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Review

Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent

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Intensive cultivation of plants in the monoculture field system in order to feed the continuously growing human population creates a need for their protection from the variety of natural competitors such as: bacteria, fungi, insects as well as other plants. The increase in the use of chemical substances in the 20th century has brought many effective solutions for the agriculture. However, it was extremely difficult to obtain a substance, which would be directed solely against a specific plant pathogen and would not be harmful for the environment. In the late 1900's scientists began trying to use natural antagonisms between resident soil organism to protect plants. This phenomenon was named biocontrol. Biological control of plants by microorganisms is a very promising alternative to an extended use of pesticides, which are often expensive and accumulate in plants or soil, having adverse effects on humans. Nonpathogenic soil bacteria living in association with roots of higher plants enhance their adaptive potential and, moreover, they can be beneficial for their growth. Here, we present the current status of the use of *Bacillus subtilis* in biocontrol. This prevalent inhabitant of soil is widely recognized as a powerful biocontrol agent. Naturally present in the immediate vicinity of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and promote their growth. In addition, due to its broad host range, its ability to form endospores and produce different biologically active compounds with a broad spectrum of activity, *B. subtilis* as well as other *Bacilli* are potentially useful as biocontrol agents.

Keywords: biocontrol, *Bacillus subtilis*, multicellular behaviour, antimicrobial agents

OVERVIEW

Biological control of plants by microorganisms is a very promising alternative to the extended use of pesticides, which are often expensive and accumulate in plants, having adverse effects on humans. Such chemicals can also be lethal to the beneficial residents of soil (Leroux, 2003). Moreover, detection of undesirable chemical compounds in the food chain connected with the emergence of fungicide-resistant strains of pathogens (El-Ghaouth, 1997) calls for an alternative, non-polluting strategy for controlling plant diseases. There is a significant number of rhizobacteria present in the soil, on average at 10^8 cells/g (Priest, 1993). Nonpathogenic soil bacteria living in association with roots of higher plants enhance the adaptive potential of the hosts and increase their growth. In 1980, Kloepper called them **P**lant **G**rowth-**P**romoting **R**hizobacteria (PGPR), to stress their beneficial effect on plants. PGPR have numerous traits, which allow them to act as biocontrol agents: suppression of diseases caused by phytopathogens thanks to the production of a wide range of antimicrobial compounds (Ongena *et al*.,

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Abbreviations: EPS, exopolysaccharide; ISR, induced systemic resistance; PGPR, plant growth-promoting rhizobacteria; NRPS, non-ribosomal peptide synthetases.

2005b), competition in colonization of the niche and for the nutrients with species non-stimulating for plant growth or even pathogenic (Bais *et al*., 2004; Timmusk *et al*., 2005), and activation of the host defense system by induced systemic resistance (ISR) (Ongena *et al*., 2005a). Plant stimulatory effects may be also achieved by an increased availability for the uptake from the soil of nutrients such as nitrogen, phosphorus, amino acids (Idriss *et al*., 2002).

For a long time, Gram-negative bacteria, especially *Pseudomonas* strains, have been intensively investigated as biological control agents (Kraus & Loper, 1995). However, recently the attention has switched to the Gram-positive members of the aerobic, spore-forming genus *Bacillus*. Among them, *B. subtilis* — a Gram-positive model organism (Moszer, 1998), and a prevalent soil inhabitant is now widely recognized as a powerful tool in biocontrol. As a soil-dwelling rhizobacterium, naturally present in the immediate vicinity of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and promote their growth. In addition, due to its broad host range, its ability to form endospores and produce different antibiotics with a broad spectrum activity, *B. subtilis* as well as other members of *Bacillus* genus are potentially useful biocontrol agents.

ANTIBIOTIC PRODUCTION — INVALUABLE WEAPON IN FIGHTING COMPETITORS

According to the present state of knowledge, several mechanisms can explain the promotion of plant growth by bacteria existing in the rhizosphere. One of the major aspects of this stimulation is certainly the suppression of diseases caused by phytopathogens (Toure *et al*., 2004). Production of antimicrobial agents by PGPR plays here a principal role (Leclere *et al*., 2005). *B. subtilis,* the most common representative of the genus, has been found to have broad suppressive properties for more than 23 types of plant pathogens *in vitro* due to its ability to produce a great abundance of antibiotics with an amazing variety of structures and activities (Stein, 2005). Those compounds include predominantly peptides that are either of ribosomal origin or are generated non-ribosomally. The features which determines their effectiveness are the wide spectrum of action and resistance to hydrolysis by peptidases and proteases. Their activity is also resistant to high temperatures and a wide range of pH (Souto *et al*., 2004). All the genes involved in the antibiotic syntheses in *B. subtilis* combined amount to 350 kb. However, as no strain has all of them, an average of about 4–5% of the *B. subtilis* genome is devoted to antibiotic production (Stein, 2005).

A major fraction of the *B. subtilis* antibiotics suppressing plant pathogens are non-ribosomally synthesized peptide derivatives, mainly lipopeptides. They are formed by large multienzymes — non-ribosomal peptide synthetases (NRPSs), which govern all necessary steps in their biosynthesis (Finking & Marahiel, 2004). Their structures are highly flexible. Natural rearrangements occur very often in these systems, permitting natural selection of compounds that offer the selective advantage (Stein, 2005). Thus, NRPSs are easily accessible to genetic manipulations, providing powerful tools for generation of novel antibiotics with new properties (Sieber & Marahiel, 2003). This might be a promising way for future development of new strategies in the field of biocontrol of plants.

Lipopeptides are amphipathic, cyclic antibiotics widespread in the *Bacillus* genus. Variations in length and branching of the fatty acid chains and amino-acid substitutions allow the lipopeptides identified so far to be divided into three groups: the surfactin (Peypoux *et al*., 1999; Mulligan, 2005), iturin (Tsuge *et al*., 2005) and fengycin families (Ongena *et al*., 2005b). They are composed of seven α-amino acids (iturins and surfactins) or ten α-amino acids (fengycins). The length of the fatty acid chains is also variable and ranges from C13 to C18 (Stein, 2005). Moreover, some lipopeptides are also produced with a huge diversity in length of the acyl side-chains, e.g.: fengycin A possesses isomers from C14 to C18 (Ongena *et al*., 2005b). It has been shown that lipopeptides with longer hydrocarbon side chains (C17 and C18) are potentially more bioactive (Toure *et al*., 2004). Their fungitoxicity increases with the number of carbon atoms in the fatty acid moiety, i.e., the C17 homologues are 20-fold more active than the C14 forms (Leclere *et al*., 2005). In the case of *B. subtilis* some of these antibiotics have been found to be produced by a great variety of strains, e.g. subtilosin A, sublancin. Others are expressed strain-specifically, for example, ericin is produced by *B. subtilis* A1/3 only (Stein *et al*., 2002). In addition, a wide range of antimicrobial substances is also produced by *Brevibacillus brevis, B. licheniformis, B. pumilus* (Munimbazi & Bullerman, 1998), *B. amyloliquefaciens* (Souto *et al*., 2004), *B. cereus, Paenibacillus polymyxa* (Timmusk *et al*., 2005).

THE VARIETY AND COMPLEXITY OF THE ITURINS AND FENGYCINS

Lipopeptides from the iturin and fengycin families display a potent antifungal activity and suppress the growth of a wide range of plant pathogens (Toure *et al*., 2004). Iturins are produced by *B. subtilis* and other closely related *Bacilli*, e.g., *B. amy-*

loliquefaciens (Souto *et al*., 2004). The iturin group comprises iturins A–E, bacillomycins D, F, and L, and mycosubtilin (Stein, 2005). Those molecules disrupt the yeast plasma membrane by forming small vesicles and by aggregating membrane-spanning particles. They also release electrolytes and high molecular mass products and degrade phospholipids. Members of the iturin family exhibit also a rather limited antibacterial activity (Maget-Dana & Peypoux, 1994). Overproduction of mycosubtilin, one of the best known members of the iturin family, by a recombinant *B. subtilis* strain BBG100, has significant antagonistic properties against phytopathogenic fungi, *Botrytis cinerea, Fusarium oxysporum* and *Pythium aphanidermatum*, and yeasts, *Pichia pastoris* and *Saccharomyces cerevisiae* (Leclere *et al*., 2005). This strain is a derivative of *B. subtilis* ATCC6633 and has a 15-fold higher mycosubtilin production rate than the parental strain. The authors demonstrated that pretreatment of tomato seeds with vegetative cells of the mycosubtilin-overproducing strain prior to planting in soil infected with *P. aphanidermatum* led to enhanced germination rates of seeds compared with the treatment with the wild-type strain ATCC6633. Besides the antifungal activities, mycosubtilin is also involved in *Bacillus* spreading. Leclere *et al*. (2006) demonstrated that overproduction of mycosubtilin is directly related to an enhanced invasive behaviour. Addition of the purified lipopeptide to the medium caused the enhance of swarming motility of *B. subtilis* 168, which is known as a non-spreading strain (Julkowska *et al*., 2004) (this phenomenon is described in a further part of the review). Numerous studies have shown the potential of the iturin family as alternative antifungal agents. *B. amyloliquefaciens* strain B94 suppresses *Rhizoctonia solani* and other fungal plant pathogens. Isomers of iturin A purified from culture broth were responsible for inhibition of *R. solani* growth *in vitro*. Moreover, Souto *et al*. (2004) indicated that those excreted secondary metabolites efficiently inhibited mycelia growth of *F. oxysporum* f. sp. *lycopercisi, R. solani, Fusarium solani* and *Sclerotinia sclerotiorum*.

Fengycins play an important role in plant disease reduction. Direct evidence derives from experiments of Ongena *et al*. (2005b), who showed the ability of *B. subtilis* strain M4, an important producer of a wide variety of fengycin-type lipopeptides, to protect wounded apple fruits against mold disease caused by *B. cinerea*.

SURFACTANTS – WIDE-RANGING SURFACE-ACTIVE COMPOUNDS

The group of biosurfactants (surface-active agents of microbial origin) are molecules that partition preferentially at the interface between two phases, such as vapour and liquid interface. The reason that causes biosurfactants to localize at interfaces is that they are amphipathic, i.e., they contain both hydrophobic and hydrophilic moieties (Przestalski *et al*., 2000; Mulligan, 2005). What determines the effectiveness of surfactants is their ability to reduce the surface tension (Ron & Rosenberg, 2001). Surfactin, the best known member of this group, is the most powerful biosurfactant ever discovered — a 20 μM solution decreases the surface tension of water from 72 to 27 mN m^{-1} (Carrillo *et al*., 2003). For a long time a surfactin was the only lipopeptide with a proven surfactant capacity. Main producers include strains of *B. subtilis*, about 20 strains from private or public collections have been categorized as surfactin-positive (Peypoux *et al*., 1999). *B. licheniformis* and *B. pumilus* produce peptidic variants of surfactin, named lichenysin and pumilacidin, respectively (Naruse *et al*., 1990; Yakimov *et al*., 1996).

Naturally occurring surfactin is a mixture of different types of molecules, which are classified according to the variation in the chain length and branching of its β-hydroxy fatty acid as well as differences in amino-acid sequence (Kowall *et al*., 1998). Biosynthesis of surfactin is a property of bacteria from the genus *Bacillus*. Surfactin prevents platelet aggregation leading to an inhibition of fibrin clot formation (Lim *et al*., 2005), is also able to remove heavy metals from contaminated soil and sediments (Mulligan, 2005), increases solubilization and biodegradation of hydrophobic compounds. Recently it was found that the colonizing behaviour and biofilm formation of *B. subtilis* strains depend on production of different families of lipopeptides (Leclere *et al*., 2006). The architecture of the colony on a swarming medium as well as the flotation and the thickness of the pellicle formed at the air/liquid interface is influenced by the pattern of the lipopeptides produced. Generally, exolipids promote swarming motility but also influence biofilm structure. Leclere *et al*. (2006) showed that addition of a purified antifungal compound, mycosubtilin, enhanced the spreading of *B. subtilis* 168 on B-medium. The role of mycosubtilin in this process is based on an increase of the wettability and a decrease of the surface tension of the medium. This double activity could be considered as a synergistic effect towards phytopathogenic fungi in the field of biocontrol, by increasing the ability of the bacteria to colonize target surfaces, connected with the strong antifungal properties of mycosubtilin. An aspect of great importance, which seems to have a remarkable potential for biocontrol of plant diseases is the surfactin's antiadhesive properties (Ron & Rosenberg, 2001). Surfactin exhibits strong antibacterial and antifungal properties. This is probably due to its capability of permeabilizing cellular

membranes (Heerklotz & Seelig, 2007). Surfactin displays an array of amazing activities, although the underlying mechanisms remain unclear and need to be established. Deciphering of the genetic organization of the operon responsible for surfactin synthesis, research of lipopeptide's molecular structure and chemical relationship between the residues are important advantages in capacity to engineer new modified compounds with improved properties. Thimon *et al*. (1994) described chemically modified (Glu-γ-methyl ester) surfactin with enhanced surface-active properties. Another interesting variant was achieved by replacing amino acids at positions 2, 4 and 7 with isoleucyl residues. This (Ile-2, -4, -7) surfactin had improved surfactant together with haemolytic and cytolytic activities (Grangemard *et al*., 1997). Genetic modifications of the surfactin biosynthesis machinery resulted in the production of a lipohexapeptide with reduced toxicity against erythrocytes and an increased inhibition of bacterial cells, including those of *B. licheniformis* (Symmank *et al*., 2002). Molecular manipulations in surfactin structure offer a possibility of constructing new derivatives with potent surfactant and antimicrobial activity. In the field of biological control of plant diseases, rhizobacteria capable of producing such compounds are a very tempting alternative for syntetic pesticides, being easily biodegradable and safe, with no adverse effects on humans.

ANTIBIOTICS — 'PROPER TIME AND COMBINATION'

During the bacterial growth surfactins are the first to be synthesized (Cosby *et al*., 1998). Secondary metabolites, such as iturin A, are generally produced after the logarithmic growth phase, when the cells have exhausted one or more essential nutrients (Mizumoto *et al*., 2006). In the moment of transition between exponential and stationary growth, maximal production of mycosubtilin was observed (Toure *et al*., 2004). Apparently, at every step of bacterial growth antimicrobial activities are present. It is another argument explaining the great potential of bacteria possessing a wide range of produced antibiotics. Simultaneously, the excretion of surfactin and other lipopeptides is often observed in *Bacillus* spp. (Souto *et al*., 2004). Mixtures of surfactin and iturin produced by *B. subtilis* RB14 and *B. amyloliquefaciens* BNM122 increase the antifungal activity, since the former compound is able to form mixed micelles with iturin and thereby improve its activity (Thimon *et al*., 1992). *B. subtilis* GA1 is a producer of a wide variety of lipopeptides: iturin A, surfactins and fengycins with various lengths of the fatty acid chains from C14 to C18 (Toure *et al*., 2004). Another example is *B. subtilis* ATCC6633 with the ability to produce subtilin, subtilosin, and lipopeptides surfactin and mycosubtilin (Leclere *et al*., 2005). In addition, studies of the kinetics of production of those compounds suggest that it might be some kind of a synergistic effect in eliminating the competitors in the habitat. Increasing the diversity of antibiotics excreted by the organism to the soil might result in an increase of the range of action on different phytopathogens. Thimon *et al*. (1992) demonstrated that co-production of surfactin, which has a strong surfactant activity, with iturin, enhances antifungal properties of iturin A. Stein (2005) speculates that the frequent occurrence of *B. subtilis* among other *Bacilli* in the natural environment might be due to the selective advantage conferred by the produced metabolites.

No single *Bacillus* strain produces all of the antibiotics depicted above. However, simultaneous production of some of these compounds by specific strains is often observed. A list of the antibacterial and antifungal compounds produced by different *B. subtilis* and *B. amyloliquefaciens* strains, including their mechanism of action and targets, is displayed in Table 1.

CAN PLANTS BENEFIT FROM MULTICELLULAR COOPERATION OF BACTERIA?

The role played in the soil by *B. subtilis* is poorly understood. This is mainly due to the fact that we got used to examining the activities and life cycle of microbes in laboratory conditions, which are far from the natural environment. Since soil is a mixture of sand, silt and a variety of mineral compounds, it is hard to put an equals sign between a natural niche and a laboratory medium. Unfortunately, the social behaviour of microorganisms in natural environment is poorly understood. The phenomena such as a quorum sensing, biofilm formation as well as detaching, different mode of movement seem to be elaborated processes a highly connected with each other. They are also believed to play a crucial role in the adaptative strategies in microbial life (Shapiro, 1998).

So far, most of the studies on *B. subtilis* have focused primarily on the 168 strain (Marburg), cultivated in the laboratory conditions for several decades, which led to the accumulation of numerous mutations. First of all, those DNA changes of domesticated strain are responsible for swarming phenotype different from natural isolates (Kearns *et al*., 2004). What is more, the 168 Marburg is unable to produce lipopeptides, e.g., surfactin, fengycin and iturin. A frameshift mutation in the *sfp* gene coding for 4'-phosphopantetheine transferase, which is

responsible for conversion of nascent antibiotic synthetases to their active holoforms, causes this defect (Nakano *et al*., 1992). Interestingly, introduction of a native *sfp* gene into *B. subtilis* 168 provoked surfactin and fengycin production (Tsuge *et al*., 1999). In the case of iturins, on the basis of whole-genome sequence data, *B. subtilis* strain 168 does not have the iturin group operon (Kunst *et al*., 1997). Analogically, Tsuge *et al*. (2005) demonstrated that conversion of the non-iturin A producer (*B. subtilis* 168) into an iturin producer requires the introduction of both the region containing the iturin A operon and the *sfp* gene. The genetic changes described above explain, at least partly, the different social behaviour of strain 168 in comparison to wild-type isolates of *B. subtilis* (Kearns & Losick, 2003; Julkowska *et al.,* 2005; Calvio *et al.,* 2005; Leclere *et al.,* 2006).

'STEP BY STEP' — TRANSLOCATION OF PGPR

When analyzed within the context of biocontrol, the translocation processes of PGPR bacteria seems to warrant more attention. Motility on surfaces is an important mechanism for bacterial colonization of new environments. Furthermore, the ability to move in a directional manner may confer distinct advantages upon host-adapted prokaryotes.

There are few investigations reporting that motility is essential for the initial steps of development of microbial biofilms, which are often basic condition of beneficial effects of PGPR (Kinsinger *et al*., 2003). Motility definitely helps to establish a stable relationship with the plant surface, as it favoures rapid and effective colonization. Avoidance of antimicrobial compounds, produced either by the host or by competitors inhabiting the same niche, also seems to be important for maintaining this contact. Better access to nutrients and translocation to favourable colonization sites are an additional advantage of the active movement of bacteria within the rhizosphere. Rapid colonization on the host surface means winning the competition with antagonists (Shapiro, 1998; Kinsinger *et al*., 2003). Taking into account that phytopathogenic fungi as well as other soil-dwelling competitors of PGPR bacteria are highly motile organisms, it is easy to realize how important is motility for survival in the environment. From a more global point of view, it becomes clearly visible how motility can influence host colonization, which is a crucial step in biocontrol.

Since swimming, perceived as a basic mode of movement, is connected with a single cell, which reacts and respond to particular chemical signals in the environment, swarming is a multicellular, coordinated movement, generated by successive waves of moving cells on a solid surface. Rapidly spreading dendritic structures are typical for this flagellum-driven motility (Julkowska *et al.,* 2005). In contrast to swimming, where chemotaxis is the basic response of the cell to environmental stimuli, it is unknown if the chemotaxis sensory system plays a role in swarming, too (Calvio *et al*., 2005). However, swarming is thought to be dependent on cell density signals. Furthermore, a role of powerful surfactants in switching from swimming to swarming motility has also been established. This is not surprising, since regulation *via* a quorum-sensing system is strictly connected with production of the mentioned lipopeptides (Connelly *et al.,* 2004; Morikawa, 2006). A swarming cell undergoing morphological changes becomes elongated and hyperflagellated in comparison to planktonic swimmers. This morphological differentiation is reversible, since swarmers can revert into shorter and less flagellated swimming cells under certain conditions (Leclere *et al.,* 2006). In both cases, a functional movement apparatus is required. However, genes governing flagella synthesis and assembly as well as the environmental signal transduction leading to flagella formation are poorly understood. Recently, it was concluded that protein SwrAA has a pivotal role in regulating the degree of cell flagellation, whereas SwrAB seems to be essential for differentiation in response to bacterial contact with a solid surface. Because of the presence of conserved interaction module PDZ, the SwrAB may contribute to the processing of SwrAA protein (Jeleń *et al*., 2003; Calvio *et al.,* 2006). Additionally, *efp* and *swrC* genes were found to be crucial for massive migration through solid, but not in liquid media. The protein encoded by *swrC* is required for resistance to the antimicrobial activity of surfactin as SwrC prevents its accumulation in the cell (Kearns *et al.,* 2004). As it was underlined above, laboratory strains harbouring frameshift mutations in some genes (*sfp*, *swrAA*) displayed a swarming phenotype different from the normal one (Kearns *et al*., 2004). It was discovered, partly in our laboratory, that wild-type strain 3610, in contrast to the domesticated strain 168, was able to swarm on a synthetic, fully defined medium. In the case of 3610 strain, incredible patterns generated by successive waves of moving cells could be obtained on plates (Fig. 1). The domesticated strain 168, which does not produce surfactin, swarms only on Luria-Bertani (LB) agar, displaying less swarming activity and reduction in speed of colonization (Julkowska *et al*., 2004; 2005). An experiment consisting in introduction of sfp^+ allele into the chromosome showed that this biosurfactant is needed for the swarming behaviour because many features observed for 3610 on LB medium were restored in the transgenic 168. Lowering the surface tension is one of the many

Table 1. Active substances produced by *Bacillus* strains and their mechanism of action.

B. subtilis strain	Mechanisms of antagonistic interactions – basic information	Antagonist (plant host)
1. **CE1** (Cavaglieri *et al*., 2005)	inhibition of *Fusarium verticillioides* growth and fumonisin B_1 accumulation *in vitro*	*Fusarium verticillioides* (maize root pathogen)
2. **B_1** (Okigbo, 2005)	inhibition of mycelia of primary rot producing fungus *in vitro*; total inhibition of rot (99–100%) in postharvest storage	*Penicillium oxalicum, Aspergillus niger, Fusarium solani* (rot of yam in storage barns)
3. **BBG100** (Leclere *et al*., 2005)	increase in germination rate of seeds tomato; mycosubtilin and surfactin production	*Botrytis cinerea, Fusarium oxysporum, P. aphanidermatum, Pichia pastoris, S. cerevisiae*
4. **ZJY-116** (Zhang *et al*., 2005)	effective suppression of *Fusarium* head blight (in field experiments); competition for space and resources; releasing secondary metabolites that inhibit growth of *F. graminearum*	*Fusarium graminearum* (FHB- *Fusarium* head blight of wheat and barley)
5. **IFS-01** (Földes *et al*., 2000)	production of either a broad spectrum of antimicrobial agents or several compounds with different activities	filamentous fungi (*Aspergillus wentii, Penicillium chrysogenum)* yeasts *(Yarrowia lipolytica, Rhodotorula mucilaginosa*) and Gram-positive bacteria (*Listeria monocytogenes, Staphylococcus aureus)*
6. **$B2_g$** (Marten *et al*., 2000) Phytovit — commercially available from Prophyta GmbH		pathogenic fungi: *Pythium ultimum, Rhizoctonia solani*
7. **QST 713** www.agraquest.com/products	Serenade® — produces over 30 different lipopeptides that work synergistically to destroy disease-causing pathogens; Rhapsody® controls a wide array of foliar and soil diseases on turf and ornamentals	
8. **A30** (Chen *et al*., 1997) **A014** (Liu *et al*., 1991) **SO113** (Lin *et al*., 2001)	production of anti-*Xoo* peptide acting through different molecular mechanisms	Gram-negative bacteria, vascular pathogen causing bacterial blight of rice — *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)
9. **6051** (Bais *et al*., 2004)	formation of protective and antimicrobial biofilms on root surface of *Arabidopsis*; secretion of lipopeptide antibiotic surfactin	*Pseudomonas syringae* pv. *tomato* DC3000 (potent leaf pathogen in *Arabidopsis*)
10. **M4** (Ongena *et al*., 2004; 2005a; 2005b)	ISR (induced systemic resistance) — activates host defense system; fengycin production which induced synthesis of plant phenolics	*Colletotrichum lagenarium* (cucumber — *Cucumis sativus* disease), *Pythium aphanidermatum* (damping-off of tomato), *Botrytis cinerea* (bean diseases)
11. **GA1** (Toure *et al*., 2004)	grey mould disease reduction (inhibition of mycelial growth) — production of antifungal lipopeptides: iturins, fengycins type A; B, surfactin families	*Botrytis cinerea* (grey mould disease of apples)
12. **L-forms** of *B. subtilis* (Walker *et al*., 2002)	symbiotic relationship with Chinese cabbage; production of antibiotic in pure culture active against *B. cinerea in vitro*	*Botrytis cinerea* (grey mould disease of Chinese cabbage)
13. **B2; B5; B7; B8** (Li *et al*., 2005)	strong nematicidal activity — killing second stage larvae; production of active factors (heat stability, resistance to extreme pH values — putative antibiotic character)	*Rhizoctonia solani* SX-6, *Pythium aphanidermatum* ZJP-1, *Fusarium oxysporum* f.sp. *cucumerinum* ZJE-2 (root-knot nematode and soil-borne fungi); larvae *Meloidogyne javanica*
14. **RC8; RC9; RC11** (Cavaglieri *et al*., 2004)	inhibition of fumonisin B_1 production (mycotoxin); strong fungal growth inhibition	*Fusarium verticillioides*
15. **RRC101** (Bacon *et al*., 2001)	reduction of mycotoxin accumulation; 'ecological homolog' of *F. moniliforme*-competition	*Fusarium moniliforme* Sheldon (facultative fungal endophyte of *Zea mays*)
16. **AF 1** (Manjula & Podile, 2001; Manjula *et al*., 2004)	significant root-colonizing ability and survival when introduced into rhizosphere; reduced incidence of wilt in pigeon pea; potential biocontrol agent of *Aspergillus niger* through chitynolysis (β-1,4-*N*-acetyloglucosaminidase production — chitinase) and induction of host defense response	*Aspergillus niger* (crown rot of groundnut, soft rot in lemons); *Puccinia arachidis* (rust in groundnut); *F. udum* (wilt of pigeon pea)

17. **JA**; **JA026** (Liu *et al*., 2005)	antifugal lipopeptides (1400 –1500 Da) from fengycin family	*Gibberella zeae* (anamorph of *Fusarium graminearum*) — *Fusarium* head blight (FHB) in wheat, barley, ear rot in corn
18. **BS21; BS22; BS23** (Adebanjo & Bankole, 2004)	reduction of seeding infection by anthracnose; severity of anthracnose disease; reduction of pathogen inoculums and displacement of pathogen	*Colletotrichum lindemuthianum* (anthracnose disease of cowpea)
19. **PY79** (Stöver & Driks, 1999)	TasA-secreted protein with broad spectrum antibacterial activity and inhibition of growth of competitor bacteria in nature	plant pathogens: *Agrobacterium tumefaciens* GV3101, *Pseudomonas* sp., *Klebsiella* sp., *Erwinia* sp.; animal pathogens: *Enterococcus faecalis, Escherichia coli, Staphylococcus epidermis*; clinical isolates including human pathogens: *Enterobacter* sp., *Klebsiella pneumoniae, Pseudomonas aeruginosa*
20. **Natural isolate** (Kawulka *et al*., 2004; Thennarasu *et al*., 2005)	subtilosin A — bactericidal activity	diverse range of Gram-positive and Gram-negative bacteria
21**. UMAF6614**; **UMAF6619**; **UMAF6639**; **UMAF8561** (Romero *et al*., 2007)	excreted antibiotics (surfactin, fengycin, iturin A, bacillomycin) major factors involved in biocontrol activity	*Podosphaera fusca* (cucurbit powdery mildew)
22. **BS 107** (Sharga & Lyon, 1998)	antibiotic production	*Erwinia carotovora* subsp. *atroseptica* and *Erwinia carotovora* subsp. *carotovora* (causal agents of potato blackleg and tuber soft rot)
23. **C1** (Singh & Cameotra, 2004)	biosurfactant – lipopeptide N1	*Mycobacterium smegmatis*, *Staphylococcus aureus*
24. **GB03** (Raupach & Kloepper, 1998)	use of GB03 in combination with two other PGPR strains caused intensive plant growth promotion; disease reduction (better plant colonization, large number of pathogen-suppressive mechanisms)	*Colletotrichum orbiculare* (anthracnose), *Pseudomonas syringae* pv. *lachrymans* (angular leaf spot), *Erwinia tracheiphila* (cucurbit wilt disease)
25. **RB14–CS** (Mizumoto *et al*., 2006)	lipopeptide production: iturin A	*Rhizoctonia solani* (causal agent of damping-off of tomato)
26. **LEV-006** (Hou *et al*., 2006)	strong, stable antifungal activity; production of fengycins A nad B	*Rhizoctonia solani* (seedling blight and root rot), *Sclerotinia sclerotiorum* (stem rot), *Alternaria brassicae* (black spot), *Leptosphaeria maculans* (black leg)
27. **ATCC 21332** (Symmank *et al*., 2002)	novel lipohexapeptide after engineering of *B. subtilis* surfactin synthetase resulted in reduced toxicity against erythrocytes and enhanced lysis of *B. licheniformis* cells	*B. licheniformis*
***B. amyloliquefaciens* strain**		
28. **MET0908** (Kim & Chung, 2004)	ß-1,3-glucanases — decomposition of fungal hyphal walls	*Colletotrichum lagenarium* (watermelon anthracnose)
29. **RC-2** (Hiradate *et al*., 2002)	lipopeptide production: iturins A-2–A-8	*Colletotrichum dematium* (mulberry anthracnose)
30. **B94** (Yu *et al*., 2002)	antibiotic production (iturin A) involved in disease-suppression	*Rhizoctonia solani* (pre- and post-emergence damping-off of soybean)
31. **FZB45** (Idriss *et al*., 2002)	stimulated growth of maize seedlings under phosphate limitation in the presence of extracellular phytate — production of phytases	*Zea mays*
32. **BNM 122** (Souto *et al*., 2004)	co-production of surfactin and iturin-like antibiotics against various plant pathogenic fungi	*Fusarium oxysporum* f. sp *lycopersici, Fusarium solani, Rhizoctonia solani, Sclerotinia sclerotiorum*

activities of surfactin, which is secreted at the edges of a growing colony. The production of extracellular lipopeptide was proved to be stimulated by K^+ ions, but the precise mechanism remains unknown (Kinsinger *et al*., 2003). The important finding of this investigation was also that apart form structural changes of the cell and specialization of particular groups of swarmers the process can also be characterized as time-dependent. Analyzing the behaviour of a swarmers' community indicated

Figure 1. Swarming pattern generated by *B. subtilis* 3610.

Figure 2. Biofilm formed on solid media by *B. subtilis* 3610.

that two waves of migration can be distinguished. The first swarm is detected at 11 h from inoculation, whereas the second one appears at 18–22 h (Julkowska *et al*., 2005).

During swarming, the number of interactions between factors, often unidentified, causes the nature of this process to be extremely elaborated. One of them is the production of surfactin, a powerful compound serving a wide variety of different functions in bacterial vegetation (Mulligan, 2005). It has been shown that lipopeptide production can be essential for motility also in other groups of bacteria. The poorly known surface translocation phenomenon of *Pseudomonas* sp. DSS73, a strain isolated from the rhizoplane of sugar beet seedlings, depends on the presence of amphisin (Andersen *et al*., 2003). Production of cyclic lipopeptides in combination with the presence of flagella allows the growing colony of bacteria to translocate effectively through the plant surfaces, thus colonizing it. A synergistic effect of the mentioned mode of movement and production of a wide range of toxic substances seems to inhibit growth of pathogen fungi such as *Pythium ultimum* and *R. solani.* Amphisin as well as tensin and viscosin belong to the group of bifunctional substances. They not only have an antifungal potential, but they can also influence surface properties. The specific morphology of swarmers combined with the production of biosurfactants and the process of water removal from the surface reduces friction forces leading to effective expansion of the surface (Matsuyama *et al*., 1992; Bees *et al*., 2000). It is worth pointing out that a close correlation between the production of the dual-functioning compounds and translocation through the surface of low-percentage agar was proved also in the case of other Gram-negative bacteria such as *Serratia* spp. and *Pseudomonas* spp. (Matsuyama *et al*., 1992; Nielsen *et al*., 2002).

EXTRACELLULAR PROTEOLYTIC ACTIVITY PLAYS A CENTRAL ROLE IN MULTICELLULAR BEHAVIOUR

Another common feature of multicellular processes is participation of extracellular proteases. An obvious role of those enzymes is degradation and acquisition of nutritional factors from the surrounding environment. However, on the basis of some recent reports it can be predicted that those extracellular proteases could also act either on the bacterial cell proteins itself or on proteins secreted by the cell, which means that their role need not be limited to nutritional only (Connelly *et al*., 2004). The *B. subtilis* PRY strain presents very robust swarming behaviour, which is a result of high serine protease production (Park *et al*., 2006). Those results are in agreement with the results published at the same time by the Murudkar's group (Murudkar *et al*., 2006). They showed that Epr, a minor extracellular serine protease, plays a significant role in swarming motility of *B. subtilis* 168. One of the functions of those extracellular proteases is cleavage of signal peptides which participate in quorum-sensing communication, which could be easily linked to swarming. It was also suggested that they modify the cell surface and release some peptides involved in swarming by digesting cell surface proteins. However, the proteolytic activity of Epr was not required for swarming (Murudkar *et al*., 2006). Considering the extracellular proteolytic activity, contribution of mainly subtilisin (AprE) and neutral metalloprotease E (NprE) is predominant (Connelly *et al*., 2004). Eight main extracellular proteases reach the highest level of expression at the end of the logarithmic phase of growth. In spite of the fact that their expression is tightly controlled, they do not seem to be essential for either sporulation or bacterial growth. Several studies

have taken into account the role of those enzymes in swarming. It was shown that *sigF*, *nprE*, *aprE* or *amyE* have only very limited function in this process. However, it is worth pointing out that a strain harbouring mutations in all listed genes displayed a completely non-swarming phenotype (Connelly *et al*., 2004). On the basis of those results, it can be concluded that the complexity of motile behaviour and its interaction as well as influence on other multicellular processes like biofilm formation needs to be established in more detail.

'BIOFILMS — EASY WAY TO RECOVER'

Biofilms are viewed as highly structured multispecies communities, a prevalent form of existence of microorganisms in every ecosystem (Fig. 2). The process of surface adhesion and biofilm development is an effective survival strategy employed by virtually all bacteria and refined over millions of years (Shapiro, 1998). Among other bacteria, *B. subtilis* is also believed to form a robust biofilm on biotic as well as abiotic surfaces. However, in laboratory conditions the most common form of this structure are pellicles, which are robust floating biofilms formed at the air-liquid interface. At the point of entering the stationary phase of growth motile cells of *B. subtilis* migrate to the air-medium interface. Once they reach the surface, cells start to differentiate into aligned chains of attached cells that eventually produce aerial structures or fruiting bodies serving as preferential sites for sporulation (Branda *et al*., 2001). When analyzed within the context of highly structured, surface-associated communities (biofilms), active movement was discovered to have a crucial meaning for the initial steps of biofilm development (Mireles *et al*., 2001). As it was pointed out, biofilm and swarming share some features, such as a quorum-sensing communication, active movement, as well as morphological changes of the cells. What is more, in both cases an increase in slime production is observed (Toguchi *et al*., 2000). Another evidence for a direct link between those phenomena is the fact that certain factors which reduce swarming motility also inhibit biofilm formation. (5*Z*)-4-Bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone of the marine alga *Delisea pulchra* has been shown to be an antimicrobial compound, as it can affect multicellular activities of *B. subtilis*. Probably due to structure similarity between furanone and one of the quorum sensing signals, AI-2, a significant defect of swarming motility as well as biofilm formation could be observed. The presence of the furanone caused reduction in the swarming speed in a dose-dependent manner and at a concentration of 40 μg ml^{-1} led to a 25% decrease in biofilm thickness (Ren *et al*., 2002). A connection between different mode of movement and biofilm formation was established in the case of *B. subtilis* (Branda *et al*., 2001). For instance, a protease- and surfactin-deficient strain, which displayed a significant defect in swarming, showed at the same time reduction in biofilm formation: the mutant formed very thin and flat pellicles in comparison to wild type. This is another step in confirming the hypothesis that biofilm formation and swarming can have overlapping control mechanisms (Connelly *et al*., 2004). A convincing support for this hypothesis is delivered also by authors examining the function of the *waaE* gene which participates in the biosynthesis of the inner-core LPS in Gram-negative bacteria (Izquierdo *et al*., 2002). Lack of the *waaE* gene was found to affect both swarming motility and biofilm development in S*erratia marcescens* and *P. mirabilis*. This observation can explain, at least partially, the fact that the *waaE* mutant displayed a drastic reduction in the ability to infect and colonize the urinary tract of rat (Izquierdo *et al*., 2002). As a collective bacterial process, swarming is not only associated with biofilm formation, but also with expression of virulence by pathogenic bacteria. Generally, it should be underlined that both processes are strongly related to diseases.

In natural environments bacteria establish symbiotic or pathogenic biofilms on plant or animal body surfaces. Effective colonization of plant roots by PGPR plays an important role in plant growth promotion. Plant roots secrete a vast array of compounds into the rhizosphere, which are determinative factors promoting bacterial colonization on the plant root. There is a number of investigations reporting a relationship between *B. subtilis* and the rhizoplane as well as rhizosphere of tea bushes (Pandey *et al*., 1997). *B. subtilis* 430A was also isolated from the *Vernonia herbacea* (Vell Rusby) rhizosphere, where it produces an exocellular inulinase (Vullo *et al*., 1991). Results obtained recently indicate that *B. subtilis* is a prevalent inhabitant of the rhizosphere of many plant species due to the fact that it forms robust biofilms on the root surface (Ongena *et al*., 2005a). The Gram-negative soil bacterium *Pseudomonas fluorescens* was also found to form biofilms on the root surface of particular species of plant. Unfortunately, a relation between biofilm formation and plant protection ability has not been established (Bianciotto *et al*., 2001). It is postulated that this kind of relationship can be beneficial to both partners. On the root surface, protection from environmental dangers and cooperation in order to gain nutrient are favoured by this mode of development (Danhorn & Fuqua, 2007). A good example of this kind of relationship is *B. subtilis* 6051, a natural inhabitant of soil, which was found to form robust biofilms on *Arabidopsis* roots. Mortality of plants caused by *Pseudomonas syringae*

pv *tomato* DC3000 was reduced from 85% to 10% due to co-cultivation with 6051 strain both *in vitro* and in a sterile soil. Furthermore, it was proved that the presence of surfactin effectively prevents root colonization by planktonic cells of a pathogen. Bais *et al*. (2004) showed that *B. subtilis* strain defective in surfactin production (strain M4) is not able to colonize roots plant effectively and the biofilm formed is much thinner in comparison to wild type. Furthermore, this kind of mutation makes the analyzed M4 strain ineffective against *P. syringae.*

It has also been reported that surfactin inhibits biofilm development by *Salmonella enterica* as well as *Escherichia coli* and *Pseudomonas mirabilis* (Mireles *et al*., 2001).

ANOTHER PHENOMENON POTENTIALLY USEFUL IN BIOCONTROL

It was proved that colonization of cucumber and tomato roots by *B. subtilis* M4 could influence expression of the plant's genes. This kind of action, known as ISR (induced systemic resistance), is believed to be responsible for the reduction of disease incidence caused by *Colletotrichum lagenarium* and *P. aphanidermatum* (Ongena *et al*., 2004). The experiments performed revealed that *B. subtilis* M4 is effective against pathogenic fungi due to induction of plant host resistance. Recently, the ISR phenomenon and the bacterium causing it have received a great deal of interest. Although this is a rather new phenomenon and still a lot needs to be discovered in this field, some information concerning the molecular mechanism of the process is available. For instance, an increase in lipoxygenase activity in tomato cells after plant treatment with a *B. subtilis* strain overexpressing both surfactin and fengycins was observed (Ongena *et al*., 2007). Together with fengycin, surfactin was found to be the signal recognized by the plants which in response initiated defense mechanisms. What is interesting, an induction of plant resistance has been shown, because treatment with either vegetative cells or endospores of *B. subtilis* M4 leads to a significant reduction in anthracnose, which is a devastative disease of cucumber (Ongena *et al*., 2005a). Similarly, *Methylobacterium* sp. PPFM-Ah isolated from groundnut leaves was found to increase the level of enzymatic substances such as chitinases and β-1,3-glucanases in the plant, which showed a synergistic antifungal activity, and of enzymes participating in cell-wall lignification (Madhaiyan *et al*., 2006). Defense-gene activation in pea (*Pisum sativum*), normally susceptible to pathogen infection, was confirmed for the endophytic bacterium *B. pumilus* strain SE34. The host defence reactions included strengthening of the epidermal and cortical cells, which contain large amounts of callose. These changes associated with the cell wall allowed the plant to defend effectively against the pea root-rotting fungus *Fusarium oxysporum* f. sp. Pisi (Benhamou *et al*., 1996). Furthermore, ISR is phenotypically similar to systemic acquired resistance (SAR), which is activated either by the first pathogenic infection or treatment with some chemical substances. However, it is believed that the signal transduction as well as the molecular basis of those processes is different. So far, ISR was associated mainly with Gram-negative bacteria belonging to the genera *Serratia* and *Pseudomonas* (van Loon *et al*., 1998). This kind of microorganism–plant interactions was reported also for *B. pumilus* and *B. amyloliquefaciens* (Benhamou *et al*., 1996).

Bacteria belonging to the PGPR group can stimulate growth of many plants due to facilitated assimilation of additional phosphorus from the surrounding soil, lack of which is an important limiting growth factor (Kerovuo *et al*., 1998). Phosphorus in soil, apart from polyphosphates, is present as phytate, a salt form of phytic acid (*myo*-inositol hexakisphosphate), which accounts for 20–50% of total soil organic phosphorus. In general, phosphomonoesterases, unlike phosphatases, are able to hydrolyse phytate (Idriss *et al*., 2002). Few studies have taken into account plant phytases and it was found that these enzymes exhibited only a very low level of activity in organs, including roots. It is also suggested that plants can not acquire phosphorus directly from soil phytate. Several attempts of cloning and using plant genes encoding phytases have failed (Maugenest *et al*., 1999). In contrast, the desired effect was obtained when purified protein with phytase activity from a fungus — *Aspergillus* sp. was added to the root system (Richardson *et al*., 2001). It is also known that phytases are produced and secreted by a wide range of both Gram-negative and Gram-positive bacteria, including *B. subtilis* (Kerovuo *et al*., 1998), *B. amyloliquefaciens* DS11 (Kim *et al*., 1998), *Klebsiella terrigena* (Greiner *et al*., 1997), *Pseudomonas* spp. (Richardson & Hadobas, 1997). In this context, it is tempting to speculate that bacteria which make phytate available for the plant under phosphate-starvation condition can contribute to plant growth. So far, there are few reports confirming this idea. Idriss *et al*. (2002) showed that phytases secreted by some strains from the *Bacillus* genus promote growth of maize seedlings under limiting soil phosphorus conditions. To date, only few phytases excreted by *B. subtilis* strains have been isolated and characterized. For instance, strain VTT E-68013 produces a novel enzyme, which does not contain the highly conserved RHGXRXP sequence typical for the active site of known phytases. Phytase PhyC is produced when inorganic phosphate is a limiting factor (Kero-

vuo *et al*., 1998). Having maximal phytase activity at pH 7 and 55ºC, PhyC together with PhyA isolated from strain 168 are candidates for transgenic studies. To our knowledge, most known phytases are active only in the acidic pH, moreover, they exhibit very little enzymatic activity at neutral pH (Wyss *et al*., 1999). Therefore the *Bacillus* phytases offer advantages which seem to be crucial for future application. PhyA and PhyL exhibited broad temperature and pH optima and showed high thermostability. Other properties, like high specifity, gave credence to an idea of creating a new biochemical pathway in transgenic plants that would mobilize inorganic phosphate from phytate (Tye *et al*., 2002). However, recent work (Antelmann *et al*., 2007) gave surprising results showing that phytases were not induced under phosphate starvation conditions or by phytate addition. However, re-distribution of the major cell wall protease WprA from the cell wall to the extracellular medium in phytate-supplemented medium was observed. It was concluded that phytate is an alternative phosphorus source allowing *B. subtilis* cells to overcome phosphate starvation.

CONCLUDING REMARKS

In conclusion, the compounds produced by *Bacillus* spp. presented above and, what is more important, targets of their action are various and broad. There are many known pathogens, parasites of plants causing large losses in the agriculture. It is promising that many of them have antagonists in the *Bacillus* genus. Commercially available spores of beneficial bacteria and the development of plants genetically modified with genes from the *Bacillus* genome give advantage to scientists and farmers over the plant pathogens. But it seems that the war is not over. Evolution of resistance in targeted pests is the most pressing problem (Zhao *et al*., 2005) and needs to be solved as soon as possible. Bacteria from the *Bacillus* genus posses an additional property, profitable from the technological point of view. They form spores, whose effectiveness in the fight against phytopathogenic competitors is worth further researches. This is so because the spores are extremely resistant and stable which means that they can remain viable in spite of a long period of storage. Apart from the listed advantages, we should pay attention to the fact that spores can be produced in a relatively easy and inexpensive way (Ongena *et al*., 2004).

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