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Isolation of *Bacillus* strains from the rhizosphere of cereals and *in vitro* screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms

T. Földes, I. Bánhegyi, Z. Herpai, L. Varga and J. Szigeti

Institute of Food Science, Faculty of Agricultural Sciences at Mosonmagyaróvár, University of West Hungary, Hungary

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T. FÖLDES, I. BÁNHEGYI, Z. HERPAI, L. VARGA AND J. SZIGETI. 2000. *Bacillus* strains were isolated from the rhizosphere of cereals in order to be used as natural biocontrol agents (BCAs). They were screened for antagonism *in vitro* against various test micro-organisms. The isolates showing antagonism were identified to species level. A combination of techniques was employed for the isolation of *Bacillus* species. Using the direct method, only one of the 25 isolates screened showed antagonistic properties. This strain (IFS-01) was identified by means of API test strips and the ATB Plus computer programme. It proved to be *Bacillus subtilis* and consequently has been designated as *Bacillus subtilis* IFS-01. This strain produced either a broad spectrum antimicrobial compound or several compounds with different activities. The fungi and Gram-positive bacteria were more sensitive to the antagonistic isolate than the Gram-negative bacteria. A *Bacillus* strain producing BCAs which can be used as biopesticides or organic preservatives has been isolated and identified.

INTRODUCTION

In intensive agricultural production systems, protection against biological factors which adversely influence the efficiency of cultivation, and the microbiological quality of crops as raw materials, is of great significance. Owing to the emergence of sustainable agriculture, the protective methods available have been reassessed in the past decade. The importance of using environment-friendly and food-hygienically-safe plant-protecting methods, and plant-protecting agents of biological origin, has been greatly emphasized.

Several members of the genus *Bacillus*, including *Brevibacillus and Paenibacillus* species, produce various antimicrobial substances, e.g. antibiotics. Being capable of producing more than 70 different antibiotics, *B. subtilis* is one of the major producers of these substances in the genus. *Brevibacillus* (formerly known as *Bacillus*) *brevis* is also characterized by its capability to produce antibiotics. In addition, a wide range of antimicrobial substances are produced by *B. licheniformis*, *B. pumilus*, *B. circulans*, *B. cereus*, *Brevibacillus laterosporus*, *Paenibacillus* (formerly *Bacillus*) *polymyxa* and other species (Katz and Demain 1977; Shoji 1978; Smirnov *et al*. 1986).

Correspondence to: Mr Tamás Földes, Institute of Food Science, Faculty of Agricultural Sciences at Mosonmagyaróvár, University of West Hungary, H-9200 Mosonmagyaróvár, Lucsony u. 15–17., Hungary (e-mail: foldest@yahoo.com).

The secondary metabolites produced by certain species and strains of the genus *Bacillus* have been found to show antibacterial and/or antifungal activity against phytopathogenic and food-borne pathogenic micro-organisms (Katz and Demain 1977; Shoji 1978; Smirnov *et al*. 1986). Some authors have suggested that the use of antimicrobially-active species and strains of the genus *Bacillus*, or use of their metabolites, may be an alternative or supplementary method to chemical plant protection (Swinburne *et al*. 1975; Pusey and Wilson 1984; McKeen *et al*. 1986; Utkhede and Sholberg 1986; Fravel 1988; Gueldner *et al*. 1988; Handelsman *et al*. 1990; Klich *et al*. 1994; Potera 1994; Leifert *et al*. 1995; Berger *et al*. 1996; Sharga and Lyon 1998). Many of these bacilli are generally soil-inhabiting bacteria or exist as epiphytes and endophytes in the spermosphere (Walker *et al*. 1998) and rhizosphere (McKeen *et al*. 1986; Handelsman *et al*. 1990; Kajimura *et al*. 1995). For this reason, *Bacillus* species are ideal candidates for use as biocontrol agents (BCAs) in seed treatment programmes against soil-borne pathogens (Walker *et al*. 1998). *Bacillus* species have also been isolated from many other environments (Sneath 1986) such as apple leaf scars (Swinburne *et al*. 1975), *Brassica* leaves (Leifert *et al*. 1992), composts (Phae *et al*. 1990), alcoholic fermentation of sugar cane (Azevedo *et al*. 1993) and dried Ndagala fish (Munimbazi and Bullerman 1998).

The aims of this study were (i) to isolate *Bacillus* species from the rhizosphere of cereals which had been sown in

fallow land and showed above average development; (ii) to screen these isolates *in vitro* for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms; and (iii) to develop a novel BCA based on the use of certain antimicrobially-active *Bacillus* strains or their metabolites.

MATERIALS AND METHODS

Test organisms

In this study, eight bacteria including two Gram-positives and six Gram-negatives, 14 filamentous fungi and three yeasts were used to determine the antimicrobial activity of the isolated bacterial strains. The phytopathogenic bacteria tested were: *Erwinia carotovora* Jones (NCAIM B.01109; ATCC 15713), *Pseudomonas syringae* pv. *syringae* van Hall (NCAIM B.01398; ATCC 19310) and *Xanthomonas campestris* pv. *campestris* Pammel (NCAIM B.01071). The food-borne bacteria tested were: *Escherichia coli* Castellani and Chalmers (HNCMB 35035), *Listeria monocytogenes* Pirie (NCAIM B.01373; ATCC 19114), *Salmonella arizonae* (III) Kauffmann (HNCMB 42021), *Salmonella typhimurium* (HNCMB 10040) and *Staphylococcus aureus* Rosenbach (HNCMB 112002). The phytopathogenic filamentous fungi tested were: *Alternaria alternata* Keissler (NCAIM F.00750), *Botrytis allii* Munn (NCAIM F.00659), *Botrytis cinerea* Persoon (NCAIM F.00744), *Colletotrichum lindemuthianum* (Saccardo and Magnus) Briosi and Cavara (NCAIM F.00756), *Drechslera sorokiniana* (Saccardo) Subramanien and Jain (*Helminthosporium sativum*; NCAIM F.00745) and *Fusarium oxysporum* Schlechtendal (NCAIM F.00728). The spoilage filamentous fungi tested were: *Aspergillus niger* van Tieghem (NCAIM F.00071), *Aspergillus wentii* Wehmer (NCAIM F.00167), *Byssochlamys fulva* Olliver and G. Smith (NCAIM F.00788; DSM 5035), *Geotrichum candidum* Link (isolated from raw milk), *Penicillium chrysogenum* Thom (NCAIM F.00680), *Penicillium expansum* Link (NCAIM F.00601), *Rhizopus stolonifer* Lind (NCAIM F.00654) and *Trichothecium roseum* Link (NCAIM F.00647). The spoilage yeasts tested were: *Rhodotorula mucilaginosa* Harrison (*R. rubra* Lodder; NCAIM Y.00488), *Yarrowia* (*Candida*) *lypolitica* Wickerham *et al.* (NCAIM Y.00062) and *Zygosaccharomyces bailii* Guilliermond (NCAIM Y.00734). All these micro-organisms were purchased from the National Collection of Agricultural and Industrial Micro-organisms (NCAIM), Budapest, Hungary and the Hungarian National Collection of Medical Bacteria (HNCMB), Budapest, Hungary.

Maintenance

The thermophilic and mesophilic bacteria were maintained on slant tubes of Casein-peptone Soymeal-peptone Agar (CASO Agar), being incubated at 37 °C for 24 h and at 26 °C for 72 h, respectively. The filamentous fungi were maintained by cultivation on slant tubes of Potato Dextrose Agar (PDA) at 26 °C for at least 4–7 days, as was required for sufficient growth of conidia. The yeasts were maintained on slant tubes of Glucose-Yeast Extract-Peptone Agar (GYP Agar), being incubated at 26 °C for 72 h. The GYP Agar contained 1% D(+)-glucose, 1% yeast extract, 1% universal peptone and 2% agar (Oxoid). The pH of all culture media was adjusted to 7·0 with 1 mol l^{-1} NaOH or HCl. After incubation, the tubes were sealed with parafilm and refrigerated at 4 °C. The bacteria and yeasts were subcultured on freshly prepared slant tubes of the appropriate media (CASO Agar and GYP Agar, respectively) at monthly intervals. The filamentous fungi were subcultured on fresh slant tubes of PDA at 2-monthly intervals. The culture medium bases and chemicals, unless stated otherwise, were purchased from Merck.

Preparation of inocula

The bacterial inocula used for screening *Bacillus* isolates for antibacterial activity were prepared by suspending bacterial growth from freshly cultivated cultures in 2 ml NaCl 0·85% Medium (bioMérieux). The density of bacterial suspensions was adjusted to 0·5 McFarland unit (equivalent to $1{\cdot}5 \times 10^8$ cells ml^{-1}) by means of a calibrated densitometer (Densimat; bioMérieux, Marcy-l'Etoile, France). This suspension was used for direct inoculation of CASO Agar. The inocula of yeasts were prepared similarly to those of bacteria, but the density was adjusted to 1·0 McFarland unit, corresponding to $1{\cdot}0 \times 10^7$ cells ml^{-1}. A 1 ml aliquot of this suspension was added to 100 ml quarter-strength Ringer's solution and mixed thoroughly. This dilution was used for direct inoculation of GYP Agar. The inocula of filamentous fungi used for screening *Bacillus* isolates for antifungal activity were made from freshly prepared cultures by harvesting conidia according to the method of Leifert *et al.* (1995). The density of conidial suspensions was adjusted to approximately $1{\cdot}0 \times 10^5$ conidia ml^{-1}. This suspension was used for direct inoculation of PDA medium. The density was lower than usual in order to meet the requirements of the method.

Source of potentially antagonistic bacteria

Fifty-five soil samples thought to be a good source of antimicrobially-active *Bacillus* species were taken from the rhizosphere of 11 cereal-growing areas in the Small Plain of

Hungary (Kisalföld). The samples were taken from fields where: (i) cereals had been sown in fallow land; (ii) cereal plants were in their vegetative phase of growth; (iii) fungicides had not been used; and (iv) cereals showed above average development. All the samples were cooled to 4 °C so that any change in the original microflora would be prevented. The weight of individual samples was approximately 100 g.

Isolation and preliminary characterization of presumptive antagonistic bacteria

Isolation. The methods used for isolating various *Bacillus* strains were based mainly on the resistance of their endospores to elevated temperatures. However, bacterial spores in natural environments may have different heat sensitivities and heat activation requirements for optimal growth from those which have been cultured. In addition, the cells of certain strains may be in an exclusively vegetative state. Consequently, it was mostly not feasible to enumerate exactly the real count of *Bacillus* species present in a sample from nature (Sneath 1986).

In this study, a combination of techniques was used to increase the number of *Bacillus* species to be isolated from the samples. The samples were blended with a special enrichment medium, which also induced vegetative cells to sporulate, and were then incubated to allow formation of mature spores in large quantities. The isolation procedures involved heat treatment for the selection of spores of *Bacillus* species. The samples were subjected to the following isolation steps.

(1) Blending. Samples (20 g) were measured into sterile bags. Finley and Fields Medium without agar (180 g; Finley and Fields 1962) was poured into each bag. The mixtures were blended in a laboratory blender (Stomacher 400; Seward Medical, London, UK) at normal speed for 60 s. A 100 ml aliquot of this mixture was poured into a sterile 300 ml Erlenmeyer flask, and used for regular procedures (i.e., incubation, heat treatment etc.). The remaining 100 ml of each mixture were saved by deep-freezing and storage at −76 °C for later repetition.
(2) Incubation. The mixtures of sample and special medium were incubated at 30 °C and 150 rev min^{-1} for 5 days in a water-bath shaker (Gyrotory® Model G76D; New Brunswick Scientific, Edison, NJ, USA).
(3) Heat treatment. Test-tubes containing 1 ml of mixture were placed in a water-bath at 80 °C for 10 min so that endospores would be separated from vegetative cells (Walker *et al*. 1998). A decimal dilution series was prepared using Ringer's solution, and the dilutions selected were spread on the surface of CASO Agar plates. The streak plates were incubated at 30 °C for 96 h. The distinct single colonies were subcultured onto CASO Agar plates for preliminary characterization and testing for antagonism.

Preliminary characterization. The subcultured bacterial isolates were preliminarily characterized by means of conventional methods (Sneath 1986). The tests characterizing the genus *Bacillus* within endospore-forming rods were carried out in triplicate. These included: microscopic appearance, Gram stain, motility, growth under strictly anaerobic conditions, catalase test, oxidase test and reduction of nitrate to nitrite.

In vitro screening of isolates for antagonism

The bacterial isolates characterized as members of the genus *Bacillus* were screened for antagonism *in vitro* against the phytopathogenic, food-borne pathogenic and spoilage micro-organisms listed above, using a direct method described by Besson *et al*. (1978) with some modifications. Liquid CASO Agar, GYP Agar and PDA (20 ml) were cooled to 45 °C and inoculated with 0·5 ml bacterial, yeast and fungal dilutions or suspensions, respectively. The culture media thus seeded were poured into Petri dishes, mixed thoroughly and set in a horizontal position. After the agar had solidified, each seeded medium was inoculated with the selected isolate by a single streak of inoculum being produced in the centre of the dish, using an inoculating loop. The plates seeded with fungi, yeasts or phytopathogenic bacteria were incubated first at 37 °C for 48 h and then at 26 °C for 72 h, whereas the plates seeded with food-borne or spoilage bacteria were incubated first at 28 °C for 72 h and then at 37 °C for 24 h. The microbial interactions were analysed by the determination of the size and shape of the inhibition zone. If negative (i.e., no) results were produced at this time, the incubation of plates continued for three more days at 30 °C and analysis was carried out again then. The experiment was conducted in triplicate. Control plates, which were not inoculated with bacterial isolates, were also prepared.

Selection and identification of antagonistic bacteria down to species level

The isolates characterized preliminarily as *Bacillus* species and showing clear antagonism *in vitro* against some of the phytopathogenic, food-borne pathogenic and/or spoilage micro-organisms tested were selected and further identified on the basis of morphological, cultural and physiological characteristics and API test results.

Morphological characterization. An Eclipse E600 (NIKON Corporation, Tokyo, Japan) microscope was used for mor-

phological characterization. For microscopic examination, the antagonistic bacteria were prepared according to the method of Logan and Berkeley (1984).

Cultural characterization and physiological tests.. The methods of Logan and Berkeley (1984) and Sneath (1986) were used for cultural and physiological tests, i.e., observation of growth on various substrates and tests for physiological characteristics, during incubation at 10–55 °C for 7 days, unless otherwise stated.

API tests.. The selected antagonistic bacteria were further identified by means of API 20 E and API 50 CHB (V2.1) test strips (bioMérieux). This test system was used in conjunction with the ATB Plus (bioMérieux) computer program (V2.4.7) to identify isolates to species level. The API tests were carried out according to the instructions in the user's manual and those of Logan and Berkeley (1984). The identification results are expressed in terms of a probability percentage and *T*-value. Atypical test results and comments on the level of identification are also presented.

RESULTS

Isolation and preliminary characterization of presumptive antagonistic bacteria

Using the combination of techniques for the isolation procedures described above, 28 distinct colonies were subcultured on CASO Agar on the basis of colony morphology. Twenty-five of these isolates proved to be Gram-positive, endospore-forming, motile, catalase-positive and mostly oxidase-positive rods. Moreover, they did not grow under strictly anaerobic conditions but were capable of reducing nitrate to nitrite; they were thus preliminarily characterized as members of the genus *Bacillus*. The rest of the isolates could not be identified to genus level at this stage of characterization.

In vitro screening of isolates for antagonism

Of the 25 *Bacillus* isolates screened for antagonism *in vitro*, only one produced any zone of inhibition against some of the phytopathogenic, food-borne pathogenic and/or spoilage micro-organisms tested. The antagonistic strain has been named *Bacillus* sp. IFS-01. The results of inhibition tests are presented in Table 1. The strain IFS-01 showed clear antagonism against several filamentous fungi and yeasts. As a result, no visible growth of these micro-organisms was observed within the zone of inhibition. The growth of *Erw. carotovora*, *Ps. syr. syringae*, *X. camp. campestris*, *E. coli* and *Salm. arizonae* was inhibited slightly. Inhibition was ambiguous at first but was becoming more and more pronounced as incubation continued. The growth of *L. monocytogenes* and *Staph. aureus* was inhibited to a high degree. *Salmonella typhimurium*, *G. candidum* and *Rhizopus stolonifer* were the most resistant species to the inhibitory effect of *Bacillus* sp. IFS-01. In the case of *P. chrysogenum*, a second zone with inhibited mycelial growth and malproduction of conidia around the clear zone of inhibition was observed (Fig. 1). This phenomenon suggests that either more than one substance is produced by strain IFS-01, or that the mode of action of the substance depends on its concentration. These alternative explanations need to be further analysed.

Selection and identification of antagonistic bacteria down to species level

On the basis of the results obtained during preliminary characterization and *in vitro* screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms, the isolate IFS-01 was selected for further identification.

The morphological and physiological characteristics of the selected strain are listed in Table 2. The solitary colonies of strain IFS-01 on CASO Agar after 3 days of incubation were opaque, dull, circular with an irregular

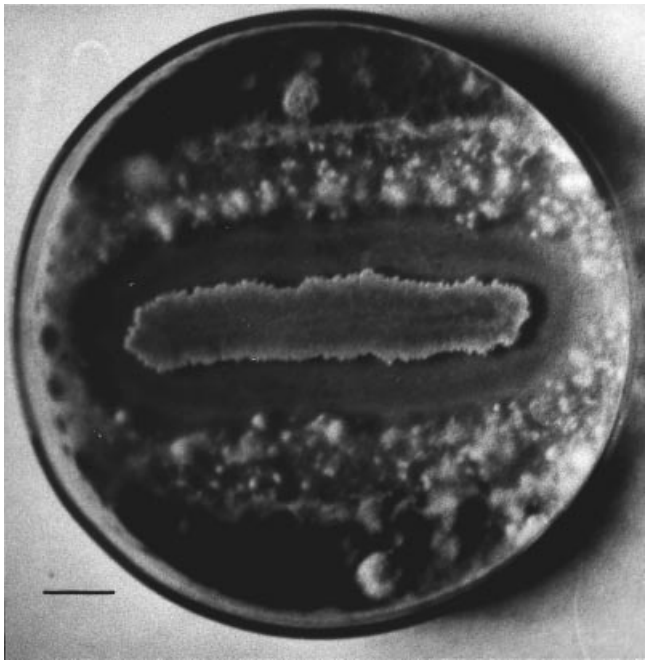

Fig. 1 Inhibitory effect of *Bacillus* isolate IFS-01 on *Penicillium chrysogenum*. Bar = 10 mm

Table 1 *In vitro* antagonism of *Bacillus* sp. IFS-01 against phytopathogenic, food-borne pathogenic and spoilage micro-organism, tested by means of the direct method

Micro-organism tested	Type of inhibition		Effect of prolonged incubation
	Microbicidal effect	Reduction in growth	
Filamentous fungi			
Alternaria alternata	+	−	nt
Aspergillus niger	+	−	nt
Aspergillus wentii	++	+	nt
Botrytis allii	+	?	nt
Botrytis cinerea	+	?	nt
Byssochlamys fulva	+/−	++	nt
Colletotrichum lindemuthianum	+	−	nt
Drechslera sorokiniana	+	?	nt
Fusarium oxysporum	+	−	nt
Geotrichum candidum	−	−	−
Penicillium expansum	+	−	nt
Penicillium chrysogenum	++	++	nt
Rhizopus stolonifer	+/−	−	−
Trichothecium roseum	++	−	nt
Yeasts			
Rhodotorula mucilaginosa	+	−	nt
Yarrowia (*Candida*) *lipolytica*	+	++	nt
Zygosaccharomyces bailii	+	++	nt
Bacteria			
Erwinia carotovora	−	−	−/+
Escherichia coli	−	−	−/+
Listeria monocytogenes	++	+	nt
Pseudomonas syringae pv. *syringae*	+/−	+/−	+
Salmonella arizonae	−	−	−/+
Salmonella typhimurium	−	−	−
Staphylococcus aureus	++	+	nt
Xanthomonas campestris pv. *campestris*	−	−	−/+

++, Strong effect; +, unambiguous effect;+/−, decreasing effect; −/+, increasing effect; −, no zone of inhibition; ?, doubtful effect; nt, not tested. As for the size of effect, if the difference between the average width, i.e. diameter, of inhibition zone and that of streak colony of antagonist is > 4 mm: +; > 12 mm: ++ (fungi), > 8 mm: ++ (bacteria).

circumference, and cream-coloured with a darker centre. No diffusible pigment was produced and the strain did not require any growth factors. During cultivation in broth without agitation, a dull, wrinkled pellicle formed on the surface but no turbidity was observed. The identification of the isolate to species level according to the profiles of API tests was carried out by means of interpretation software. The results showed that isolate IFS-01 was far more closely related to *Bacillus subtilis*, with an identification percentage of 97·6 and a *T*-value of 0·84, than to any other species. Strain IFS-01 differs from *B. subtilis* in only two characteristics, i.e., it is capable of producing acid from lactose and it cannot utilize citrate as the sole carbon source. The comment made by the interpretation software was 'Good identification'. On the basis of the experimental results, the isolate has been designated as *B. subtilis* IFS-01.

DISCUSSION

The isolation technique used was appropriate for finding almost exclusively aerobic spore-forming bacteria. Antagonism was tested by means of the direct method of Besson *et al.* (1978) with some minor modifications. Thus, it was easy to carry out successful screening because direct interactions were clearly visible. This method was less sensitive to bacteria than to fungi, therefore giving doubtful results for bacteria; this was because there could not be an effec-

Table 2 Morphological and physiological characteristics of *Bacillus* isolate IFS-01

Characteristics	*Bacillus* sp. IFS-01
Cell shape	Non-vacuolated straight rod
Cell size	0·7–0·9 × 2·0–6·0 μm
Motility	Positive
Form of spore	Ellipsoidal
Swelling of cell at spore formation	Negative
Position of spore	Subterminal
Gram stain	Positive
Nitrate reduced to nitrite	Positive
Denitrification	Negative
Methyl red test	Negative
Voges-Proskauer test	Positive
Formation of	
Indole	Negative
H_2S	Negative
Utilization of citrate	Doubtful or negative
ONPG	Positive (weakly)
Gas from glucose	Negative
Growth factor requirement	Negative
Arginine dihydrolase	Negative
Lysine decarboxylase	Negative
Ornithine decarboxylase	Negative
Urease	Negative
Oxidase	Positive
Catalase	Positive
Degradation of	
Agar	Negative
Cellulose	Negative
Hydrolysis of starch	Positive (strong)
Liquefaction of gelatin	Positive
Range of growth	
pH	5·5–9·0
(Optimum)	(6·0–8·0)
Temperature	15–45 °C
(Optimum)	(28–37 °C)
Tolerance to NaCl	< 10%

tive difference between the two incubation temperatures used. The degree of antagonism under the conditions described depended on the difference in growth rate between the bacteria tested and the *Bacillus* strains. For this reason, the level of inoculation of the test organisms was lower, whereas that of *Bacillus* isolates as streaks was higher than usual and thus, the sensitivity of the method and the rate of production of antibacterial compound(s) increased. The results suggest that this method was appropriate for the screening of isolates for antagonism against fungi. However, when the antagonistic effect of isolates on bacteria was tested, the method only worked if *Bacillus* isolates were given an advantage, by pre-incubation, over the test bacteria with respect to their optimal growth temperature so that various metabolites could be produced. The sensitivity of the method was basically influenced by the degree of this advantage. Prolonged incubation proved to be an appropriate way of determining the inhibitory effect on Gram-negative bacteria at late stages of their growth.

The isolate identified as *B. subtilis* IFS-01 displayed antagonism against some phytopathogenic, food-borne pathogenic and spoilage bacteria and fungi owing to the production of antimicrobial compound(s). The filamentous fungi, yeasts and Gram-positive bacteria tested were found to be more sensitive to the antimicrobial compound(s) produced by strain IFS-01 than the Gram-negative bacteria tested. The difference in microbial sensitivity may have been due to the difference in experimental conditions. Antagonism was fungicidal and bactericidal in nature. Inhibition of growth and of production of conidia was also observed. These results suggest that strain IFS-01 produced either a broad spectrum antimicrobial compound, or several compounds with different activities. It remains to be seen whether the various forms of antagonism depend on the concentration of the active substance(s). Future research will provide an answer to these questions.

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