

Potential applications of *Bacillus subtilis* strain SR/B-16 for the control of phytopathogenic fungi in economically relevant crops

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REVIEW

ABSTRACT

Countries all over the world have experienced the negative impact that phytopathogenic fungi and oomycetes have on food security. Controlling these organisms remains a daunting task due to their genetic plasticity and the large temporal and geographic variability of their populations, which enables them to evolve and develop pesticide-resistant variants despite the considerable effort spent on developing disease-resistant varieties. One strategy for the control of plant diseases is that of biological control using natural enemies of these pests, such as rhizobacteria of the *Bacillus* and *Pseudomonas* genera. *Bacillus subtilis*, in particular, is characterized by the extracellular secretion of a number of antibiotics, microbial lipopeptides and hydrolytic enzymes such as chitinases and proteases that can be harnessed for the control of phytopathogens. The present review describes and examines the advantages and potential applications of *B. subtilis* strain SR/B-16, originally isolated from the rhizosphere of organically farmed ornamental plants, for the biological control of fungal phytopathogens attacking commercially important crops. *In vitro* challenging of phytopathogenic fungi with SR/B-16 has demonstrated that the antifungal activity of the latter has a broad spectrum, due to the secretion of metabolites producing structural and ultrastructural changes on the fungal cell. In addition, strain SR/B-16 efficiently colonizes the rhizosphere, which confers it advantages as a potential biopesticide and biofertilizer. Therefore, this microorganism may promote plant growth both by increasing the availability of nitrogen and phosphorous in agricultural soils and by controlling fungal phytopathogens.

Keywords: *Bacillus*, antifungals, morphological alterations, phytopathogenic fungi

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RESUMEN

Potencialidades de la cepa SR/B-16 de *Bacillus subtilis* para el control de enfermedades causadas por hongos en cultivos de interés agrícola. El impacto negativo de los hongos y oomycetes fitopatógenos es una amenaza importante para la seguridad alimentaria en varios países. El control de tales microorganismos se dificulta por su mutabilidad genotípica y espaciotemporal y su capacidad adaptativa, que les permite desarrollar variedades resistentes a plaguicidas. Las estrategias en ese sentido incluyen el control biológico con el empleo de microorganismos enemigos naturales, como las rizobacterias de los géneros *Bacillus* y *Pseudomonas*. La especie *Bacillus subtilis* se puede utilizar a través de la producción extracelular de antibióticos, lipopéptidos antimicrobianos y enzimas hidrolíticas, como las quitinasas y las proteasas. En este artículo se describen las potencialidades de la cepa autóctona *Bacillus subtilis* SR/B-16, aislada a partir de rizosfera de cultivos fertilizados con substrato orgánico, para el control de hongos fitopatógenos en cultivos de importancia económica. La interacción *in vitro* de la cepa SR/B-16 con estos microorganismos ha evidenciado su actividad antifúngica de amplio espectro, que se expresó mediante la excreción de metabolitos causantes de alteraciones en la estructura y la ultraestructura fúngica. La bacteria SR/B-16 posee propiedades que le permiten colonizar la rizosfera, por lo que se puede utilizar como bioplaguicida y también como biofertilizante. Este microorganismo puede contribuir al crecimiento de las plantas, por el aumento de la disponibilidad de nitrógeno y fósforo en los suelos agrícolas y el control de enfermedades fúngicas.

Palabras clave: *Bacillus*, antifúngicos, alteraciones morfológicas, hongos fitopatógenos

Introduction

Pests and diseases attacking economically relevant crops account for losses of approximately 10 % of the world's food production. About one half of these are caused by phytopathogenic fungi and oomycetes [1, 2].

The negative effect of these organisms on agriculture is not limited to increases in production costs deriving from the need to implement strategies for their control. It also includes post-harvest losses through their impact on the storage, marketing and sanitary

surveillance of crop foods [2] and of raw materials of plant origin used for the manufacture of foodstuffs, drugs and cosmetics, among other purposes.

Crop diseases are a problem not only in the context of commercial agriculture, but in the gardening industry as well, and pose an important obstacle to environmental protection programs [2]. The impact of phytopathogens is felt most strongly in developing countries, where alimentation often relies on the predominant consumption of a single dietary staple,

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and financial and material resources for phytosanitary surveillance and the control of phytopathogens are usually scarce [1].

The incidence in economically relevant crops of diseases caused by fungi and oomycetes exhibits an upward trend, and outbreaks and reinfections caused by these pests have flared in several regions around the planet [1, 3]. The control of phytopathogens, however, is not an easy task, due among other causes to the spatial, temporal and genotypic variation exhibited by the populations of these organisms and their constant change and evolution in response to the selective pressure exerted by the use of pest-resistance varieties [1].

Strategies for the control of these organisms include quarantines, the certification of seeds and plant material to be used for propagation, the implementation of appropriate culture practices, and the use of disease-resistant varieties together with chemical and biological control agents [2]. Biological control, in particular, is an environmentally friendly strategy for dealing with plant pathogens that is based on the directed application of their natural predators. One of its advantages is that it is not circumscribed to live plants, but can be extended to the post-harvest and storage stages. In addition, biocontrol agents are biodegradable, unlike most agrochemicals currently in use [3].

Research on the development of bioproducts for phytopathogen control usually takes into account a number of issues, including the ecological preservation of plant-microorganism interactions, strategies for the application of inoculants, the isolation of new strains and the dissection of novel mechanisms of action. Emphasis is also made on the use of biocontrol agents as part of integrated, multidisciplinary programs for the fight against plant diseases and the preservation and management of soil quality [4].

Studies on bacterial organisms for the biological control of plant diseases and the stimulation of plant growth have focused mainly on rhizospheric species such as those of the *Pseudomonas* and *Bacillus* genera. While published data on members of the *Pseudomonas* abound, much less is known about the interactions of plants with members of *Bacillus* spp. and related genera, as well as their relevance for pest control [4].

The potential application of *Bacillus* spp. for the biological control of phytopathogens

Aerobic spore-forming bacteria of the *Bacilli* class (*Bacillus* spp. and related genera) play a direct role in resistance to phytopathogenic organisms through the production of extracellular antimicrobial antibiotics, toxins, hydrolases and lipopeptides [5, 6]. Bacterial lipopeptides, in particular, are not only effective against a broad range of fungal, bacterial and viral species, but are known to act as effector molecules activating the mechanisms of induced resistance in their plant host [7].

Recent studies on the potential use of members of the *Bacilli* class against phytopathogenic fungi have included the isolation of *Bacillus* sp. strains secreting antifungal lipopeptides, chitinases and proteases, including representatives from *Bacillus amyloliquefaciens* and *B. subtilis* [8-13] as well as from undefined

species of said genus [14]. Other genera of rhizospheric bacteria, of which *Pseudomonas* and *Burkholderia* are the main representatives, also synthesize compounds exhibiting a wide antimicrobial spectrum, such as pyrrolnitrin, phenazine and pyoluteorin, although the efficacy of the latter class of compounds in agricultural ecosystems has not been conclusively proved due to the many biotic and abiotic factors that modulate antibiotic production in natural conditions [6].

Proteases, chitinases and antimicrobial lipopeptides are among the metabolites responsible for the antifungal and antibacterial activity of *B. subtilis* strains. For instance, *B. subtilis* strain 21, an isolate from strawberry rhizosphere shown to be effective for the control of phytopathogenic fungi in economically relevant crops and pathogenic bacteria responsible for food poisoning, is known to secrete such types of compounds [10].

Many *B. subtilis* and *B. amyloliquefaciens* strains that exhibit a strong antifungal activity owe their properties to the non-ribosomal production of high amounts of chemically homogeneous iturins, surfactins and fengycins. One example is the HC8 endophytic isolate of *B. subtilis*, which inhibits fungal growth and produces morphological deformities in hyphae grown from spores that have been pretreated with the metabolites excreted by this bacterium [13].

Isolate C9 of *B. subtilis* subsp. *subtilis* has also been shown to synthesize volatile compounds inhibiting mycelial growth and sporulation in phytopathogenic fungi, one of which is an acetylbutanediol stereoisomer that activates plant defense mechanisms. This compound binds the DNA molecule, inhibiting transcription and protein synthesis in fungi and affecting spore germination and the biosynthesis of components of the fungal cell wall [12].

The first commercially available biopesticides prepared from strains of *B. subtilis*, branded as Quantum®, Kodiak® and Epic®, appeared in the US market in 1985. Their success in the control of soil-dwelling phytopathogenic microorganisms laid the foundation for extending the application of *Bacillus*-based biopreparations to commercially important crops [15].

Currently, the US remains the market leader in the production of biopesticides based on rhizospheric bacteria, including species of the *Bacillus* genus. Most formulations are produced from *Bacillus pumilus* (QST 2808 Sonata™ and GB34 Yield Shield®) or *B. subtilis* (GBO3 Kodiak®) [15, 16]. A total of 18 bioproducts produced from *Bacillus* spp. were registered during 2012 in China [6], and the European Community has implemented a strategic plan to increase the number of available microbial pesticides for agricultural use in that market [17]. Strain FZB42 of *B. amyloliquefaciens*, marketed as inoculant by Bayer CropScience and Abitep GmbH Berlin, has been shown to be highly beneficial for a number of potato varieties from diverse regions, providing protection against pests such as potato's stem canker and black scurf, among others [17, 18].

The development of inoculants from aerobic spore-forming bacteria has pushed forward research on the biodiversity, distribution and physiology of this microbial group. Selecting new strains as candidates for

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the formulation of novel biopesticides demands a thorough knowledge of the factors ensuring a successful colonization of the rhizosphere, and the implementation of efficient methodologies to evaluate the effects of the interactions these biopesticides establish not only with phytopathogenic microorganisms, but with beneficial members of the local microflora. Another issue to be taken into account is the contribution of candidate biopesticides to the induction of disease resistance mechanisms in the target crop [19].

The multifactorial nature of the mechanisms whereby plant-associated bacteria stimulate plant health is one of the difficulties associated with current research on the biological control of phytopathogenic agents, and despite growing awareness of the need for an integrated, multidisciplinary approach to this field of study, many research groups have remained focused on a single biocontrol mechanism. Although this state of affairs has yielded a large number of publications describing microbial isolates with antagonistic *in vitro* and *in vivo* activities, the metabolites responsible for these activities and even the relevant mechanisms through which they counteract specific phytopathogens [20], it has failed to produce sufficient data on the efficacy of these bio-preparations under field conditions.

***Bacillus subtilis* SR/B-16 as a potential agent for the biological control of phytopathogenic fungi**

Bacillus subtilis SR/B-16 is an autochthonous strain from the microbiota of Cuban soils that was isolated from rhizospheric samples of ornamental plants, cultured in an organic substrate of compost and livestock manure supplemented with urea [21]. Research on SR/B-16 was first addressed at its taxonomic identification by means of ribosomal 16S rRNA sequencing, and revealed an identity of 99 % between the resulting partial sequence (GenBank accession number HQ025917) and that of reference isolate B23052 of *B. subtilis* subsp. *inaquosorum* [22].

Further studies aimed at dissecting whether SR/B-16 could be used as a biological control agent demonstrated that this strain exhibited *in vitro* inhibitory activity for the growth of phytopathogenic fungi of the species *Curvularia lunata*, *Curvularia gudauskasii*, *Fusarium oxysporum* and *Fusarium solani* as well as members of the *Colletotrichum* genus, isolated from ornamental plants and sugar cane seed banks. These results suggested that the metabolites from this antagonistic bacterium might have a broad antifungal spectrum [21, 23].

Attempts to elucidate the biocontrol mechanisms of *B. subtilis* SR/B-16 have been performed *ex situ*, as is also true of most research on microbiological control agents against phytopathogens [24]. *In vitro* challenges of phytopathogenic fungi with this bacterium demonstrated that SR/B-16 and its extracellular metabolites produce growth-inhibiting alterations in the morphology and structure of *C. gudauskasii* [23]. Ultrastructural studies of the hyphae of this pathogen in the presence of SR/B-16 evidenced changes in the width and regeneration of its cell walls, hyphal constrictions in the region of the transversal septum and the induction of secondary branching in the fungal cell.

The periodical swelling, torsion and formation of bulbs in hyphae from *C. gudauskasii* was causally linked to the excretion by SR/B-16 of antimicrobial lipopeptides of the iturin and fengycin families [24], which have previously been shown to be present in *B. subtilis* strains with antifungal activity [25]. Bacterial lipopeptides bind to actin filaments in the cytoskeleton of the target cell, producing changes in the apical growth pattern of the hyphae that ultimately result in hyphal swelling and the inhibition of fungal growth [26]. It must be stressed that the apical elongation patterns of fungal hyphae play an important role in the pathogenicity of endophytic fungi attacking plant tissues [27], representing therefore a potential target for fungal inhibition strategies.

The hyphae of *Curvularia* and *Fusarium* interacting with SR/B-16 also exhibited intense vacuolization, evidencing the presence of antifungal compounds of bacterial origin in their cytoplasm. It has been shown that vacuoles play an active role in the intracellular degradation of foreign compounds in the cytoplasm of eukaryotic cells [28]. The observed variations in the thickness and regeneration of the fungal cell wall have been interpreted as alternative growth patterns developed by the target fungi in the presence of SR/B-16. Together, these changes evidence that the pathogenicity of surviving fungi increases as part of their response to the biotic stress represented by their interaction with antagonistic bacteria and the metabolites they secrete [24]. A similar phenomenon was described for phytopathogenic strains of *F. oxysporum* and *Botrytis cinerea* when challenged with antagonistic isolates of *Pseudomonas* spp. [29]. However, it should be stressed that not every pathogenic fungus sits idly waiting to be “victimized” by a biocontrol agent, as many fungi develop counter measures conferring resistance to the antagonistic action of antagonistic bacteria, including the inactivation of inhibiting metabolites and the modifications of the structures serving as the target for these bacterial toxins [30]. Taking into account that antimicrobial peptides can easily cross the fungal cell wall thanks to their relatively low molecular weight [25], the thickening of cell walls noticed in *Curvularia gudauskasii* when interacting with SR/B-16 might represent a strategy of structural modification to create a physical barrier limiting the entry of lipopeptides into the hyphal cytoplasm.

An important element when evaluating the efficacy of biological control agents is their specificity [31]. The fungal growth inhibition mechanisms exhibited by SR/B-16 seem to be unspecific, as they target structures shared among all filamentous fungi and eukaryotic cells such as the cytoplasmic membrane, the cytoskeleton and the secretory apparatus [28]. Not surprisingly then, *B. subtilis* SR/B-16 has a wide antifungal spectrum that includes diseases caused by members of the *Fusarium* genus, such as *F. oxysporum*, whose main pathogenicity factor consists on the presence of a taxonomic category within the species, denominated *formae speciale* (f. sp.) [24]. *Formae speciale* are specific to each plant host, thereby providing these organisms with a huge potential for ecophysiological variability that limits considerably any attempts at chemical or biological control [32, 33].

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The target spectra of biocontrol agents with broad host specificities can cover even entire orders, classes and even kingdoms [31]. In the case of SR/B-16, its antifungal activity *in vitro* encompassed several genera (*Fusarium*, *Curvularia* and *Colletotrichum*) and species of the fungal kingdom [34] that cause plant diseases among members of the *Rosidae* [35], *Asteraceae* [36], *Agavaceae* [37] and *Poaceae* [38] families. *B. subtilis* SR/B-16 can, therefore, be classified as a generalist species with a broad specificity for plant pathogens, thus representing an excellent candidate for the formulation of a bioinoculant based on its efficacy, ease of production at industrial scale and market appeal. Generalist microorganisms usually employ many different sources of nutrients and can easily switch their target host [31]. The presence of broad-spectrum antifungal activity in rhizospheric strains of *B. subtilis* has been described only recently [39, 40].

The main obstacle for determining target pathogen specificities in the case of biocontrol agents is the fact that most research on this topic has employed *in vitro* experiments, thus obviating two fundamental elements of the agricultural ecosystem: environmental conditions and the host plant. Many authors have acknowledged that the target pathogen specificities of microbial control agents under field conditions can be very different from those observed *in vitro* [31].

It is not uncommon to find variability in the target pathogen specificity of biocontrol agents, even within the same species [31]. Therefore, strong preference is given to the *in situ* selection of autochthonous strains in direct interaction with their intended targets [40] in order to maximize the efficacy of the isolated strains. Such is the case of strain SR/B-16. This bacterium can eliminate pathogenic fungi by both direct competition for nutrients in the same ecological niche and the excretion of antifungal metabolites [23]. Its ability to form endospores, which confers this strain the capacity to survive adverse environmental conditions, enables SR/B-16 to tolerate edaphoclimatic variation and even persist at low population densities [41].

Thanks to the broad specificity for target pathogens exhibited by SR/B-16 during *in vitro* studies, the commercial appeal of this candidate biopesticide equals or surpasses that of equivalent broad-range chemical formulations. From an industrial viewpoint, the production of a bioinoculant from this bacterium is more cost-effective, and the ease of application of such a product makes it appealing to the farmers [31], who can effortlessly integrate it into existing pest control programs for commercial crops.

The interactions established between different microorganisms in a complex environment such as that of the plant rhizosphere are decisive for the success of bioinoculants in agricultural ecosystems [42]. Biological control agents are exposed to competition and antagonism from the endogenous microbiota, which can dramatically diminish their population densities and affect the physiological activity of the inoculant [43]. In turn, the introduction of exogenous microorganisms to agricultural soils may damage their ecological equilibrium, affecting local microbial populations that are actually beneficial to crop production. *In vitro*, *Bacillus subtilis* SR/B-16 inhibits over 60 % of

the growth of many different phytopathogenic fungi, and exhibits an antagonist effect to Gram-negative bacteria [22]. In addition, excessive amounts of even a properly chosen inoculant may favor disproportionately the growth of this exogenous population, which competes with the endogenous microbiota for nutrients, oxygen and physical space [44]. When combined with the broad inhibitory spectrum of SR/B-16, such a combination of circumstances may damage the agricultural ecosystem. This disadvantage is minimized, however, if this bacterium is applied to crops cultured in artificial, low-organic-matter substrates poor in endogenous microflora, such as hydroponics.

B. subtilis SR/B-16 can also be employed for the biological control of oomycetes, which cause a number of very common and also emerging plant diseases constituting a serious threat to food security in several countries [1]. This is a very important finding, as the antifungal activity of many other well-known strains is based on the secretion of chitinases, making them totally ineffective against oomycetes due to the lack of chitin in the cell walls of the latter. The structures targeted by SR/B-16 and its antifungal metabolites reside within the fungal cell, conferring this bacterium a significant advantage for the treatment of diseases caused by members of the *Phytophthora* and *Pythium* genera in some very important crops, such as corn and potato [41, 42]. Previous studies have demonstrated that lipopeptides produced by the endophytic fungus *Acremonium* spp. have an antagonistic effects on *Pythium ultimum* [44], and a strain of the tobacco pathogen *Phytophthora nicotianae* was recently shown to be sensitive *in vitro* to bacteria of the species *Bacillus altitudinis*, *Bacillus licheniformis* and *Brevibacillus* spp. [45], all phylogenetically close to *B. subtilis* [46].

The data available so far indicate that *B. subtilis* SR/B-16 is an excellent candidate agent for the biological control of pests affecting commercially relevant crops, as it interferes with the elements of the disease triangle. This last is a simple model describing the interactions between pathogen, host and the environment [41]. Plant growth-promoting rhizobacteria may influence any of these three elements, thereby modulating the course of an infectious disease due to their multiple effects.

As explained above, strain SR/B-16 directly inhibits the growth of phytopathogenic fungi through the excretion of antifungal compounds, while concurrently favoring the development of the host by increasing the availability of nitrogen and phosphorous in the soil through the degradation of urea [20] and the solubilization of calcium phosphate [24]. These properties confer this bacterium an advantage in the biological control of diseases caused by opportunistic phytopathogens that are associated with nutritional deficiencies. One example is the fungus *Cercospora* spp., which attacks adult coffee plants under conditions of nitrogen limitation [41], and whose effects can be eliminated by increasing the ureolytic activity of several bacterial species of the rhizosphere, similar to SR/B-16 [20].

B. subtilis SR/B-16 also secretes lytic enzymes (cellulases and pectinases) [24] enabling this bacterium to obtain nutrients from decaying plant matter in the soil, which it uses as a source of carbon and

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energy [5]. In soils rich in organic matter, such as the artificial ecosystems created in organoponic units, the application of SR/B-16 formulations may stimulate the growth of its populations in the rhizosphere, as well as its antagonist effects, contributing to disease control and plant growth promotion.

Directions for future research on *B. subtilis* SR/B-16

Three main questions concerning the physiology of *B. subtilis* SR/B-16 remain to be addressed: 1) whether one of the mechanisms through which it exerts its biological control over phytopathogens is the stimulation of mechanisms of induced resistance in the host plant; 2) a thorough characterization of its capacity for colonizing the rhizosphere and the endophytic environment of commercial crops (rhizocompetence) and 3) the efficacy of this bacterium in the biological control of plant diseases under field conditions, where SR/B-16 establishes complex relationships to other microbial populations inhabiting the rhizosphere and many other plant species.

Bacterial lipopeptides have previously been shown to activate mechanisms of induced resistance in plants [5] and, as mentioned above, one of the possible modes of action explaining the *in vitro* effect of SR/B-16 on phytopathogenic fungi is indeed the excretion of this type of compounds. The morphophysiology of SR/B-16 enables it to colonize the rhizosphere: it is shaped as a bacillus, is motile, and forms biofilms when cultured on nutritive media [24]. These characteristics confer SR/B-16 a larger metabolic rate and growth speed, facilitating chemoattraction in the rhizospheric environment and aggregation into more complex biofilms. Motility, in particular, is a physiological attribute that enhances the competitiveness of *Pseudomonas* spp. in rhizospheric biofilms [47]. Biofilm formation is a fundamental requirement for bacterial colonization in the rhizosphere, as it increases the

concentration of antimicrobial metabolites excreted by member bacteria, forming a physical and chemical barrier to the entry of pathogens into root tissues [48].

The studies on SR/B-16 as a biological control agent for fungal plant diseases are not circumscribed to providing data on the *in vitro* interactions of this bacterium with phytopathogenic fungi [20, 22], but also illustrate how pest control depends on the simultaneous interaction of different biotic and abiotic elements in the environment [23]. Using *B. subtilis* SR/B-16 and its extracellular products for the development of bioinoculants requires more experimental data to properly assess its practical benefits in the biological control of fungal crop diseases. In addition, a large scale process for producing SR/B-16-based inoculants with a consistent and dependable effect under field conditions is yet to be developed, not to mention that the selection of the adequate microorganism and the optimization of its culture conditions must take into account the physical media to be used for their storage and release [49]. It must be noticed, nevertheless, that SR/B-16 is an endospore-forming organism, which confers it a considerable advantage for the formulation, storage, preservation and application of biopesticides manufactured from this bacterium.

The USA alone spends over 5000 million dollars each year on fungicidal compounds for corn, soy, wheat, potato, coffee and rice [50], and the expenditure on seeds and biopesticides has doubled in the last two years [51]. These facts illustrate the need to develop plant growth promotion strategies that rely not on one, but several mechanisms, as done by members of the *Bacillus* spp. genus [52, 53]. The potential advantages of *B. subtilis* SR/B-16 make it, therefore, a prime candidate for integration into prioritized actions for the careful design of strategies for increasing crop yields in a sustainable manner while decreasing agricultural production costs and gradually eliminating the use of chemical pesticides [51].

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