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Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect

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Several *Bacillus* strains belonging to the *B. subtilis*/*amyloliquefaciens* group isolated from plant-pathogen-infested soil possess plant-growth-promoting activity [Krebs, B. *et al.* (1998) *J Plant Dis Prot* 105, 181–197]. Three out of the four strains investigated were identified as *B. amyloliquefaciens* and were able to degrade extracellular phytate (*myo*-inositol hexakisphosphate). The highest extracellular phytase activity was detected in strain FZB45, and diluted culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate. The amino acid sequence deduced from the phytase *phyA* gene cloned from FZB45 displayed a high degree of similarity to known *Bacillus* phytases. Weak similarity between FZB45 phytase and *B. subtilis* alkaline phosphatase IV pointed to a possible common origin of these two enzymes. The recombinant protein expressed by *B. subtilis* MU331 displayed 3(1)-phytase activity yielding D/L-Ins(1,2,4,5,6)P5 as the first product of phytate hydrolysis. A phytase-negative mutant strain, FZB45/M2, whose *phyA* gene is disrupted, was generated by replacing the entire wild-type gene on the chromosome of FZB45 with a *km*::*phyA* fragment, and culture filtrates obtained from FZB45/M2 did not stimulate plant growth. In addition, the growth of maize seedlings was promoted in the presence of purified phytase and the absence of culture filtrate. These genetic and biochemical experiments provide strong evidence that phytase activity of *B. amyloliquefaciens* FZB45 is important for plant growth stimulation under phosphate limitation.

Keywords: phytate, phosphate limitation, *phyA*, plant-growth-promoting rhizobacteria (PGPR)

INTRODUCTION

Plant-growth-promoting rhizobacteria (PGPR) are free-living bacteria that have a beneficial effect on plants, as they enhance emergence, colonize roots, and stimulate growth. In addition to bacteria present on the root surface (rhizoplane) and in the rhizosphere, there are significant numbers of bacteria present in the root interior (McInroy & Kloepper, 1995) that are also beneficial for plant growth. In the last decade, the concept of PGPR for promotion of plant growth has gained acceptance (Kloepper *et al*., 1989), and several possible mechanisms have been proposed, including suppression of diseases caused by plant pathogens (Smith *et al*., 1999), competition with pathogenic micro-organisms by colonizing roots (Dekkers *et al*., 1998), production of plant-growth-regulating substances such as indole-3-acetic acid (IAA) (Steenhoudt & Vanderleyden, 2000) and lowering ethylene levels in root cells (Li *et al*., 2000). Plant-stimulatory effects exerted by PGPR might also be due to an enhanced availability of limited plant nutrients such as nitrogen, phosphorus, B-vitamins and amino acids in the rhizosphere caused by phosphate-solubilizing and diazotrophic bacteria (Nautiyal *et al*., 2000; Rozycki *et al*., 1999). Improved

Abbreviations: IAA, indole-3-acetic acid; PGPR, plant-growth-promoting rhizobacteria.

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phosphorus nutrition is achievable by 'mobilization' of phosphorus fixed as insoluble inorganic polyphosphates and/or phytate, which accounts for 20–50 % of the total soil organic phosphorus (Richardson *et al.*, 2001a). A number of PGPR are efficient in phytostimulation and biofertilization, and as biocontrol agents, but in most cases difficulties in obtaining successful formulations and insufficient knowledge of the basic molecular principles of their action has hindered their commercial use (Bloemberg & Lugtenberg, 2001).

Gram-positive bacteria offer a biological solution to the formulation problem due to their ability to form heat- and desiccation-resistant spores, which can be formulated readily into stable products (Emmert & Handelsman, 1999). Enhancement of plant growth by root colonizing *Bacillus* and *Paenibacillus* strains is well known (Broadbent *et al.*, 1977; Timmusk & Wagner, 2001). Some biocontrol products based on *Bacillus subtilis* and other *Bacillus* species are commercially available (http://www.barc.usda.gov/psi/bpdl/bpdlprod/bioprod.html). Unfortunately, apart from their well-characterized capacity to produce antibacterial and antifungal lipopeptides (Steller *et. al.*, 1999), and a single report suggesting implication of levan production in the aggregation of root-adhering soil (Bezzate *et al.*, 2000), very little is known about the beneficial action of the *Bacillus* group of PGPR at the molecular level. The likely hypothesis that those bacteria contribute to improved plant growth by making available additional nutrients that are limiting (e.g. phosphorus) has not been rigorously proved up to now.

In general, phosphatases are not able to hydrolyse phytate (Reddy *et al.*, 1989). However, a special group of phosphomonoesterases, capable of hydrolysing phytate to a series of lower phosphate esters of *myo*-inositol and phosphate, has evolved in prokaryotic and eukaryotic organisms (for a review see Wodzinski & Ullah, 1996). Plants producing 3- and 6(4)-phytases (Greiner & Larsson Alminger, 2001) display only low activity in roots and other plant organs, and occurrence of plant-secreted phytase within the rhizosphere is not documented. This suggests that plant roots may not possess an innate ability to acquire phosphorus directly from soil phytate. The possible role of microbial phytases produced by PGPR in supporting plant growth under phosphate limitation has not yet been investigated, but phytase has been isolated and characterized from a few Gram-positive and Gram-negative soil bacteria, e.g. *B. subtilis* (Kerovuo *et al.*, 1998), *Bacillus amyloliquefaciens DS*11 (Kim *et al.*, 1998), *Klebsiella terrigena* (Greiner *et al.*, 1997), *Pseudomonas* spp. (Richardson & Hadobas, 1997) and *Enterobacter* sp. 4 (Yoon *et al.*, 1996).

We hypothesize that, besides other factors, the ability of some root-colonizing bacteria to make the phytate phosphorus in soil available for plant nutrition under phosphate-starvation conditions might contribute to their plant-growth-promoting activity. Another beneficial effect due to bacterial phytase activity in the rhizosphere is elimination of chelate-forming phytate, which is known to bind nutritionally important minerals (Reddy *et al.*, 1989). In this study we used an artificial sterile system consisting of maize seedlings and culture filtrates of PGPR to establish the contribution of secreted phytases to the observed plant growth promotion by *B. amyloliquefaciens*.

METHODS

Chemicals, bacterial strains and plasmids. Phytate, dodecasodium salt, was purchased from Sigma. All other chemicals were of the analytical grade commercially available. Descriptions of the bacterial strains, plasmids and primers used are given in Table 1.

Growth of the bacteria. *Escherichia coli* and *B. subtilis* were grown in solid or liquid Luria–Bertani broth (LB) at 37 °C. When necessary, media for *E. coli* were supplemented with ampicillin (100 mg l^{-1}) and media for *Bacillus* strains with kanamycin (10 mg l^{-1}) or chloramphenicol (5 mg l^{-1}). For phytase production, *Bacillus* strains were cultured initially in ASW medium [containing, per litre: 5 g $MgCl_2.6H_2O$, 5 g $MgSO_4.7H_2O$, 500 mg KCl, 500 mg $CaCl_2$, 20 g NaCl, 3 g casein peptone (pancreatic digest), 1·5 g soybean tryptone, 5 g glucose (filter-sterilized), pH 6·8] or in wheat bran medium [containing, per litre: 20 g fine milled wheat bran, 0·4 g $(NH_4)_2SO_4$, 0·2 g $MgSO_4.7H_2O$, 20 mM $CaCl_2$, pH 6·5]. Cultures were grown with shaking (210 r.p.m.) for 2 days at 37 °C.

Morphological and phenotypic characterization of *Bacillus* strains. These were performed according to Parry *et al.* (1983). Parameters investigated were cell and spore morphology, production of enzymes (catalase, oxidase, lecithinase, nitrate reductase, arginine dihydrolase and urease), growth characteristics (anaerobic growth, and growth in the presence of NaCl), sensitivity to lysozyme, carbon source utilization (glucose, arabinose, xylose, mannitol, fructose and lactose), utilization of citrate and propionate, hydrolysis of starch, gelatin, casein, Tween 80 and aesculin, indole formation, and Voges–Proskauer reaction.

Ribotyping. Fragments of genomic bacterial DNA digested by *Eco*RI were separated by agarose gel electrophoresis and probed with the partial 16S *rrnE* gene from *B. subtilis* covering nucleotides 46 to 540. The resulting patterns were digitized and compared with a database of known riboprint patterns. This analysis was performed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Assay of phytase and phosphate content. Phytase measurements were carried out at 50 °C and pH 6·5 by a modification of the ammonium molybdate method (Greiner *et al*, 1997). Fifty microlitres of culture filtrate were incubated with 100 μl phytate (0·4 % dodecasodium salt from rice, P3168, dissolved in 100 mM sodium acetate buffer pH 6·0 containing 2 mM $CaCl_2$). After 1 h the reaction was stopped by adding 300 μl ammonium heptamolybdate/ammonium vanadate reagent solution. The samples were centrifuged for 1 min at 13 000 r.p.m. Supernatant samples of 100 μl were used for measuring A_{415} with a SPECTRA max 340 spectrophotometer. One unit (U) of activity was expressed as 1 mol inorganic orthophosphate liberated min^{-1}. The phosphate content was assayed with the ammonium heptamolybdate/ammonium vanadate reagent as described for assay of phytase activity.

Table 1. Bacterial strains, plasmids and primer sequences

Restriction enzyme recognition sites within primer sequences are underlined.

Strain, plasmid or primer	Description	Source* or reference
E. coli		
DH5α	(φ80d*lacZ*ΔM15)Δ(*lacZYA–argF*)*U169 glnV44 deoR gyrA96 recA1 relA91 endA1 thi-1 hsdR17*	Lab. stock
B. subtilis		
168	*B. subtilis* subsp. *subtilis trpC2*	BGSC 1A1
SB19	*B. subtilis* subsp. *subtilis*	BGSC 1A101
FZB37	Wild-type	FZB Berlin
MU331	Lysogenic for φ105MU331 prophage, Em^{R}	J. Errington
ATCC 6633	*B. subtilis* subsp. *spizizenii* wild-type	ATCC
W23	*B. subtilis* subsp. *spizizenii* wild-type	BGSC 2A1
B. amyloliquefaciens		
DSM7 (F)	Type strain: ATCC 23350, S19	DSMZ
S23 (N)	Wild-type	F. G. Priest
ATCC 15841	Wild-type	Lab. stock
ZIMET 10639	Wild-type	Lab. stock
ZF178	Wild-type	FZB Berlin
FZB24	DSM 10271	FZB Berlin
FZB42	Wild-type	FZB Berlin
FZB45	Wild-type	FZB Berlin
FZB45/M1	Δ*phy*45/1, Km^{R}	This work
FZB45/M2	Δ*phy*45/2, Km^{R}	This work
***Bacillus* sp.**		
ILAT G697/1		ILAT Berlin
Plasmids		
pUK19	pUC19 derivative, Amp^{R} Km^{R}	Ju *et al.* (1998)
pGEM-T	Cloning vector	Promega
PGEMKm	With amplified *apha3* gene from pUK19	This work
pGEM-T-45	With amplified *phyA* gene from FZB45	This work
PGEMKm45	*apha3* cassette inserted within the *phyA* gene	This work
pSG1112	Derivative of pSG703 integrative expression vector	J. Errington
Primers		
FAR22 (*Nde*I FW)	ATACTAGTCATATGAATCATTCAAAAACACTTTG	This work
FAR06 (*Bam*HI REV)	AAGGATCCTTATTTCCGCTTCTGTCGG	This work
PRB1601	GGATCCTAATACATGCAAGTCGAGCGG	Goto *et al.* (2000)
PRB1602	GGATCCACGTATTACCGCGGCTGCTGGC	This work
KM1 (*Eco*RI FW)	TAGAATTCAAGGAACAGTGAATTGG	This work
KM2 (*Hin*dIII REV)	AGATGTCTAAAAAGCTTGTAGTTAAACC	This work
ABa3	GCACGCTGATGCAGTATTTTGAATGG	This work
ABa4	GGAGCTGTCACCTTCCCTCGTCC	This work
ABs3	ACAGGATCCTCCAGTCTTCACATC	This work
ABs4	GTTCATCATTAAGGATCCTTCCAGG	This work
OTA1	ATGAGATTCGACAACTCAGAGG	Tam & Borriss (1998)
OTA2	AAAGGCAGCATTAAACCGC	Tam & Borriss (1998)

* FZB, FZB Biotechnik GmbH, D-12489, Berlin, Germany; J. Errington, University of Oxford, UK; F. G. Priest, Heriot-Watt University, Edinburgh, UK.

DNA amplification and sequencing. Chromosomal DNA was isolated from growing *Bacillus* cultures (Cutting & Vander Horn, 1990). For amplification and sequencing of the hypervariant region (HV region) of 16S rDNA, we followed the method described by Goto *et al.* (2000). The forward primer PRB1601 corresponds to nucleotide positions 47–69 and the reverse primer PRB1602 to nucleotide positions 520–540 of the *B. subtilis rrnE* gene. Amplification of amylase genes was performed with primers ABa3 (forward) and ABa4 (reverse) annealing with the *B. amyloliquefaciens* amylase gene at

+100 and +1346 from the translation start, and primers ABs3 (forward) and ABs4 (reverse) annealing with the *B. subtilis* amylase gene at −114 and +2164, respectively. PCR amplification of the *thyA* gene was performed with primers annealing within conserved regions of the *B. subtilis thyA* gene at positions 601–622 and 1309–1291 (Tam & Borriss, 1998). After cloning of the amplified DNA fragments into pGEM-T, sequence analysis was performed by the chain-termination method (Sanger *et al.*, 1977), using an ALF DNA sequencer (Amersham Pharmacia Biotech).

Macrorestriction of genomic DNA and electrophoretic separation of large DNA fragments by PFGE. High-molecular-mass DNA was prepared from 3 ml of mid-exponential-phase *Bacillus* cells (Birren & Lai, 1993). Cells were resuspended in 1 ml 20 mM Tris/HCl pH 8, 20 mM $MgSO_4$, 20% sucrose, 1 mM EGTA and treated with lysozyme (1 mg) for 30 min at 37 °C. Plugs were prepared by mixing with 1% low-melting-point agarose. Aliquots of 20 µl were allow to harden on ice. For each digest, two plugs were placed for 1 h in 150 µl ice-cold buffer (20 mM Tris/HCl pH 7·5, 5 mM $MgSO_4$, 50 mM NaCl, 1 mM EGTA, 1 mM DTT). After removing the buffer and replacing it with the appropriate digestion buffer, restriction with *Sfi*I (Pharmacia) was performed overnight as recommended by the manufacturer. The plugs were then loaded into the wells of 1% agarose gel (peqLab) in TBE buffer (0·045 M Tris/borate, 0·01 M EDTA). PFGE was done in a Rotaphor Type V (Biometra) for 18 h at 13 °C.

Transformation of *Bacillus* strains. Competent *B. amyloliquefaciens* FZB42 and FZB45 cells were obtained by applying a two-step protocol (Kunst & Rapoport, 1995) with slight modifications. Cells were grown in GCHE medium containing 1% glucose, 0·2% potassium L-glutamate, 100 mM potassium phosphate buffer (pH 7), 3 mM trisodium citrate, 3 mM $MgSO_4$, 22 mg ferric ammonium citrate l^{-1} and 0·1% casein hydrolysate. At the time of transition from exponential growth to the stationary phase (T_0, OD_{600} 2·1), the culture was diluted with an equal volume of GE medium (GCHE medium without casein hydrolysate). After dilution, incubation was continued for 1 h at 37 °C with shaking. DNA (0·3 µg pUK19 harbouring homologous phytase gene fragment) was added, and selection for transformants was carried out after overnight incubation at room temperature on kanamycin-containing agar plates. Applying these methods transformation rates of 4×10^3 for FZB42 and of $7{\cdot}1\times10^1$ for FZB45 per µg plasmid DNA were obtained.

Cloning of the phytase gene. The FZB45 phytase gene coding region including signal sequence was amplified with the oligonucleotide primer pair FAR06 and FAR22, derived from the *B. amyloliquefaciens* DS11 phytase gene sequence (Kim *et al.*, 1998). PCR was performed in 30 cycles (1 min 94 °C, 1 min 50 °C and 2 min 72 °C) with 2·5 U *Taq* polymerase (Appligene) and 1·0 U Pwo (PeQLab) in a PTC 100^{TC} programmable thermal controller (Biozym). The amplified DNA was isolated after running on 1·2% agarose gel with gel extraction kit QiAEX (Qiagen) and cloned within pGEM-T. DNA sequences were determined as described above. Comparisons with database sequences were done with the BLAST program package.

Construction of pGEMKm45. The *aphA3* (AF060241) gene was amplified from pUK19 plasmid DNA using primers KM1 and KM2 (Table 1). Then the *aphA3* gene was used to replace the FZB45 *phyA* internal 312 bp *Eco*RI–*Eco*RV fragment. The resulting kanamycin-resistance (*km*) cassette pGEMKM45 consists of the *aphA3* gene flanked by the remaining 5′ and 3′ sequences of the FZB45 phytase gene harboured in pGEM vector plasmid.

Overexpression, purification and characterization of FZB45 phytase. To overexpress phytase in *B. subtilis* the phytase gene was subcloned into the integrative *Bacillus* expression vector pSG1112, a derivative of pSG703 (Thornewell *et al.*, 1993) using the *Nde*I and *Bam*HI cloning sites of the vector plasmid. In this construct, the coding region of the FZB45 *phyA* gene is preceded by strong *Bacillus* phage ϕ105 holin promoter (Leung & Errington, 1995). After linearizing with *Sca*I the recombinant plasmid was used to transform competent *B. subtilis* MU331 containing defective prophage ϕ105 sequence (Thornewell *et al.*, 1993). The defective MU331 prophage has been deleted in a region needed for cell lysis, and contains a *ts* mutation in the phage immunity repressor allowing induction by a shift in temperature without concomitant cell lysis (Gibson & Errington, 1992). Transformed cells were selected on LB agar plates containing 5 mg chloramphenicol l^{-1}. Cm^R clones were screened by PCR using FZB phytase-specific primers FAR22 and FAR06. Extracellular phytase activity which was under control of the ϕ105 promoter and its ts repressor protein, was expressed in LB medium supplemented with 3% glucose after shifting the temperature to 50 °C for 10 min. Cells were harvested 5 h after thermo-induction. At this time phytase activity in culture fluid was about 1000 U l^{-1}. Phytase was purified to apparent homogeneity from the culture filtrate by cation-exchange chromatography. *myo*-Inositol phosphate isomers obtained after enzymic hydrolysis of phytate were separated by high-performance ion chromatography as described previously (Greiner *et al.*, 2000).

Plant culture. Surface-sterilized maize (*Zea mays* cv. Elita) seeds were grown in sterile agar supplied with phytate/low-phosphate medium. The aseptic 4-day-old seedlings were transferred into sterile tubes each containing 20 ml of a low-phosphate nutrient solution with the following composition per litre: $Ca(NO_3)_2.4H_2O$ 0·88 g, $NaH_2PO_4.2H_2O$ 0·006 g, K_2SO_4 0·39 g, $MgSO_4.7H_2O$ 0·31 g, FeEDTA ($C_{10}H_{13}O_8N_2Fe$) 0·0031 g and 1 ml micro-element solution. The pH was adjusted to 6·5 with 2 M NaOH.

Phytate adjusted to pH 6·5 was added to each tube, resulting in 1·12 mM final concentration. To 20 ml plant nutrient solution, 20 µl culture filtrates of wild-type FZB45, containing 3 mU phytase activity, and of mutant strain FZB45/M2, both grown for 24 h ASM medium, were added. Controls did not contain bacterial culture filtrates, but in order to study the effect of phytase alone in some cases 3 mU purified phytase was added to the plant nutrition medium. Plants were grown in a controlled-environment growth chamber (30 °C/16 h light period, 20 °C/8 h dark period and relative humidity 65/75 day/night). After 15 days, plants were harvested and dried, and plant growth parameters were determined.

Chlorophyll content. Chlorophyll content of the whole plant was measured after extraction with 80% ethanol at 90 °C, and filtration, by its absorbance at 663 and 645 nm. The chlorophyll content was calculated in mg l^{-1}.

RESULTS

Taxonomic position of PGPR *Bacillus* strains FZB24, FZB37, FZB42 and FZB45

Bacillus strains FZB24, FZB37, FZB42 and FZB45 were isolated from plant-pathogen-infested soil and shown to promote plant growth (Krebs *et al.*, 1998). Microscopic observation revealed that all strains were rods 0·6–0·8 µm wide and 2·0–4·0 µm long, able to form oval endospores. Growth characteristics and physiological

Table 2. Characterization of PGPR strains FZB24, FZB37, FZB42 and FZB45 in comparison with the type strains of *B. subtilis* 168 (*B. s.*) and *B. amyloliquefaciens* DSM7 (*B. a.*)

All strains were able to grow at 50 °C and in the presence of 7% NaCl but unable to grow at 55 °C; they were positive for lecithinase and catalase formation, nitrate reduction, Voges–Proskauer reaction, in using citrate as sole carbon source, in forming acid from glucose, xylose, arabinose and mannitol, and in hydrolysing starch, gelatin, casein and haemoglobin; they were unable to grow anaerobically, to produce gas from glucose, to deaminate phenylalanine or to produce urease, oxidase and indole.

Characteristic	FZB24	FZB37	FZB42	FZB45	*B. s.*	*B. a.*
Colonial morphology	rough	rough	rough	rough*	smooth	rough
Acid from lactose	+	−	+	+	−	+
Hydrolysis of Tween 80	−	+	−	−	+	−

* With a distinct centre.

Table 3. Comparison of 16S rDNA sequences of FZB24, FZB37, FZB42 and FZB45 with *B. subtilis* (*B. s.*) and *B. amyloliquefaciens* (*B. a.*)

Nucleotides different from the *B. subtilis* 168trpC2 *rrnE* gene sequence are shown in bold letters. Presence of the *thyA* gene (*thyA*) and the amylase-encoding genes *amyS* from *B. subtilis* and *amyA* from *B. amyloliquefaciens* is also indicated. Partial 16S rDNA sequences were amplified from chromosomal DNA isolated from *B. subtilis* FZB37 (FZB37), *B. amyloliquefaciens* DSM7 (AMYF), *B. amyloliquefaciens* S23 (AMYN), and *B. amyloliquefaciens* ATCC 15841, FZB24, FZB42 and FZB45.

Strain	16S rDNA	181	203	286	466	473	484	*thyA*	*amyA*	*amyS*	Phy*
B. s.											
168	AJ276351	G	A	A	A	G	T	+	−	+	−
FZB37	AY055222	G	A	A	A	G	T	+	−	+	−
B. a.											
AMYF†	AY055225	**C**	**G**	**G**	**G**	**A**	**C**	−	+	−	+
AMYN	AY055223	**C**	**G**	**G**	**G**	**A**	**C**	−	+	−	+
ATCC 15841	AY055226	**C**	**G**	**G**	**G**	**A**	**C**	−	+	−	+
FZB24	AY055219	G	**G**	**G**	**G**	**A**	**C**	−	+	−	+
FZB42	AY055221	G	**G**	**G**	**G**	**A**	**C**	−	+	−	+
FZB45	AY055224	G	**G**	**G**	**G**	**A**	**C**	−	+	−	+

* Expression of phytase.

† Type strain of *B. amyloliquefaciens* (DSM7).

investigations such as carbon source utilization (e.g. acid production from sugars) and biochemical reactions suggested that the strains are closely related to the *Bacillus subtilis*/*amyloliquefaciens* group. *B. amyloliquefaciens* and FZB24, FZB42 and FZB45 are distinguished from *B. subtilis* and FZB37 by their ability to produce lipase and acid from lactose (Table 2). The last property has been proven useful to distinguish *B. subtilis* and *B. amyloliquefaciens* (Priest *et al.*, 1987). Previous phage-susceptibility studies indicated that except FZB37, which clearly belongs to *B. subtilis*, the three other FZB strains are most related to *B. amyloliquefaciens* (Krebs *et al.*, 1998). In order to clarify this point further we performed a more extensive analysis involving 16S rDNA sequencing, ribotyping, macrorestriction profiling, and occurrence of the *thyA* and liquefying amylase genes.

In order to distinguish PGPR strains, 16S rDNA according to nucleotide positions 47–540 of the *B. subtilis rrnE* gene was amplified from all FZB strains using sequence-specific oligonucleotide primers, and sequenced. This region, which includes two hypervariant 5′ regions of the 16S *rrnE* gene (HV regions) has been shown to be a useful index for the identification or grouping of related *Bacillus* spp. (Goto *et al.*, 2000). Comparison between FZB37 and the *B. subtilis rrnE* gene sequence revealed a perfect match in this area. In contrast, the plant-growth-promoting strains FZB24, FZB42 and FZB45 share five nucleotide changes between positions 181 and 484 within the HV 5′ 16S rDNA sequence, underlining their different taxonomic positions. Since 16S rDNA sequences from *B. amyloliquefaciens* are not well represented in the public databases, we decided to determine the HV regions of *B.*

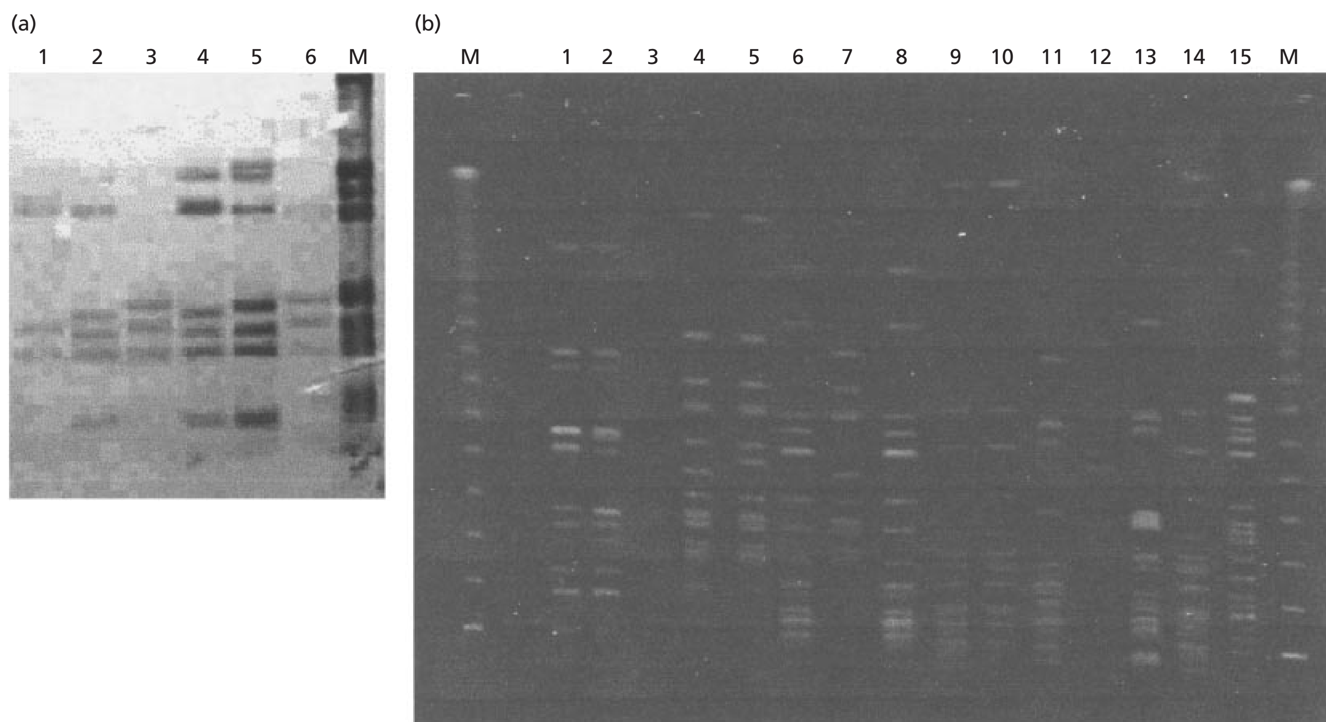

Fig. 1. (a) Riboprint analysis from *B. amyloliquefaciens* DSM7 (1), FZB24 (2), FZB37 (3), FZB42 (4), FZB 45 (5), *B. subtilis* 168 *trpC2* (6). M, *Eco*RI/*Hin*dIII digest of phage lambda DNA (21·2, 5·1, 4·3, 3·5, 2·0, 1·9, 1·6, 1·4, 0·95, 0·83, 0·56 kb). (b) Genomic DNA macrorestriction profiles of *Bacillus* strains produced by PFGE after *Sfi*I digestion. Strains: *B. subtilis* ATCC 6633 (1), *B. subtilis* W23 (2), *B. subtilis* SB19 (3), *B. subtilis* 168 *trpC2* (4,5), FZB24 (6), FZB37 (7), FZB42 (8), FZB45 (9), FZB45/M1 (10), *B. amyloliquefaciens* ATCC 15841 (11), *B. amyloliquefaciens* ZIMET 10639 (12), *B. amyloliquefaciens* ZF178 (13), FZB45/M2 (14), *Bacillus* sp. ILAT G 697/1 (15). M, molecular mass markers (48·5, 97, 145, 194, 242, 291, 339, 388, 436, 485, 582, 630, 679 kb).

amyloliquefaciens strains F and N. Except for nucleotide 181, their sequences were found to be almost identical with that of the above-mentioned plant-growth-promoting strains, suggesting that these *Bacillus* strains are closely related to *B. amyloliquefaciens* and clearly distinct from the *B. subtilis* group (Table 3).

The ribotype patterns of FZB24 and FZB42 obtained with *Eco*RI-digested genomic DNA probed with a 16S rDNA sample (see Methods) are nearly identical but clearly distinct from those of FZB37 and *B. subtilis* 168. FZB45 displays a unique pattern which is more related to FZB24 and FZB42 than to the type strain *B. amyloliquefaciens* DSM7 (ATCC 23350) (Fig. 1a). RiboPrinter Microbial Identification Systems (Qualicon) and analysis of cellular fatty acids of FZB24 (DSM 10271) performed by the DSMZ confirmed similarity with the type strain of *B. amyloliquefaciens* ATCC 23350 (similarity coefficient 0·63). According to those data FZB24 and FZB42 form a common ribogroup with *B. amyloliquefaciens* DSM 1065.

In addition, we analysed *Bacillus* strains by macrorestriction of genomic DNA with the restriction endonuclease *Sfi*I and electrophoretic separation of large DNA fragments with PFGE. The patterns of the plant-growth-promoting FZB strains were compared with the profiles obtained from representatives of *B. subtilis* and *B. amyloliquefaciens*. The method is more discriminating than 16S rRNA analysis and the FZB strains could easily be grouped as follows: FZB37 together with *B. subtilis* subsp. *subtilis* strains (group II) but distinct from *B. subtilis* subsp. *spizenii* (group I). FZB24 and FZB42 were not distinguishable in their restriction pattern and form group III. FZB45 and its mutants were unique in their restriction profiles (Fig. 1b).

Moreover, the *thyA* gene, which encodes a second thymidylate synthase in *B. subtilis* 168 and some strains related to *B. subtilis* but is not common within *B. amyloliquefaciens* (Tam & Borriss, 1998), was detected in FZB37 but not in FZB24, FZB42 and FZB45. Remarkably, *thy* genes which are very similar to the *thyA* gene reside within the genomes of some *B. subtilis* phages such as ϕ3T and SPβ22 (Kenny *et al*., 1985). Probably, the occurrence of the *thyA* gene is restricted to the *Bacillus* strains accessible for horizontal gene transfer by *B. subtilis* phages. These data coincide well with results previously obtained by phage-sensitivity studies, demonstrating that *B. subtilis* 168 and FZB37 share most features, whereas FZB24 and FZB42 form a different group similar to other representatives of *B. amyloliquefaciens* (Krebs *et al*., 1998).

Despite their close taxonomic relationship, *B. subtilis* and *B. amyloliquefaciens* possess very different α-

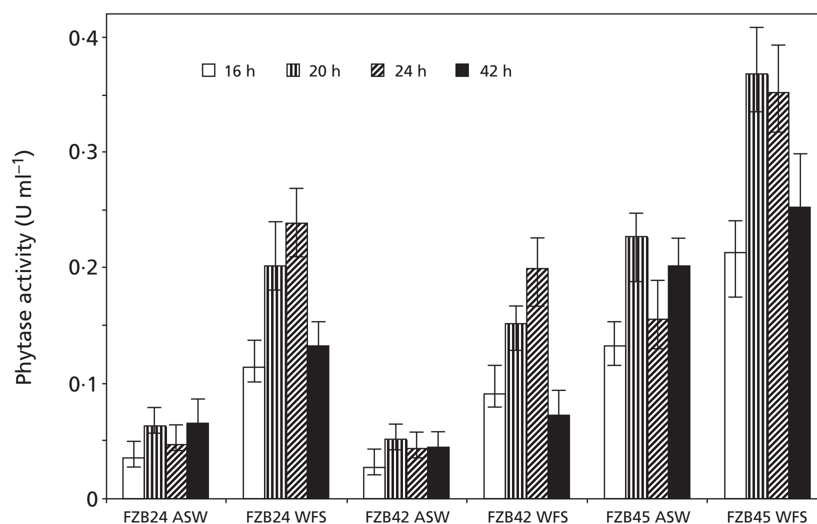

Fig. 2. Extracellular phytase activities expressed in of FZB24, FZB42 and FZB45 grown in wheat bran (WFS) and synthetic (ASW) medium. Culture filtrates were assayed for phytase activity after 16, 20, 24 and 42 h cultivation. Mean values ± SD of three independent experiments are shown. No activity was found in FZB37 and *B. subtilis* 168 under all conditions tested.

amylase genes. Whereas the amylase genes from *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* are highly homologous, the *B. subtilis* α-amylase is most closely related to the enzyme of *Butyrivibrio fibrisolvens* and displays no homology to the *B. amyloliquefaciens* amylase (Ferrari *et al.*, 1993). Therefore, determination of the amylase gene is a useful marker to distinguish the two species. Sequence-specific primers derived from the *B. amyloliquefaciens* liquefying α-amylase gene were successfully used to amplify the amylase genes from *B. amyloliquefaciens* DSM7 (control) FZB24, FZB42 and FZB45 but failed in FZB37 and *B. subtilis* 168 (control). Conversely, primers derived from the *B. subtilis* 168 amylase gene were successfully used in amplification of FZB37 amylase but did not amplify sequences from FZB24, FZB42 and FZB45, suggesting again that except FZB37 the plant-growth-promoting strains are most related to *B. amyloliquefaciens*.

In summary, our results strongly support the view that PGPR strains FZB24, FZB42 and FZB45 are clearly different from *B. subtilis*. Furthermore, we conclude that PGPR FZB37 belongs to *B. subtilis* subsp. *subtilis* (Nakamura *et al.*, 1999) whereas strains FZB24 and FZB42 are members of *B. amyloliquefaciens* (Priest *et al.*, 1987). Based on the features described in Table 3 and according to the results of ribotyping and macrorestriction profiling, FZB45 should also be classified as a member of the *B. amyloliquefaciens* group.

Ability to secrete phytase activity is restricted to the *B. amyloliquefaciens* strains

A survey of the spectrum of extracellular enzymes revealed that macromolecule-hydrolysing enzymes such as amylases, proteases and glucanases were secreted by all of the PGPR strains investigated (data not shown). In addition, *B. amyloliquefaciens* strains FZB24, FZB42 and FZB45 exhibited extracellular phytase activity (Fig. 2) whereas *B. subtilis* FZB37 and strain 168 (control) did not. Phytase activity was mainly produced during the late stage of exponential growth and during the transition to stationary growth phase, suggesting that similar to other extracellular depolymerases phytase acts as a 'scavenger' enzyme after exhaustion of rapidly metabolized nutrient sources. Thus, we conclude that under phosphate starvation in soil, the ability of phytase-secreting *B. amyloliquefaciens* strains to make phytate phosphorus available for plant nutrition might contribute to their plant-growth-promoting activity. Phytase was constitutively produced largely independent of the composition of the nutrient medium, and no correlation between the concentration of phosphate and the presence of phytate could be detected. For most strains a soybean digest (0·3 % w/v), casein peptone (0·3 % w/v) medium supplemented with 10 % (w/v) soil extract and 0·5 % (w/v) starch was most favourable. Strain FZB45 displayed the highest phytase activity and was chosen for further genetic and biochemical experiments.

B. amyloliquefaciens phytase genes might have evolved from an alkaline phosphatase ancestor molecule

The high degree of sequence homology with *B. amyloliquefaciens* DS11 permits amplification of the FZB45 phytase-encoding sequence, including the signal peptide, using genomic DNA isolated from FZB45 as a target with an oligonucleotide primer pair derived from the 5′ and 3′ regions of *B. amyloliquefaciens* DS11 phytase (Kim *et al*, 1998). The cloned 1·1 kb fragment enabled the *B. subtilis* MU331 host strain, which displays no apparent phytase activity, to express *Bacillus* phytase activity. The FZB45 phytase gene encodes a 383 aa polypeptide with high similarity to the deduced amino acid sequences of the *B. amyloliquefaciens* DS11 phytase (98 % identical residues) and the *B. subtilis* VTT E-68013 phytase (93 % identical residues; Kerovuo *et al.*, 1998). The *Bacillus* phytases do not share sequence homology with other phytases and do not possess the

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PHY_DS11      KHKLSDPYHFTVNAAAETEPVDTAGDAADDPAIWLDPKNP------QNSKLITTNKKSGL
PHY_FZB45     KHKLSDPYHFTVNAAAETEPVDTAGDAADDPAIWLDPKNP------QNSKLITTNKKSGL
APASE         KKQDKAEIRNVIVMIGDGMGTPYIRAYRSMKNNGDTPNNPKLTEFDRNLTGMMMTHPDDP
              *:: .   : .:   .:   .       .       *:**      :* . :  .: ..
                      ↓
PHY_DS11      AVYSLEGKMLHSYHTGKLNNVDIRYDFPLNGKKVDIAAASNRSEGKNTIEIYAIDGKNGT
PHY_FZB45     AVYSLEGKTLHSYHTGKLNNVDIRYDFPLNGKKVDIAAASNRSEGKNTIEIYAIDGKNGT
APASE         DYNITDSAAAGTALATGVKTYNNAIGVDKNGKKVKSVLEEAKQQGKSTGLVATSEINHAT
                   :.    :  :  ::. :   ..  *****. .  . :.:**.*  : : : ::.*
                     ↓
PHY_DS11      LQSITDPNRPIASAIDEVYGFSLYHSQKTGKYYAMVTGKEGEFEQY------ELNADKNG
PHY_FZB45     LQSITDPDRPIASAIDEVYGFSLYHSQKTGKYYAMVTGKEGEFEQY------ELNADKNG
APASE         PAAYGAHNESRKNMDQIANSYMDDKIKGKHKIDVLLGGGKSYFNRKNRNLTKEFKQAGYS
                :    :..  .  : . .:   : : . *  .:: * :. *::       *::    .

PHY_DS11      YISGKKVRAFKMNSQTEGMAAD----------------------------DEYGSLYIA
PHY_FZB45     YISGKKVRAFKMNSQTEGMAAD----------------------------DEYGSLYIA
APASE         YVTTKQALKKNKDQQVLGLFADGGLAKALDRDSKTPSLKDMTVSAIDRLNQNKKGFFLMV
              *:: *:.   : :.*. *: **                             :: * : :.

PHY_DS11      EEDEAIWKFSAEPDGGSNGTVIDRADGRHLTPDIEG-----LTIYYAADGKGYLLASSQG
PHY_FZB45     EEDEAIWKFSAEPDGGSNGTVIDRADGRHLTPDIEG-----LTIYYAADGKGYLLASSQG
APASE         EGSQIDWAAHDNDTVGAMSEVKDFEQAYKAAIEFAKKDKHTLVIATADHTTGGFTIGANG
              * .:  *    :   *: . * *  :. : : ::       *.*  * . .* :  .::*

PHY_DS11      NSSYAIYERQGQNKYVADFQITDGPETDGTSDTDGIDVLGFGLGPEYPFGLFVAQDGENI
PHY_FZB45     NSSYAIYERQGQNKYVADFQITDGPETDGTSDTDGIDVLGFGLGPEYPFGLFVAQDGENI
APASE         EKNWHAEP-ILSAKKTPEFMAKKISEGKPVKDVLARYANLKVTSEEIKSVEAAAQADKSK
              :..:       . * ..:* .. .* . ..*. .  .     . *      .** .:.
                   ↓                                                ↓
PHY_DS11      DHGQKANQNFKMVP----------------WERIADKIGFHPQVN--KQVDPRKMTDRSG
PHY_FZB45     DHGQKVNQNFKMVP----------------WERIADKIGFHPQVN--KQVDPRKLTDRSG
APASE         GASKAIIKIFNTRSNSGWTSTDHTGEEVPVYAYGPGKEKFRGLINNTDQANIIFKILKTG
              . .:   : *:  .                :   ..*  *:  :*  .*.:      ::*

PHY_DS11      K
PHY_FZB45     K
APASE         K
              *
```

Fig. 3. Sequence comparison of mature phytases of DS11 (sp O66037) and FZB45 (gb AY055220) with alkaline phosphatase PhoA IV of *B. subtilis* (P19406). Residues different in the two phytases are marked by vertical arrows. Stars on the consensus line indicate identical residues; colons indicate similar residues in phosphatase and phytase.

RHGXRXP active-site motif characteristic of acid phosphomonoesterases and phytases of fungal or plant origin. Notably, 50 residues identical with the *B. subtilis* alkaline phosphatase PhoA IV (Hulett *et al.*, 1991) and yielding 14 % sequence homology are distributed within the entire sequence of the mature phytase (Fig. 3). The central NGKKV motif is present in both groups of enzymes. However, the D-S-A/G triad known as the phosphorylation site in alkaline phosphatases is only partially conserved as an H-S-Y sequence motif and most of the functional residues reported to be involved in high-affinity and low-affinity calcium-binding sites in *Bacillus* phytase (Ha *et al.*, 2000) do not occur in alkaline phosphatase.

Expression and properties of *B. amyloliquefaciens* FZB45 3-phytase

The purified phytase protein expressed in recombinant *B. subtilis* MU331 (see Methods) exhibited a specific activity of 86 U (mg protein)$^{-1}$ and was characterized by high-performance ion chromatography analysis as a 3-phytase (EC 3.1.3.8). Initial hydrolysis of the phosphate ester occurred at the D/L-3 position of Ins(1,2,3,4,5,6)P6 to yield D/L-Ins(1,2,4,5,6)P5. Final products of hydrolysis were *myo*-inositol triphosphates (R. Greiner, unpublished results) as reported for *B. subtilis* phytase (Kerovuo *et al.*, 2000). In contrast to the phytases from *E. coli* (Greiner *et. al.*, 2000) and *Klebsiella terrigena* (Greiner *et al.*, 1997) the phytase of *B. amyloliquefaciens* FZB45 did not hydrolyse the general phosphatase substrates *p*-nitrophenyl phosphate, 1-naphthyl phosphate and 2-naphthyl phosphate to a significant extent. Phytase of FZB45 is clearly more thermostable than the phytases of *B. amyloliquefaciens* FZB24 and FZB42 and has a temperature optimum near to 70 °C (results not shown). Other biochemical properties of the thermostable enzyme, which is resistant to pancreatin but not to pepsin and displays its highest activity at pH 7, are as described for the native enzyme from FZB45 (Igbasan *et al.*, 2000). Most of these properties, such as substrate specificity, temperature and pH behaviour, are shared by the *Bacillus* phytases from VTT E-68013 (Kerovuo *et al.*, 1998) and DS11 (Kim *et al.*, 1998).

Insertional inactivation of the 3-phytase-encoding gene of FZB45

To investigate the role of phytase of *B. amyloliquefaciens* FZB45 in growth promotion, the phytase-encoding gene was inactivated in the chromosome by a gene-replacement strategy using DNA transformation and homologous recombination with a linear DNA fragment consisting of a *km* cassette flanked by FZB45 *phyA* sequences. The isogenic mutant generated was only altered in its ability to express extracellular phytase.

Plasmid pGEMKm45, containing the amplified phytase gene of FZB45 with the *phyA* internal 312 bp *Eco*RI–*Eco*RV fragment replaced with a kanamycin resistance (*km*) cassette was constructed (see Methods). After digestion of the recombinant plasmid with *Pst*I

Table 4. Effect of *B. amyloliquefaciens* spores and culture filtrates, and of purified phytase, on maize seedlings grown in phosphate-limited nutrient solution

Seedlings were grown in 20 ml low-phosphate (LP) medium, with and without 4 mM sodium phytate (see Methods), and with the following additions as shown in the table: 1×10^9 spores of FZB45; 0·1% (w/v) culture filtrate (CF) of FZB45 and of its phytase-negative mutant FZB45/M2; purified phytase. The data are mean values ± SD of 14 seedlings grown in at least three independent experiments. Addition of *meso*-inositol (4 mM) to the plant growth medium did not affect plant growth.

	LP control	LP + 4 mM *meso*-inositol	LP + 4 mM phytate	LP + 4 mM phytate FZB45 spores	LP + 4 mM phytate CF FZB45	LP + 4 mM phytate CF FZB45/M2	LP + 4 mM phytate phytase*
Phytase†	0	0	0	720 mU	3 mU	0	3 mU
Phosphate‡	0·01 (1), 0·00 (2)	0·01 (1), 0·01 (2)	0·01 (1), 0·01 (2)	0·81 (1), 1·58 (2)	0·19 (1), 0·19 (2)	0·02 (1), 0·02 (2)	0·18 (1), 0·20 (2)
Shoot weight (mg)	142 ± 7	133 ± 6	114 ± 9	214 ± 20	202 ± 17	142 ± 11	198 ± 16
Root weight (mg)	141 ± 9	132 ± 8	112 ± 7	226 ± 19	183 ± 8	130 ± 7	193 ± 9
Root length (mm)	188 ± 12	178 ± 10	178 ± 8	260 ± 13	239 ± 17	192 ± 12	249 ± 9
Chlorophyll (mg l^{-1})	ND	ND	0·6	ND	3·6	1·2	4·2

ND, Not determined.

* Recombinant phytase of FZB45 expressed in MU331 and purified to homogeneity as described in the text.

† Phytase activity present in 20 ml plant nutrient medium.

‡ mM, in plant nutrition medium after one (1) and two (2) weeks cultivation of the seedlings.

and *Sph*I, the *phyA*::*km* insert was isolated and at least 1 µg of the linear DNA was used to transform competent FZB45 cells. Three Km^R transformants generated by a double crossover event were isolated and shown not to express phytase activity. Chromosomal DNA of the phytase-deficient FZB45 transformants (FZB45/M1 and FZB45/M2) was used to confirm the presence and the correct integration of the *km* cassette by amplification of the kanamycin-resistance-encoding *aphA3* gene and by Southern hybridization using plasmids pGEMKm and pGEMKm45 as probes (data not shown).

Establishment of an efficient transformation system was necessary for application of this strategy. Surprisingly, it turned out that by using a slight modification of the two step protocol (Kunst & Rapoport, 1995) competent *B. amyloliquefaciens* FZB42 and FZB45 cells were obtained (see Methods). Except for phytase activity, no physiological and morphological differences were detected between the wild-type and mutant strains. Riboprinting and PFGE confirmed the identity of the wild-type and mutant strains (Fig. 1b).

FZB45 culture filtrate promotes growth of phosphate-limited maize seedlings

Growth of maize seedlings in liquid medium under low-phosphate conditions in the presence of phytate was greatly enhanced when inoculated with spores of strain FZB45. Especially, the development of the root system was stimulated. Phytase activity was detected in the combined bacteria–plant system but not in control experiments performed with maize seedlings without bacteria. Moreover, the phosphate content measured in the presence of FZB45 was sufficient to support plant growth (Table 4). Control experiments revealed that the deficiency of available phosphorus in the plant nutrition medium retarded plant growth in various parameters such as root length, and masses of the root and shoot systems. Presence of *meso*-inositol hexaphosphate (phytate) or of its dephosphorylated form (*meso*-inositol) did not improve growth under conditions of low phosphate, suggesting that only the increase in the available phosphorus supports growth of plants (Table 4).

To address the influence of secreted phytase on plant growth conferred by treatment with PGPR, a gnotobiotic model system was established (see Methods). Growth of seedlings in phosphate-limited medium containing phytate was stimulated by dilute culture supernatant of strain FZB45 (Fig. 4a). Despite the fact that phytase activity present in the culture filtrate was comparatively low (3 mU) the phosphate content in the plant nutrition medium was strikingly enhanced, sufficient to support plant growth in the low-phosphate medium The chlorophyll content was increased sixfold compared with maize seedlings grown in presence of phytate without FZB45 culture filtrate (Table 4).

It is tempting to speculate that the growth promotion observed in presence of the culture filtrate of FZB45 was due to its extracellular phytase activity. Growth improvement could be explained by increased supply with additional phosphorus and/or reduction of harmful effects by binding of cations essential for plant metabolism to phytate, due to phytate hydrolysis. Reduction in the chlorophyll content might be due to the formation of phytate–cation complexes preventing uptake of e.g. Mg^{2+}.

Phytase activity contributes to the plant-growth-promoting effects of FZB45

Culture filtrates obtained from wild-type (phytase plus) and mutant strains (phytase minus) were used in a sterile test system to compare their effects on maize seedlings grown under phosphate limitation in the presence of phytate (see Methods).

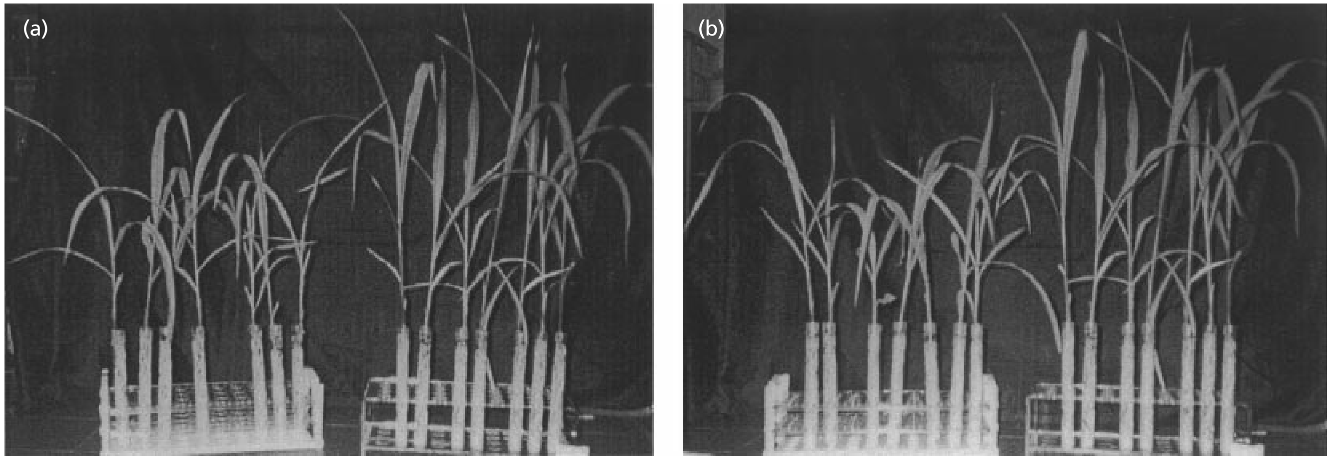

Fig. 4. Influence of culture filtrates of FZB45 grown for 24 h in synthetic (ASW) medium on development of maize seedlings. (a) Left, seedlings grown in the presence of phytate (1·12 mM) and under phosphate limitation, without culture filtrate; right, seedlings grown in the presence of 0·1 % culture filtrate of FZB45 (*phyA*$^{+}$, expressing phytase activity). (b) Seedlings grown with culture filtrates of: (left) FZB45/M2 (Δ*phyA*, without phytase activity) and (right) FZB45 (*phyA*$^{+}$, expressing phytase activity).

Whereas plants treated with the culture filtrate of the wild-type were improved in all the parameters measured as described above, addition of the culture filtrate of the phytase-minus mutant resulted in poor root growth and reduced chlorophyll content. Control experiments performed without culture filtrates but containing FZB45 phytase purified from recombinant *B. subtilis* MU331 cells supported the idea that under phosphate limitation, but in the presence of phytate, the phytase activity secreted by *B. amyloliquefaciens* FZB45 is beneficial for plant growth in making phytate phosphorus available (Fig. 4b). As shown in Table 4, small amounts of the *Bacillus* phytase activity (3 mU per 20 ml plant nutrient medium) present in diluted culture filtrates or as the purified enzyme are sufficient to cleave off orthophosphate from phytate and to enhance plant growth. The phytase activity developed by *B. amyloliquefaciens* FZB45 upon inoculation of maize seedlings in the low-phosphate plant nutrition medium was about 20 times higher, suggesting that the phytase productivity of FZB45 might be sufficient to support plant growth under low-phosphate conditions in the natural environment (Table 4).

DISCUSSION

Using a genetic approach, we have obtained evidence that secreted phytase activity present in the culture filtrate of the PGPR *B. amyloliquefaciens* FZB45 promotes growth of maize seedlings in a sterile system consisting of a phosphate-limited plant nutrient medium in the presence of phytate, which accounts for up to 80 % of total phosphorus in cereals and legumes (Reddy *et al*., 1989). Culture filtrates of isogenic mutant strains with a *km* cassette inserted within the central region of the *phyA* gene, and not capable of producing extracellular phytase, did not stimulate plant growth. Given that these results can be confirmed after introduction of the isogenic *phyA* and *phyA*$^{+}$ strains in a system more closely related to the complex natural soil system, our findings support the observation that soil microorganisms possessing phytase activity do contribute significantly to plant phosphorus nutrition (Richardson *et al*., 2001b).

Although plants have developed numerous mechanisms to increase the availability of soil phosphorus, utilization of phytate phosphorus from the soil is very limited due to low phytase activity present within rhizosphere. Phytase activity in *Arabidopsis* roots contributes only 0·5 % of the total phosphomonoesterase activity and to our knowledge no extracellular plant phytase has been detected to date. Limited bioavailability of phytate phosphorus was also observed for a range of agricultural plants, including wheat and various forage species (Richardson *et al*., 2000; Hayes *et al*., 2000). Two phytase genes were cloned from maize but again the phytases encoded by these gene are not secreted (Maugenest *et al*., 1999). Due to their lack of extracellular phytase activity, plants utilize soil phytate, a major component of organic phosphorus, only poorly. In contrast, transgenic *Arabidopsis* plants expressing extracellular *Aspergillus* phytase were shown to obtain their phosphorus requirement directly from phytate (Richardson *et al*., 2001a). Addition of purified phytase to the rooting medium has the same effect (Findenegg & Nelemans, 1993; this study), underlining the importance of phytate availability for plant nutrition.

In this study we have demonstrated that numerous *B. amyloliquefaciens* strains have evolved a functional phytate-degrading enzyme from an ancestor which was most likely alkaline phosphatase, an enzyme common in Gram-positive and Gram-negative bacteria (Fig. 3). Besides *B. amyloliquefaciens* FZB45, PGPR FZB24 and

FZB42 are able to secrete phytase activity. Sequence analysis revealed that FZB24 and FZB42 harbour phytase genes very similar to that of FZB45 but clearly different from that of *B. subtilis* 168 (unpublished results). Extracellular phytase activity was also detected in *B. amyloliquefaciens* strains ATCC 15841, DSM7 and BE20/71 (unpublished observations), suggesting that production of extracellular phytase is a common property in PGPR and other *B. amyloliquefaciens* strains, whereas *B. subtilis* strains FZB37 and 168 did not express functional phytase. Failure to express phytase activity was unexpected since the genome of *B. subtilis* 168 contains a *phy* gene (http://genolist.pasteur.fr/SubtiList/P42094) that could be functionally expressed in recombinant *E. coli* and *B. subtilis* hosts strains if fused with heterologous promoters (A. Farouk, unpublished observation). The biocontrol strain FZB24, which is related to FZB45, is commercially available (FZB Biotechnik GmbH, Berlin, Germany). Application of strain FZB45 under conditions of limited availability of phosphate might be favourable because of its high phytase productivity and the high stability of the secreted enzyme. Moreover, we have obtained first experimental hints that FZB45 is able to colonize plant roots and to express a high local concentration of phytase at the surface of the roots (E. E. Idriss, unpublished observation), making this bacterium very suitable for application as a PGPR.

Extracellular *Bacillus* 3-phytase belongs to the group of 3-phytases, since it hydrolyses phytate first at the D/L-3 position. The end-products of phytate degradation have been identified as *myo*-inositol trisphosphates and orthophosphate (Kerovuo *et al.*, 2000; this paper). Those lower inositol derivatives may provide a nutrient source for soil bacteria. Recently, two inositol transporters acting in *B. subtilis* have been identified (Yoshida *et al.*, 2002). Enzymes responsible for inositol catabolism are encoded by a gene family clustered within the *iol* operon. They are induced in the presence of inositol (Yoshida *et al.*, 1997). *Rhizobium* strains that are capable of utilizing certain inositol derivatives are better colonizers of their host plants. Interestingly, a functional inositol dehydrogenase was shown to be required to establish symbiotic nitrogen fixation of *Sinorhizobium fredii* in soybeans (Jiang *et al.*, 2001), a finding that suggests a general role of inositol derivatives in plant–bacteria interactions. However, several questions still remain concerning the fate of phosphorylated inositol derivatives and regulation of phytase expression:

1. Do the phytase-producing *Bacillus* spp. take up lower phosphorylated inositol derivatives, and how is expression of the phytase-encoding gene regulated?

2. Are other (intra- or extracellular) phosphomonoesterases able to split off the remaining phosphate groups of *myo*-inositol triphosphates produced by the *Bacillus* 3-phytase?

3. Are there 'synergistic' effects of several extracellular enzyme activities produced by different rhizosphere micro-organisms (bacteria and fungi) that could lead to complete breakdown of *myo*-inositol phosphates?

This study can only be considered as a starting point in the molecular analysis of factors responsible for plant growth stimulation exerted by the PGPR *Bacillus* strains here identified as *B. amyloliquefaciens*. Clearly, the identification of the *phyA* gene whose expression influences plant growth in a model system established in this study calls for verification under conditions which better mimic the natural environment. Moreover, identifying phytase activity as a factor responsible for phytostimulation by the plant-root-colonizing *B. amyloliquefaciens* strains does not exclude that other metabolic activities of *Bacillus* spp. contribute to this process. Representatives of *B. subtilis* such as FZB37 not expressing phytase also have the capacity to stimulate plant growth and to act as biocontrol strains (Krebs *et al.*, 1998).

An analysis of the possible role of low-molecular-mass IAA-like compounds secreted by PGPR *B. amyloliquefaciens* in phytostimulation is in progress.

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