [22] do not exhibit these activities. Evidence for siderophore function was found in the *B. simplex* 30N-5 draft genome.

Next, anti-fungal activity was investigated for *B. simplex* 30N-5 and two different *B. subtilis* strains: BAL218 and 30VD-1 (Table 1). BAL218 was used to compare the activity of a typical laboratory strain with that of a newly isolated *B. subtilis*. The *Bacillus* strains and various *Fusarium* species [46,47] were co-cultured first on Potato Dextrose Agar (PDA) because this medium is routinely used. However, *B. subtilis* BAL218 growth was poor on PDA, so we tried Czapek Dox agar and Malt Extract agar (MEA), but neither *B. simplex* nor *B. subtilis* BAL218 grew robustly on these two media. Also, PDA did not support *B. simplex* growth as well as that of *B. subtilis* 30VD-1, making it difficult to evaluate the antagonistic activity of strain 30N-5 towards *Fusarium* species. For example, in tests against *F. oxysporum* f. sp. *conglutinans* PHW 808 (FOC) and *F. verticillioides* FV3 X0042 (Fv), *B. subtilis* 30VD-1 significantly inhibited Fv radial growth compared to the uninoculated control whereas *B. simplex* 30N-5 did not (Figure 6a). By contrast, *B. simplex* reduced Fv growth, but again it was not as effective at doing so as was *B. subtilis* 30VD-1 (Figure 6b). This result led us to use V8 agar and solidified ISP2, both of which support bacterial and fungal growth better than PDA [56,57].

B. simplex 30N-5 grew well on V8 medium, either filtered or unfiltered, and exhibited its most active anti-fungal activity on this substrate. However, the laboratory strain BAL218 was ineffective on all media tested, and so it was dropped from further tests. In subsequent experiments, two other fungi were included along with FOC, namely *F. oxysporum* f. sp. *matthioli* PHW 726 (FOM) and the pea pathogen *Nectria haematococca* 77-13-4 (Nh) (Table 1). As can be seen in Figures 6c–e, the mean radial growth of all uninoculated fungi was much improved in V8 agar compared to PDA (Figures 6a,b). In addition, each fungal species responded differently to this medium with FOM expanding more extensively than Nh, which grew better than FOC.

On V8 agar, an inhibitory effect of *B. simplex* on FOM radial expansion was apparent 72 hours after the start of the experiment and was obvious by day 4 (Figure 6c). Nevertheless, *B. subtilis* 30VD-1 remained a more potent antagonistic agent, restricting fungal growth by more than 31% compared to 9% inhibition for *B. simplex* after 3 days (Figure 6d). By day 6, *B. simplex* had reduced FOM radial expansion by ca. 28% whereas *B. subtilis* 30VD-1 inhibited growth by 53% (cf. Figure 6c,d). FOC growth was inhibited by *B. simplex* on V8 agar 4 dpi (Figure 6e) in contrast to PDA, where *B. simplex* grew poorly (Figure 6a), and ISP2 media. Conversely, *B. subtilis* 30VD-1 was a robust fungal antagonist, reducing fungal growth by 50% or more after 6 days of co-cultivation. In general, *B.*

simplex 30N-5 was not as effective an antagonistic agent as strain 30VD-1 in part because of its reduced growth on many of the co-cultivation media. *B. simplex* 30N-5 limited fungal radial expansion to about one-half the level of that of *B. subtilis* 30VD-1, and on V8 agar, Nh fungal hyphae became transparent (Figure 7), most likely due to altered hyphal growth on the side adjacent to the bacteria. SEM studies observed that *Colletotrichum acutatum* hyphae appeared lysed and spores degraded in response to *B. subtilis* inoculation [20]. Whether Nh transparency in response to *B. simplex* 30N-5 resulted from cell lysis or an inhibition of aerial hyphae needs further study.

Figure 6. Effect of *B. simplex* 30N-5 (red line) and *B. subtilis* 30VD-1 (green line) on the growth of fungi on either PDA (**a,b**) or V8 agar (**c**–**e**). The fungal species tested were: **(a)** *Fusarium oxysporum* f. sp. *conglutinans* PHW 808 (FOC); **(b)** *Fusarium verticillioides* FV3 X0042 (Fv); **(c)** *Fusarium oxysporum* f. sp. *matthioli* PHW 726 (FOM); **(d)** *Nectria haematococca* 77-13-4 (Nh); and **(e)** *Fusarium oxysporum* f. sp. *conglutinans* PHW 808 (FOC). The blue line illustrates the radial growth of the fungus in the absence of bacteria over a 6 or 7-day period. Standard errors are illustrated.

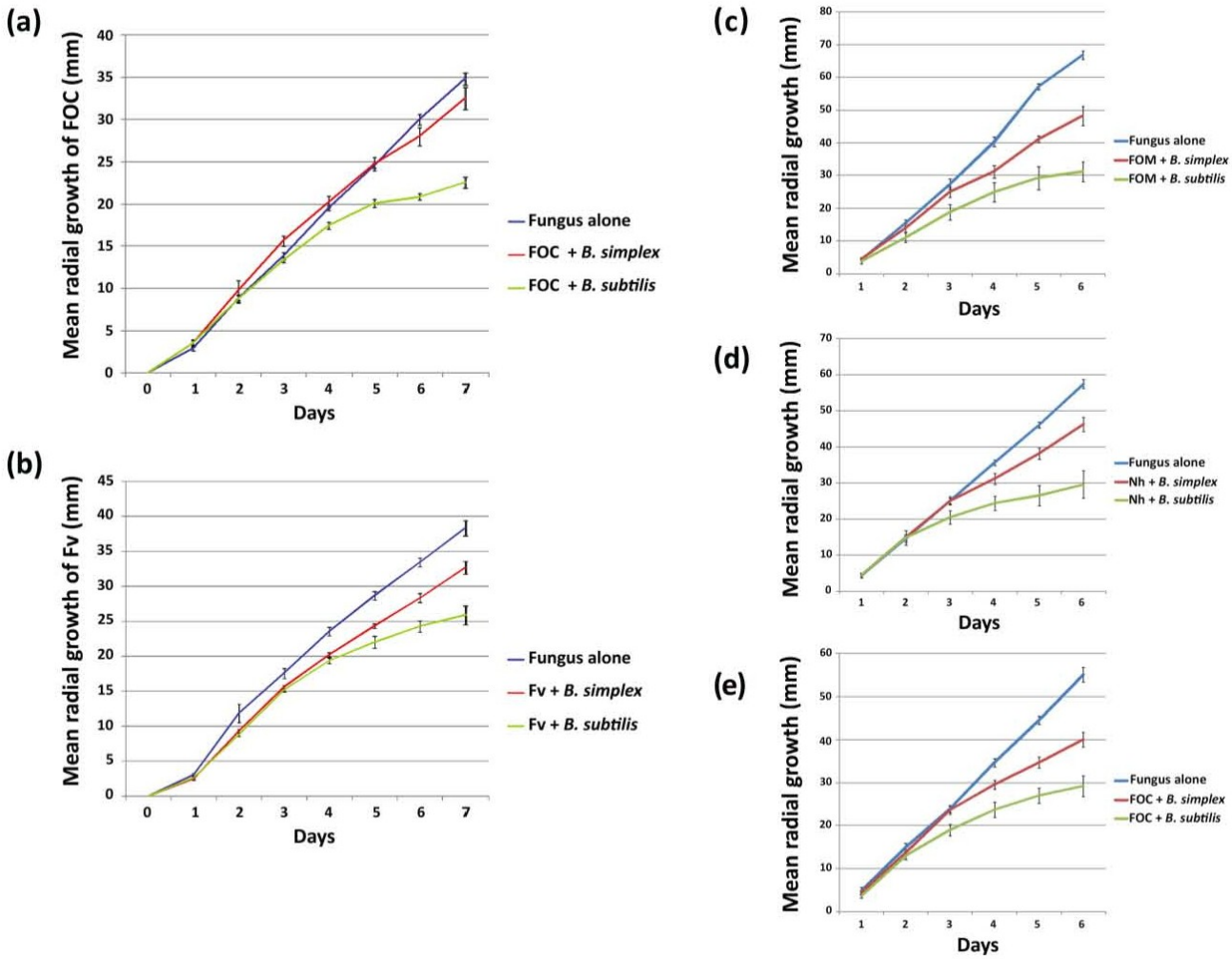
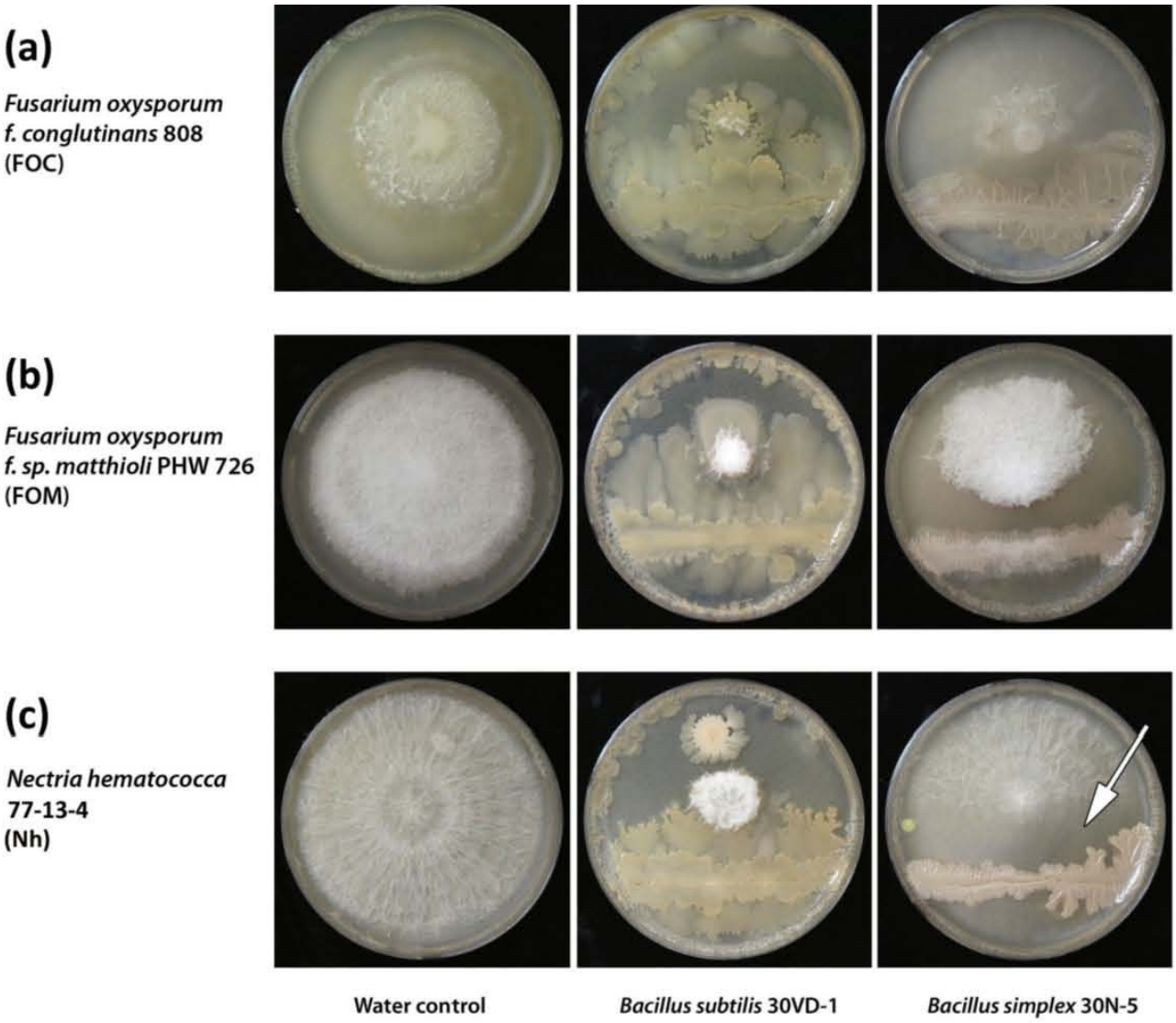


Figure 7. Effect of *Bacillus* strains on fungal growth. Responses of FOC **(a)** and FOM **(b)** to water (negative control), *B. subtilis* 30VD-1, and *B. simplex* 30N-5 following 13 days of culture on filtered V8 agar. *B. subtilis* 30VD-1 has almost completely surrounded the fungal culture. *B. simplex* limits the growth of both FOC and FOM, but not as much as the 30VD-1 strain does (middle column); **(c)** Response of *Nectria haematococca* 77-13-4 to the negative control and to the *Bacillus* strains on filtered V8 agar. An increase in transparency of the fungus is observed on the side closest to the *B. simplex* 30N-5 streak (arrow), whereas for the uninoculated control, the appearance of the fungal colony is the same throughout. *B. subtilis* 30VD-1 has almost completely surrounded the fungus.



In summary, both *B. simplex* 30N-5 and *B. subtilis* 30VD-1 exhibited anti-fungal activity and thus have potential for use in biocontrol. Our next step will be to determine in greenhouse experiments whether these *Bacillus* strains limit plant disease in response to fungal inoculation. Taken together, these data demonstrate that an approach of mixing nitrogen-fixing rhizobia and bacteria with PGP and anti-fungal activity increases legume growth and biomass production. Such a strategy should prove efficacious under field conditions, but more testing is needed.

3. Experimental Section

3.1. Routine Bacterial Growth Conditions

Soil samples were collected from the rhizospheres of plants growing in UCLA MEMBG (Table 1). For the first isolate, serial dilutions of the soil were plated on an agar-solidified modified BAP medium without nitrogen [58] and incubated at 30 °C for several days. Repeated streak plating of individual colonies was conducted on the same medium until purified isolates, named 30N-5, were obtained. The second strain, strain 30VD-1, was isolated from soil in a different section of the MEMBG (Table 1) and originally propagated on VXylA medium [59] in the dark at 30 °C. Unless otherwise noted, *B. simplex* 30N-5 and the *B. subtilis* strains were grown on LB (Luria-Bertani) [60] or TY [61] media, at either 30 °C or 37 °C. The rhizobial strains were grown in Rhizobium Defined Medium (RDM) [62] and prepared for inoculation as described for *B. simplex* (Section 3.8). Antibiotics were used at the following concentrations: 50 μg/mL streptomycin (str), 10 μg/mL tetracycline (tet), and 100 μg/mL ampicillin (amp).

3.2. Amplification, Purification, and Sequencing of the 16S rRNA Gene

Bacteria were suspended from a single *B. simplex* colony grown on LB plates into 20 μL of sterile-filtered water. For *B. subtilis* 30VD-1, genomic DNA was extracted from two independent colonies and the 16S DNA was amplified as described below.

The gene for a 16S rRNA was amplified by PCR using the forward primer fD1 and the reverse primer rD1 [63] (Appendix Table A1). Amplification was performed in a total volume of 20 μL containing 15 μL sterile-filtered water, 2 μL of bacterial sample, 2 μL of 10× Taq Buffer ($MgCl_2$), 0.2 μL fD1 and rD1 (3.2 pmole/μL), 0.2 μL dNTPs, and 0.2 μL *Taq* DNA polymerase. Amplified 16S rDNA products were visualized with ethidium bromide both in the gel and in the gel electrophoresis running buffer, and purified from a 0.8% low-melting point agarose gel (100 V, 400 mA, 1 h). The gel extraction was performed with the Invitrogen Quick Gel Extraction Kit according to the manufacturer's directions.

To sequence the DNA fragments, the dideoxynucleotide chain-termination method was used. The sequencing reaction was performed in a total volume of 10 μL, containing 5.8 μL purified DNA, 2 μL 5× Seq buffer, 2 μL BigDye®, and 0.2 μL fD1 or rD1. The conditions were: 2 min at 96 °C, 40 cycles of 10 s at 96 °C, 5 s at 57 °C, and 4 min at 60 °C. DyeEx2.0 Spin Columns were used to clean the sequencing reactions. The reaction mixture was sequenced at the UCLA GenoSeq Core sequencing facility, and analyzed using the nucleotide BLAST program [64].

3.3. Phylogenies

Phylogenetic analyses were carried out using MEGA5 [65] on two different datasets. One dataset was a set of 16S ribosomal RNA genes, aligned using the Muscle algorithm and trimmed to 836 positions. A second dataset consisted of a concatenated set of four protein-coding genes: glyceraldehyde-3-phosphate dehydrogenase (*gapA*), RNA polymerase beta subunit (*rpoB*), excision nuclease subunit A (*uvrA*), and phosphoglycerate kinase (*pgk*) [31,40]. Nucleotide sequences

for each protein-coding gene set were individually aligned using the Muscle algorithm on the amino-acid translation of the nucleotide sequences. Aligned amino acid sequences were back translated into the original nucleotide sequences after alignment and manually concatenated into a single alignment of 2966 nucleotide positions. Phylogenetic reconstruction for each nucleotide alignment (16S and concatenated protein-coding) was carried out using the Maximum Likelihood algorithm with the following options: Jukes-Cantor model; gamma + invariant; 5 gamma categories; all sites used; heuristic = NNI. Bootstraps (500) were calculated for each tree using Maximum Likelihood (identical parameters to tree calculation) and neighbor joining with the following parameters: Maximum composite likelihood distances, gamma parameter = 1; pairwise deletion. Both sets of bootstrap values are shown in each tree (ML/NJ) and bootstraps are only reported if the clade has >50% support.

3.4. Amplification, Purification, and Sequencing of a Putative nifH *Gene*

Degenerate primers designed to target *nifH* were based on those reported in [41] (Appendix Table A1). Amplification was described as above, but with *nifH* primers and 0.2 µL DMSO. Amplified gene products running at the same distance as an *S. meliloti nifH* PCR product were visualized and purified from 1% low-melting point agarose gels (100 V, 1 h, 400 mA) and sequenced as described.

Cloning was performed using the Invitrogen TOPO® TA Cloning Kit with the pCR®2.1-TOPO® vector. To confirm successful cloning, the four samples of DNA obtained from the mini-prep were cut with *EcoRI* to separate the putative *nifH* insert from the cloning vector. After confirmation of the presence of a band of the expected size for *nifH*, the DNA from the miniprep samples was prepared for sequencing as described above. The sequencing reactions were taken to the UCLA GenoSeq facility, and sequence outputs were analyzed using NCBI nucleotide BLAST.

3.5. PCR Amplification of a Putative acdS *Gene*

Degenerate PCR primers [48] (Appendix Table A1) and amplification as described above were used to detect the *acdS* gene. The putative *acdS* gene products were visualized and purified from a 1% low-melting point agarose gel. The PCR product was extracted, cloned, and sequenced as described above in Section 3.4.

3.6. ACC Utilization Assay

For testing whether *B. simplex* 30N-5 could utilize ACC as a sole N source, the bacteria were grown on either solid or liquid Dworkin and Foster (DF-N) minimal medium without nitrogen [45]. To make DF medium N-limiting, $(NH_4)_2SO_4$ was omitted and 30 µmol of ACC (Calbiochem-Novobiochem Corp., La Jolla, CA, USA) were added. The plates were incubated for 48 h at 30 °C because temperatures higher than 30 °C may inhibit ACC deaminase activity.

3.7. Detecting IAA in B. simplex *30N-5 Growth Medium Supernatant*

To determine whether *B. simplex* produces the phytohormone IAA, the procedure of [52] was followed. 5 mL of DF salts minimal medium [45] were inoculated with *B. simplex* for overnight

culture. 20 µL of this culture were transferred into 5 mL DF medium supplemented with varying concentrations of L-tryptophan (from a sterile-filtered 2 mg/mL stock prepared in warm water) at 0, 50, 100, 200, 500, 800 µg/mL, and incubated for 42 h with shaking at 37 °C. The density of each culture was measured at OD_{600}. The cells were removed from the culture medium by centrifugation (5500× *g* for 10 min), and the supernatant retained. 1 mL of each supernatant was mixed vigorously with 4 mL Salkowski's reagent (150 mL of concentrated H_2SO_4, 250 mL distilled H_2O, 7.5 mL 0.5 M $FeCl_3 \cdot 6H_2O$). The reaction proceeded at room temperature for 20 min before the absorbance at 535 nm was measured. To quantify IAA production, absorbance at 535 nm was divided by the optical density at 600 nm to take into account the variability of culture growth.

3.8. Pisum sativum *Growth Experiments and GUS Staining*

DR5::GUSA pea plants were inoculated with bacteria or left uninoculated (sterile water only) 2 days after the seeds were planted in Magenta jars [66]. The seeds were sterilized in 50% ethanol for 5 min, in 10% commercial bleach for 10 min, and rinsed 5–10 times with sterile water before planting. *B. simplex* was grown in liquid TY until stationary phase when the cells were harvested by centrifugation (8000× *g*, 10 min), and the pellets washed with sterile water three times to remove the growth medium. The cell pellets were diluted to OD_{600} = 0.2 in 1/4 strength Hoagland's N-free medium [67] or sterile water. For inoculation of pea, Rlv/pHC60 (Table 1) was used. This strain was constructed via a triparental mating using the plasmid pHC60, which carries GFP [68]. 2 mL of either *B. simplex* 30N-5 or Rlv/pHC60 were added to the Magenta jars. Coinoculated pea plants were inoculated with 1 mL *B. simplex* 30N-5 and 1 mL Rlv/pHC60. The uninoculated control peas were watered with 2 mL of sterile water or 1/4 strength Hoagland's N-free medium. Ten or more pea plants per treatment were set up per experiment, and the plants were harvested 14 dpi or later.

For GUS staining, plant roots were removed from the Magenta jars and washed to remove all perlite and vermiculite particles. Tissues were prepared as described by [69].

A pot growth experiment was done in the UCLA Plant Growth Center. After sterilization, *P. sativum DR5::GUSA* seeds were planted in pots containing a perlite/vermiculite mixture [66] topped with sterile beads to minimize contamination. *B. simplex* 30N-5, Rlv, and *B. subtilis* 30VD-1 were grown in liquid culture to a final cell density of OD_{600} = 0.3–0.4. At germination, the plants were inoculated with 1 mL of *B. simplex* 30N-5 or Rlv or *B. subtilis* 30VD-1. Coinoculated pea plants were inoculated with 0.5 mL *B. simplex* 30N-5 and 0.5 mL Rlv, or with 0.5 mL *B. subtilis* 30VD-1 and 0.5 mL Rlv. The uninoculated control peas were watered with 1 mL of sterile water or 1/4 strength Hoagland's N-free medium. The plants were watered every 3–4 days with 150 mL of 1/4 strength Hoagland's medium (with or without nitrogen) or with sterile water, and harvested 3 weeks post inoculation.

At varying times, plants were harvested and shoot and root length, lateral root number, nodule number (where appropriate), and dry biomass were measured.

3.9. Nodules Squashes and Microscopy

Pea roots were coinoculated as described. Fourteen dpi or later, root nodules were surface-sterilized in 10% bleach for 10 min, washed with sterile water 10 times, and then squashed in 20 µL of sterile

water with a sterile glass rod. The nodule squashate was diluted 1:10, 1:100, and 1:1000. 20 µL of each dilution was spread on TY agar. The plates were incubated at 30 °C.

Roots and nodules were examined directly after staining with a Zeiss Axiophot Microscope. Nodules, either singly inoculated or coinoculated, were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) and embedded in methacrylate. Plastic-embedded material was sectioned for light microscopy at 3 or 4 µm, affixed to polylysine-coated slides, and stained with 1% aqueous acid fuchsin or as described in [51]. GUS-stained nodules were also embedded in methacrylate, sectioned as described, and counter-stained with safranin O.

3.10. Phosphate Solubilization Assay

Pikovskaya phosphate medium (PVK) was made according to [70]. For inoculation onto plates, the bacterial strains were grown in liquid medium until stationary phase at which time the cells were harvested by centrifugation (8000× *g*, 10 min), and the pellets washed with sterile water three times to remove any traces of the medium. The cell pellets were diluted to OD_{600} = 0.2 in sterile water. 5 µL droplets were spotted onto the plates and allowed to dry right side up for 20 min. The plates were incubated upside down at 30 °C for 10 days and the size of the clearing zone around the colony was measured. The averages and standard deviations were calculated for ca. 20 colonies of Rlv, *B. simplex*, and *B. subtilis*. The experiment was repeated three times.

3.11. Siderophore Assay

CAS agar medium devoid of nutrients was used as an indicator of siderophore presence. The components needed for a liter of the overlay medium were: 0.5 M MOPS buffer, 10 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 50 mL of Solution I (Chrome Azurol S (CAS) 0.065 g in 50 mL H_2O), 10 mL of Solution II (0.135 g $FeCl_3 \cdot 2H_2O$ in 500 mL H_2O), 40 mL of Solution III (0.0729 g CTAB in 40 mL H_2O) and 9 g Bacto-Agar [71]. 10 mL of the gel was spread as an overlay on culture plates of *B. simplex* on grown for 4 days on several solid media, including LB, TY, and BAP + N [58] medium, or over *B. simplex* and *B. subtilis* spotted onto TY plates. After a maximum period of 15 min, a color change in the blue medium was observed around the colonies. The experiment was repeated three times.

3.12. Analysis of Anti-Fungal Activity

B. simplex 30N-5 and the two *B. subtilis* strains were grown in liquid TY or LB. 1 mL of bacteria was transferred separately to autoclaved Eppendorf tubes and centrifuged at 14,000 rpm for 10 min at 22 °C. The supernatant was discarded and the pellet was re-suspended in sufficient sterile water to dilute the bacteria to a final cell density of OD_{600} = 0.3–0.4.

50 µL of the bacterial suspension or sterile water were inoculated onto PDA, V8, or ISP2 [57] agar plates and incubated at 30 °C. After 0 (*B. subtilis* 30VD-1) or 24 h (*B. simplex* 30N-5 and *B. subtilis* BAL218) of incubation at 30 °C, fungal plugs were placed 2.5 cm away from the site of bacterial inoculation. Measurements of fungal radial growth with a ruler were taken every 24 h using a dissecting microscope or a Quebec® Dark-Field Colony counter. The percentage of growth inhibition

was calculated using the equation: % growth inhibition = $F_C - F_{BS}/F_C$ where F_C = Fungus control and F_{BS} = Fungus inoculated with *B. simplex* or *B. subtilis* [72]. Photos were taken with Olympus digital camera FE-370. Graphs illustrating fungal radial growth were done in Excel and standard errors were calculated.

4. Conclusions

Taken together, the data presented herein strongly suggest that *B. simplex* 30N-5 is an effective PGPB on legumes and operates via a mechanism that involves fungal antagonism, phosphate solubilization, siderophore production, and auxin secretion. However, we cannot conclude whether the source of IAA triggering the expression of the *DR5::GUS* construct is exclusively from *B. simplex* 30N-5. The possibility exists that auxin synthesized by the plant is also involved. For example, although auxin-induced GUS staining was detected in Arabidopsis in response to *Phyllobacterium brassicacearum* inoculation, this PGPB strain produced very low levels of IAA and did not cause an increase in IAA content in plant roots [73]. By employing Arabidopsis lines mutated in IAA production, transport, or signaling, these authors determined that *P. brassicacearum* inoculation stimulated auxin production by the plant. Additional experiments will be needed to determine whether IAA synthesis is similarly up-regulated in legumes upon PGPB inoculation.

We also conclude that *B. subtilis* 30VD-1 has the potential of being an effective biocontrol agent against *Fusarium*, but additional tests on plants are required. A multi-species consortium of nodulating and growth-promoting bacteria is proposed as a better way to inoculate legume crops for growth improvement in poor soils.

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Conflicts of Interest

The authors declare no conflict of interest.

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Appendix

Figure A1. Colorimetric detection of auxin production from culture medium supplemented with L-tryptophan. The amounts indicated (µg/mL) of tryptophan were added to each culture. The bacteria were grown as described in the Materials and Methods, and the growth medium was collected for analysis. Each data point represents 7 independent samples. Auxin bound to iron is detected at OD_{535}, and that value is divided by the bacterial concentration detected at OD_{600}. The slope of the line was calculated by the equation: $Y = 0.0793X + 0.0478$, $R^2 = 0.9696$, and the standard deviations are indicated.

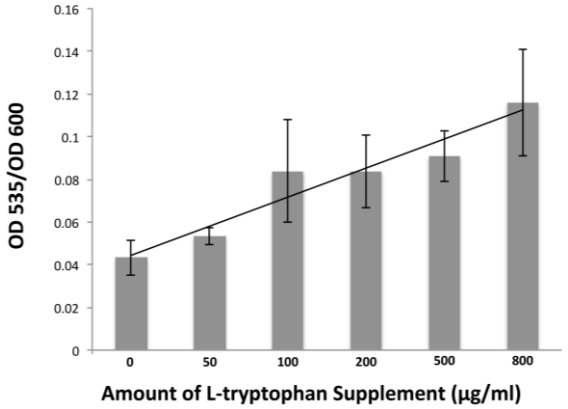

Figure A2. Iron-binding (**A**–**C**) and phosphate solubilization assays; (**A**) *B. simplex* grown on LB with Chrome Azurol S overlay; (**B**) Similar to **A**, but TY medium was used, the black arrows indicate *B. simplex* whereas the white arrows indicate *B. subtilis* BAL218, the halos indicate siderophore production; (**C**) CAS assay for *B. subtilis* 30VD-1; (**D**) *B. subtilis* 30VD-1 is able to degrade rock phosphate; The arrow delimits the edge of the phosphate-solubilization zone.

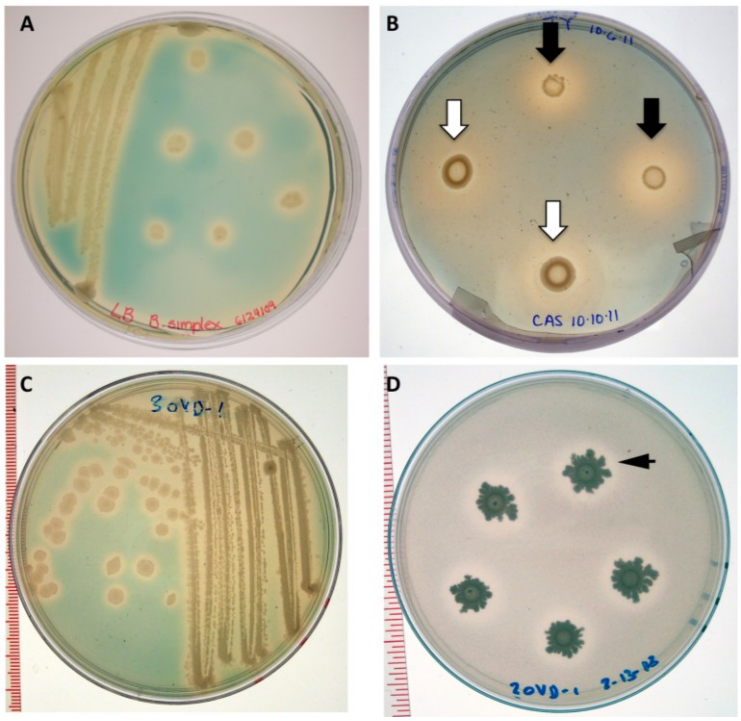

Table A1. PCR primers and conditions used in this study.

Gene	Sequence	PCR Conditions	Size (bp)	Reference
16S rRNA	5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3' 5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3'	5 min at 95 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1.5 min at 68 °C, and 10 min at 68 °C	832	[64]
nifH	5'-CTGVGCCTTGTTYTCGCGGGATSGGCATGGC-3' 5'-GGCTGCGATCCVAAGGCCGAYTCVACCCG-3'.	same as above	ca. 300	[42]
acdS	5'-TACAAAGCTTATGAACCTGCAACGATTCC-3' 5'-AATGGATCCTTAGCCGTTGCGGAAAAATG-3	same as above	1017	[49]