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Mechanism of phosphate solubilization and antifungal activity of *Streptomyces* spp. isolated from wheat roots and rhizosphere and their application in improving plant growth

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The application of plant-growth-promoting rhizobacteria (PGPR) at field scale has been hindered by an inadequate understanding of the mechanisms that enhance plant growth, rhizosphere incompetence and the inability of bacterial strains to thrive in different soil types and environmental conditions. Actinobacteria with their sporulation, nutrient cycling, root colonization, bio-control and other plant-growth-promoting activities could be potential field bio-inoculants. We report the isolation of five rhizospheric and two root endophytic actinobacteria from *Triticum aestivum* (wheat) plants. The cultures exhibited plant-growth-promoting activities, namely phosphate solubilization (1916 mg l^{-1}), phytase (0.68 U ml^{-1}), chitinase (6.2 U ml^{-1}), indole-3-acetic acid (136.5 mg l^{-1}) and siderophore (47.4 mg l^{-1}) production, as well as utilizing all the rhizospheric sugars under test. Malate (50–55 mmol l^{-1}) was estimated in the culture supernatant of the highest phosphate solublizer, *Streptomyces* mhcr0816. The mechanism of malate overproduction was studied by gene expression and assays of key glyoxalate cycle enzymes – isocitrate dehydrogenase (IDH), isocitrate lyase (ICL) and malate synthase (MS). The significant increase in gene expression (ICL fourfold, MS sixfold) and enzyme activity (ICL fourfold, MS tenfold) of ICL and MS during stationary phase resulted in malate production as indicated by lowered pH (2.9) and HPLC analysis (retention time 13.1 min). Similarly, the secondary metabolites for chitinase-independent biocontrol activity of *Streptomyces* mhcr0817, as identified by GC-MS and ^{1}H-NMR spectra, were isoforms of pyrrole derivatives. The inoculation of actinobacterial isolate mhce0811 in *T. aestivum* (wheat) significantly improved plant growth, biomass (33 %) and mineral (Fe, Mn, P) content in non-axenic conditions. Thus the actinobacterial isolates reported here were efficient PGPR possessing significant antifungal activity and may have potential field applications.

INTRODUCTION

Root exudate, comprising 5–21 % of the carbon fixed by plants, consists of varied organic compounds such as amino acids, fatty acids, nucleotides, organic acids, phenolics, plant growth regulators, sugars, sterols and vitamins. The portion of soil in the close vicinity of roots, characterized by high microbial activity due to exudate nutrients, is defined as the rhizosphere (Hiltner, 1904). Rhizosphere-colonizing bacteria that stimulate plant growth, increase yield and reduce pathogens as well as biotic or abiotic stresses, are broadly classified as plant-growth-promoting rhizobacteria (PGPR) (Vessey, 2003).

Streptomyces sp. and other filamentous actinobacteria have been explored extensively over several decades for antibiotic production. However, in the last few years this group of bacteria, owing to its soil dominance and strong antimicrobial potential, has received fresh attention for plant-growth promotion (Franco-Correa *et al.*, 2010). Actinobacteria degrade a wide array of biopolymers by secreting various hydrolytic enzymes, are effective colonizers of plant root systems and endure unfavourable conditions by forming spores (Alexander, 1977). Actinobacteria, especially *Streptomyces*, also exhibit immense biocontrol action against a range of phytopathogens (Wang *et al.*, 2013).

Abbreviations: DAP, diaminopimelic acid; IAA, indole-3-acetic acid; ICL, isocitrate lyase; IDH, isocitrate dehydrogenase; MS, malate synthase; NCBI, National Center for Biotechnology Information; qRT-PCR, real-time quantitative reverse transcription PCR; RT, retention time.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the seven actinobacteria isolates are KF414970–KF414976.

Two supplementary figures are available with the online version of this paper.

Actinobacteria can solubilize phosphate and promote plant growth, as well as producing siderophore and phytohormone [indole-3-acetic acid (IAA)] (Jog *et al.*, 2012). Phosphorus (P) is the second most important plant-growth-limiting nutrient after nitrogen, which due to its complex inorganic mineral phosphate and organophosphate (phytate) form remains unavailable to plants. Soil micro-organisms capable of releasing free phosphate from bound forms by mechanisms including acidification through production of organic acids such as gluconic acid, citric acid, succinic acid and oxalic acid, depending on carbon source and metabolic pathways, are termed as phosphate solubilizers (Rajput *et al.*, 2013). Actinobacteria are rarely reported for production and quantification of organic acid (Rózycki & Strzelczyk, 1986), whilst, to the best of our knowledge, there are no reports that describe the mechanisms involved in organic acid production in phosphate-solubilizing actinobacteria. Actinobacteria produce a variety of antibiotics possessing polyketides, β-lactams and a peptide moiety, in addition to a variety of other secondary metabolites that have antifungal, anti-tumour and immunosuppressive activity (Běhal, 2000). Taking into consideration the diversity of secondary metabolites produced by actinobacteria, it is imperative to recognize them as antimicrobial agents and characterize the antimicrobial metabolites that are responsible for the inhibition of plant pathogens.

However, in spite of their high soil population, secondary metabolite production and ability to survive in adverse conditions, *Streptomyces* and other actinobacteria are surprisingly under explored for plant-growth promotion, as compared to *Pseudomonas* or *Bacillus* spp. (Doumbou *et al.*, 2001). In the present study, we have screened actinobacterial isolates for plant-growth-promotion activities, while focusing on elucidating the pathway responsible for phosphate solubilization through gene expression, as well as enzyme activities, and identifying the purified antifungal secondary metabolites produced by the most effective *Streptomyces* sp. The actinobacterial isolates were inoculated into plants for evaluating their potential in improving the growth parameters and mineral content of wheat plants in pot scale experiments.

METHODS

Sample collection and characterization. Rhizospheric soil samples and roots from 3-month-old *Triticum aestivum* (wheat var. Lokwan) plants (five plants each from ten fields) grown in the Mehsana region of Gujarat, India (latitude 23° 42′ N and longitude 72° 33′ E), were collected in sterile polyvinyl bags separately by following the composite sampling method and transported under ice-cold conditions. The loosely adhered soil was removed to obtain the rhizosphere soil associated with plant roots. Soil samples were sieved through 2 mm mesh for determination of physico-chemical characteristics, namely pH, conductivity and salinity, using a multi-parameter electrode tester (model PCSTestr 35; Eutech Instruments). The carbon and nitrogen content of the soil was determined with a C-H-N analyser (model 2400 series II; Perkin-Elmer). The iron, phosphorus and magnesium contents of soil were determined by inductively coupled plasma atomic emission spectroscopy (AES-ICP) (model Optima-330RL; Perkin-Elmer).

Isolation, characterization and identification of actinobacteria. Actinobacteria from wheat rhizosphere were selectively isolated by pre-treatment and enrichment methods that preclude other bacterial genera. Spore forming bacteria other than actinobacteria were selectively eliminated on nutrient depleted humic acid vitamin agar (HV agar) containing antibacterial and antifungal agents. The soil samples (1 g) were separately treated with dry heat (120 °C, 15 min), 1 % phenol, 2 % calcium carbonate, 0.1 % SDS, 5 % yeast extract for 15 min as prescribed (Hayakawa *et al.*, 1991, 1995; Otoguro *et al.*, 2001; Hayakawa & Nomomura, 1989). Appropriate dilutions (10^{-3}, 10^{-4}) of pre-treated soil suspensions were spread onto HV agar (pH 7.4) supplemented with nalidixic acid (10 mg l^{-1}), trimethoprim (20 mg l^{-1}) and cycloheximide (20 mg l^{-1}) to preclude bacterial and fungal growth. Plates were incubated at 30 °C for 1 week in the dark (Williams & Davies, 1965; Hayakawa & Nonomura, 1987).

For isolation of endophytic actinomycetes, surface-sterilized wheat roots were crushed to obtain root sap, which was appropriately diluted and spread onto actinomycete isolation agar, casamino glucose extract agar, starch casein agar and water yeast extract agar for isolation of endophytic actinobacteria (Crawford *et al.*, 1993). All the media and antibiotics were supplied by Hi-media.

Actinobacterial isolates thus obtained were morphologically characterized for substrate, aerial mycelia colour, sporulation, pigmentation, microscopic and colony characteristics on International *Streptomyces* Project (ISP) media numbers 1 to 7 (Hi-media) as mentioned by Shirling & Gottlieb (1966). Isomers of diaminopimelic acid (DAP) in cell-wall hydrolysates were determined by TLC (Boone & Pine, 1968) and cultures were characterized up to the genus level using *Bergey's Manual of Systematic Bacteriology* (Cross, 1989). The genomic DNA, isolated by a combination of bead-beating and the SDS-lysozyme method (Kieser *et al.*, 2000), was amplified with an actinobacteria-specific (F243 and R1378) primer pair (Heuer *et al.*, 1997) using a GC-rich DNA polymerase (Invitrogen) on a MasterCycler Personal thermal cycler (Eppendorf). The 16S rDNA amplicons were purified (QIAquick PCR purification kit; Qiagen) and sequenced using an ABI 310 DNA sequencer (BigDye termination cycle sequencing ready reaction kit; Perkin-Elmer). The 16S rDNA sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and compared to published sequences of species with standing in nomenclature available from the EMBL/GenBank/DDBJ databases using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The DNA sequences of the isolates were deposited in GenBank at the National Center for Biotechnology Information (NCBI).

Phosphate solubilization and organic acid production. The actinobacterial isolates were screened on soluble phosphate deprived Tris-buffered (100 $mmol^{-1}$) tricalcium phosphate (TCP) agar (pH 8.0) and rock phosphate agar (RP agar) (pH 8.0) to determine phosphate-solubilization ability (Joseph & Jisha, 2009; Hamdali *et al.*, 2008). The actinobacterial isolates capable of solubilizing rock phosphate were studied quantitatively for soluble phosphate estimation using the phosphomolybdate-ascorbate method (Ames, 1964). The organic acid responsible for the phosphate solubilization of the two most effective solubilizers was determined as follows. The cultures were grown on minimal medium [0.2 g $(NH_4)_2SO_4$ l^{-1}, 0.06 g $MgSO_4.7H_2O$ l^{-1}, 50 g PEG 6000 l^{-1}] supplemented with glucose (20 g l^{-1}), 1 ml trace salts solution (1.0 g $ZnSO_4.7H_20$ l^{-1}, 1.0 g $MnCl_2.4H_2O$ l^{-1}, 1.0 g $CaCl_2$ l^{-1}) for 7 days (Hopwood *et al.*, 1985). The cell-free extract obtained after centrifugation of 1-week-old broth (stationary phase) was filter-sterilized (0.22 μm) and analysed using HPLC (Waters) with an organic acid specific Supelcogel C-610H column (Sigma-Aldrich). The mobile phase consisted of 0.1 % phosphoric acid, with a flow rate of 1 ml min^{-1}, whilst maximum organic acid absorbance was detected at 210 nm. The organic acids produced were identified and quantified by

comparing the retention times (RTs) and peak areas with a solution of pure organic acid standards.

Putative pathway for malate overproduction in mhcr0816. Since malate overproduction was identified in the highest phosphate-solubilizing isolate *Streptomyces* mhcr0816, the putative pathway for organic acid (malate) overproduction was determined by gene expression and assay of key glyoxalate shunt enzymes viz. isocitrate dehydrogenase (IDH), isocitrate lyase (ICL) and malate synthase (MS), where *Streptomyces* strain mhce0811, a non-malate producer, served as a control. The flasks containing minimal media (inoculated separately with test and control isolates) supplemented with 100 mmol glucose l^{-1} were removed on the third day (exponential phase, before malate production) and seventh day (stationary phase, maximum malate production and phosphate solubilization) of incubation. The cultures were prepared for gene expression and enzyme assay by partial lysis. The cells were centrifuged, washed, resuspended in 100 mmol phosphate buffer (pH 7.5) l^{-1} and incubated with lysozyme for 1 h prior to rapid freeze (−20 °C) and thaw (65 °C) cycles as recommended for difficult to lyse cells like actinobacteria (Kieser *et al.*, 2000). The partially lysed cell suspension thus obtained was divided into two halves for gene expression analysis and enzyme assays of IDH, ICL and MS.

Gene expression studies

Primer design. The nucleotide gene sequences encoding the enzymes (IDH, ICL and MS) were procured for all available *Streptomyces* spp. from the NCBI. The sequences were aligned and a contiguous sequence was prepared using Codon Aligner software (CodonCode). Primers for contig sequences of the enzyme-encoding DNAs were designed using Oligoanalyser 3.1 (Integrated DNA Technology – http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) (Table 1) and the primer binding was confirmed by *in silico* PCR (http://insilico.ehu.es/PCR/).

RNA isolation and real-time quantitative reverse transcription PCR (qRT-PCR). Total RNA was isolated from lysozyme-treated cell supernatant with a High Pure RNA isolation kit (Roche) according to the manufacturer's protocol. RNA was quantified (Nanophotometer; Implen) and 1 µg RNA was used to obtain cDNA using a RevertAid first strand cDNA synthesis kit (Thermo Scientific). Gene expression analysis by qRT-PCR was carried out with SYBR Premix Ex *Taq* II (Takara) using 100 mmol l^{-1} forward and reverse primers specific for ICL, IDH and MS to generate PCR products (200 bp). PCR cycling was performed in a StepOnePlus real-time PCR system (Applied Biosystems) typically at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) as per the supplier's software (StepOnePlus; Applied Biosystems). DNA gyrase expression was used as an internal control, while the IDH, ICL and MS gene expression profile of the other phosphate-solubilizing *Streptomyces* strain, mhce0811 (non-malate producer), was used as an experimental control for relative quantification.

Enzyme assays. The lysozyme-treated cell suspensions of test (mhcr0816) and control (mhce0811) cultures were separately sonicated with a 650 Hz pulse of 30 s for 10 min in a Syclon ultrasonic cell crusher (Ningo Haishu Sklon Instruments). The cell lysate, cleared by centrifugation, was used for measuring the enzyme activities of IDH, ICL and MS by continuous spectrophotometric rate determination as described by Brent & Bergmeyer (1974), Chell *et al.* (1978) and Silverstein (1975), respectively. Units of activity were defined as µmoles of β-NADPH, glyoxylate formed and acetyl coenzymeA cleaved min^{-1} for IDH, ICL and MS, respectively, under standard assay conditions.

Inhibition of fungal growth. Standard strains of pathogens that commonly infected wheat plants in the sampling region were obtained from the Microbial Type Culture Collection (MTCC) (IMTECH, Chandigarh, India). The antifungal activity of all the actinobacterial cultures was assessed against plant pathogens *Aspergillus niger* MTCC 282, *Penicillium chrysogenum* MTCC 160 and *Microsporum gypseum* MTCC 7675 by the cross streak method (Lemos *et al.*, 1985). Actinobacterial isolates were single streaked in a line on starch casein agar and incubated (30 °C, 3–4 days) to obtain abundant ribbon-like growth. Fungal pathogens were streaked perpendicular to line of actinobacterial growth and the plates were further incubated (30 °C, 7 days). The cultures that inhibited fungal growth were quantified for chitinase production on minimal medium containing (20 g l^{-1}) colloidal chitin (Hsu & Lockwood, 1975). The reducing equivalents of chitinase hydrolysate were estimated by the 3,5-dinitrosalicylic acid method (Miller, 1959) and expressed as µmoles *N*-acetylglucosamine released min^{-1} under standard assay conditions.

Production and characterization of antifungal metabolite. *Streptomyces* mhcr0817 inhibited fungal growth but lacked chitinase activity and therefore was tested for production of low molecular mass antifungal secondary metabolites. Complex production medium (solution A – 16 g tryptophan medium l^{-1}, 0.5 g $(NH_4)_2PO_4$ l^{-1}, 0.05 g $MgSO_4.7H_2O$ l^{-1}, 0.05 g $FeSO_4.7H_2O$ l^{-1}, 15 ml glycerol l^{-1}; solution B – 3 g $CaCO_3$ l^{-1}; for 1 l medium – 800 ml solution A was mixed with 100 ml solution B and the volume was adjusted with distilled water up to 1 l, pH 7.4) was inoculated with *Streptomyces* mhcr0817. After 8 days of incubation, cell-free extract from 2.5 l medium was collected by centrifugation. The extract was mixed with an equal volume of ethylacetate, evaporated under a vacuum and reconstituted in 50 % methanol. The aqua-methanolic extract was filtered through 0.22 µ membrane and analysed by GC-MS (model QP-2010; Shimadzu). The extract was further purified with silica

Table 1. Primers used for gene expression

Primer*	Target	Sequence (5′→3′)	Reference
F-idh	IDH	CCAACATCATCAAGCTGCCGAACA	This study
R-idh	IDH	AGACCTTCATCATCGTGGCCTTCA	
F-icl	ICL	TTCGAGCTGACCAAGGCGATGAT	This study
R-icl	ICL	CCAGGGTGATGAACTGGAACTTGT	
F-ms	MS	ACTTCGGCCTGTACTTCTTCCACA	This study
R-ms	MS	TCGTAGAGGATCTCCTCCATCTCGAA	
F-gyr	DNA gyrase	GAAGTCATCATGACCGTTCTGCA	Lee *et al.* (2011)
R-gyr	DNA gyrase	AGCAGGGTACGGATGTGCGAGCC	

*F, Forward primer; R, reverse primer.

(silica 60; Merck) gel chromatography (chloroform : methanol, 9 : 1) and each fraction was checked for antifungal activity against test pathogens by well diffusion assay. The fraction with maximum activity was filtered through 0.22 μ, dried and redissolved at 10 mg ml^{-1} concentration in 50 % methanol. ^{1}H-NMR spectra of the purified antifungal product were recorded in a δ scale given in p.p.m. on a 500 MHz Avance II spectrophotometer (Bruker).

Plant-growth-promotion activities. The actinobacteria were screened for traits like IAA, siderophore and phytase production on tryptophan agar, chrome azurol sulphonate agar and sodium phytate agar, respectively (Bric *et al.*, 1991; Schwyn & Neilands, 1987; Quan *et al.*, 2001). The positive cultures were studied quantitatively for IAA, siderophore production and phytase activity (Gordon & Weber, 1951; Meyer & Abdallah, 1978; Engelen *et al.*, 1994). The ability of actinobacterial isolates to utilize common rhizospheric sugars was also determined by plant-sugar-utilization profile, wherein isolates were separately grown on minimal media supplemented with sugars (20 g l^{-1}: arabinose, fructose, glucose, mannose, sucrose, xylose), whilst minimal medium without any carbon source served as a control. The plant sugar utilization profile was determined by observing actinobacterial growth after 1 week of incubation at 30 °C.

Plant inoculation assay. All seven actinobacteria isolates were tested in plant growth studies using *T. aestivum* (var. Lokwan) in pot experiments. The seeds were surface sterilized with 0.2 % $HgCl_2$ and 95 % ethanol for 3 min, followed by several washes with sterile distilled water. The surface sterilization was checked by rolling seeds on nutrient agar plates. A set of three pots containing sieved, air-dried soil (2.5 kg per pot, obtained from sampling wheat fields of the Mehsana region) mixed with actinobacterial inoculum (10^8 spores per pot) separately for each culture treatment were prepared and individually sown with eight to ten seeds per pot. Five plants per pot were maintained after germination and the uninoculated plants served as controls. The experimental and control pots were randomly incubated in a plant growth chamber (Milestone) at 21 °C and 45 % relative humidity with a 14 h day and 10 h night cycle. Four-week-old plants were carefully removed to measure root length, shoot length, the number of lateral roots, shoot branches and plant biomass. The dried plants were also analysed for carbon and nitrogen (C-H-N analyser; Perkin-Elmer), and iron, manganese and phosphorus content (ICP; Perkin-Elmer).

Statistical analysis. The data were analysed by sigma plot (Windows version 11.0; Systat Software). A one-way ANOVA was used to determine statistical significance, which was assumed to be different when the comparison showed a significance level of $P \leqslant 0.05$. Results were reported as the mean ± SEM.

RESULTS

Rhizosphere soil characterization

The wheat rhizospheric soil was typically deep brown–black sandy loam fertile alluvial soil with a total content of 35 g carbon l^{-1}, 54 g nitrogen l^{-1}, 0.15 g phosphorus l^{-1}, 4.8 g iron l^{-1} and 0.8 g magnesium l^{-1}. The soil had pH 7.4, low electrical conductivity (1.2 dS m^{-1}) and low to moderate salinity (2–3 p.p.m.).

Isolation, characterization and identification of actinobacteria

Seven (five rhizospheric, two endophytic) morphologically distinct and abundant actinobacteria cultures were selected from numerous colonies obtained on isolation media. The isolates were characterized based on morphology and chemotaxonomy (Table 2). The 16S rDNA amplicons were sequenced and identified using the BLAST algorithm. The identified cultures mainly belonged to the genus *Streptomyces* (rhizosphere, four *Streptomyces* and one *Rhodococcus*; root endophyte, one *Streptomyces* and one *Nocardiopsis* sp). The gene sequences were deposited in GenBank (NCBI) with accession numbers as follows: *Streptomyces lomondensis* mhcr0810 (NCBI accession no. KF414974), *Streptomyces cellulosae* mhcr0816 (NCBI accession no. KF414971), *Streptomyces werraensis* mhcr0817 (NCBI accession no. KF414972), *Streptomyces cheonanensis* mhcr0824 (NCBI accession no. KF414973), *Rhodococcus rhodochrous* mhcr0825 (NCBI accession no. KF414976), *Streptomyces tricolor* mhce0811 (NCBI accession no. KF414975) and *Nocardiopsis lucentensis* mhce0814 (NCBI accession no. KF414970). All the actinobacterial strains showed 100 % similarity with their respective standard strain.

Phosphate solubilization and organic acid production

From seven actinobacterial strains analysed, two *Streptomyces* isolates efficiently solubilized phosphate on Tris-buffered (100 $mmol^{-1}$) TCP agar (pH 8.0) and RP agar (pH 8.0). The isolates released significant free phosphate (*Streptomyces* mhcr0816, 1916 ± 10 mgl^{-1}, pH 2.9; *Streptomyces* mhce0811, 950 ± 09 mg l^{-1}, pH 4.6) by acidification. The organic acid responsible for phosphate solubilization was identified and quantified by HPLC analysis of cell-free supernatant by comparing with the RT values of standard organic acids. *Streptomyces* mhcr0816 produced high amount of malic acid (RT 13.1 min, 50–55 mmol l^{-1}) (Fig. 1a), whilst *Streptomyces* mhce0811 produced a derivative of gluconic acid (RT 12.2 min, 10–15 mmol l^{-1}) when grown on minimal medium supplemented with glucose (15–20 g l^{-1}) (Fig. 1b, and Figs S1 and S2, available in the online Supplementary Material).

Gene expression studies

The activities and gene expression of key glyoxalate shunt enzymes responsible for malate overproduction in the most efficient phosphate solubilizer mhcr0816 was estimated, while mhce0811, the non-malic acid producer, was used as an experimental control. The gene expression of IDH, ICL and MS was monitored using specifically designed primers (Table 1) during the exponential phase (third day) and the malate-producing stationary phase (seventh day). The qRT-PCR analysis showed no significant change in IDH expression during the malate-producing stationary phase (seventh day) in either mhcr0816 or mhce0811, as compared to the growth phase (third day) (Fig. 2a). A several fold increase in expression of ICL and MS (Fig. 2a) in the stationary phase of mhcr0816 correlated with high malate production (50–55 mM, pH 2.9) and phosphate solubilization (1916 mg l^{-1}). Expression of ICL and MS in

Table 2. Morphological and chemotaxonomic characterization of actinobacteria isolates

Strain	Genus	Morphological and chemotaxonomic characteristic				
		Aerial mycelia	Aerial spore mass	Soluble pigment	DAP isomer	Gram staining
mhcr0810	*Streptomyces*	Chalk white	Grey	Dark brown	L-DAP	Positive, filamentous, branched
mhcr0816	*Streptomyces*	Pale white	Light grey	Brown	L-DAP	Positive, filamentous, branched
mhcr0817	*Streptomyces*	White	White	Brown	L-DAP	Positive, filamentous, branched
mhcr0824	*Streptomyces*	White	Light pink	Yellow	L-DAP	Positive, filamentous, branched
mhcr0825	*Rhodococcus*	Rare pink colonies	None	None	L-DAP	Positive, coccoid
mhce0811	*Streptomyces*	White	White	Black	*m*-DAP	Positive, filamentous, branched
mhce0814	*Nocardiopsis*	Pale white	None	Yellow	L-DAP	Positive, short irregular filamentous

mhce0811 did not alter drastically during the acid-production phase (Fig. 2a).

Enzyme assays

The enzymes assays correlated with the gene expression studies of IDH, ICL and MS in both the test (mhcr0816) and control (mhce0811) isolates. The IDH activity lacked significant alteration in both the cultures before (third day) and during acid production (seventh day). However, significantly elevated level of ICL (fourfold) and MS (tenfold) explained the malic acid overproduction and high phosphate solubilization in mhcr0816 as compared to mhce0811 (Fig. 2b).

Inhibition of fungal growth

All the actinobacterial cultures tested inhibited *Penicillium* sp., while six (mhcr0811, mhcr0816, mhcr0817, mhcr0824, mhce0811, mhce0814) inhibited *Aspergillus* sp. The growth of *Microsporum* was inhibited by the endophytes (mhce0811, mhce0814) and rhizospheric actinobacteria (mhcr0817). Five actinobacterial cultures produced chitinase and the maximum chitinase activity (6.2 U ml^{-1}) was recorded with *Streptomyces* mhce0811 (Table 3).

Production and characterization of antifungal metabolite.

Streptomyces mchr0817, which inhibited growth of all three test fungal pathogens despite non-significant chitinase activity (0.6 U ml^{-1}), was cultured for production of the low molecular mass metabolites responsible for chitinase-independent antifungal activity. The production media was centrifuged and antifungal metabolites were extracted from the supernatant by ethylacetate (1 : 1, v/v). The ethylacetate extract yielded pink coloured pellets upon vacuum evaporation, which were dissolved in a small quantity of 50 % methanol and filtered through a 0.2 μ membrane. The GC-MS profile of the extract confirmed production of low molecular mass compounds. The RT values of the peaks obtained were compared to the corresponding RT values of standard compounds in the GC-MS software library. The extract of mhcr017 may contain compounds that corresponded to isomers of pyrrolo[1,2-α]pyrazine-1,4 dione, hexahydro-3-(2-methylpropyl) (RT 12.085, 12.74 min) and pyrrolo[1,2-α]pyrazine-1,4 dione, hexahydro-3-(phenylmethyl) (RT 14.61, 14.79 min) (Fig. 3). The extract was further purified by silica gel column chromatography. Elutes obtained from fractions 21–25 showed maximum antifungal activity. NMR analysis of the purified product gave the following major relevant peaks in ^{1}H-NMR (500 Hz): 1 δ, 3.30–3.39 (m, 2H, -CH_2); 2 δ, 4.91–5.01 (s, 1H, -SH). The predicted structure from ^{1}H-NMR (as shown in Fig. 3) confirmed the compounds identified through GC-MS.

Plant-growth-promoting activities

All actinobacterial isolates assessed for various other plant-growth-promoting activities produced chitinase and siderophores, while five cultures produced IAA and three produced phytase. *Streptomyces* mhcr0816 was the highest IAA (136.5 mg l^{-1}) and phytase (0.86 U ml^{-1}) producer, whereas *Streptomyces* mhce0811 produced a significantly high quantity of siderophores (47.4 mg l^{-1}) (Table 3). Actinobacterial cultures flourished on rhizospheric sugars with abundant growth during plant-sugar-utilization profiling (Table 4).

Plant inoculation assay

Plant-growth-promoting actinobacterial cultures significantly improved wheat growth upon inoculation as compared to an uninoculated control after 4 weeks under identical experimental conditions (Table 5). The inoculated plants had a higher number of branches, a higher number of lateral roots (30 % for *Streptomyces* mhcr0817 and mhce0811) and a significantly higher biomass (33 and

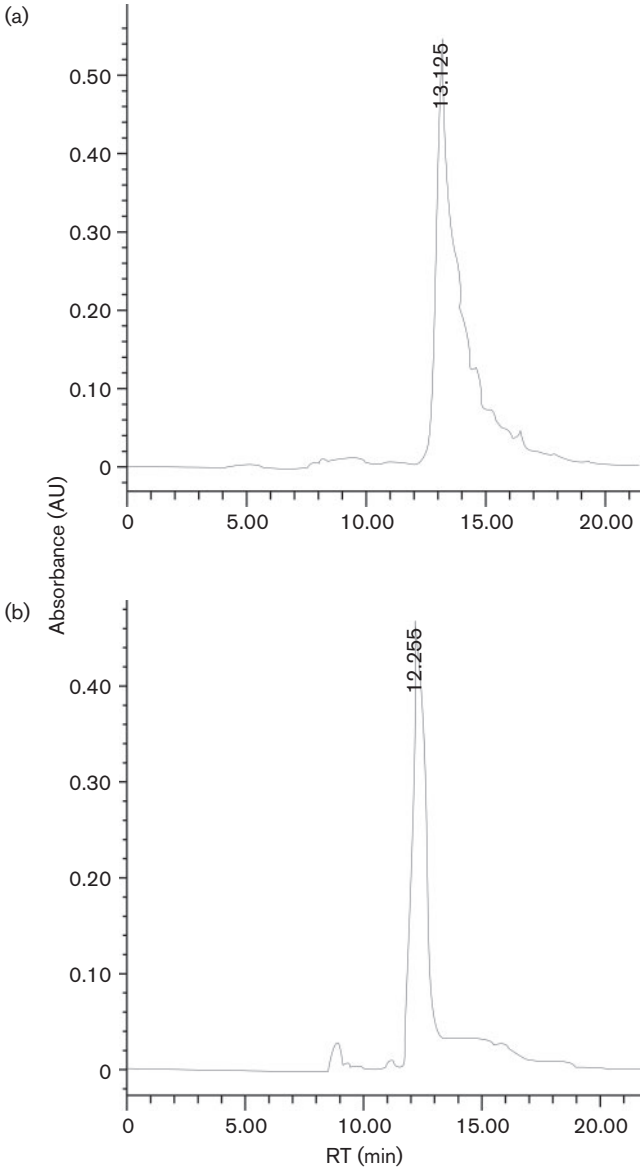

Fig. 1. HPLC profile for organic acid production in *Streptomyces*. (a) mhcr08116, RT 13.12 min corresponds to malate; (b) mhce0811, RT 12.25 min corresponds to a gluconate derivative.

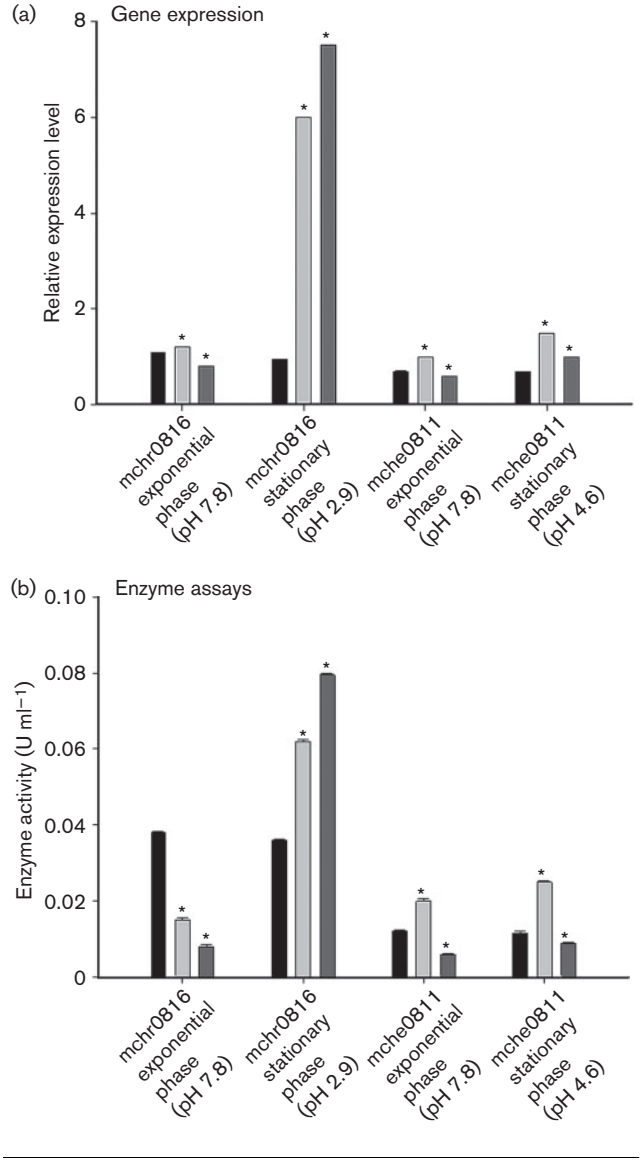

Fig. 2. Gene expression (a) and enzyme assays (b) of IDH (black bars), ICL (light grey bars) and MS (dark grey bars) in mhcr0816 and mhce0811. The asterisks indicate means that were significantly different as compared to the controls ($P \leqslant 0.05$).

15 % higher for *Streptomyces* mhcr0816 and mhcr0817, respectively) as compared to an uninoculated control. An increase in shoot length by more than 50 % was recorded in wheat plants inoculated with *Streptomyces*, mhcr0816, mhcr0817 and mhce0811, while no significant effect of actinobacterial inoculation was observed on root length (Table 5). Actinomycete (root endophyte mhce0811) inoculation significantly increased mineral content (Fe, Mn and P) in inoculated plants, with plants recording higher Fe [1062 μg (g biomass)$^{-1}$], Mn [135 μg (g biomass)$^{-1}$] and P [43 750 μg (g biomass)$^{-1}$] content as compared to the control.

DISCUSSION

The rhizosphere soil profile of the Mehsana region had high carbon, nitrogen and other mineral contents. However, some of these nutrients and minerals, especially phosphate, remain in complex or precipitated forms that cannot be utilized by plants. Actinobacteria, that are abundantly distributed in the rhizosphere and colonize plant roots as endophytes, may play a crucial role in plant-growth promotion by increasing nutrient availability. In the present study, we have used different pre-treatment and enrichment methods to selectively obtain actinobacteria. Seven morphologically and phenotypically distinct actinobacterial cultures (including two endophytes) were selected

Table 3. Plant-growth-promoting activities of actinobacteria

Data are means ± SEM for three determinants (n=3).

Strain	Plant-growth-promoting activities				
	Chitinase production (U ml^{-1})	Phytase production (U ml^{-1})	Siderophore production (mg l^{-1})	IAA production (mg l^{-1})	Phosphate solubilization (mg l^{-1})
mhcr0810	3.20 ± 0.02*	NA	47.4 ± 0.33*	56.51 ± 0.81†	NA
mhcr0816	4.60 ± 0.01*	0.68 ± 0.01	33.4 ± 0.24*	136.52 ± 0.9†	1916.12 ± 10.35†
mhcr0817	0.62 ± 0.04	0.42 ± 0.02	09.1 ± 0.31*	30.21 ± 1.20†	NA
mhcr0824	4.53 ± 0.03*	NA	10.2 ± 0.16*	10.23 ± 0.41†	NA
mhcr0825	0.91 ± 0.01	NA	13.0 ± 0.42*	NA	NA
mhce0811	6.23 ± 0.02*	0.57 ± 0.01	10.3 ± 0.38*	43.72 ± 1.50†	950.23 ± 9.20†
mhce0814	4.32 ± 0.03*	NA	09.3 ± 0.23*	NA	NA

NA, No significant activity.

*Mean values were significantly different ($P \leqslant 0.01$).

†Mean values were significantly different ($P \leqslant 0.001$).

after rigorous isolation and characterization of wheat rhizosphere and root samples. *Streptomyces* species, which constituted the majority of the total actinobacterial colonies of the wheat rhizosphere [10^6 c.f.u. (g soil)$^{-1}$] (data not shown), were amongst the abundant rhizosphere microflora and effective colonizers as reported earlier (Coombs & Franco, 2003). The actinobacterial isolates were screened for plant-growth-promoting activities and underlying mechanisms of phosphate solubilization, as well as antifungal activity.

The organic acid profile of the two best phosphate-solubilizing isolates revealed malate (mhcr0816) and gluconate (mhce0811) production. The gluconic acid derivative in mhce0811 could not be identified and the acid was not produced with other hexoses or at lower glucose concentrations (data not shown). *Streptomyces* mhcr0816 produced a high amount of malate (50–55 mmol l^{-1}) and solubilized 1916 mg inorganic phosphate l^{-1}, which was significantly higher than reported for phosphate-solubilizing *Bacillus* (957 mg l^{-1}) and other actinobacteria (*Arthrobacter*, 519 mg l^{-1}) (Mehta *et al.*, 2000; Chen *et al.*, 2006). Since malate production remained consistent at lower glucose concentrations (50 mmol l^{-1}), as well as with other hexoses (data not shown), the mechanism of malate overproduction was explored in mhcr0816. We hypothesized that *Streptomyces* mhcr0816 produced malate by glyoxalate bypass, which was confirmed through analysis of the relative expression of IDH, ICL and MS, using specifically designed primers, and enzyme activities during exponential (third day) and stationary (seventh day) phase. Actinobacteria usually utilize glucose by the Embden–Meyerhof–Parnas (glycolysis) or pentose phosphate pathway (PPP) followed by TCA cycle (Hodgson, 2000). Key enzymes that regulate TCA metabolic flux are citrate synthase and IDH. It has been reported in enteric bacteria that IDH activity is regulated by IDH kinase/phosphatase, thus acting as a switch between TCA and glyoxalate bypass (Nimmo & Nimmo, 1984). In our study, inhibition of IDH activity and gene expression was not observed with either of the cultures, and IDH activity was inherently higher in mhcr0816. However, a significant increase in ICL and MS activity, as well as expression, during stationary phase (seventh day) correlated with overproduction of malate in mhcr0816, which was not observed with mhce0811. A 10-fold increase in MS activity and expression in *Streptomyces* mhcr0816 compared to the control (mhce0811) indicated the presence of MS isoforms that may have been active during the glyoxalate phase. Similar isoforms have been reported in cephalosporin-producing *Streptomyces clavuligerus* (Chan & Sim, 1998) and the excess malate thus produced could be secreted out of the cell probably via secondary transporter SAV1515 (Ikeda *et al.*, 2003), which has a 1.5 kb nucleotide sequence [annotated in the whole genome sequence of *Streptomyces avermitilis* (Omura *et al.*, 2001)]. SAV1515 is a secondary membrane transporter of the auxin efflux carrier (AEC) family assigned for IAA secretion. Thus, high malate and IAA secretion in *Streptomyces* mhcr0816 (Fig. 1, Table 3) may be interlinked. Future studies involving the cloning of the unusual MS, as well as the malate transporter, in the best PGPR that lacks phosphate solubilization ability may be beneficial for increasing its efficiency as a bio-fertilizer.

Besides phosphate solubilization, biocontrol is one of the most desirable traits for inoculants. The endophyte *Streptomyces* mhce0811 produced the maximum chitinase (6.2 U ml^{-1}), which probably degraded the fungal cell wall and limited growth of test fungal pathogens. *Streptomyces* endophytes with chitinolytic activity have been reported to significantly reduce the risk of pathogenic infection thus enhancing plant growth (Quecine *et al.*, 2008). The non-significant chitinase producer mhcr0817 inhibited all tested fungal pathogens indicating a chitinase-independent antifungal activity. The probable low molecular mass antifungal

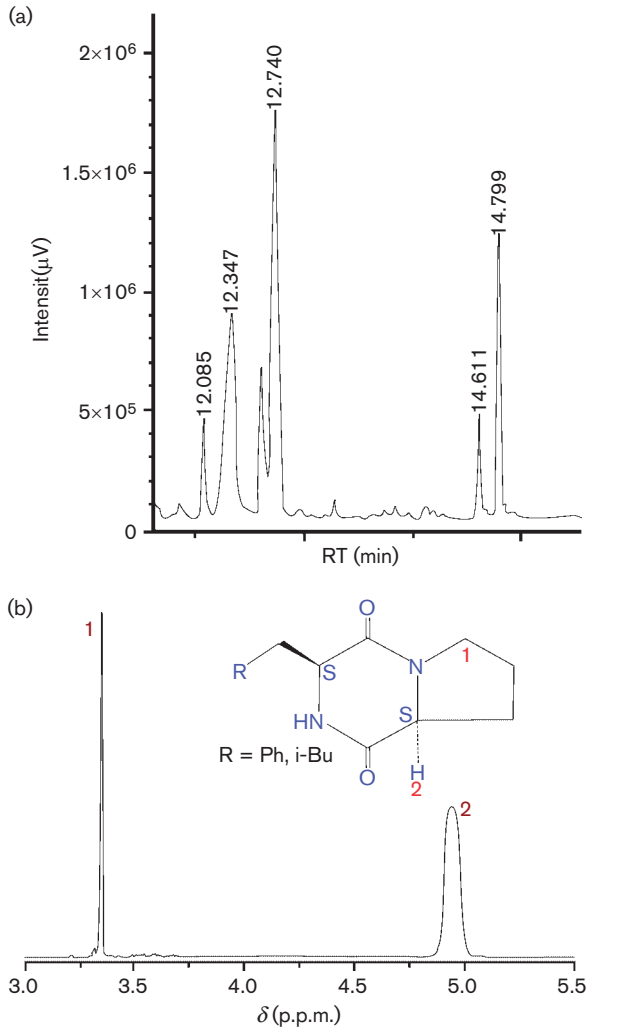

Fig. 3. Characterization of antifungal secondary metabolites in extract of *Streptomyces* mhcr0817: (a) GC-MS profile, (b) ^{1}H-NMR spectra.

metabolites produced by *Streptomyces* mhcr0817, as determined by GC-MS and ^{1}H-NMR spectra, were a mixture of isoforms of pyrrolo ring derivatives (phenylmethyl and methylpropyl). Similar pyrrolo compounds with methylpropyl and phenylmethyl derivatives from *Gillisia* sp. (Flavobacteria) and *Vibrio* sp. have been implicated in antimicrobial activities (Dash *et al.*, 2009; Pandey *et al.*, 2010).

The isolated actinobacteria were also screened for other plant-growth-promoting activities, namely siderophore, IAA and phytase production. All isolates produced siderophores, which are low molecular mass iron chelator peptides that form a Fe^{3+}-siderophore complex for plant uptake under iron-deficient conditions (Vessey, 2003). Similarly, five isolates, when grown on tryptophan-supplemented medium, produced IAA, which is a highly active plant-growth stimulator widely produced by soil micro-organisms including actinobacteria (Farina *et al.*, 2012). IAA induces rapid cell division, and enlargement and extension of plant tissues. The highest IAA production (136 mg l^{-1}) recorded for *Streptomyces* mhcr0816 was comparable to the reported values of standard strains *Rhizobium* sp. (142 mg l^{-1}) (Ghosh *et al.*, 2013) and *Bacillus* sp. (55 mg l^{-1}) (Yasmin *et al.*, 2009), whilst lower than the excellent IAA producer *Pseudomonas fluorescens* CHAO (195 mg l^{-1}) (Beyeler *et al.*, 1999). The enzyme phytase that mineralizes phytate, the predominant form of organic phosphorus unavailable to plants, is produced by sporulating *Bacillus*, *Streptomyces* and several other bacteria (Richardson & Simpson, 2011; Jog *et al.*, 2012). Three actinobacterial isolates in this study produced a moderate amount of phytase (mhcr0816, 0.86 U ml^{-1}), whilst all tested actinobacterial cultures utilized every tested rhizospheric sugar, indicating a higher probability of rhizosphere survival.

The actinobacterial cultures, when used for plant inoculation, significantly improved plant growth and development in terms of root and shoot length, and number of

Table 4. Plant-sugar-utilization profile of actinobacterial isolates

Strain	Plant sugar (2 %) added*					
	Glucose	Fructose	Mannose	Sucrose	Arabinose	Xylose
mhcr0810	+ +	+	+ +	+ +	+	+ +
mhcr0816	+ +	+ +	+ +	+ +	+	+ +
mhcr0824	+ +	+ +	+ +	+ +	+ +	+ +
mhcr0825	+	+	+	+	+	+
mhcr0817	+ +	+ +	+ +	+ +	+	+ +
mhce0811	+ +	+ +	+ +	+ +	+ +	+ +
mhce0814	+ +	+ +	+ +	+ +	+ +	+ +

+, Presence of growth; + +, significant growth.
*The control with only MM showed no growth.

Table 5. Effect of actinobacteria inoculation on plant parameters

Data are means ± SEM for three determinants ($n=3$).

Strain	Root length (cm)	Shoot length (cm)	No. of shoot branches	No. of lateral roots	Plant biomass (mg)	Elemental analysis [µg (g biomass)$^{-1}$]		
						Fe	Mn	P
Control	5.87 ± 0.1	20.12 ± 0.1	05	09	165 ± 12.3	250	63	19900
mhcr0810	5.80 ± 0.2	22.50 ± 0.1*	05	10	164 ± 11.4	337*	75	20750
mhcr0816	6.20 ± 0.1*	36.10 ± 0.1†	07*	11*	385 ± 13.2†	854*	130*	40541†
mhcr0824	5.89 ± 0.4	19.23 ± 0.3	05	10	169 ± 10.1	261	78	20120
mhcr0825	5.81 ± 0.2	18.12 ± 0.4	05	09	153 ± 12.2	252	60	18700
mhcr0817	5.95 ± 0.2	27.01 ± 0.2*	07*	12*	276 ± 20.9†	812*	128*	29375†
mhce0811	6.10 ± 0.1*	38.89 ± 0.3†	07*	13*	395 ± 14.2†	1062*	135*	43750†
mhce0814	5.76 ± 0.2	24.32 ± 0.2*	05	10	173 ± 09.3	300*	80	20438

*Mean values were significantly different as compared to the control ($P \leqslant 0.05$).
†Means values were significantly different as compared to the control ($P \leqslant 0.01$).

branches and lateral roots, as well as plant biomass. Inoculation of efficient plant-growth-promoting actinobacteria (mhce0811, mhcr0816, mhcr0817) significantly improved the Fe, Mn and P content of wheat plants when compared to an uninoculated control. The nutrients mobilized by the actinobacteria probably contributed to higher plant growth, development and mineral content.

Thus, we conclude that actinobacteria possess a wide range of plant-growth-promoting activities that can directly or indirectly benefit plant growth. Actinobacterial cultures were rhizosphere competent as they were able to utilize all plant sugars commonly available in the rhizosphere, as well as being able to promote plant growth upon inoculation under natural conditions. The results were encouraging and we propose three significantly beneficial *Streptomyces* (mhcr0816, mhcr0817 and mhce0811) as potential bio-inoculants. We hope actinobacteria with their nutrient cycling, antifungal activity and ability to thrive in harsh conditions would be rated as first choice PGPR for the development of successful bio-inoculants for a wide range of crop plants.

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