High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan

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Summary
Soil salinization is increasing steadily in many parts of the world and causes major problems for plant productivity. Under these stress conditions, root-associated beneficial bacteria can help improve plant growth and nutrition. In this study, salt-tolerant bacteria from the rhizosphere of Uzbek wheat with potentially beneficial traits were isolated and characterized. Eight strains which initially positively affect the growth of wheat plants in vitro were investigated in detail. All eight strains are salt tolerant and have some of the following plant growth-beneficial properties: production of auxin, HCN, lipase or protease and wheat growth promotion. Using sequencing of part of the 16S rDNA, the eight new isolates were identified as Acinetobacter (two strains), Pseudomonas aeruginosa, Staphylococcus saprophyticus, Bacillus cereus, Enterobacter hormaechei, Pantoae agglomerans and Alcaligenes faecalis. All these strains are potential human pathogens. Possible reasons for why these bacteria present in the rhizosphere and establish there are discussed.

Introduction
Salinization of soils and groundwater is a serious land-degradation problem in arid and semi-arid regions and causes major problems for crop productivity (Keren, 2000). Salt-affected soils cover about 10% of the world’s agricultural area. The Republic of Uzbekistan is also facing this ecological problem as well as that of drying up of a substantial part of the Aral Sea.
Wheat is the most important staple crop in the world (Khush and Toenniessen, 1991). In Uzbekistan alone, an area of 1.1 million hectares is used for wheat growth. Substantial areas under wheat cultivation in the arid and semi-arid regions of the country are affected by soil salinity. Plant productivity in saline soils is considerably reduced due to improper nutrition of plants as well as osmotic and drought stress (Munn, 1993). The most appropriate solution in such conditions is to use salt-tolerant bacterial inoculants that may prove useful in developing strategies to facilitate plant growth in saline soils (Mayak et al., 2004). Garcia and Hernandez (1996) reported that salinity negatively affects biological activity by high osmotic strength (low water potential) which can be attributed to the toxic effect on microbial growth except tolerant halophytic bacteria (Brown, 1976). Therefore, salt-tolerant root-colonizing bacteria that have managed to survive adverse environmental factors could greatly help in harnessing them for their beneficial properties in such environments in which other microorganisms hardly survive (Mayak et al., 2004). Some of them may be able to increase plant growth, increase the rate of seed germination, improve seedling emergence and responses to external stress factors, and protect plants from disease (Lugtenberg et al., 2001).
Understanding the highly complex nature of the microbial adoption and response to alterations in the biological, chemical and physical environment of the rhizosphere remains a significant challenge for plant biologists and microbiologists (Lugtenberg et al., 2002). Studies on microbial diversity in such environments may provide valuable information on the physiology of these microbes and on their role in the ecology of plants and soil (Atlas, 1984). However, detailed studies on beneficial salt-tolerant bacteria and understanding of their physiology and properties in arid salinated soils are scarce. The interactions among these microbes in field applications under different environments are still not well understood. Therefore plant–microbe interactions, the indigenous microorganisms and their physiological adaptation under ecologically stressed conditions have to be studied. The objectives of our work were to isolate salt-tolerant, plant...
growth-promoting bacteria from the rhizosphere of wheat grown in salinated soils from a semi-arid climate, and to characterize their physiological and taxonomic properties.

Results

Natural bacterial colonization of wheat grown on salinated soil

Bacteria were isolated from the rhizosphere soil strongly attached to the wheat root and their numbers were compared with those of the phyllosphere and surrounding bulk soil. Bacterial numbers typically were $10^8 \pm 5.4$ colony-forming units (cfu) per gram of fresh root for the rhizosphere, $1.3 \times 10^7 \pm 2.6$ cfu g$^{-1}$ for bulk soil and $8 \times 10^8 \pm 0.8$ cfu per gram of leaf.

Preliminary screening of wheat rhizosphere bacteria for plant growth stimulation

Two hundred and ten bacterial isolates from the wheat rhizosphere were examined in a plate bioassay for their ability to promote the length of roots and shoots. Thirty-one bacterial strains increased root and shoot growth, and from these we selected the best eight strains (Table 1) which in preliminary experiments appeared to stimulate seedling growth in vitro (> 20%).

Characterization of strains

Molecular characterization based on 16S rDNA homology of a partial sequence (1440 bp) with the sequences in GenBank nucleotide sequencing of amplified 16S rDNA fragments obtained after colony polymerase chain reaction (PCR), and comparative analysis using DNA databases, revealed that the isolated strains belong to the genera Acinetobacter, Pseudomonas, Bacillus, Alcaligenes, Pantoae, Enterobacter and Alcaligenes (Table 1). The 16S rDNA sequences of the isolates show very high homology with the strains listed in Table 1. Consultation of a classification list of bacteria in safety risk groups (Anonymous, 1998) showed that all eight isolates fall in risk group 2, indicating that it cannot be excluded that they are pathogens.

Salt and temperature tolerance

To test their salt tolerance, the bacterial strains were grown in liquid KB, BM minimal medium and BM minimal medium supplemented with a series of NaCl concentrations. From the resulting growth curves it was concluded that all eight strains grow well in the presence of high salt. Staphylococcus saprophyticus and Bacillus cereus are the least resistant strains whereas the Pseudomonas aeruginosa strain grew well even in the presence of the highest salt concentration tested, 4.0% NaCl in KB and 3.0% NaCl in BM (Table 2). All eight strains grow well at 37°C.

Phenotypic properties

Strains were tested for production of the volatile HCN and for exoenzymatic activities (Table 2). The strains P. aeruginosa TSAU145 and Enterobacter hormaechei TSAU2 produce hydrogen cyanide. Five strains secrete the exoenzymes lipase and protease (Table 2). None of the strains secretes cellulase or $\beta$-glucanase (results not shown). In addition to being antagonistic against three Fusarium strains, F. oxysporum, F. culmorum and F. solani, TSAU145 is antagonistic towards the other phytopathogens Pythium ultimum, Alternaria alternate, Botrytis cinerea and Phytophthora cryptogena on two tested media PDA and WA (data not shown). Alcaligenes faecalis TSAU3 inhibits growth of only P. ultimum (data not shown). The other six strains did not show antagonistic activity towards phytopathogens.

Auxin production

Auxin production was tested in the absence and presence of 500 $\mu$g ml$^{-1}$ of the auxin precursor tryptophan. The results obtained from 8-day-old cultures are shown in Table 2. All eight newly isolated strains appeared to produce auxin. Production by the three strains A. faecalis TSAU3, B. cereus TSAU80, S. saprophyticus TSAU415 was the lowest. A time-course (not shown) showed that the presence of tryptophan strongly stimulated auxin production in the two Acinetobacter strains as well as in E. hormaechei, P. agglomerans and P. aeruginosa (Table 2).
Inoculation of wheat with bacterial strains A. faecalis TSAU3, Acinetobacter sp. TSAU16, P. agglomerans TSAU26 and S. saprophyticus TSAU415 significantly increased plant shoot and root growth compared with the control (Fig. 1). Strain Acinetobacter sp. TSAU132 did not demonstrate stimulatory activity, whereas strain P. aeruginosa TSAU145 slightly decreased root growth of wheat.

**Discussion**

**Beneficial properties**

In an attempt to eventually improve wheat yield in salinated soils, eight bacteria were isolated from the rhizosphere of wheat plants grown in salinated soil which initially stimulated wheat growth in vitro. They were identified as the Gram-negative bacteria A. faecalis, Acinetobacter (two strains), E. hormaechei, P. agglomerans and P. aeruginosa, and the Gram-positive bacteria B. cereus and S. saprophyticus (Table 1). All strains grow fast in medium supplemented with 3% and/or 4% NaCl (Table 2). Salt-tolerant bacteria have convergently evolved to cope with environments of elevated osmolarity by the accumulation of a restricted range of low molecular mass molecules, termed compatible solutes owing to their compatibility with cellular processes at high internal concentrations (Sleator and Hill, 2001). The ability to adapt to changes in the osmolarity of the external environment is therefore of fundamental importance for growth and survival, and as such, prokaryotic cells have evolved a number of osmoadaptive strategies to cope with fluctuations in this important environmental parameter (Csonka, 1989; Sleator and Hill, 2001).

Considering the fact that most crops in Uzbekistan are cultivated on agricultural lands where the soil salinity level varies from 0.5% to 1%, this result indicates that these bacteria can easily stand the local salt stress. Nautiyal and colleagues (2000) also reported that bacterial strains with their genetic potential for increased tolerance to high salt and high temperature can enhance crop production in semi-arid and arid regions of the world (Nautiyal et al., 2000).

*Fig. 1.* The effect of bacterization of seeds on shoot and root length of wheat grown in salinated soil. Plants were either not inoculated (‘Untreated control’) or inoculated with bacteria of which the identity is shown in Table 1. (Dry weight gram per plant; control – 0.51; TSAU26 – 0.53*; TSAU2 – 0.57*; TSAU3 – 0.59*; TSAU80 – 0.55*; TSAU16 – 0.58*; TSAU415 – 0.55*; TSAU132 – 0.48; TSAU145 – 0.43). The asterisks (*) indicate a statistically significant difference between untreated control and inoculated plants according to the Student’s test at 95% confidence level.

### Table 2. Overview of properties of eight selected bacterial strains isolated from the rhizosphere of wheat grown in salinated soil.

<table>
<thead>
<tr>
<th>Bacterial test straina</th>
<th>Salt toleranceb</th>
<th>Capacity for growth at 37°C</th>
<th>HCN</th>
<th>Lipase</th>
<th>Protease</th>
<th>Auxin concentrationc (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcaligenes faecalis TSAU3</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>5.6</td>
</tr>
<tr>
<td>Acinetobacter spp. TSAU132</td>
<td>2</td>
<td>2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>8.0</td>
</tr>
<tr>
<td>Acinetobacter spp. TSAU16</td>
<td>2</td>
<td>2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>10.0</td>
</tr>
<tr>
<td>Bacillus cereus TSAU80</td>
<td>2</td>
<td>2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>4.0</td>
</tr>
<tr>
<td>Enterobacter hormaechei TSAU2</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>17.5</td>
</tr>
<tr>
<td>Pantoae agglomerans TSAU26</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>9.7</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa TSAU145</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4.7</td>
</tr>
<tr>
<td>Staphylococcus saprophyticus TSAU415</td>
<td>2</td>
<td>1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>6.0</td>
</tr>
</tbody>
</table>

a. Identification based on 16S rDNA sequence (see Table 1).
b. Highest salt concentration (%) which did not affect growth rate.
c. All eight strains were negative for extracellular production of cellulase and β-glucanase.
d. Auxin (IAA) level after 8 days of cultivation in µg ml⁻¹; detection limit 2 µg ml⁻¹.
All eight strains appeared to produce auxin (Table 2). No correlation was found between the level of auxin production in vitro and significant shoot and root length stimulation (Fig. 1). This is not surprising as plant growth stimulation requires more traits than auxin production alone, e.g. root colonization. Moreover, conditions in the rhizosphere and in vitro are very different.

Six out of the eight strains significantly increased shoot and/or root growth (Fig. 1). In the test in salinated soil P. aeruginosa TSAU145 appeared to decrease plant growth, in contrast to the result of the initial in vitro screening, in which it stimulated wheat growth. This negative effect in soil is consistent with the results of Rahme and colleagues (1995) which indicate that P. aeruginosa can act as a plant pathogen in in vivo experiments.

The best-performing strains in pot experiments in salinated soil were A. faecalis TSAU3, Acinetobacter spp. TSAU16, P. agglomerans TSAU26 and S. saprophyticus TSAU415 (Fig. 1). At first glance these strains show promise as plant growth-promoting agents. However, they were classified as potential pathogens (Table 1) belonging to the risk group 2 (Anonymous, 1998).

The plant growth-stimulating isolates are potential pathogens

A remarkable observation was that all eight strains which initially showed plant growth stimulation were identified as belonging to species that harbour human pathogens (Table 1). The presence of P. aeruginosa in the rhizosphere of wheat has been reported previously (Morales et al., 1996; Germida and Siciliano, 2001). The presence of A. faecalis, Acinetobacter, P. agglomerans, E. hormaechei, S. saprophyticus and B. cereus in the rhizosphere of wheat has not been reported. These strains have been isolated from the rhizosphere of other plants: Acinetobacter from the rhizosphere of strawberry (Berg et al., 2005); P. agglomerans from strawberry (Berg et al., 2002), potato (Sessitsch et al., 2004), cucumber, tomato and paprika (Kamilova et al., 2005; Validov et al., 2006); E. hormaechei from a sand dune plant (Park et al., 2005) and B. cereus from oilseed rape and potato (Berg et al., 2005). To our knowledge this is the first time when S. saprophyticus is reported as a free-living wheat-associated rhizobacterium. Besides among clinical isolates, it was only identified among carrot root endophytes (Surette et al., 2003) and phyllosphere isolates of chestnut (Valverde et al., 2005).

It is known that various bacterial genera contain root-associated strains that encounter bivalent interactions with both plant and human hosts (reviewed by Berg et al., 2005). Our observation that all eight bacteria that initially promoted plant growth in vitro belong to species that also harbour human pathogens not necessarily means that they are pathogens indeed.

Pseudomonas aeruginosa is a dominant pathogen infecting the lungs of cystic fibrosis patients and it is capable of infecting various tissues and organs, and causing severe, damaging and often fatal cases (Knapp et al., 2005; Sadikot et al., 2005). Seifert and colleagues (1994) and Bergogne-Berezin and Towner (1996) reported that some Acinetobacter strains are significant nosocomial pathogens, especially in patients with impaired host defences and in intensive care unit patients. Some Alcaligenes species cause bacteraemia, meningitis, pneumonia and peritonitis (Vu-Thien et al., 1998) whereas S. saprophyticus can cause urinary tract infections (Fihn et al., 1998). Bacillus cereus can cause food-borne gastroenteritis and opportunistic infections and some B. cereus strains can cause severe pneumonia (Hoffmaster et al., 2004). Enterobacter hormaechei has been recognized increasingly frequent as the cause of nosocomial infections and has been isolated from blood, wounds and sputum of adult hospital patients (O’Hara et al., 1989). Cruz and colleagues (2007) describe paedi atric cases of P. agglomerans infections in patients, from which the bacterium was isolated from the bloodstream, abscesses, urinary tract, peritoneum and the thorax.

Some authors reports that P. aeruginosa can also cause serious diseases in animals, such as equine placentitis (Hong et al., 1993), chronic bovine mastitis (McLennan et al., 1997) and mink haemorrhagic pneumonia (Hammer et al., 2003), whereas Staphylococcus can cause mastitis in cattle, sheep, goats and horses and botryomycosis in pigs and horses (Rich, 2005). Bacillus cereus may cause mastitis of varying severity and abortion in cattle herds (Frank, 1997). Acinetobacter contributed to the death of dogs and cats (Francey et al., 2000).

A key question is whether the eight strains are from human origin or whether the conditions in the analysed rhizosphere select for strains which share characteristics with human pathogens.

The rhizosphere, which is relatively rich in organic substrates, stimulates microbial growth and can contain up to 10^{11} bacteria per gram of root. The composition of exudates is thought not only to attract beneficial bacteria to colonize the roots, but also human pathogens that have also evolved to respond to the same signals (Roberts et al., 2000; Ji and Wilson, 2002). In this way, potentially pathogenic species are supposed to survive and become enriched in the rhizosphere where they rapidly utilize simple carbon sources (Gilbert et al., 1993). Hence, they would be expected to compete effectively with the indigenous microflora in the field (Jablonske et al., 2005). Another explanation for the observation that human pathogens can colonize the rhizosphere is that bacteria that colonize plant roots and bacteria that colonize human
tissue share many pathogenicity factors (Rahme et al., 1995) and colonization genes and traits (Lugtenberg et al., 2001; Berg et al., 2005). Further support for the notion that strains from human origin can thrive in the rhizosphere came from the observation by Troxler and colleagues (1997) who showed that the opportunistic human pathogen *P. aeruginosa* can function as a biocontrol agent of the wheat disease caused by the oomycete *P. ultimum*. Moreover, several studies support the view that environmental strains are indistinguishable from clinical isolates in terms of genotypic, taxonomic or metabolic properties (Kiewitz and Tuemmler, 2000; Finnan et al., 2004). Although there is no clear evidence that strains from the rhizosphere directly colonize the human body, Parke and Gurian-Sherman (2001) interpret the appearance of unique clones in individual patients as evidence that these may be acquired independently of the environment.

We believe that in the case we studied, it is possible that the potential pathogens originate from warm-blooded animals or from human beings. Especially in regions where a sewage system does not exist, manure is collected in holes in the ground and may leak towards the groundwater which is used for irrigation. Alternatively, manure is directly used to fertilize the nutrient-poor soil. In both cases pathogens can reach the rhizosphere. Further support for this notion comes from the observation that bacteria that colonize plant roots and bacteria that colonize human tissue share many colonization genes and traits (Lugtenberg et al., 2001). Finally, the high temperature of the soil in arid regions will create conditions that are favourable for bacteria that originate from warm-blooded animals. However, this is not a prerequisite for temperature-resistant bacteria to colonize the rhizosphere as many temperature-resistant bacteria have also been reported to be present in the rhizosphere of oilseed rape and strawberry in Northern Germany (Berg et al., 2005).

Assuming that the selected bacteria are indeed from warm-blooded origin, another question is how they can successfully compete with the local indigenous rhizosphere microflora. To our knowledge, this first question has never been answered. Our observation that the numbers of cfu in the analysed rhizosphere (10^6 cfu per gram fresh weight) are approximately 1000-fold lower than those found in non-salinated rhizospheres (Ji and Wilson, 2002) may be significant. The low numbers of the indigenous rhizosphere microflora make it relatively easy for the incoming microbes to compete for nutrients and niches in the rhizosphere.

Whatever the reason is for the observation that the rhizosphere of wheat under the studied conditions contains many potential pathogens, this fact should be investigated further and this knowledge should be incorporated in the agricultural management strategies in relation to environmentally acquired infections. Finally, attempts should be made to manage the studied rhizosphere in such a way that it becomes more difficult for pathogens to colonize the rhizosphere and easier for beneficial bacteria to do this. It is tempting to speculate that beneficial bacteria that have been selected as enhanced colonizers (Kamilova et al., 2005; Validov et al., 2006) may be able to outcompete the pathogens thereby creating a more environmentally friendly agriculture which is healthier for farmers and users.

**Experimental procedures**

**Study site, soil sampling and soil characterization**

Deep tillage (0–40 cm) irrigated cotton fields affected by salinity (based on EC) were selected for the collection of soil samples at Sayhunobod district (41°00′N, 64°00′E), Syrdarya province, in the North-East of Uzbekistan. The field has EC values of 560 ± 61 mS per metre of soil. In strongly salinated fields, salt accumulation at the surface was evident. According to the FAO (Decker et al., 1998) classification, the soils are Calciisol (silt loam seirozem) which are formed from loess, eluvial and proluvial parent materials. The soil surface horizon is calcareous saline, and the deeper horizons are mild alkaline in nature. The salts that moved to the surface evidently have higher Na, CO3 and Cl contents, thereby increasing the salinity of the soil. On average, soils contained 43 ± 9 g of sand kg⁻¹, 708 ± 12 g of silt kg⁻¹, and 25 ± 13 g of clay kg⁻¹ with cation exchange capacity of 23.6 ± 1 (cmol+) kg⁻¹, exchangeable Na percentage (ESP) of 4.41 and Na absorption rario (SAR) of 0.32. A significantly higher concentration of Ca, K and Na associated with CO3 and Cl reflects a dominance of carbonate and chloride associated salts in soil (Egamberdiyeva et al., 2007). Among the salt types, Ca, Mg and Na associated with sulfates, chlorides and bicarbonates were dominant in this soil. Among the salt types, a large amount of Na2SO4 (> 40%) had accumulated within the soil profile (0–120 cm depth) followed by CaSO4 (~29%), NaCl (18%) and MgCl2 (10%). Seventy per cent of these salts, namely Mg and Na sulfates and chlorides, are highly toxic for plant growth. In this region, the groundwater is saline and contains 5–24 g per litre of salts (Egamberdiyeva et al., 2007).

The climate is semi-arid with an average annual rainfall of 200 mm, mostly falling in autumn and spring. The average air temperatures in January and July are 0°C and 36–38°C respectively. Typical characteristics of the summer climate of Uzbekistan are drought, abundance of high temperatures and high light conditions. The soils in semi-arid areas are drought, saline and contains 5–24 g per litre of salts (Egamberdiyeva et al., 2007).

Winter wheat (*Triticum aestivum*, cv. Turon) was used for the bacterial inoculation experiments. Before isolating bacterial
strains, we determined the natural level of bacterial colonization in the rhizosphere and phyllosphere of wheat and bulk soil. The seeds of wheat were sterilized by immersion in 70% ethanol for 5 min and subsequently in 0.1% HCl, after washing with water sown in pots, and plants were grown for 28 days. In the laboratory, whole plants were gently removed from the soil, and the roots and shoots were separated from bulk soil. For the determination of soil bacterial colonization, bulk soil samples were gently sieved through a 2 mm mesh, visible pieces of crop residues and roots were removed subsequently. 1 g of moist bulk soil was shaken with 9 ml of sterile water pre-mixed with 1 ml of 40% TMTD (tetramethylthiuram disulfide) for 30 min. For determination of rhizosphere colonization, 1 g of soil strongly attached to the roots was removed and was shaken with 9 ml of sterile water. To determine phyllosphere colonization, 1 g of leaf was macerated and subsequently shaken with 9 ml of sterile water. All samples were taken in threefold. Subsequently the suspensions were serially diluted on solidified King’s medium B (KB) (King et al., 1954). The number of cfu was determined. Purified strains were frozen in 30% glycerol at –80°C. Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98.

Seedling growth promotion

Two hundred and ten rhizosphere isolates were picked up from agar plates and tested for their effect on growth yield by placing wheat seeds on sterile filter paper moistened with sterile distilled water in Petri plates. Bacterial strains were grown in liquid KB medium for 3 days. All used seeds were sterilized by immersion in 70% ethanol for 5 min and subsequently in 0.1% HCl for 1 min. Finally, they were soaked in a bacterial suspension of 10⁷ cfu ml⁻¹ and control seeds were soaked in sterile distilled water. Twenty seeds per plate were put in Petri dishes containing wet filter paper. Plates were incubated for 7 days at room temperature. The shoot and root length of seedlings were measured after 7 days. Bacteria that stimulated shoot and root growth of seedlings more than 20% were considered as plant growth-stimulating bacteria. The experiments with the best strains were repeated twice.

Antagonistic activity

To test which strains have inhibitory effects against the phytopathogens F. culmorum 556, F. oxysporum f.sp. radicis-lycopersici ZUM2407, F. solani 558, P. ultimum LBOP17, A. alternate ZUM2372, P. cryptogea and B. cinerea ZUM2076, the bacterial isolates were tested in vitro using a plate bioassay with potato dextrose agar PDA (Difco Laboratories) and Waxman agar (WA) (Opelt and Berg, 2004). The strains of phytopathogens were grown on solid medium at 28°C for 5 days and the disks of a fresh culture (5 mm in diameter) were cut out and placed in the centre of a Petri plate. Bacteria grown on KB agar were streaked on the test plates perpendicular to the fungal inoculum. Plates were incubated at 30°C for 7 days, until the phytopathogens had completely covered the surface of control plates without bacteria. Antifungal activity was recorded as the width of the zone of growth inhibition between the phytopathogens and the test bacterium.

Identification, DNA isolation, PCR amplification

Bacterial strains were grown at 28°C under vigorous aeration on Luria–Bertani medium amended with 10 mM MgSO₄. Total DNA from both Gram-negative (strains TSAU2, TSAU3, TSAU16, TSAU26, TSAU132 and TSAU145) and Gram-positive (strains TSAU80 and TSAU415) was isolated using the technique of de Souza and colleagues (2003). An approximately 1440 bp DNA fragment encoding part of the 16S rDNA sequence was amplified with primers 27fm (5′-AGAGTTTGTATCMTGCTAG-3’) and r1522 (5′-AAGGAGGTGATCCAGCCGCA-3’) using a PCR. Total DNA of the strains was used as a source of template DNA. The nucleotide sequence of the PCR fragments was determined by ServiceXS (Leiden, the Netherlands). Sequences were assembled with DNAMAN Software. Homology searches with 16S rDNA sequences in GenBank were performed with the BLASTN program (version 2.2.1) (Altschul et al., 1997).

Salt tolerance and temperature resistance

For the determination of salt tolerance, liquid KB and minimal medium BM (Lugtenberg et al., 1999) supplemented with 0%, 1%, 2%, 3% and 4% of NaCl was used. The optical density (620 nm) of the bacterial isolates was determined with spectrophotometrically after 2, 4, 6, 8 and 24 h. Temperature resistance ability of strains were observed in KB medium after incubation at 37°C for 5 days.

Phenotypic characterization of the strains

Production of hydrogen cyanide (HCN) by the tested strains was detected using cyanide indicator paper (Castric, 1975), of protease with 3% milk agar plates (Brown and Foster, 1970), of β-glucanase on plates containing lichenan (Sigma, St Louis, MO, USA; Walsh et al., 1995), of cellulose on plates containing 1-carboxymethylcellulose (Fluka, Steinheim, Germany; Hankin and Anastasakis, 1977) and of lipase with Tween 80 agar plates (VWR International Prolabo; Howe and Ward, 1976).

Auxin production

The production of IAA (indole acetic acid) was determined using the colorimetric method. Briefly, the tested strains were inoculated in KB without and with tryptophan (500 µg ml⁻¹) and incubated at 28°C at 150 r.p.m. min⁻¹. After 2, 4, 6 and 8 days of cultivation, aliquots of bacterial cultures were centrifuged at 13 000 r.p.m. for 10 min. Two millilitres of supernatant fluid was added to a tube with 100 µl of 10 mM orthophosphoric acid and 4 ml of Salkowski reagent (Gordon and Weber, 1951). The mixture was incubated at room temperature for 25 min and the absorbance of the developed pink colour was read at 530 nm. The IAA concentration in culture was determined by using a calibration curve of pure IAA as a standard.

Plant growth promotion

Bacteria were grown in KB medium for 48 h. Wheat seeds were coated with bacteria by dipping the seeds in a bacterial
suspension of 10⁶–10⁷ cfu ml⁻¹. To study the effect of bacterial strains on shoot and root growth of wheat, plastic pots (10 cm in diameter; 15 cm deep) containing 350 g of soil were used. Non-inoculated control plants and plants inoculated with bacteria were grown for 4 weeks at a temperature of 32–34°C during the day and 18–20°C at night. The inoculation treatments were set up in a randomized design with 12 replications (two plants per pot). Four seeds were sown per pot and after germination the number of plants was thinned to two per pot.

Statistical procedures

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98; comparisons were performed using a Student’s t-test. Mean comparisons were conducted using a least significant difference (LSD) test ($P = 0.05$). Standard error and a LSD result were recorded.

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