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Heavy metal tolerant *Pseudomonas* sp. isolates closely related to *P. protegens* from agricultural well water in northeastern Algeria with plant growth-promoting, insecticidal and antifungal activities

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Abstract

The application of plant growth-promoting bacteria (PGPB) with biocontrol activities as inoculants of crops plants against phytopathogenic fungi and insect pests provides a biological alternative to the use of agrochemicals. Two *Pseudomonas* sp. strains were isolated from agricultural well water in the area of Bejaia, northeastern Algeria, located rather closely to a lead mine deposit. The isolates S4LiBe and S5LiBe had 16S rRNA gene sequence similarities of 99.4% to 99.7% with *Pseudomonas protegens* CHAO^T and other *P. protegens* strains. The phenotypic profiles tested with BIOLOG-GN2-microplates were very similar, but showed also some remarkable differences. The isolates S4LiBe and S5LiBe showed plant growth-promoting potential based on the production of the phytohormone indole acetic acid and siderophores and the solubilization of insoluble phosphate. In addition, they produced chitinase and other polymer degrading enzymes. Interestingly, while S4LiBe and S5LiBe were resistant against heavy metals (2.0 mM $K_2Cr_2O_7$ and 3.0 mM $CoSO_4$, $HgSO_4$, $CdSO_4$ $8H_2O$ and $PbCl_2$), the reference strain *P. protegens* CHAO^T was very sensitive to Hg^{2+} and Cd^{2+} and had lower tolerance towards Co^{2+} and Pb^{2+}. The isolates S4LiBe and S5LiBe were very active in mycelial growth inhibition assays against *Botrytis cinerea, Verticillium dahlia, Fusarium graminearum, Aspergillus niger* and *A. flavus* (growth inhibition between 88% and 48%). Furthermore, S4LiBe and S5LiBe showed effective insecticidal activities, when tested in the *Galleria* injection assay and they were tested positive for the insect toxin gene *fitD* alike the reference strain CHA0^T. Finally, inoculation of barley seeds with S5LiBe resulted in significantly stimulated germination rate and growth of seedlings, with increased shoot length, shoot and root fresh weight, shoot and root dry weight as compared to non-inoculated plants. Thus, the heavy metal tolerant isolates S4LiBe and S5LiBe harbor a diverse potential as beneficial bacteria for agricultural application. They may be very useful even in polluted soils for the stimulation of e.g. biomass crops. The demonstration of successful isolation from agricultural well water may open more ready access for a wide variety of this kind of beneficial bacteria for agricultural application.

Keywords: *Pseudomonas protegens*, heavy metal tolerance, plant growth-promoting bacteria, biocontrol, insecticidal activity, enzymatic activities, barley

1. Introduction

Inoculation with plant growth-promoting bacteria (PGPB) represents an agrobiotechnology designed to improve growth, yield and health of agricultural crops [49, 51]. The use of these beneficial microorganisms appears to be a cost-effective, ecosystem friendly and healthy alternative to the extensive use of chemicals such as fungicides, herbicides and insecticides, which could have negative impacts on the environment and human health.

The potential of PGPB to increase crop production involves the solubilization of inorganic phosphate and ferric iron minerals, the increased uptake of mineral nutrients [33], the ability to reduce stress ethylene production in plants [19], N_2 fixation [31], and the production of plant hormones such as auxins, gibberellins and cytokinins [18]. Another mechanism used by the PGPB to improve plant health is the increase of resistance against phytopathogenic microorganisms. PGPB are able to control pathogens by several mechanisms: competitive root colonization, production of antimicrobial compounds [11], production of hydrolytic enzymes, siderophores, HCN, ammonia, and by inducing systemic plant resistance [49]. In addition, insecticidal activities were reported for some PGPB in addition to the existing biocontrol repertoire with perspectives for application against insect crop pests [48]. Recent reports suggest that PGPB also enhance the tolerance of plants towards abiotic stresses such as drought [54, 58], chilling injury [3], salinity [22], metal toxicity [13] and elevated temperature stress [1, 2].

A high diversity of PGPB have been identified which enhance plant growth by several mechanisms. These PGPB belong for example to the bacterial genera *Pseudomonas, Azospirillum, Cellulosimicrobium, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* [10, 18, 21, 27, 35, 36, 39].

Among PGPB, *Pseudomonas* spp. have been broadly studied for their roles in plant growth promotion and biological control [16, 17, 21, 25, 30, 46, 59]. Plant growth promoting activities of *Pseudomonas* spp. include production of indole acetic acid (IAA) [16], phosphate solubilization [60], degradation of toxic compounds [38] and production of biocontrol agents against fungal phytopathogens like the production of siderophores [7, 14] or antibiotics [21, 24] as well as against insect pests by the production of insect toxins [41].

Due to industrialization, environmental pollution by heavy metals is a common problem all over the world. However, heavy metal contamination of soils and water may also originate from natural occurring geominerals. Nevertheless, in many sites anthropogenic activities have

enriched or deposited huge amounts of heavy metal containing rocks, soil and debris, which are the sources of pollution plumes into the surrounding soil and water bodies. In heavy metal-polluted sites, heavy metal-resistant bacteria have been found, which face the pollution and even have the ability to reduce it [38].

In the present investigation, agricultural well water was tested for the presence of PGPB, because this water was fed from agricultural soil and it was used for irrigation. In addition, there was a lead mineral containing rock deposit in some distance, origination from lead mining, which is no longer in operation. An emphasis was laid on the *Pseudomonas* group of bacteria, because these have a wide spectrum of plant-beneficial activities ranging from antagonistic activities against a wide range of phytopathogenic fungi, insecticidal activity, and phytohormone, siderophore and extracellular enzyme production. One of the isolate was further tested for plant growth promotion effects on barley seedlings.

2. Materials and Methods

2.1 Isolation and screening of bacteria

PGPB were isolated from well water (receiving effluents from agricultural soil) in the region of Bejaia (northern Algeria). Samples were serially diluted in sterile distilled water and 0.1 ml of each dilution was seeded onto nutrient broth agar in triplicates. The agar plates were incubated at 28°C for 1 week and colonies with different morphologies were selected, re-streaked on nutrient agar medium, and checked for purity.

2.2 Phenotypic and molecular phylogenetic characterization

The isolates and reference strains were phenotypically characterized using the Biolog GN2 MicroPlate™ according to the manufacturer´s instruction. For the phylogenetic characterization, genomic DNA extraction from pure bacterial colonies was carried out using the FastDNA® SPIN kit in conjunction with the FastPrep FP120 instrument (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. The genomic DNA was further PCR amplified for 16S rDNA gene sequencing using the flanking primer pair 616F (5´AGA GTT TGA TYM TGG CTCAG 3´) and 630R (5´ CAK AAA GGA GGT GAT CC 3´). The amplification program was performed in a thermocycler peqSTAR 96 Universal, with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of 45s denaturation

at 94°C, 45s annealing at 55°C, 1 min extension at 72°C, and a final extension step at 72°C for 10 min. Correct amplification was tested with standard horizontal agarose gel electrophoresis followed by ethidium bromide staining. Amplification products were cloned using the StrataClone PCR cloning system. Clones were then sequenced using the Big Dye Terminator Labeling Kit (Applera, Europe BV) with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, USA). For phylogenetic analyses the obtained 16S-sequences were aligned with the *Sina Aligner V1.2.11* on the Silva website (www.arb-silva.de) and phylogenetical allocated with the software package ARB [32]. Phylogenetic tree construction was performed by using the Maximum-Likelihood [40].

2.3 Plant growth-promoting properties:

Production of indole acetic acid (IAA)

IAA production was measured using the method described by Bric et al. [5]. The isolates were grown in LB liquid medium supplemented with 0.5% glucose and 500 µg/ml tryptophan and incubated under shaking conditions at 30°C for 72h. 5 ml of culture was centrifuged at 9000 rpm for 20 min and 2 ml of supernatant was transferred to a fresh tube to which 100µl of 10 mM orthophosphoric acid and 4 ml of reagent (1 ml of 0, 5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated at room temperature for 25 min and the absorbance of pink color developed was read at 530 nm.

Phosphate solubilization

The isolates were tested for inorganic phosphate solubilization. Freshly grown bacterial culture was inoculated to Pitkovskaya agar containing inorganic phosphate and incubated at 30°C for 5 days. A clear halo around the bacterial colony indicates solubilization of mineral phosphate [53].

Cellulase activity

Bacteria were inoculated in CMC (Carboxy Methyl Cellulose) agar containing (g/l): Na_2HPO_4 6, KH_2PO_4 3, NaCl 0.5, NH_4Cl 1, yeast extract 3, CMC 7, agar-agar 15 and incubated at 30°C for 8 days. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was inundated with a solution of Congo red (1% w/v) for 20 min. Congo red solution was then poured off, and the plates were further treated with 1 M NaCl [8]. Clear zones around the colony indicated the production of extracellular cellulase.

Esterase activity

The isolates were inoculated in the media which is described by Sierra [56], containing (g/l) peptone 10, NaCl 5, $CaCl_2$ $2H_2O$ 0.1, agar-agar 18, and 1% of sterilized tween 80. After incubation at 30°C for 48 h a clear halo around the colonies demonstrated esterase activity.

Lipolytic activity

Lipolytic activity was determined as described above for the determination of esterase activity [56]. In this experiment, tween 80 was replaced by tween 20. Clear halos around the colonies indicate lipolytic activity.

Protease activity

Protease activity tests were carried out in skimmed milk agar medium. After incubation at 30°C for 48 h, the presence of a clear zone around colony was the indication of protease production [26].

Siderophore production

The experiments were carried out in Chrome Azurol S agar, according to the method of Schwyn and Neilands [55]. CAS agar was prepared from four solutions, which were sterilized separately before mixing: the Fe-CAS indicator solution, buffer solution, nutrient solution and casamino acid solution. After autoclaving and at a temperature of about 50ºC, nutrient solution and casamino acid solution were added to the buffer solution. The indicator solution was added last with sufficient stirring to mix the ingredients. The isolates were streaked on the surface of the blue agar plates, incubated at 30°C for 3 days, and examined for growth and production of red to orange halos surrounding the colonies, indicating siderophore production.

Chitinase activity

Chitinase activity was determined as described by Kopečný et *al*. [28]. Bacterial isolates were inoculated in minimal salt medium containing 0,8% of colloidal chitin as sole carbon and energy source and the following incredients (g/l): K_2HPO_4 2.7, KH_2PO_4 0.3, $MgSO_4$ $7H_2O$ 0.7, NaCl 0.5, KCl 0.5, yeast extract 0.13, agar-agar 15. The plates were incubated at 30°C for 7 days. Clear zone around the colonies indicate extracellular chitinase activity.

2.4 Biocontrol activities:

Antifungal activity

The isolates were assayed for antifungal activities against *Botrytis cinerea, Verticillium dahlia*, *Fusarium graminearum, Asperillus niger* and *A. flavus* as described by Sagahón et al. [52]. An -cm^2 fungal plug was placed in the center of Luria Bertani (LB) plate and each bacterial isolate was inoculated at a distance of 2.5 cm from the fungal inoculum. Plates without potential bacterial antagonist served as negative control. Three replicates were performed for each confrontation experiment. The plates were then incubated at 25±2°C for 5 days and verified every day. The percentage of growth inhibition (PGI) of the fungus was recorded and calculated using the formula: PGI (%) = KR-R1/KR-100 where KR corresponds to the distance from the point of inoculation to the colony margin on the control dish (mm). R1 represents the distance (mm) of fungal growth from the point of inoculation to the colony margin on the treated dishes.

Insecticidal activity

As biological insecticidal activitiy test, the *Galleria mellonella* virulence assay was performed. The injection assays for virulence determination used last-instar larvae of *G. mellonella* (Entomos AG, Grossdietwil, Switzerland) as described before by Péchy-Tarr et al., [41] with 18 larvae per tested bacterial strain.

To test the presence of the *fitD*-toxin gene *fitD*-specific primers were applied with DNA extracted from the bacteria. The polymerase chain reaction was performed using the GoTaq DNA Polymerase kit (Promega) according to the manufacturer´s instructions and the primer pairs fitD-screen-F: 5´-CCTGCTCAATACCCTGATCG-3´ and fitD-screen-R: 5´-GTGGTTGGCGAAGTACTGCTC-3`.

2.5 Heavy metal tolerance

Heavy metals incorporated media were used to examine the ability of the isolates to resist heavy metals. Cells of overnight grown cultures were inoculated on nutrient agar plates supplemented with different heavy metals (K_2CrO_7, $HgSO_4$, $CdSO_4$ $8H_2O$, $CoSO_4$, $PbCl_2$), The concentration of each heavy metal solution (0.5, 1, 1.5, 2, 2.5 and 3 mM) was prepared in sterile deionized water and sterilized by autoclaving at 121ºC for 15 min. After incubation for 24-48 h at 30°C, the plates were examined for cell growth [23].

2.6 Plant growth stimulation tests

Barley (*Hordeum vulgare* L.) seeds were surface-sterilized as described by Götz et al. [20]. First, the seeds were treated with 70% ethanol for 1 min and then with 12% acidified hypochlorite for 15 min. The seeds were washed thoroughly in sterile water. Then the seeds were germinated on LB agar plates in the dark at room temperature for 2 days.

For seed inoculation, S5LiBe was grown in LB medium overnight at 30ºC. The bacterial culture was pelleted by centrifugation and the supernatant was discarded. The cell pellet was washed twice with 20 ml phosphate buffered saline (PBS, pH 7.2), and suspended in PBS. The optical density of the bacterial suspension was adjusted to 0.1 at 620 nm, corresponding to a cell density of 10^8 cells/ml [15]. Surface sterilized barley seeds were incubated with the bacterial suspension for 1 h at room temperature. Control seeds were incubated in sterile distilled water under the same conditions [35]. The seeds were planted in pots filled with agricultural soil with the following characteristics: pH (7.04), granulometry (clay 17.07%, fine silt 23.0%, coarse silt 7.09%, fine sand 12.35%, coarse sand 28.92%), active limestone 0.38%, conductivity 200 µS/cm, organic carbon 6.38%, exchangeable K_2O 0.11 g/kg, exchangeable CaO 3.99 g/kg, exchangeable MgO 5.24 g/kg.
The experiment consisted of seven lots and each lot was composed of seven seeds, inoculated or non-inoculated with bacterial suspension. The seeds were sown at a depth of approximately 1 cm. The experiment was performed under natural dark/light cycles (16 h of light and 8 h of dark) at a temperature of 25-35°C for one to two weeks [50]. Seed germination was determined by counting germinated seeds at 3 and 7 day after sowing the seeds. Results corresponding to final counts were reported as percentage of germination [26]. Plant growth response parameters were measured after 15 days including shoot length, fresh and dry weight of shoot, fresh and dry weight of root.

2.7 Statistical analysis

Data obtained from the plant growth responses were subjected to analysis of variance by the least significant difference (LSD) test at $p \leq 0,05$ with statistical software XLSTAT version 2009.1.02.

3. Results

3.1 Phylogenetic and phenotypic characterization of the isolates

Two bacterial strains (S4LiBe and S5LiBe) were obtained from the well water after plating and purifying on NB-agar. The colonies had a greenish yellow color, resembling *Pseudomonas* spp. Bacterial cells were motile and stained Gram-negative.

For molecular phylogenetic characterization of the isolates, 16S rDNA sequence analysis was performed. PCR-amplified 16S rDNA of the bacterial strains was sequenced and blasted with the NCBI database. Comparative analysis with whole 16S rDNA data base sequences suggested that the isolates S4LiBe and S5LiBe were most closely related to *Pseudomonas protegens*. The 16S rDNA sequences of the two isolates were 99.8% similar to each other. S4LiBe showed 99.8% 16S rRNA similarity to *Pseudomonas sp*. AF521651, 99.7% to *P. protegens* Pf-5 (sequence AJ417073), 99.6% to *P. protegens* PGNR1 (sequence AJ417071), and 99.5% to *P. protegens* CHA0^T (sequence AJ278812). The 16S rDNA of S5LiBe had 99.4% similarity with *Pseudomonas* sp. AF521651, 99.6% with *Pseudomonas protegens* AJ 417073 and 99.5% with *P. protegens* CHA0^T (sequence AJ278812) (see table S2). The phylogenetic position of the isolates based on 16S rDNA similarity is also shown in the dendrograms based on maximum likelihood tree calculation (Figure 1).
The metabolic characteristics of the isolates S4LiBe and S5LiBe were obtained using the Biolog GN2 MicroPlates (Table S1) and compared with the type strain of *P. protegens* CHA0^T and *P. protegens* Pf-5. From 115 carbon sources tested with the isolates S4LiBe and S5LiBe, 89% were identical with the type strain CHA0^T, while 84% were identical with the strain Pf-5. Differences in the utilization pattern between CHA0^T and the isolates were found for m-inositol, D-psicose, sucrose, formic acid, hydroxybutyric acid, itaconic acid, succinamic acid, D-alanine, L-alanine, L-alanylglycine, hydroxy-L-proline and L-threonine (for more information see table S1).

Figure 1: Phylogenetic relationship of the 16S rDNA sequences of S4LiBe and S5LiBe and different reference strains. The calculated dendrogram is based on maximum likelihood tree calculation.

3.2 Plant growth promotion traits

Table 1 shows the PGP traits of the isolates in comparison with the *P. protegens* type strain CHA0^T. The production of IAA ranged from 3.1 to 4.0 µg ml^{-1} in the presence of 500 µg/ml

L-tryptophan. The isolates and the reference strain produced urease, lipase, protease, esterase and cellulose at different levels. However, $CHA0^T$ failed to produce chitinase under the applied test conditions. The abilities to solubilize precipitated phosphate and to produce siderophores were common in S4LiBe, S5LiBe and $CHA0^T$.

3.3 Heavy metal tolerance

Among all heavy metals tested, lead and cobalt were the least toxic to all isolates. S4LiBe, S5LiBe and CHA0 were able to grow in the presence of K_2CrO_7 at concentrations up to 2 mM. The isolates S4LiBe and S5LiBe showed a very high degree of tolerance to up to 3 mM of $HgSO_4$, $CdSO_4$, and $PbCl_2$. In contrast, CHA0 was very sensitive to $HgSO_4$ and less tolerant towards $CdSO_4$ and $PbCl_2$ (Table 2).

3.4 Biological control activities

Fungal antagonistic activity

Figure 2 shows the antifungal activity of the isolates against the pathogenic fungi *F. graminearum*, *V. dahliae* and *B. cinerea* as well as *Asperillus niger* and *A. flavus* tested in an agar plate confrontation assays. All five fungi were inhibited to different extents by the *P. protegens* isolates S5LiBe and S4LiBe.

Figure 2: Percentage Growth Inhibition (PGI %) of plant pathogens in the presence of the isolates S4LiBe and S5LiBe

Insecticidal activity

The *Galleria mellonella* virulence assay of the isolates S4LiBe and S5Libe as well as the reference strain $CHA0^T$ clearly demonstrated that the isolates exhibited equal effective insecticidal activity as the *P. protegens* reference strain (Figure 3). In both isolates also the *fitD* gene for the Fit insecticidal toxin could be detected.

Figure 3: Insecticidal activity of the isolates S4LiBe and S5LiBe compared to the reference strain $CHA0^T$.

3.5 Plant growth-promoting effect of isolate S5LiBe

The seed germination and growth promotion tests with barley showed that two weeks after inoculation strain S5LiBe significantly increased germination percentage and growth of barley. S5LiBe increased significantly the shoot length compared to the control. Bacterial

inoculation also had a significant effect on fresh and dry weight of shoot and root (0.01; 0.075; 0.04 and 0.03), respectively, compared to the controls (0.06; 0.047; 0.025 and 0.016).

Figure 4: Germination percentage in non-inoculated and S5LiBe-inoculated barley seeds after 3 and 7 days.

Figure 5a, b, c: Stimulation of growth of barley by inoculation with strain S5BiLe after 15 days of growth.

4. Discussion

Fluorescent pseudomonads and other rhizobacteria are well known for their abilities to successfully colonize plant roots and to promote plant growth by biological control [21] and plant growth promotion activities [42]. In the present study, two *Pseudomonas* sp. isolates from agricultural well water indeed showed a wide variety of different features of plant growth promoting traits. The isolates S4LiBe and S5LiBe appeared to be related to fluorescent pseudomonads based on 16S rDNA gene similarity analysis (Figure 1) and carbon utilization pattern (Table S1). The isolates S4LiBe and S5LiBe are very similar (99.8% 16SrRNA gene similarity) to each other and are very closely related (99.4 to 99.7%) to the *P. protegens* strains CHA0^T and Pf-5 [44]. However, a definite phylogenetic clarification would need further analysis based on concatenated alignments of several household genes [30, 42], because 16S rRNA analysis alone cannot conclusively resolve very closely related species, like in the *Pseudomonas fluorescens* cluster.

Biological control of phytopathogens by fluorescent pseudomonads reduces the severity of many plant diseases [49]. In this study, both isolates showed clearly *in vitro* antagonistic potential against three plant pathogenic fungi *Botrytis cinerea, Verticillium dahliae* and *Fusarium graminearum* (88%, 80% and 82% respectively) as well as towards two *Aspergillus* spp. (Figure 2). Srinivasan et al. [57] had characterized five members of the genus *Pseudomonas*, *P. putida* FC-6B, *P.* sp. FC-7B, *P. putida* FC-8B, *P.* sp. FC-9B and *P.* sp. FC-24B, which showed antifungal activity against *F. oxysporum* sp and *F. oxysporum*. f.sp. *lycopersici*. Sagahón et al. [52] reported that *Pseudomonas* spp. 11 inhibited up to 70% of *Stenocarpella maydis* and *Stenocarpella macrospora* and the filtrates obtained in logarithmic growth phase from the *P. fluorescens* 16 inhibited 54% of the growth of *S. maydis.* In addition to siderophore and chitinase production, the observed biological control activity of the isolates is probably also due to the production of several antibiotic compounds, like 2,4-

diacetylphloroglucinol and pyoluteorin, as is well known for fluorescent pseudomonads [21, 44]. Currently, genomic analysis of the two isolates is in progress, which will demonstrate which biosynthetic gene clusters for antibiotic production and other antagonistic activities are present in the two well water isolates from Algeria. Like the reference strain *P. protegens* CHA0^T and related strains [41, 47], the isolates S4LiBe and S5LiBe also harbor insecticidal activities, as could be demonstrated by the positive *Galleria mellonella* injection assay and the presence of the *fitD* gene (Figure 3). Since the infection and growth within insect larvae constitutes a second growth cycle of these bacteria - apart from plant roots , it may be hypothesized, that these types of bacteria were enriched in the well water also harboring insect larvae. Therefore, the isolation of PGPB with insecticidal activities from agricultural well water may be a quite straightforward approach, which may be useful in future isolation attempts.

S5LiBe-inoculated plants significantly increased germination rate, shoot length, fresh and dry biomass of barley comparing with non-inoculated plants after two weeks of growth. Plant growth-promoting strains of *Pseudomonas* spp. have been already described in multiple studies, recently e.g. by Rosas et al. [46], who reported that the strain *P. aurantiaca* SR1, when applied on maize and wheat seeds showed a significant plant growth-promoting effect. The beneficial effect of *Pseudomonas* was also confirmed by Egamberdieva et al. [15] who tested the co-inoculation of *Pseudomonas* spp. with *Rhizobium* on the growth of fodder galega (*Galega orientalis* Lam.). Co-inoculation of plants showed increased shoot and root dry matter compared to the inoculation with *R. galega* HAMBI 540 alone. The isolates S4LiBe and S5LiBe as well as the reference strain CHA0 had IAA production activity in the presence of L-tryptophan in the range of 3-4 µg/ml, which is in the range of other PGPBs. Naik and Sakthivel [37] suggested that the plant growth-promoting ability of *Pseudomonas* sp. PUP6 could be based on the production of phytohormone IAA, siderophores and phosphate-solubilizing enzymes. Wahyudiet al. [59] reported that the capability to increase plant growth parameters in germination seed bioassays was highly related to the IAA level produced by *Pseudomonas* spp. applied. The growth-promoting effect of S5LiBe can also be due to another mechanism, which is linked to its phosphate-solubilizing activity. Phosphorus (phosphate) is one of the major essential macronutrients for biological growth and development and the formation of insoluble forms of phosphorus limits soil phosphorus accessibility. Rodriguez and Fraga [45] reported that *Pseudomonas* and other phosphate-solubilizing bacteria are capable to increase the availability of phosphate in soil. Siderophore

production is another important characteristic of efficient plant growth promotion which was observed with the isolates as well as the applied reference strain CHA0. Siderophores are widely studied as one biocontrol mechanism against plant pathogenic microbes, but they also may support iron nutrition of plants. Siderophores can stimulate plant growth indirectly by the inhibition of phytopathogenic microorganisms competing for the growth-limiting, mostly insoluble ferric iron resource in soils [15, 29, 50].

Furthermore, the isolates S4LiBe and S5LiBe produce several exo-cellular enzymes (protease, cellulose, chitinase and urease) which are potentially relevant for soil fertility. The degradation of protein by microbial peptidases is important in N-cycling in soils by making organically bound nitrogen accessible for plants [4]. Chitinases are well known to lyse the fungal cell wall [9] and thus could effectively contribute to control plant-pathogenic fungi. As reported by Egamberdieva et al. [15], cellulose production of bacteria can enhance nodule formation. Co-inoculation of the cellulose-producing strain *Pseudomonas trivialis* 3Re27 with *Rhizobium galegae* HAMBI 540 significantly increased nodulation and nitrogen content of fodder galega, whereas cellulose-negative *Pseudomonas extremorientalis* TSAU20 showed no significant stimulation.

Since heavy metals cannot be biologically degraded to harmless products and hence persist in the environment indefinitely, heavy metal contamination of agriculture soil is a significant environmental problem and has several disadvantages on human health and agriculture [61]. The selection of metal-tolerant plant growth-promoting microorganisms can be advantageous to speed up the recolonization of the plant rhizosphere in polluted soils [12]. Both isolates in this study were characterized by considerable tolerance to rather high levels (up to 3 mM) of the heavy metals Co, Pb, Hg and Cd, and up to 2 mM of chromium (Table 3). Dell'Amico et al. [13] showed that inoculation with cadmium-resistant strains of *Pseudomonas tolaasii* and *Pseudomonas fluorescens* enabled *Brassica napus* to grow under cadmium stress and they suggested that the bacterial production of indole acetic acid (IAA), siderophores and ACC (1-aminocyclopropane-1-carboxylate) deaminase was involved in plant protection against cadmium stress. Since the *P. protegens* reference strain CHA0^T (like related PGPB) is rather sensitive to these heavy metals (Table 3), the heavy metal tolerance of S4LiBe and S5LiBe can be regarded as a distinct novel feature among this group of bacteria. Probably, the vicinity of the heavy metal deposit had resulted in soil contamination, which fostered the distribution of heavy metal tolerance. It is well known that metal resistance transposons or plasmids are shared between Gram-negative bacteria [34]. Further detailed investigation of the actual

contamination in the area of isolation and the well water as well as of the genetic basis in the isolated bacteria are necessary.

In conclusion, the isolates S5LiBe and S4LiBe revealed plant growth-promoting potentials, as shown by the presence of many key features of plant growth promotion and by the stimulation of germination and growth of barley seedlings upon inoculation. Furthermore, the estimation of the antagonistic effect against pathogenic fungi and also insecticidal activity add further valuable activities for possible plant beneficial bacterial inoculants. To find out most efficient biological control agents against plant diseases under given application conditions, an increasing number of isolates is still to be investigated [6]. The observed heavy metal tolerance of the new isolates towards mercury, cadmium, cobalt and lead may enable the newly isolated *P. protegens* strains as superior candidates for inoculation of biomass crops in heavy metal contaminated soils.

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Table 1: Plant growth-promoting traits of the isolates S4LiBe, S5LiBe, and *P. protegens* CHA0^T

Traits	S4LiBe	S5LiBe	CHA0^T
IAA (µg/ml)	4.0	3.1	3.5
Chitinase	+	++	-
Protease	+	+	++
Esterase	+	+	+
Cellulase	+	+	+
Lipase	+	+	++
Urease	++	+++	+
Phosphate solubilization	++	++	++
Siderophore production	++	+++	++

+++: High activity; ++: average activity; +: moderate activity; -: no activity

Table 2: Heavy metal tolerance of the isolates S4LiBe and S5LiBe and *P. protegens* CHA0^T

		Isolates		
		S4LiBe	**S5LiBe**	**CHA0^T**
K_2CrO_7 (mM)	0.5	+++	+++	+++
	1	+++	+++	++
	1.5	++	++	++
	2	+	+	+
	2.5	-	-	-
	3	-	-	-
$HgSO_4$(mM)	0.5	+++	+++	-
	1	+++	+++	-
	1.5	++	++	-
	2	++	++	-
	2.5	++	++	-
	3	++	++	-
$CdSO_4 8H_2O$ (mM)	0.5	+++	+++	++
	1	++	++	+
	1.5	+	++	-
	2	+	+	-
	2.5	+	+	-
	3	+	+	-
$CoSO_4$ (mM)	0.5	+++	+++	+++
	1	+++	+++	+++
	1.5	+++	+++	++
	2	++	++	++
	2.5	+	+	+
	3	+	+	-
$PbCl_2$ (mM)	0.5	+++	+++	+++
	1	+++	+++	+++
	1.5	+++	+++	++
	2	+++	+++	++
	2.5	+++	+++	+
	3	+++	+++	+

+++: High resistance; ++: average resistance; +: resistance; -: sensitive

Legends to Figures:

Figure 1: 16S rRNA gene similarity tree, based on maximum likelihood analysis

Figure 2: Percentage Growth Inhibition (PGI %) of plant pathogenic fungi (plate confrontation assays) by the isolates S4LiBe and S5LiBe

Figure 3: Insecticidal activity of the isolates S4LiBe and S5LiBe compared to the reference strain $CHA0^T$.

- PCR-analysis of the *fitD* gene
- *Galleria mellonella* virulence assay, according to Péchy-Tarr et al. [41];
  Black circles: *P. protegens* $CHA0^T$ (positive control)
  Blue diamonds: *Pseudomonas* sp. S4LiBe;
  Red squares: *Pseudomonas* sp. S5LiBe;
  Gray triangles: 0.9% NaCl solution (negative control)

Figure 4: Germination percentage in non-inoculated and S5LiBe-inoculated barley seeds after 3 and 7 days. Different letters (a and b) indicate significant differences obtained by the Fischer LSD test ($p \leq 0,05$).

Figure 5: Stimulation of growth S5LiBe-inoculated and non-inoculated (control) barley seedlings 15 days after inoculation. Different letters (a and b) indicate significant differences obtained by the Fischer LSD test ($p \leq 0,05$).

A) Effect on shoot length,

B) Effect on fresh and dry weight of shoots,

C) Effect on fresh and dry weight of roots.

Figure 1:

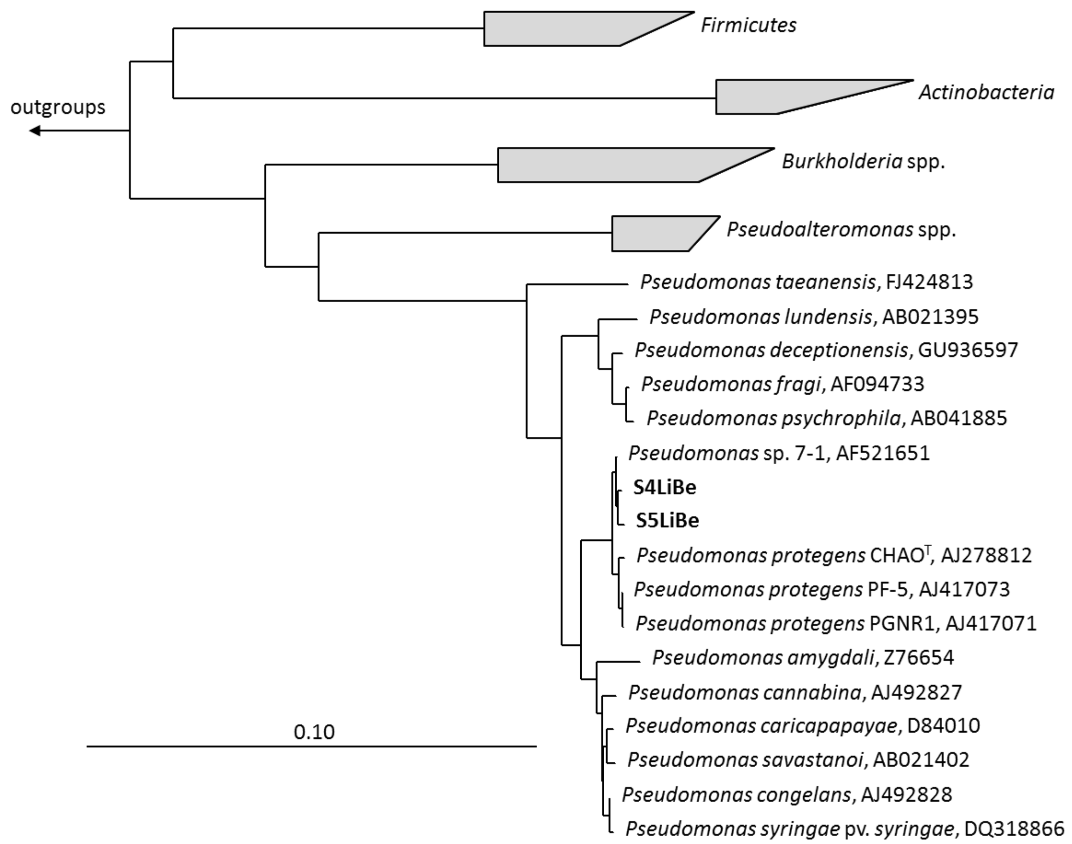


Figure 2:

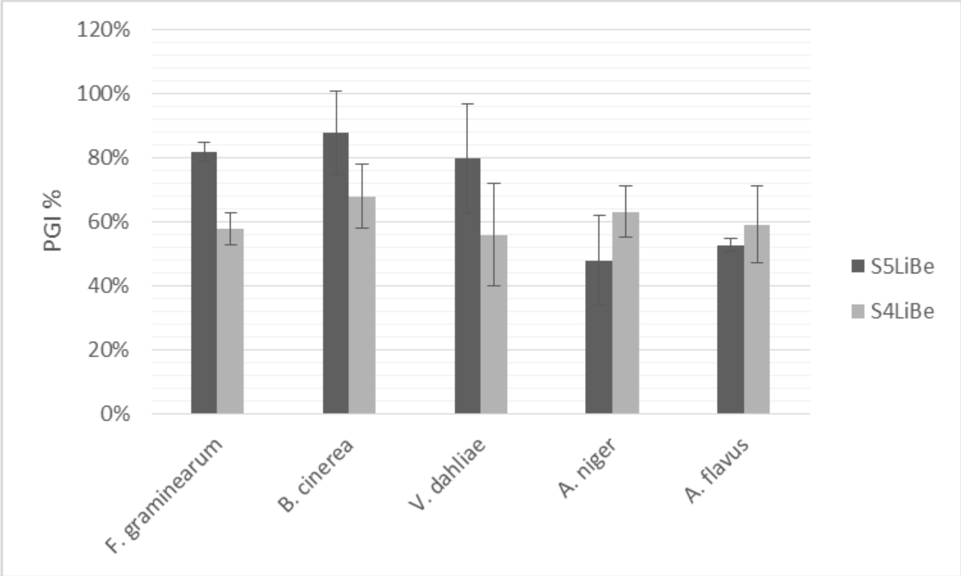


Figure 3:

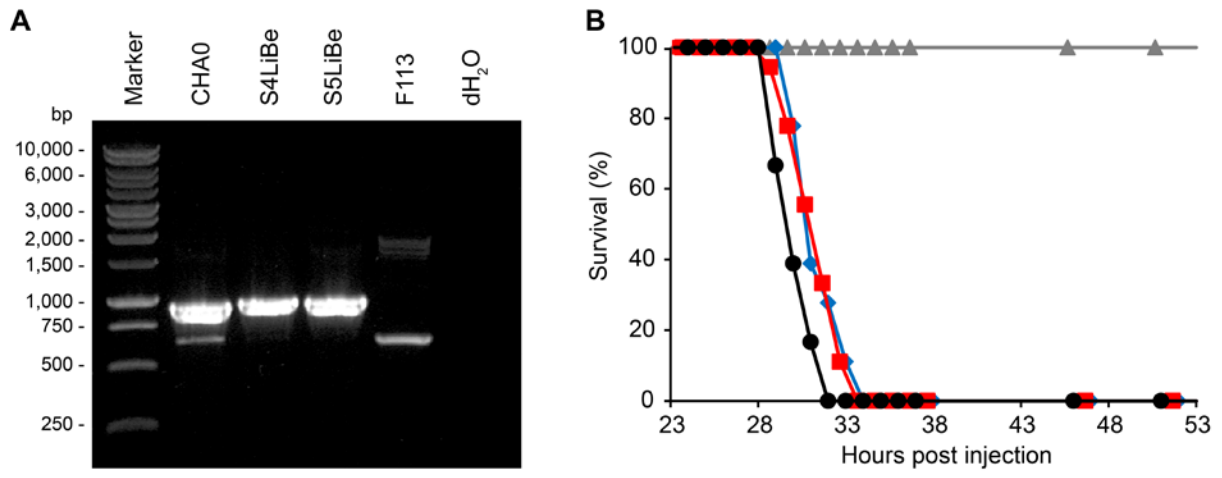


Figure 4:

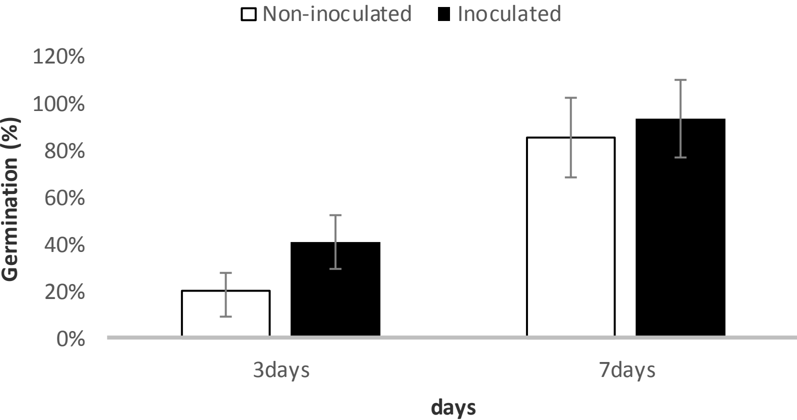
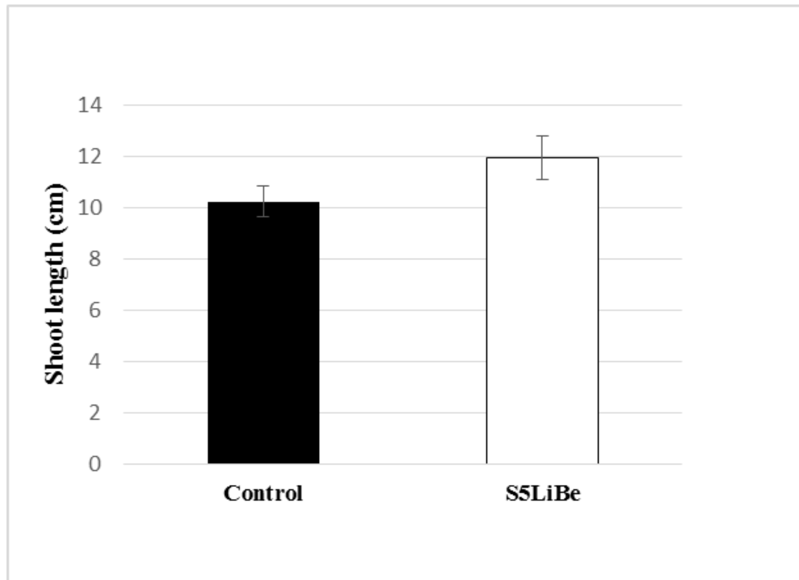
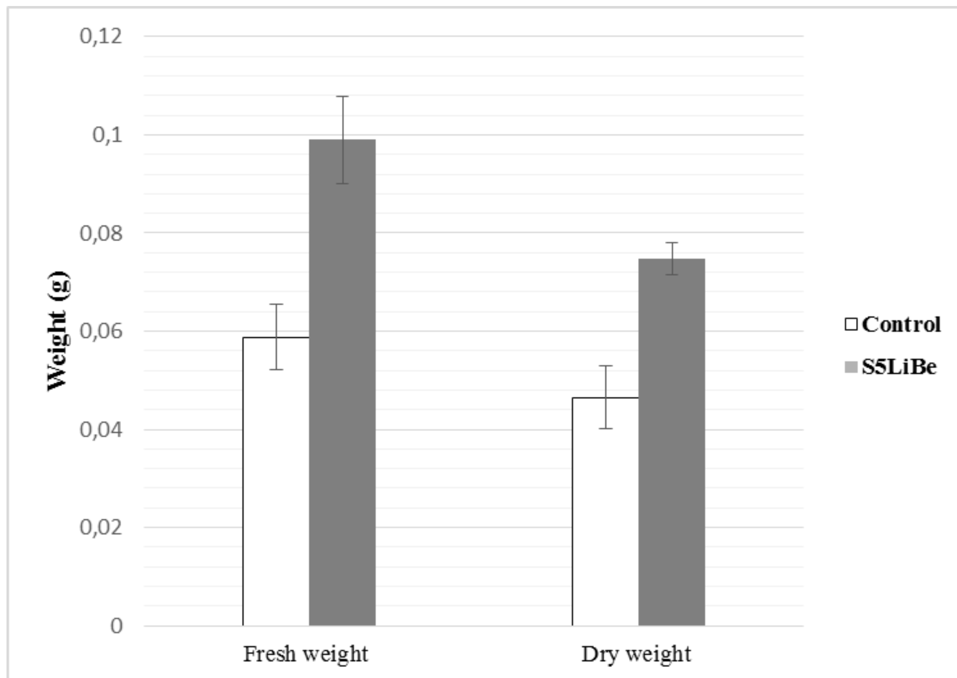


Figure 5:

A)



B)



C)

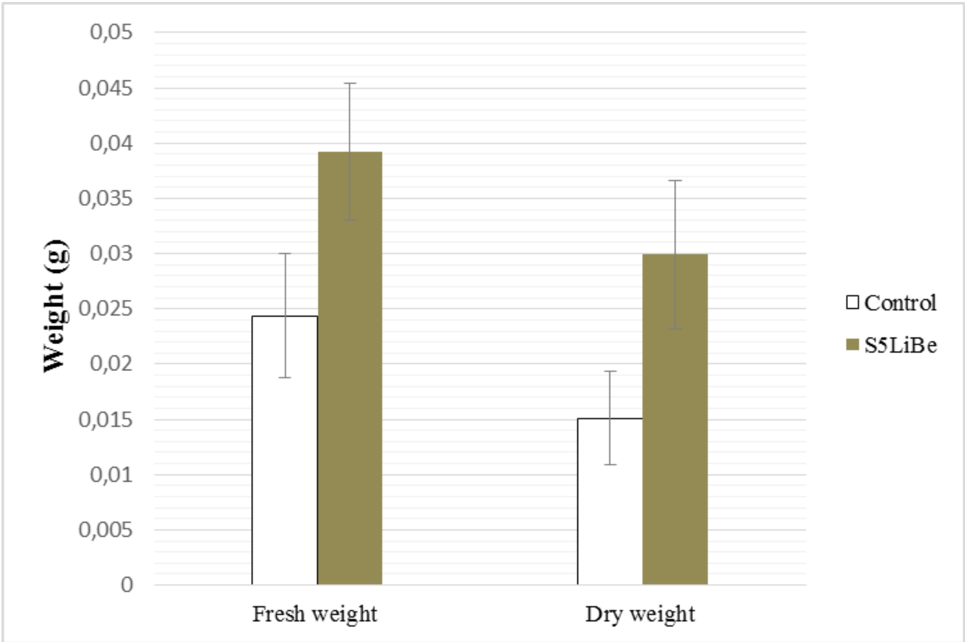


Table S1: 16S rRNA-gene similarities between the isolates S4LiBe and S5LiBe and different *Pseudomonas protegens* and other *Pseudomonas* spp. strains

	Pseudomonas taeanensis. FJ424813	*Pseudomonas lundensis*. AB021395	*Pseudomonas deceptionensis*. GU936597	*Pseudomonas fragi*. AF094733	*Pseudomonas psychrophila*. AB041885	*Pseudomonas* sp.. AF521651	**S4LiBe**	**S5LiBe**	*Pseudomonas protegens* CHAO^{T}. AJ278812	*Pseudomonas protegens* PF-5. AJ417073	*Pseudomonas protegens* PGNR1. AJ417071	*Pseudomonas amygdali*. Z76654	*Pseudomonas cannabina*. AJ492827	*Pseudomonas caricapapayae*. D84010	*Pseudomonas savastanoi*. AB021402	*Pseudomonas congelans*. AJ492828	*Pseudomonas syringae* pv. *syringae*. DQ318866
P. taeanensis, FJ424813	100																
P. lundensis, AB021395	96.8	100															
P. deceptionensis, GU936597	96.6	99.3	100														
P. fragi, AF094733	96.8	99.1	99.4	100													
P. psychrophila, AB041885	96.2	99.2	99.4	99.8	100												
P. sp., AF521651	95.8	97.6	97.5	97.2	97.1	100											
S4LiBe	**95.7**	**97.5**	**97.4**	**97.1**	**97.0**	**99.9**	**100**										
S5LiBe	**95.6**	**97.4**	**97.3**	**97.0**	**96.9**	**99.8**	**99.8**	**100**									
P. protegens CHAO^{T}, AJ278812	95.6	97.6	97.5	97.2	97.0	99.6	**99.5**	**99.4**	100								
P. protegens Pf-5, AJ417073	95.8	97.6	97.5	97.2	96.9	99.7	**99.7**	**99.5**	99.9	100							
P. protegens PGNR1, AJ417071	95.7	97.6	97.5	97.2	96.8	99.7	**99.6**	**99.5**	99.8	100	100						
P. amygdali, Z76654	95.4	97.4	97.2	97.0	97.1	97.9	**97.8**	**97.7**	97.9	97.9	97.9	100					
P. cannabina, AJ492827	95.9	98.1	97.7	97.5	97.4	98.3	**98.2**	**98.1**	98.0	98.1	98.0	98.7	100				
P. caricapapayae, D84010	96.2	97.9	97.6	97.3	97.4	98.6	**98.5**	**98.4**	98.6	98.6	98.6	98.6	99.5	100			
P. savastanoi, AB021402	96.2	98.1	97.7	97.4	97.6	98.7	**98.6**	**98.5**	98.7	98.7	98.7	98.7	99.5	99.8	100		
P. congelans, AJ492828	95.9	98.1	97.7	97.5	97.4	98.6	**98.6**	**98.4**	98.3	98.4	98.4	98.8	99.5	99.7	99.7	100	
P. syringae pv. *syringae*, DQ318866	95.7	98.0	97.6	97.3	97.5	98.6	**98.4**	**98.4**	98.3	98.4	98.3	98.7	99.5	99.6	99.7	99.9	100

Table S2: Carbon source utilization patterns (BIOLOG GN2 microplate tests) of S4LiBe, S5LiBe and *P. protegens* CHA0^{T} and Pf-5:

Metabolite	**S5LiBe**	**S4LiBe**	**CHA0^{T}**	**Pf-5**
α-Cyclodextrin	-	-	-	-
Dextrin	+	+	+	+
Glycogen	-	-	-	-
Tween 40	+	+	+	+
Tween 80	+	+	+	+
N-Acetyl-D-Galactosamine	-	-	-	-
N-Acetyl-D-Glucosamine	+	+	+	-
Adonitol	-	-	-	-
L-Arabinose	-	-	-	-
D-Arabitol	-	-	+	-
D-Cellobiose	-	-	-	-
i-Erythritol	-	-	-	-
D-Fructose	+	+	+	+
L-Fucose	-	-	-	-
D-Galactose	-	-	-	-
Gentiobiose	-	-	-	-
α-D-Glucose	+	+	+	+
m-Inositol	+	+	-	-
α-D-Lactose	-	-	-	-
Lactulose	-	-	-	-
Maltose	-	-	-	-
D-Mannitol	+	+	+	-
D-Mannose	+	+	+	-
D-Melibiose	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-
D-Psicose	-	+	+	-
D-Raffinose	-	-	-	-
L-Rhamnose	-	-	-	-
D-Sorbitol	-	-	-	-
Sucrose	+	+	-	+
D-Trehalose	+	+	+	-
Turanose	-	-	-	-
Xylitol	-	-	-	-
Methyl pyruvate	+	+	+	-
Mono-Methyl-succinate	-	-	-	-
Acetic Acid	+	+	+	+
Cis-Aconitic Acid	+	+	+	+
Citric Acid	+	+	+	+
Formic Acid	+	-	+	-
D-Galactonic Acid Lactone	-	-	-	-
D-Galacturonic Acid	-	-	-	-
D-Gluconic Acid	+	+	+	+
D-Glucosaminic Acid	-	-	-	-
D-Glucuronic Acid	-	-	-	-
α-Hydroxybutyric Acid	-	-	-	-
β-HydroxybutyricAcid	+	+	+	+
γ-Hydroxybutyric Acid	-	-	+	+
p-HydroxyPhenylaceticAcid	+	+	+	-
Itaconic Acid	-	-	+	+
α-Keto Butyric Acid	-	-	-	-
α-KetoGlutaric Acid	+	+	+	+
α-KetoValeric Acid	-	-	-	-
D,L-Lactic Acid	+	+	+	+
Malonic Acid	+	+	+	+

Propionic Acid	+	+	+	+
Quinic Acid	+	+	+	+
D-Saccharic Acid	-	-	-	-
Sebacic Acid	-	-	-	-
Succinic Acid	+	+	+	+
Bromosuccinic Acid	+	+	+	+
Succinamic Acid	(+)	-	+	-
Glucuronamide	-	-	-	-
L-Alaninamide	-	-	-	-
D-Alanine	-	(+)	-	-
L-Alanine	+	(+)	-	-
L-Alanylglycine	+	-	-	-
L-Asparagine	+	+	+	+
L-Aspartic Acid	+	+	+	+
L-Glutamic Acid	+	+	+	+
Glycyl-L-Aspartic Acid	-	-	-	-
Glycyl-L-Glutamic Acid	+	+	+	-
L-Histidine	+	+	+	+
Hydroxy-L-Proline	+	+	+	-
L-Leucine	+	+	-	-
L-Ornithine	-	-	-	-
L-Phenylalanine	-	-	-	-
L-Proline	+	+	+	+
L-Pyroglutamic Acid	+	+	+	-
D-Serine	-	-	-	-
L-Serine	+	+	+	+
L-Threonine	-	-	+	-
D,L-Carnitine	-	-	-	-
γ-Amino Butyric Acid	+	+	+	+
Urocanic Acid	+	+	+	+
Inosine	+	+	+	-
Uridine	-	-	-	-
Thymidine	-	-	-	-
Phenyethylamine	-	-	-	-
Putrescine	+	(+)	+	+
2-Aminoethanol	+	+	+	+
2,3-Butanediol	-	-	-	-
Glycerol	+	+	+	+
D,L-α-Glycerol Phosphate	+	+	+	-
Glucose-1-phosphate	-	-	-	-
Glucose-6-phosphate	+	+	+	+

"+": Positive reaction, "(+)": Weak positive reaction, "**-**": Negative reaction