Screening of plant growth promoting traits of *Bacillus thuringiensis*

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Abstract - This study aimed to evaluate the plant growth promoting (PGP) potential of *Bacillus thuringiensis*. In this context, several genetic determinants of factors implicated in PGP potential were investigated by polymerase chain reaction (PCR) in 16 *B. thuringiensis* strains of different origin and belonging to different subspecies. PCR screening was performed on acid phosphatase, phytase, siderophore biosynthesis protein, 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indolpyruvate decarboxylase (*ipdC*). Production of indol acetic acid (IAA)-like compounds and of ACC deaminase, and capability of solubilising mineral phosphate were investigated by phenotypic tests. All the strains were PCR positive for the presence of the siderophore biosynthesis protein, ACC deaminase and acid phosphatase genes. Five and seven strains gave an amplicon with the expected length for the phytase and *ipdC* genes respectively. All the strains produced IAA compounds and seven had a high capacity to solubilise inorganic phosphorous. Qualitative phenotypic test for ACC deaminase activity showed that seven strains are able to grow on salt minimal medium containing ACC as sole nitrogen source, indicating the expression of the *accd* genes. Our screening results in thirteen strains having more than one PGP trait and showed that *B. thuringiensis* harbours and expresses several PGP determinants that could be very interesting in field application to enhance the plant growth. To our knowledge, this is the first report on the multiple plant growth promoting potential of *B. thuringiensis*.

Key words: *Bacillus thuringiensis*, PGPR, IAA, ACCD

INTRODUCTION

Bacillus thuringiensis is a Gram-positive spore-forming bacterium commonly known as an important biocontrol agent. It is widely used as bioinsecticide for the control of many agricultural insect pests and vectors of human diseases (Chattopadhyay *et al.*, 2004). This is owing to its ability to produce during sporulation characteristic proteinaceous crystalline toxins (Δ-endotoxins) exhibiting specific activities against different orders of insect and nematodes (Schnepf *et al.*, 1998). *Bacillus thuringiensis* could also act as a potential biocontrol agent against different fungal and bacterial species owing to production of antimicrobial molecules such as zwittermicin A (Stabb *et al.*, 1994; Cherif *et al.*, 2003), chitinases (Arora *et al.*, 2003), glucanases, HCN (Raddadi *et al.*, 2007; Raddadi *et al.*, unpublished), bacteriocins (Cherif *et al.*, 2003), and the quorum-sensing-quenching enzymes N-acyl homoserine lactone-lactonases (Dong *et al.*, 2000).

Plant growth-promoting bacteria are endophytic and free-living soil bacteria that can either directly or indirectly facilitate the growth of plants. Indirect stimulation of plant growth includes a variety of mechanisms by which the bacteria prevent phytopathogenic microorganisms from inhibiting plant growth and development. Direct stimulation may include providing plants with fixed nitrogen, iron that has been sequestered by bacterial siderophores, soluble phosphate and other nutrients, and the ability to produce suitable amounts of the plant hormones such as indole-3-acetic acid (IAA), gibberellic acid and cytokinins (Bloemberg and Lugtenberg, 2001) and to lower the levels of the plant ethylene hormone mediating 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Glick, 2005).

In this study, for the first time, an investigation of the potential of *B. thuringiensis* as a biostimulator and biofertiliser bacterium that could promote the plant growth was carried out on 16 strains representing different subspecies (ssp.) and wild isolates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Bacillus* strains used in this study were routinely maintained at 4 °C after growth at 30 °C on Tryptic soy broth (TSB) or Nutrient agar. For longer-term maintenance, stock cultures were stored in 25% (v/v) glycerol, 75% (v/v) TSB at -20 and -80 °C. The *Bacillus thuringiensis* strains used in this work were obtained from different collections and are listed in Table 1.

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TABLE 1 - Screening of plant growth promoting traits in *Bacillus thuringiensis* isolates

Strains*	Organic P solubilisation[a]		Inorganic P solubilisation[b]	Siderophore shynthesis gene[a]	*ipdC*[a]	IAA[c] (μg ml^{-1})	*accd*[a]	ACCD[d]
	Phytase	Acid phosphatase						
BMG1.7 ssp. *thuringiensis*	+	+	+++	+	+	1.60 ± 0.02	+	+
HD9 ssp. *entomocidus*	+	+	++	+	–	9.71 ± 0.22	+	–
HD22 ssp. *thuringiensis*	+	+	++	+	+	2.77 ± 0.05	+	+
HD110 ssp. *entomocidus*	+	+	+	–	–	8.18 ± 0.21	+	–
HD125 ssp. *tolworthi*	+	+	+	+	+	6.95 ± 0.1	+	–
HD868 ssp. *tochigiensis*	–	+	+++	+	+	3.11 ± 0.03	+	+
HD932 ssp. *dakota*	–	+	+++	+	–	2.49 ± 0.14	+	–
HD1012 ssp. *shandogiensis*	+	+	+	+	+	1.88 ± 0.23	+	–
H45	–	+	++	+	+	1.61 ± 0.3	+	–
H51	–	+	+/–	+	–	2.22 ± 0.07	+	–
H77	–	+	+++	–	+	1.73 ± 0.01	+	+
H112	–	+	+	+	–	1.71 ± 0.07	+	+
H150	–	+	+/–	+	–	2.06 ± 0.14	+	–
H152	–	+	+	+	–	2.25 ± 0.1	+	–
H156	–	+	+	+	–	1.53 ± 0.4	+	+
H172	–	+	+/–	+	–	2.04 ± 0.36	+	+

* Sources of *B. thuringiensis* strains. HD strains: Bacillus Genetic Stock Center, strains provided by D.R. Zeigler (HD 868) and B.M. Hansen; H strains: Department of Biological Sciences, University of Jordan, Amman, Jordan, strains were provided by H. Khyami-Horani; strain BMG1.7: LMBA University of Tunis, Tunis Tunisia.
[a] + indicates a PCR product of the expected size; –, no PCR product was formed, or the size of the product deviated significantly from the expected size.
[b] inorganic P solubilisation was qualitatively estimated based on the increasing decolouration (from +/– to +++) of bromophenol blue due to the pH drop of the medium.
[c] The concentration of IAA in each culture medium was determined by comparison with a standard curve. Results presented here are means ± SD of three independent experiments.
[d] Qualitative ACC deaminase estimation based on the $OD_{600\ nm}$ of the bacterial culture after 72 h of incubation in DF mineral medium containing ACC as unique nitrogen source. +, OD was stable or increased over time; –, the cells were lysed and an OD decrease over time occurred.

PCR analysis. Total DNA was extracted from 12-14 h cultures in Luria-Bertani (LB) medium by sodium dodecyl sulphate-proteinase K treatment (Daffonchio *et al.*, 2000). PCR was performed as follows: two microlitres (about 60 ng) of DNA extract were amplified with 1.0 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Milan, Italy), 1 μM of each primer, 2.5 mM of $MgCl_2$, 1x Taq polymerase buffer (Amersham Pharmacia Biotech) and 0.12 mM of dNTPs in a 25-ml reaction mixture in an I-cycler (Bio-Rad, Milan, Italy). Thermal protocol consists of 30 cycles of denaturation at 94 °C for 1 min, annealing for 45 s at the suitable temperature (see Table 2), and extension at 72 °C for 2 min. PCR products were analysed by 1.2% agarose gel electrophoresis in 0.5 Tris-borate-EDTA buffer and stained for 30 min in an 0.5-mg l^{-1} solution of ethidium bromide. PCR products were sequenced with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) as described by the manufacturer. The primers used to generate the PCR products were used to sequence the DNA on both strands in an ABI Prism 310 DNA capillary sequencer (Applied Biosystems, Monza, Italy) equipped with a 47 cm capillary. Each sequence was checked manually and searched for sequence similarities in databases with the assistance of the BLAST facilities. Nucleotide sequences of the primers used in this study are shown in Table 2.

Phenotypic tests. Inorganic phosphorous solubilisation was carried out as described by Mehta and Nautiyal (2001). In brief, strains were grown for 16 h on Nutrient broth (NB), then 100 μl of bacterial culture was inoculated in triplicate into 5 ml NBRI-BPB medium containing (in g l^{-1}) glucose 10, $Ca_3(PO_4)_2$ 5, $MgCl_2 \cdot 6H_2O$ 5, $MgSO_4 \cdot 7H_2O$ 0.25, KCl 0.2, $(NH_4)_2SO_4$ 0.1, and bromophenol blue (BPB) 0.025. The medium was adjusted to pH 7.0 before autoclaving. Qualitative estimation of phosphate solubilisation (based on the qualitative estimation of BPB) was carried out by measuring the absorbance at 600 nm of the culture supernatant after 72 h of incubation. NBRI-BPB uninoculated medium was used as blanc.

For quantification of IAA production, bacterial strains were propagated overnight in 5 ml of NB medium. Then 20 μl aliquots were transferred into 5 ml of NB medium supplemented with D-L-tryptophan (BDH, Biochemicals, England) at a final concentration of 500 μg ml^{-1} (from a filter-sterilised 2-mg ml^{-1} stock prepared in warm water). After incubation for 72 h, the bacterial cells were removed from the culture medium by centrifugation (3500 rpm, 10 min). A 1-ml aliquot of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent made of 150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$ (Gordon and Weber, 1951) and allowed to stand at room temperature for 20 min before the

TABLE 2 - Results from PCR and/or phenotypic screening of plant growth promoting traits in the *Bacillus thuringiensis* isolates

Primer set	Primer name[a]	Annealing temperature (°C)	Primer sequence (5´→ 3´)[b]	Target gene	Position (GenBank Accession N.)
Sider	F-Sider	55	GAGAATGGATTACAGAGGAT	Siderophore biosynthesis protein	1901958-1901977
	R-Sider		TTATGAACGAACAGCCACTT		1903687-1903668
					(AE017355)
Auxin	F-ipdC	50	CAYTTGAAAACKCAMTATACTG	Indole pyruvate decarboxylase	92483-92462
	R-ipdC		AAGAATTTGYWKGCCGAATCT		90806-90827
					(AE017272)
ACCD	F-accd	52	GTGAACCACCTGAATGTA	ACC deaminase	3074786-3074803
	R-accd		AAACGAGATGATTTACTTGG		3075641-3075622
					(AE017355)
Ac Pho	F-AcPho	55	AAGAGGGGCATTACCACTTTATTA	Acid phosphatase	4456184-4456207
	R-AcPho		CGCCTTCCCAATCRCCATACAT		4456916-4456895
					(AE016877)
phy	F-phy	48	TATGATTTTCCGTTGAAC	Phytase	406-42
	R-phy		ATTCCGTCTGTATCGCTTGT3		1040-1021
					(AF292103)

[a] F, forward strand; R, reverse strand.
[b] In the degenerate primers, the sequence is given according to the degenerate DNA genetic code: K = G or T; M = A or C; W = T or A; Y = C or T.

absorbance at 535 nm was measured. The uninoculated Trp-containing medium mixed with the Salkowski reagent was used as blank. The concentration of IAA in each culture medium was determined by comparison with a standard curve. Results presented are means of three independent experiments.

Qualitative ACC deaminase activity was carried out as described by Penrose and Glick (2003). Briefly, strains were grown on 15 ml TSB for 4 h, then cells were harvested by centrifugation (6000 *x g*, 10 min, 4 °C) and the pellet was washed three times with 5 ml of DF (Dworkin and Foster, 1958) salt minimal medium. Cells were then resuspended in 7.5 ml of DF salt minimal medium in a fresh culture tube. Just prior incubation, ACC (Sigma) was added at a final concentration of 3 mM (from a 500 mM-stock prepared in H_2O and stored at -20 °C). Strains were then incubated for 72 h (30 °C, agitation) and bacterial growth was followed by measuring the OD at 600 nm.

RESULTS AND DISCUSSION

The inorganic phosphate solubilisation and the phytohormone and siderophore production activities were screened by PCR and/or phenotypic tests on 16 strains of *B. thuringiensis*. Two primer pairs were used to amplify genes encoding acid phosphatase and phytase, the two enzymes implicated in organic phosphate and phytate solubilisation respectively. All the strains gave an amplified fragment of the expected MW (734 bp) for the first enzyme, and only strains HD9, HD110, HD125, HD1012 and BMG1.7 were positive for the phytase gene (Table 1). Partial sequencing of the acid phosphatase gene from strains HD9, HD125 and BMG1.7 revealed a 99% nt iden-

tity to acid phosphatase from *Bacillus cereus* ATCC 14579 (data not shown). Phenotypic tests for inorganic P solubilisation, on plates and in liquid NBRI-BPB medium using tricalcium phosphate as the only phosphate source, resulted in strains H77, BMG1.7, HD868, HD9, H45, HD22 and HD932 as the most potent phosphate solubilises.

In soil, phosphate is present in two forms: organic phosphates represented essentially by inositol phosphates (or phytates) and phosphoesters; and mineral phosphates in the form of calcium phosphates, hydroxyapatites and rock phosphates (Rodriguez and Fraga, 1999; Nautiyal *et al.*, 2000). To become available for plants, both mineral and organic phosphates should be solubilised and phosphate solubilising bacteria play an important role in this process (Rodriguez and Fraga, 1999; Nautiyal *et al.*, 2000) and hence in plant growth promotion. Although the genetic basis of mineral phosphate solubilisation is not well understood yet, the main mechanism for mineral phosphate solubilisation is the production of organic acids, which results in acidification of the microbial cell and its surroundings leading to the release of ionic phosphate by proton substitution for Ca^{2+} (for review see Rodriguez and Fraga, 1999). Our strains were shown to have mineral phosphate solubilising activity. This indicates that *B. thuringiensis* strains were able to produce organic acids which lead to the pH drop and to tricalcium phosphate solubilisation. The different strains were able to solubilise phosphate both in liquid and solid media, although observation of the halos on solid medium was slower (14 days of incubation) with respect to the decolourisation in the liquid medium (3 days).

Interestingly, P-solubilising strains BMG1.7, HD868, HD9, H45, HD22 and HD932 were also siderophore pro-

ducers. Hence they could be considered as good candidates for elaboration of biofertilising inoculants that could provide plant with both soluble iron and phosphates. However, whether these strains exert biofertilising potential in soil remain to be demonstrated, since not all strains that are effective *in vitro* retain the capacity in the field due to the influence of many factors on their activity such as ionic strenght, temperature and pH (Nautiyal *et al.*, 2000).

The genetic determinants for siderophore production were investigated by PCR with primers targeting a siderophore biosynthetic protein gene. Apart from strains H77 and HD110, all the other strains were PCR-positive for this gene (Table 1). Siderophore production is a common character of the *B. cereus* group as was shown for *B. cereus* (Park *et al.*, 2005) and *Bacillus anthracis* (Cendrowski *et al.*, 2004) and recently for *B. thuringiensis* (Wilson *et al.*, 2006). In the case of *B. thuringiensis* this character could be relevant for biocontrol of phytopathogenic fungi due to competition effects for iron, but also for providing the plant with iron. Indeed, a number of plants possess heterologous iron uptake mechanism for acquisition of iron through iron-bacterial siderophore complex (Yehuda *et al.*, 1996; Sharma *et al.*, 2003). The role of soil microbial activity in iron acquisition and plant growth has been reported by Masalha *et al.* (2000) who found that under non-sterile soil system, plants show no iron-deficiency symptoms and have fairly high iron level in roots, in contrast to plants grown in sterile system.

We also investigated the capacity of *B. thuringiensis* to interfere with phytohormone metabolism, in particular by producing IAA and ACC deaminase (Table 1). Examination of the auxin production potential was carried out by PCR screening of the *ipdC* gene that codify for indole pyruvate decarboxylase, a key enzyme implicated in the biosynthesis of IAA from tryptophane via the indole pyruvate pathway (IPyA, Fig. 1) (Schutz *et al.*, 2003). We amplified *ipdC* gene from strains BMG1.7, HD22, HD125, HD868, HD1012, H45 and H77. In phenotypic test, carried out using the Salkowski's reagent, development of IAA-characteristic pink colour was observed in the culture supernatants of all the *B. thuringiensis* strains. Quantitative analysis of the IAA production in each culture medium revealed different IAA concentrations varying from 1.53 to 9.71 µg ml^{-1}, with HD9 (9.71 µg ml^{-1}), HD110 (8.18 µg ml^{-1}) and HD125 (6.9 µg ml^{-1}) having the highest amounts (Table 1). The IAA production capacity and the *ipdC* gene have been reported in the genomes of *B. thuringiensis* ssp. *konkukian* str. 97-27 (Accession N. AE017355), *B. cereus* E33L (Accession N. CP000001), *B. cereus* ATCC 10987 (Rasko *et al.*, 2004), *B. cereus* ATCC 14579^T (Ivanova *et al.*, 2003), *B. cereus* G9241 (Hoffmaster *et al.*, 2004), and *B. anthracis* Ames (Read *et al.*, 2003). These observations support the hypothesis that *B. cereus*/*B. thuringiensis*/*B. anthracis* were originally soil bacteria that evolved to occupy different ecological niches. In fact, production of IAA, a plant hormone that does not apparently function as a hormone in bacterial cells, may have evolved in bacteria because it is important in the bacterium-plant relationship. The *ipdC* gene was chosen as target because it has been reported that beneficial plant associated bacteria frequently synthesise IAA via the IPyA pathway (Schutz *et al.*, 2003). Interestingly, some of the strains that responded positively to the Salkowski's reagent did not give amplification of the *ipdC* gene, possibly because IAA production occurs in these strains via the indole acetamide (IAM) pathway (Fig. 1).
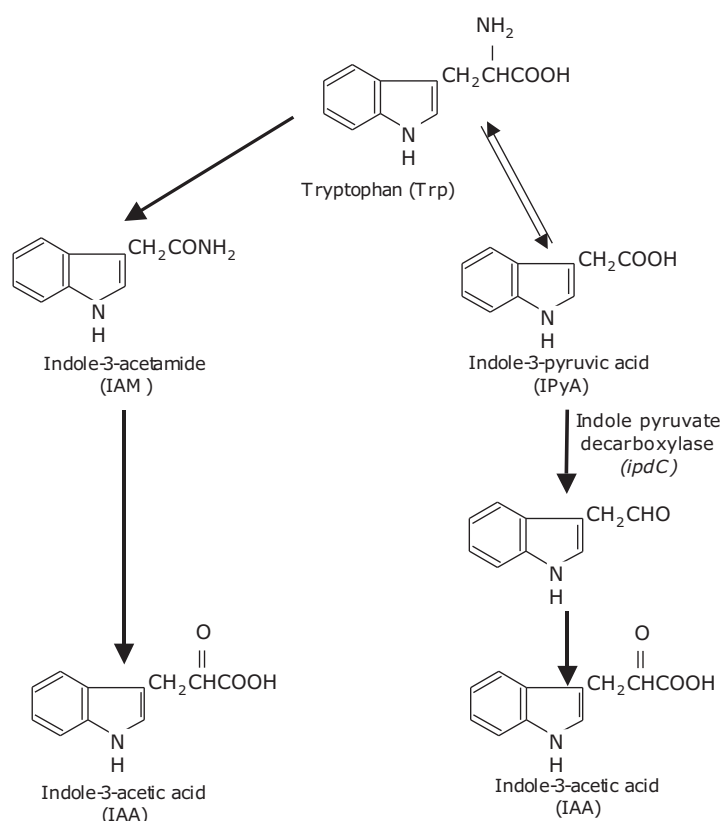

FIG. 1 - Tryptophan-dependent pathways of IAA biosynthesis based on existing litterature.

The highest IAA quantities (6.9-9.71 µg ml^{-1}, Table 1) could be considered in the range of beneficial IAA concentrations that could not cause plant pathogenesis but allow plant growth promotion. First, they are comparable to the quantities produced by the non pathogenic endophytic bacterium *Erwinia herbicola* 299R (Brandl and Lindow, 1996) but lower than those produced by phytopathogenic bacteria like the olive pathogenic species *Pseudomonas savastanoi*, used as control in our study, and which produced 73.29 µg ml^{-1} in the same conditions. These amounts are also lower with respect to the quantities usually produced by *Pseudomonas syringae* pathovars (70 µg ml^{-1}) that induce necrotic diseases (Glickmann *et al.*, 1998) or by *Agrobacterium tumefaciens* (Schwalm *et al.*, 2003). Second, the IAA concentrations produced by the *B. thuringiensis* strains are near to those produced by the plant growth promoting bacterium *Pseudomonas putida GR12-2* (Patten and Glick, 2002).

Such concentrations could not have an inhibitory effect on plant root elongation by influencing the plant ethylene levels. In fact, it is important to note that IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division, or indirectly by influencing bacterial ACC deaminase activity. ACC deaminase, produced by many plant growth-promoting bacteria, is involved in the stimulation of root elongation in seedlings by hydrolyzing plant ACC, the immediate precursor of the phytohormone ethylene, and thereby prevents the production of plant growth-inhibiting levels of ethylene (Penrose *et al.*, 2001).

All the strains were PCR positive for the *accd* gene encoding ACC deaminase enzyme (Table 1). Partial sequencing of the PCR products from strains HD9, HD110, HD125 and BMG1.7 showed identity between 97 and 99% to the *B. cereus* ATCC 14579^T *accd* gene. The high frequence of the presence of this gene could be explained by considering that this enzyme could be implicated in the deamination of substrates other than ACC as was found for the ACCD enzymes from *Pseudomonas putida* UW4 (Hontzeas *et al.*, 2004) and *Pyrococcus horikoshii* (Fujino *et al.*, 2004). Seven strains (HD22, BMG1.7, H77, H172, HD868, H156, H112) out of the 16 *B. thuringiensis* isolates tested were able to grow on DF mineral medium containing 3 mM of ACC as the sole nitrogen source as was shown by following the OD$_{600nm}$ until 72 h of incubation (data not shown), and hence could have ACCD activity. Interestingly, HD22, BMG1.7 and H77 showed a higher growth rate with respect to the remaining strains, suggesting a higher active ACC deaminase for these strains.

Besides lowering the level of ethylene mediating the hydrolysis of its precursor, ACC deaminases have also been shown to protect plants from the deleterious effects of some environmental stresses including heavy metals (Belimov *et al.*, 2005), flooding (Grichko and Glick, 2001), salt (Mayak *et al.*, 2004a), drought (Mayak *et al.*, 2004b) and phytopathogens (Wang *et al.*, 2000), which induce ethylene synthesis. Accordingly, finding of ACC deaminase *B. thuringiensis* producers could be of great interest for field application and especially under stress conditions. Additionally, the resistance of *B. thuringiensis* spores make from this bacterium a feasible plant growth promoting agent under extreme environments (high temperatures, high salt concentrations), and this is an advantage with respect to the non sporulating and/Gram-negative PGP bacteria which are usually more sensitive to these stresses.

We demonstrated that *B. thuringiensis* isolates has the genetic determinants for siderophore and acid phosphatase production, and is capable of hydrolyzing mineral phosphates. This suggests that *B. thuringiensis* could be used as an important bioinoculant that provides the plant with both soluble iron and phosphorous. We also demonstrated that *B. thuringiensis* isolates are capable of releasing IAA, and suggest that IAA production may proceed via the indole-3-pyruvic acid and/or indole-3-acetamide pathways since the *ipdC* gene were found in some, but not in all of the strains. Finally, we showed that *B. thuringiensis* produces the ACC deaminase suggesting that it can interfere with plant hormone balance in a complex way. Taken together, all these results with those of previous studies (Raddadi *et al.*, 2005, 2007, unpublished) confirm *B. thuringiensis* as a polyvalent biocontrol, biofertilising and biostimulating bacterium whose plant growth promoting properties should be further explored in green-house and field conditions especially for its ability to compete with the endogenous soil and rhizosphere microbial communities.

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