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Identification of Heterotrophic Zinc Mobilization Processes among Bacterial Strains Isolated from Wheat Rhizosphere (*Triticum aestivum* L.)

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ABSTRACT Soil and plant inoculation with heterotrophic zinc-solubilizing bacteria (ZSB) is considered a promising approach for increasing zinc (Zn) phytoavailability and enhancing crop growth and nutritional quality. Nevertheless, it is necessary to understand the underlying bacterial solubilization processes to predict their repeatability in inoculation strategies. Acidification via gluconic acid production remains the most reported process. In this study, wheat rhizosphere soil serial dilutions were plated on several solid microbiological media supplemented with scarcely soluble Zn oxide (ZnO), and 115 putative Zn-solubilizing isolates were directly detected based on the formation of solubilization halos around the colonies. Eight strains were selected based on their Zn solubilization efficiency and siderophore production capacity. These included one strain of *Curtobacterium*, two of *Plantibacter*, three strains of *Pseudomonas*, one of *Stenotrophomonas*, and one strain of *Streptomyces*. In ZnO liquid solubilization assays, the presence of glucose clearly stimulated organic acid production, leading to medium acidification and ZnO solubilization. While solubilization by *Streptomyces* and *Curtobacterium* was attributed to the accumulated production of six and seven different organic acids, respectively, the other strains solubilized Zn via gluconic, malonic, and oxalic acids exclusively. In contrast, in the absence of glucose, ZnO dissolution resulted from proton extrusion (e.g., via ammonia consumption by *Plantibacter* strains) and complexation processes (i.e., complexation with glutamic acid in cultures of *Curtobacterium*). Therefore, while gluconic acid production was described as a major Zn solubilization mechanism in the literature, this study goes beyond and shows that solubilization mechanisms vary among ZSB and are strongly affected by growth conditions.

IMPORTANCE Barriers toward a better understanding of the mechanisms underlying zinc (Zn) solubilization by bacteria include the lack of methodological tools for isolation, discrimination, and identification of such organisms. Our study proposes a direct bacterial isolation procedure, which prevents the need to screen numerous bacterial candidates (for which the ability to solubilize Zn is unknown) for recovering Zn-solubilizing bacteria (ZSB). Moreover, we confirm the potential of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) as a quick and accurate tool for the identification and discrimination of environmental bacterial isolates. This work also describes various Zn solubilization processes used by wheat rhizosphere bacteria, including proton extrusion and the production of different organic acids among bacterial strains. These processes were also clearly affected by growth conditions (i.e., solid versus liquid cultures and the presence and absence of glucose). Although highlighted mechanisms may have significant effects at the soil-plant interface, these should only be transposed cautiously to real ecological situations.

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Zinc (Zn) is a micronutrient involved in a variety of metabolic and physiological reactions in all forms of life (1, 2). Consequently, Zn deficiency may occur in any living organism when the intake does not match the requirements. Zn deficiency in crops reduces biomass production and quality (i.e., low Zn concentrations in edible organs) (3, 4). This is a global issue, as nearly 50% of the soils used for cereal cultivation contain very little plant-available Zn (5). In fact, the problem is usually not the lack of Zn in soil but its limited availability to plants due to, e.g., high pH or a high clay content (3). To determine the availability of Zn in soils, diethylenetriaminepentaacetic acid (DTPA) extractions are commonly used (6). Soils with levels of DTPA-extractable Zn lower than 0.5 mg · kg^{-1} are commonly considered to contain too little available Zn for optimal crop growth (5). The production of grains with low Zn concentrations further increases the risks of human Zn deficiency in countries where large parts of the populations depend on cereals as the staple food (7).

Bacteria are intimately involved in the biogeochemical cycling of Zn. Zn solubility is affected by autotrophic and heterotrophic bacterial processes, depending on the metabolism of the bacterium involved and the physicochemical conditions of the associated environment (8). Metal solubilization mediated by autotrophic bacteria (e.g., sulfur/ferric iron-oxidizing bacteria) have been extensively studied and used for the recovery of Zn, copper (Cu), and nickel from industrial ores and wastes (9). Zn solubilization by heterotrophic bacteria has also drawn attention, and strains with such properties (often referred as Zn-solubilizing bacteria [ZSB]) have been tested as inoculum for improving plant Zn accumulation in agronomic studies. For instance, in a pot experiment performed with soil containing low levels of plant-available Zn by Ramesh et al., seed inoculation with different *Bacillus aryabhattai* strains (10^8 CFU per seed) resulted in increased wheat grain Zn concentrations from 42 to 61 mg · kg^{-1} (10). This increase was partly attributed to the ability of the inoculated ZSB to mobilize soil Zn by releasing organic acids and decreasing the pH, as they did in liquid solubilization assays enriched in Zn carbonate ($ZnCO_3$), Zn oxide (ZnO), or Zn phosphate [$Zn_3(PO_4)_2$]. On the other hand, in a field study conducted in northern India, Rana et al. found no increase in wheat grain Zn concentrations in field plots inoculated with a strain of *Providencia* sp. (5×10^{13} CFU · ha^{-1}) that solubilized $ZnCO_3$ in plate assays (11). Instead, the inoculation by *Providencia* sp. increased the grain iron (Fe), manganese (Mn), and Cu concentrations, and this was attributed to plant growth promotion, e.g., via the inhibition of pathogenic fungi. Furthermore, in the context of heavy metal contamination, several pot studies reported increased Zn uptake in hyperaccumulator plants upon soil inoculation with ZSB, suggesting the potential of ZSB-assisted phytoextraction approaches for the remediation of contaminated sites (12, 13). However, also in this context, inconsistencies were found for the effects of bacterial inoculation on soil metal availability and uptake by plants (14). Key factors determining the success of these inoculation strategies include the ability of the inoculated strains to colonize, survive, and obviously to mobilize trace elements under natural conditions. Therefore, it is necessary to better understand the bacterial processes responsible for Zn solubilization to predict their repeatability in inoculation strategies. However, despite the various potential applications of ZSB, only few studies have focused in detail on the underlying bacterial processes of solubilization.

The accumulation of gluconic acid (and its keto derivatives) remains by far the most frequently reported mechanism for Zn solubilization by heterotrophic bacteria (15–19). Gluconic acid is derived from the extracellular oxidation of glucose, which for bacteria, is mainly catalyzed by the membrane glucose dehydrogenase (20, 21). The production of this acid is therefore strongly dependent on the availability of glucose in the immediate environment of bacteria. The solubilization of $ZnCO_3$, ZnO, and $Zn_3(PO_4)_2$ via gluconic acid production was described *in vitro*, e.g., for strains of *Pseudomonas* (16,

17), *Acinetobacter* (22), and *Gluconacetobacter* (18). The main solubilization process identified in these studies was medium acidification, resulting from the production of large gluconic acid quantities. In fact, organic acids may originate from other bacterial metabolic processes (i.e., the tricarboxylic acid cycle [TCA] and glycolysis) and are not specifically exuded to mobilize Zn or other trace elements (23). In a metal mobilization assay conducted by Li et al., the solubilization of Zn by *Burkholderia cepacia* was attributed to the production of four other organic acids (i.e., oxalic, tartaric, formic, and acetic acids) despite the presence of glucose in the growth medium (24). Therefore, it appears that different species of ZSB can solubilize Zn through the production of different organic acids and that gluconic acid is not the only compound possibly involved. Beside organic acids, siderophores are also produced by bacteria and are primarily involved in Fe solubilization through chelating processes (25). Some siderophores were also found to chelate Zn, but their exact role in Zn solubilization remains poorly studied (26, 27).

Before processes underlying heterotrophic bacterial Zn solubilization can be studied, it is first necessary to isolate bacterial strains that are able to mobilize Zn. To achieve this, bacterial candidates (those in which the ability to solubilize Zn is unknown) are generally first recovered from environmental samples, and their ability to solubilize Zn is then investigated (28). This screening is usually done via Zn solubilization assays in which each tested bacterial isolate is grown in an agar medium containing scarcely soluble Zn salts [e.g., ZnO and $Zn_3(PO_4)_2$]. The formation of clearing zones around the colonies demonstrates the ability of the isolate to solubilize Zn. Microbiological media used to this end include Bunt and Rovira (29, 30) and Pikovskaya (31–34) media and many more (22, 35–37). Therefore, ZSB are generally not directly recovered from environmental samples but rather are screened and identified among numerous bacterial isolates (i.e., via an indirect isolation procedure). This screening procedure may turn out to be costly and time consuming, as very often, ZSB represent only a small proportion of the tested bacteria (33, 38–40).

In this study, the first objective was to develop a so-called "direct" ZSB isolation procedure to recover quickly and directly a large number of Zn-solubilizing isolates from wheat rhizospheres, while our second objective was to investigate the bacterial processes of Zn solubilization under *in vitro* conditions. We hypothesized that different species of ZSB can dissolve Zn via the production of different organic acids or through the release of Zn-chelating siderophores.

RESULTS

Direct isolation and MALDI-TOF characterization of 115 Zn-solubilizing isolates from wheat rhizospheres. The inoculation of tryptic soy agar plus ZnO (TSA+ZnO), *Pseudomonas* agar base plus ZnO (PA+ZnO), *Bacillus* agar plus ZnO (BA+ZnO), desoxycholate citrate lactose sucrose agar no. 2 plus ZnO (DCLS+ZnO), and nutrient agar plus ZnO (NA+ZnO) plates with dilutions of rhizosphere soil suspensions enabled the direct isolation of 115 putative Zn-solubilizing isolates (Table 1, step 1). Examples of plates with Zn-solubilizing colonies are shown in Fig. S1 in the supplemental material. For each isolate, a bacterial identity number (ID; from 1 to 115) was given. These, as well as information for each strain on soil origin, medium of isolation, and taxonomic affiliations as given by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis, can be found in Table S1. Of the 115 newly isolated ZSB, 68 were recovered from the PA+ZnO solid medium. On the other hand, 34 of the isolates were recovered from NA+ZnO and only eight, three, and two Zn-solubilizing isolates were recovered from TSA, DCLS, and BA, respectively. MALDI-TOF MS fingerprints of the 115 putative Zn-solubilizing isolates were compared with a bacterial reference library (Spectral archive and microbial identification system [SARAMIS]; Mabritec AG) (41) (Table 1, step 2). Twenty-eight of the 115 analyzed ZSB were affiliated with the *Microbacterium* genus. Equal numbers of isolates (15 each) were affiliated with *Bacillus* and *Streptomyces* genera. Fewer isolates were affiliated with *Pseudomonas* (nine isolates), *Frigoribacterium* (three isolates), *Flavobacterium* (three

TABLE 1 Overall strategy used for the isolation and characterization of ZSB recovered from wheat rhizospheres

Steps	Biological material	Procedure	Main output
1. Direct ZSB[a] isolation	Wheat rhizosphere soils	Wheat rhizosphere soil suspensions plated on TSA+ZnO, PA+ZnO, BA+ZnO, DCLS+ZnO, and NA+ZnO[b]	Detection and subculture of 115 putative Zn-solubilizing isolates plus isolation of NZSB[c]
2. ZSB identification and clustering	115 Zn-solubilizing isolates	MALDI-TOF MS[d] analysis for: Mass spectrum cross-comparison with bacterial reference database (SARAMIS) Similarity comparison of the obtained 115 fingerprints by the generation of a dendrogram	 Taxonomic affiliation found for 65% of the isolates Clustering of 115 isolates and selection of 47 distinct bacterial strains (selection 1)
3. Bacterial screening	Selection 1 (plus NZSB)	Zn solubilization efficiency assay on plates Siderophore production ability assay	Selection of eight bacterial strains (including NZSB) (selection 2)
4. Precise identification of selection 2	Selection 2	16S rRNA gene sequencing and identification	Phylogenetic identification of selection 2
5. Study of the solubilization mechanisms	Selection 2	Liquid cultures (with or without glucose) supplemented with ZnO Measure of the organic acids and siderophores produced in culture supernatant	Highlight of different solubilization mechanisms depending on the strain and the availability of glucose

[a]ZSB, zinc-solubilizing bacteria.
[b]Microbiological media and description of the isolation procedure can be found in "Origin of rhizosphere soils and direct ZSB isolation procedure."
[c]NZSB, non-Zn-solubilizing bacterium (a bacterial isolate also recovered from wheat rhizosphere [on PA+ZnO], which was not surrounded by a solubilization halo).
[d]MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry.

isolates), *Cellulosimicrobium* (one isolate), and *Arthrobacter* (one isolate) genera (Table S1). No reliable identification was obtained for 40 of the 115 analyzed ZSB. A linear dendrogram was generated to compare the similarities of the mass spectral fingerprints of the 115 ZSB (see Fig. S2). Based on this dendrogram, the number of isolates to be further studied was reduced from 115 to a set of 47 ZSB, named "selection 1." These shared less than 60% similarity with their MALDI-TOF signatures and were presumed to be taxonomically distinct.

Zn solubilization efficiencies and siderophore production abilities of selection 1. The assays were performed with an additional wheat rhizosphere strain ("non-Zn-solubilizing bacterium" [NZSB]) (Table 1, step 3). This strain was not surrounded by a solubilization halo on its plate of isolation (PA+ZnO) and was identified as a *Stenotrophomonas rhizophila* by MALDI-TOF MS (Table S1). NZSB was used as a negative control in solid solubilization assays (Table 1, step 3). Not surprisingly, NZSB was not able to solubilize Zn in the solid plate assays (Table 2). Moreover, NZSB was found to be unable to release siderophores in modified M9 medium (MM9) (Table 2). The highest solubilization efficiencies were found for two strains affiliated with the genus *Pseudomonas* (strains 24 and 46), followed by two nonidentified strains (strains 81 and 5) (Table 2). *Microbacterium* strain 85 and nonidentified strains 37, 55, and 93 solubilized only ZnO, while *Pseudomonas* strain 46 and the nonidentified strains 63 and 103 solubilized only $ZnCO_3$ (details of these data not shown). Except for unaffiliated strains 4 and 71, only *Bacillus*, *Pseudomonas*, and *Streptomyces* strains released detectable siderophores in the liquid MM9 (Table 2). The quantities of siderophores released per ml of medium differed considerably between the strains, with productions ranging from 0.513 $\pm$ 0.101 (nonidentified 4) to 91.5 $\pm$ 0.853 (*Pseudomonas* 106) μmol deferoxamine mesylate (DFOM) equivalent. *Bacillus cereus* ATCC 14579, which we know can produce petrobactin and bacillibactin siderophores (42), was also found to be positive in our assays (Table 2).

For the fourth and fifth steps of this study (Table 1), eight bacterial strains within selection 1, with contrasting Zn solubilization efficiencies and siderophore production abilities, were selected. This set was designated selection 2 and consisted of the following strains: *Pseudomonas* strain 24 and nonidentified strains 5 and 81 (best

TABLE 2 Zinc solubilization efficiencies and siderophore production abilities of 47 preselected ZSB (selection 1)[a]

Bacterial ID	MALDI-TOF MS taxonomic affiliation	Zn solubilization efficiency		Siderophore production ability	
		Ratio[b]	SD	μmol/10^8 CFU[c]	SD
12	*Bacillus cereus*	5.41	0.284	40.4	2.47
48	*Bacillus cereus*	3.69	0.057	−	
69	*Bacillus cereus*	2.55	0.563	+	
110	*Bacillus megaterium*	1.68	0.079	53.2	2.37
14	*Bacillus licheniformis*	1.25	0.059	1.51	0.559
91	*Cellulosimicrobium cellulans*	6.36	0.792	−	
17	*Frigoribacterium faeni*	4.55	0.488	−	
112	*Microbacterium foliorum*	4.82	0.685	−	
83	*Microbacterium foliorum*	4.11	1.11	−	
79	*Microbacterium foliorum*	2.2	0.595	−	
86	*Microbacterium maritypicum*	1.43	0.067	−	
108	*Microbacterium paraoxydans*	1.43	0.269	−	
89	*Microbacterium foliorum*	1.15	0.039	−	
56	*Microbacterium foliorum*	+		−	
111	*Microbacterium maritypicum*	+		−	
85	*Microbacterium maritypicum*	+		−	
24	***Pseudomonas* sp.**	**12.9**	**2.27**	**22.4**	**1.42**
46	*Pseudomonas* sp.	8.98	0.344	3.49	0.727
39	*Pseudomonas* sp.	6.13	0.315	+	
106	***Pseudomonas* sp.**	**2.19**	**0.42**	**91.5**	**0.853**
115	*Pseudomonas* sp.	1.79	0.256	103.5	12
44	***Pseudomonas chlororaphis***	**1.48**	**0.4**	**3.58**	**1.72**
31	*Streptomyces* sp.	3.37	0.309	+	
68	***Streptomyces* sp.**	**3.08**	**1.41**	**67.6**	**0.414**
15	*Streptomyces* sp.	1.36	0.097	12.1	4.75
38	*Streptomyces* sp.	+		−	
81	Nonidentified	**11.5**	**1.36**	**−**	
5	Nonidentified	**7.92**	**0.454**	**−**	
80	Nonidentified	7.43	1.41	−	
36	Nonidentified	6.10	1.7	−	
42	Nonidentified	**5.99**	**0.927**	**−**	
28	Nonidentified	5.40	1.72	−	
78	Nonidentified	4.28	1.01	−	
26	Nonidentified	3.67	1.51	−	
87	Nonidentified	3.28	0.435	−	
4	Nonidentified	2.46	0.547	0.513	0.101
74	Nonidentified	2.20	0.304	−	
82	Nonidentified	2.19	0.968	−	
75	Nonidentified	2.15	0.506	−	
84	Nonidentified	1.91	0.181	−	
71	Nonidentified	1.30	0.079	8.52	6.34
35	Nonidentified	1.23	0.022	−	
37	Nonidentified	+		−	
55	Nonidentified	+		−	
63	Nonidentified	+		−	
93	Nonidentified	+		−	
103	Nonidentified	+		−	
ATCC 14579[d]	*Bacillus cereus*	2.53	0.537	28.7	8.96
NZSB[e]	***Stenotrophomas rhizophila***	**−**		**−**	

[a]Strains constituting selection 2 are indicated by bold type.
[b]Zn solubilization efficiency calculated as the average ratio of ZnO and $ZnCO_3$ solubilization assays (see Materials and Methods). −, absence of solubilization (i.e., no clarification zones underneath the agar cores); +, slight clarification zones underneath the agar cores.
[c]−, absence of siderophore production; +, production of siderophore but no CFU data available (no growth on the NA plates used for CFU measurements).
[d]Positive control for siderophore production ability assay (42).
[e]NZSB, non-Zn-solubilizing bacterium (a bacterial isolate recovered from wheat rhizospheres, which was not surrounded by a solubilization halo).

solubilizers of all ZSB), *Pseudomonas* strain 106 (best siderophore producer), and *Streptomyces* strain 68 (best siderophore producer from *Streptomyces* strains) (Table 2). Moreover, our negative-control *Stenotrophomonas rhizophila* NZSB, *Pseudomonas* strain 44 (worst *Pseudomonas* Zn solubilizer), and the nonidentified strain 42 (very similar to the nonidentified strain 5 in terms of MALDI-TOF profiles [Fig. S2] and solubilization efficiencies [Table 2]) were also included in selection 2. In this way, Zn solubilization

TABLE 3 Comparison of MALDI-TOF MS and 16S rRNA gene sequencing identifications of selection 2 strains

Bacterial ID	MALDI-TOF MS taxonomic affiliation	Most-related bacterial strain by BLAST (accession no. [% identity])[a]
81	Nonidentified	*Curtobacterium oceanosedimentum* (LN890020.1, 99%)
5	Nonidentified	*Plantibacter flavus* (HE716918.1, 99%)
42	Nonidentified	*Plantibacter flavus* (HE716918.1, 99%)
44	*Pseudomonas chlororaphis*	*Pseudomonas chlororaphis* (FJ652611.1, 100%)
106	*Pseudomonas* sp.	*Pseudomonas moraviensis* (HG000013.1, 99%)
24	*Pseudomonas* sp.	*Pseudomonas syringae* (AY574913.1, 99%)
NZSB	*Stenotrophomas rhizophila*	*Stenotrophomonas rhizophila* (CP016294.1, 100%)
68	*Streptomyces* sp.	*Streptomyces narbonensis* (KU324447.1, 99%)

[a]16S rRNA gene sequences blasted against the NCBI nr database.

performances obtained on solid solubilization assays (Table 1, step 3) were compared with those obtained under liquid conditions (Table 1, step 5). Moreover, selecting strains with various siderophore production abilities enabled us to check whether a strain potentially able to produce more siderophores would also solubilize more Zn in liquid assays.

16S rRNA gene-based phylogenetic analysis of selection 2. As a fourth step (Table 1), the phylogenetic affiliations of selection 2 were finalized by 16S rRNA gene sequence analysis. This enabled the identification to the species level of all bacterial strains of selection 2 and confirmed taxonomic affiliations obtained by MALDI-TOF MS for strain 44, strain 106, strain 24, NZSB, and strain 68 (Table 3).

Zn solubilization by selection 2. Strains from selection 2 were separately subjected to solubilization assay medium (SAM) with or without glucose, and the solubilization of ZnO was monitored over 6 days of incubation (Table 1, step 5). Soluble Zn levels and pH values from uninoculated controls in SAM without glucose and in SAM plus glucose remained very close to the initial values during the entire experiment (see Fig. S3); thus, Zn dissolution by mechanic agitation or contamination of the cultures was excluded. Only three of the eight tested strains, i.e., *Curtobacterium oceanosedimentum* strain 81 and *Plantibacter flavus* strain 5 and strain 42, were able to mobilize Zn when no glucose was added to the growth medium (Fig. 1A, B, and C). Moreover, *Plantibacter flavus* strain 5 and strain 42 generally solubilized more Zn in SAM without glucose than *Curtobacterium oceanosedimentum* strain 81 under this condition. Indeed, in SAM without glucose, *Plantibacter flavus* strain 5 and strain 42 solubilized ca. 250 mg Zn · liter^{-1} after 2 days of incubation, while Zn mobilization by *Curtobacterium oceanosedimentum* strain 81 barely exceeded 100 mg Zn · liter^{-1} at any time point (Fig. 1A, B, and C). Interestingly, Zn mobilization by *Curtobacterium oceanosedimentum* strain 81 in SAM without glucose was accompanied by a gradual pH increase from 6.8 up to pH $>$8 (Fig. 1A). In contrast, Zn solubilization by *Plantibacter flavus* strain 5 and strain 42 in SAM without glucose was associated with a pH drop throughout the incubation from 6.8 to ca. 4.8. Acidification by *Plantibacter flavus* strain 5 and strain 42 was even more important in the presence of glucose, and more Zn was dissolved in these cultures (Fig. 1B and C). For the other strains (Fig. 1D to H), Zn solubilization occurred only when glucose was added to the SAM. As for *Plantibacter flavus* strain 5 and strain 42, solubilization by these five other strains was associated with pH acidification below pH 5. There was no clear correspondence between Zn solubilization performances observed in plate assays and in liquid medium. For example, the amount of Zn solubilized in liquid medium by *Pseudomonas syringae* strain 24, which had the highest Zn solubilization efficiency in plate assays (Table 2), was similar to the amounts for the other strains of selection 2 (Fig. 1). Furthermore, while *Stenotrophomonas rhizophila* NZSB did not strictly produce any clarification zones on NA+ZnO or NA supplemented with Zn carbonate (NA+$ZnCO_3$; 0.829 g · liter^{-1}) in step 3 (Table 2), it solubilized Zn in SAM with glucose (Fig. 1H).

Study of the solubilization mechanisms used by selection 2. The concentrations of siderophores and organic acids measured in each culture filtrate after 4 days of incubation can be found in the supplemental material (Fig. S4). Low concentrations

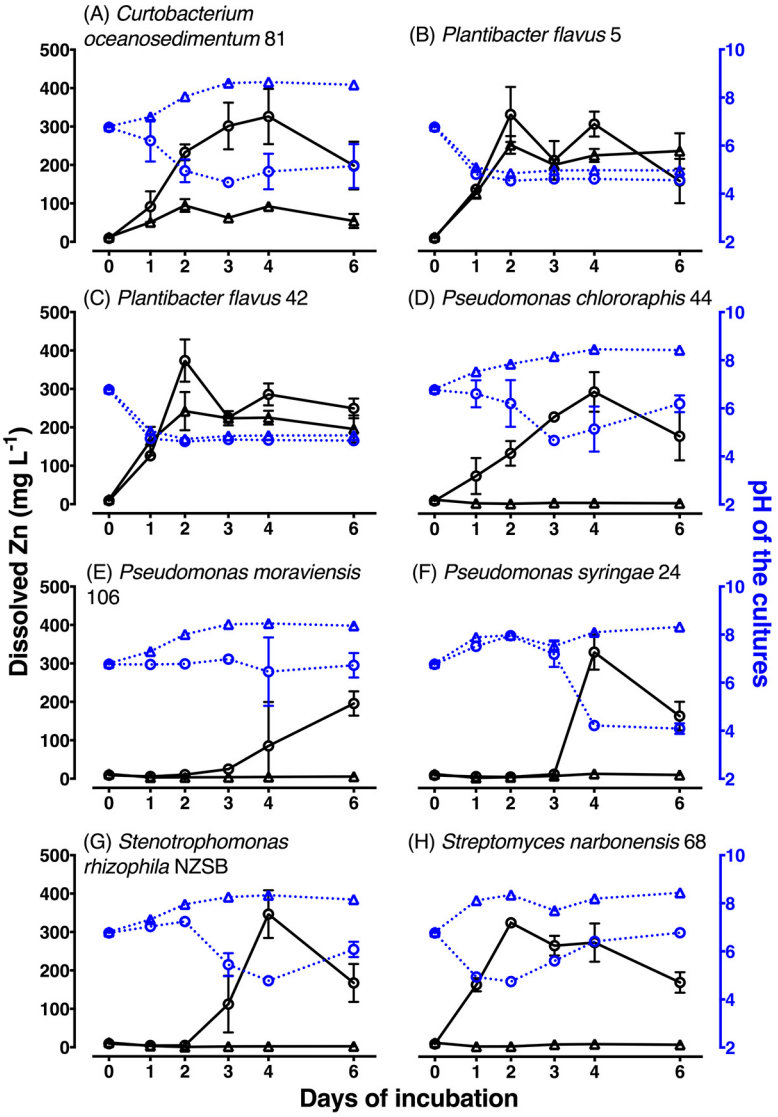

FIG 1 Zinc oxide dissolution in relation to pH change during incubation of selection 2 isolates for 6 days in SAMs. △, means for SAM without glucose; ○, means for SAM with glucose. Black lines represent dissolved Zn; blue dashed lines represent pH. Error bars show standard errors for each of the means ($n = 3$).

(below 10 μM) of malonic and 2-oxoglutaric acids were found in the uninoculated control medium (Table 4; see also Fig. S4Q). Moreover, the control medium contained >100 μM glutamic acid (Table 4).

Organic acids in SAM without glucose cultures. Among the nine organic acids measured in SAM without glucose, only glutamic acid was found in concentrations above 10 μM (Table 4). Interestingly, glutamic acid was only observed in cultures of *Curtobacterium oceanosedimentum* strain 81 and *Plantibacter flavus* strain 5 and strain 42, where a considerable amount of Zn had been solubilized (Fig. 1A to C and Table 4). Additionally, *Curtobacterium oceanosedimentum* strain 81 and *Plantibacter flavus* strain 5 released small quantities of citric and oxalic acids, respectively (Table 4). *Plantibacter flavus* strain 5 and strain 42 both released succinic acid in SAM without glucose (Table 4). Small amounts of gluconic acid were released by *Pseudomonas chlororaphis* strain 44, *Pseudomonas moraviensis* strain 106, *Pseudomonas syringae* strain 24, *Stenotrophomonas rhizophila* NZSB, and *Streptomyces narbonensis* strain 68 in SAM without glucose (Table 4). In these cultures, no glutamic acid was measured (although it was

TABLE 4 Concentrations of organic acids and siderophores measured in SAM with and without glucose after 4 days of incubation

Bacterial ID	pH[a]	Dissolved Zn (mg · liter^{-1})[a]	Organic acid[b]									Siderophores[b]
			Citric	Fumaric	Gluconic	Glutamic	Malic	Malonic	Oxalic	2-Oxoglutaric	Succinic	
SAM without glucose												
Control[c]	6.84	14.4				++		−		−		
81	8.65	92.2	−			+		−				
5	4.98	224.4				+			−	−	−	
42	4.86	225.1				+				−	−	
44	8.45	3.97			−			−				++
106	8.46	4.54			−			−				++
24	8.10	12.9			−			−				+++
NZSB	8.33	2.19			−			−				++
68	8.19	8.80										++
SAM with glucose												
81	4.93	326.2	+		−	−	−	+++	−	+	+	
5	4.61	305.9			+	++		−	+	−		
42	4.67	285.6	−		+	++		−	−	−		
44	5.14	292.6			+			+++	+			+++
106	6.46	85.3			++	−		++	+			+++
24	4.22	329.4			+++			++	+		−	+++
NZSB	4.77	346.5	−		+++			+++	+		−	+++
68	6.41	272.7	+	−	−	−	+			+	+	+

[a]Values are the means from three experimental replicates.

[b]−, from 1 to 10 μM; +, from 10 to 100 μM; ++, from 100 to 1,000 μM; +++, >1,000 μM.

[c]Control corresponds to uninoculated SAM without glucose incubated under the same conditions as the bacterial cultures.

present initially in the SAM), which indicates that it was metabolized by these five strains.

Organic acids in SAM with glucose cultures. All the strains of selection 2 solubilized Zn in SAM with glucose (Fig. 1), including *Stenotrophomonas rhizophila* NZSB, which did not produce a clear halo on NA+ZnO or on NA+$ZnCO_3$ (Table 2). Remarkably, generally much higher quantities of organic acids were recovered from SAM with glucose than from SAM without glucose (Table 4). *Pseudomonas chlororaphis* strain 44, *Pseudomonas moraviensis* strain 106, *Pseudomonas syringae* strain 24, and *Stenotrophomonas rhizophila* NZSB were distinctive from the rest of the selection 2 strains due to their nearly exclusive production of gluconic, malonic, and oxalic acids (Table 4). Similarly, *Plantibacter flavus* strain 5 and strain 42 released mostly gluconic and oxalic acids in SAM with glucose (Table 4). Relatively to the other strains of selection 2, *Curtobacterium oceanosedimentum* strain 81 and *Streptomyces narbonensis* strain 68 produced only small amounts of gluconic acid, and *Streptomyces narbonensis* strain 68 released no malonic acid in SAM with glucose. Moreover, these two strains distinguished themselves by the production of various organic acids, such as citric, fumaric, malic, 2-oxoglutaric, and succinic acids (Table 4).

Siderophore production in both media. *Curtobacterium oceanosedimentum* strain 81 and *Plantibacter flavus* strain 5 and strain 42 did not release detectable siderophores in any of the SAMs. On the other hand, *Pseudomonas chlororaphis* strain 44, *Pseudomonas moraviensis* strain 106, *Pseudomonas syringae* strain 24, *Stenotrophomonas rhizophila* NZSB, and *Streptomyces narbonensis* strain 68 released siderophores in both SAM without glucose and SAM with glucose in concentrations above 100 μM DFOM equivalent (Table 4; see also Fig. S4). While such concentrations of siderophore enabled the chelation of Fe from the chrome azurol complex in step 3, they did not enable ZnO solubilization, as these five strains were also those which did not solubilize Zn in SAM without glucose (Fig. 1). Apart from *Streptomyces narbonensis* strain 68, the production of siderophores was generally increased by the addition of glucose in the SAM (Table 4; see also Fig. S4).

DISCUSSION

In this study, the procedure used for the isolation of ZSB from wheat rhizospheres consisted of plating soil serial dilutions on various microbiological agar media (NA, TSA, PA, BA, and DCLS) supplemented with glucose and a scarcely soluble Zn salt (ZnO). ZSB were directly detected on the basis of the observation of clearing zones around the colonies. Our results showed that PA+ZnO was the most efficient ZSB isolation medium, perhaps due to the higher diffusion rates of bacterial exudates in this medium (43). Similar "direct" isolation methods have been used before with different media, such as modified Bunt and Rovira media (15) or tris-mineral salt media (44). This study confirms that such a direct ZSB isolation method can be efficiently used for the quick recovery of a large number of Zn-solubilizing isolates from rhizosphere soil samples.

The next step was to identify which of the 115 newly isolated ZSB were probably the same strain. Our study used MALDI-TOF MS for the discrimination and typing of Zn-solubilizing isolates. An analysis of 16S rRNA gene sequences of selection 2 strains supported the identification provided by MALDI-TOF MS (Table 3), which confirms the potential of this proteomic technique for the identification of unknown soil bacterial isolates. The identification of bacteria by MALDI-TOF MS approaches remains limited when applied to the soil environment, since reference spectrum databases mostly contain clinically important microorganisms (45). For this reason, no reliable affiliation was given for approximately 35% of the bacteria (40 isolates) in our study. Nevertheless, the generation of a dendrogram (see Fig. S2 in the supplemental material) enabled us to appreciate the similarities between the 115 solubilizing isolates, including those that were not successfully affiliated to any bacterial taxa. It is worth noting that unaffiliated isolates formed separate clusters, which strongly suggest that among these 40 isolates, different bacterial taxa were represented (46). The remaining 65% of the isolates were successfully affiliated with eight different bacterial genera. In the study presented here, *in vitro* Zn solubilization is reported

for strains of *Streptomyces*, *Cellulosimicrobium*, and *Arthrobacter*, as well as for strains of *Curtobacterium* and *Plantibacter*. The ability to solubilize Zn under *in vitro* conditions is therefore not restricted to a few bacterial genera. This is in agreement with previous studies, where isolates belonging to various bacterial genera were reported as Zn solubilizers, including *Acinetobacter* (47), *Exiguobacterium* (48), *Gluconacetobacter* (18), *Pseudomonas* (16, 17), *Rhizobium* (33), and *Serratia* (49).

Previous studies have shown that bacteria can solubilize and acquire Zn bound in their environment by releasing Zn-chelating siderophores (50, 51). However, in our liquid solubilization assays, strains that produced siderophores did not dissolve ZnO in SAM without glucose (Table 4). In these cultures, it is excluded that ZnO was in fact dissolved by siderophores and thereafter taken up by bacterial cells. Indeed, despite the production of siderophores in the presence of glucose, large quantities of Zn remained dissolved in SAM with glucose (Table 4). Therefore, the siderophores produced by strains of selection 2 seemed unable to chelate Zn and thus dissolve ZnO. Zn-chelating siderophores may have represented only a small proportion of the siderophores released by selection 2 strains. So far, although a wide structural diversity of siderophores has been described (27), only a few of these compounds have been clearly identified as Zn chelators (25).

The potential bacterial mechanisms of Zn solubilization include acidification (through the production of protons), chelation (via siderophores or organic acids), and chemical transformation (e.g., with redox reactions, generally under extreme conditions) (52). Protons can be either directly released by bacteria (e.g., for the acquisition of nutrients and the preservation of charge balance) or indirectly released from carboxylic groups of exuded organic acids (at pH higher than pKa). Protons can replace Zn cations at sorption sites of minerals (e.g., oxides and phosphates) and thus mobilize Zn in solution (52). Metal chelation by organic acids is mostly controlled by pH. Indeed, in neutral to alkaline environments, organic acids might occur as fully deprotonated anions and act as metal-complexing agents (53). In our study, with the exception of *Streptomyces narbonensis* strain 68 grown in SAM without glucose, all strains released organic acids in SAMs (Table 4). These were produced in larger amounts when glucose was initially added to the medium, probably by the stimulation of metabolic activity (54). Although proton extrusion may also have decreased the pH in SAM plus glucose, the observed acidification in this medium was probably due to the release of large amounts of organic acids by the inoculated ZSB. In these cultures, the expected mechanism of solubilization is rather via acidification, as complexation processes at such pH values are unlikely (17). In previous studies, the solubilization of insoluble Zn substrates [ZnO, $ZnCO_3$, and $Zn_3(PO_4)_2$] was generally linked to an increased proton concentration, resulting from the production of gluconic acid (15–18, 22). On the other hand, the production of gluconic acid in our study does not solely explain the observed solubilization. For instance, *Curtobacterium oceanosedimentum* strain 81 and *Streptomyces narbonensis* strain 68 produced only marginal quantities of gluconic acid in SAM with glucose. Instead, the nature and quantity of organic acids possibly responsible for ZnO dissolution in SAM with glucose depended on the bacterial strain initially inoculated. Indeed, the solubilization of ZnO by *Streptomyces narbonensis* strain 68 and *Curtobacterium oceanosedimentum* strain 81 may be attributed to the accumulation of six and seven different organic acids, respectively, in SAM with glucose. On the other hand, the other strains solubilized Zn in SAM with glucose mostly through the production of gluconic, malonic, and oxalic acids. This is in line with the observations of Li et al., where metal solubilization was attributed to different organic acids among *Burkholderia cepacia* and a mixture of *Microbacterium saperdae* and *Enterobacter cancerogenus* (24).

In the absence of glucose, *Plantibacter flavus* strain 5 and strain 42 both decreased the pH in the SAM to levels similar to those observed in SAM with glucose (Fig. 1). This acidification, which might be responsible for the ZnO dissolution in these cultures, cannot be explained by organic acid production, as the quantities released by *Plantibacter flavus* strain 5 and strain 42 were comparable to those measured in the uninoculated control and thus were too low (<10 μM) to acidify the medium (Table 4).

The excretion of protons and subsequent ZnO dissolution by *Plantibacter flavus* strain 5 and strain 42 in SAM without glucose might be the consequence of other metabolic processes, such as ammonia consumption or bacterial respiration (55). *Curtobacterium oceanosedimentum* strain 81 (grown in SAM without glucose) acted as an exception because, despite the progressive medium alkalization after 4 days of incubation, an average of 92.2 mg Zn · liter^{-1} was dissolved. Medium alkalization occurred probably as a consequence of amino acid hydrolysis and the subsequent release of ammonium. Since, unlike the other strains, *Curtobacterium oceanosedimentum* strain 81 did not consume all of the glutamic acid initially present in SAM without glucose (Table 4), it is proposed that the remaining glutamic acid solubilized Zn via complexation processes likely to occur at such pH values (52).

We observed inconsistencies between the Zn solubilization efficiencies in solid and liquid solubilization assays. Indeed, there was no correspondence between solubilization zone sizes in solid plates (Table 2) and the amount of Zn dissolved in liquid SAMs (Fig. 1). Therefore, the intensities of the metabolic processes responsible for Zn solubilization were affected to various magnitudes among the bacterial strains by growing conditions and the type of medium (NA for solid versus SAM for liquid assays). For example, different C/N ratios between these two media probably affected the organic acid production and thus Zn solubilization (56). A striking example was the case of *Stenotrophomonas rhizophila* NZSB (our negative control), which solubilized similar quantities of Zn compared with those of the other strains after 4 days of incubation in SAM with glucose (Fig. 1H) but did not produce clear halos on NA+ZnO or NA+$ZnCO_3$ in step 3 (Table 2). Similar cases were reported before (57). The differences in the types and the quantities of organic acids produced under solid and liquid growing conditions were considered to be responsible for the observed variability in solubilization abilities (43).

To conclude, our work describes various bacterial processes of Zn solubilization, beyond the production of gluconic acid that until now was described as a major solubilization mechanism. These processes include mostly the extrusion of protons and the production of different organic acids among bacterial strains. In this study, there is no evidence for the involvement of bacterially released siderophores in the dissolution of ZnO. The magnitudes of these processes are strongly affected by the conditions in which the bacteria are studied. Although the processes highlighted in this study might have an ecological significance, it is necessary to remain cautious when transposing these mechanisms to the rhizosphere ecosystem, where biotic and abiotic interactions are even further from laboratory conditions.

MATERIALS AND METHODS

Overall strategy. The five steps summarizing the overall strategy of this study are presented in Table 1. First, 115 Zn-solubilizing isolates were recovered from wheat rhizospheres using a direct isolation procedure (Table 1, step 1). To determine which of the 115 isolates likely belonged to the same strains, their protein mass fingerprints obtained by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) were compared. This enabled us to reduce the number of bacteria from 115 to 47 ZSB, and this set of strains, with distinct MALDI-TOF profiles, was named selection 1 (Table 1, step 2). Subsequently, eight bacterial strains (referred to as selection 2), with contrasting Zn solubilization efficiencies and siderophore production abilities, were selected from selection 1 (Table 1, step 3). Efficient and inefficient ZSB were included in selection 2 to determine later whether bacteria performing best (or worst) in step 3 (i.e., on solid plate assays) behave in a similar way in liquid solubilization assays (Table 1, step 5). Thus, the effect of bacterial growth conditions on the ability of a given strain to solubilize Zn was also investigated. As a fourth step, selection 2 strains were identified using 16S rRNA gene sequencing. Finally, selection 2 strains were grown in insoluble ZnO-containing liquid broth (SAM), and Zn dissolution was monitored over 6 days of incubation (i.e., soluble Zn and pH measured once per day) (Table 1, step 5). Additionally, siderophores and organic acids present in SAMs were measured after 4 days of incubation. These liquid assays were conducted with and without glucose to exclude gluconic acid production and investigate the potential involvement of other organic acids.

Media and chemicals. The TSA, PA, BA, and DCLS used in this study were obtained from Sigma-Aldrich (Switzerland). NA was purchased from BD Difco (Switzerland). For the isolation of Zn-solubilizing isolates from wheat rhizospheres (Table 1, step 1), TSA, PA, BA, DCLS and NA were supplemented with ZnO (0.629 g · liter^{-1}; Merck, Switzerland) and D-glucose (10 g · liter^{-1}) to obtain TSA+ZnO, PA+ZnO, BA+ZnO, DCLS+ZnO, and NA+ZnO, respectively. Cycloheximide (50 mg · liter^{-1}; Sigma-Aldrich, Switzerland) was added to all solid media to prevent fungal growth. For the determination of Zn solubilization efficiency (Table 1, step 3), the bacterial strains were subjected to NA+ZnO and NA+$ZnCO_3$. For

the assessment of siderophore production ability (Table 1, step 3), selection 1 was grown in MM9 and siderophores were detected following the chrome azurol S (CAS) solution assay, as described by Alexander and Zuberer (58). The MM9 contained (per 1,000 ml) KH_2PO_4 (0.3 g), NaCl (0.5 g), NH_4Cl (1 g), mannitol (2 g), $MgSO_4 \cdot 7H_2O$ (493 mg), $CaCl_2$ (11 mg), $MnSO_4 \cdot H_2O$ (1.17 mg), H_3BO_3 (1.4 mg), $CuSO_4 \cdot 5H_2O$ (0.04 mg), $ZnSO_4 \cdot 7H_2O$ (1.2 mg), $Na_2MoO_4 \cdot 2H_2O$ (1 mg), Casamino Acids (3 g; BD Difco, Switzerland), and PIPES {[piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]} (30.24 g; Sigma-Aldrich, Switzerland). The study of the solubilization mechanisms (Table 1, step 5) was performed in the solubilization assay medium (SAM). This liquid medium had the same chemical composition as the MM9 except that ZnO (0.629 g · liter^{-1}) was supplemented and the PIPES buffer was removed. These liquid assays were performed in SAM with or without glucose added (10 g · liter^{-1}). Nutrient broth (NB) used for routine liquid bacterial cultivation was prepared with peptone (5 g · liter^{-1}; BD Difco, Switzerland) and meat extract (3 g · liter^{-1}; Sigma-Aldrich, Switzerland).

Origin of rhizosphere soils and direct ZSB isolation procedure. The Zn-solubilizing isolates used in this study were recovered from the rhizospheres of two varieties of wheat (*Triticum aestivum* L.) at different development stages (Table 5). To further diversify the taxonomic range of the isolated bacteria, we used three distinct soils with differing chemical properties (i.e., two from Switzerland and one from Iran), since the diversity of soil bacterial populations is strongly affected by soil edaphic properties (59). The wheat plants were sampled either directly from the field or from pot experiments (Table 5). Entire wheat plants were transported to the laboratory and processed within 5 h after sampling. The bulk soil was removed from the plants by shaking and tapping the root systems gently. Only soil strongly adhering to the roots (i.e., a thin [<2-mm] soil layer) was considered rhizosphere soil. Roots, including rhizosphere soil, were cut off from the shoot with sterile blends, suspended in 30 ml of a sterile NaCl solution (9 g · liter^{-1}), and shaken horizontally for 30 min (120 rpm). Tenfold serial dilutions were prepared from the rhizosphere soil suspensions, and 100-μl aliquots of dilutions (10^{-3}, 10^{-4}, and 10^{-5}) were spread on NA+ZnO, TSA+ZnO, PA+ZnO, BA+ZnO, and DCLS+ZnO. These plates were incubated upside down at 28°C for up to 14 days until halo-forming colonies appeared on the surfaces (see Fig. S1 in the supplemental material). Colonies surrounded by clear clarification zones were selected as ZSB, streaked with a wire loop onto NA plates, and subcultured for purification. For long-term storage, the isolates were stored at −80°C in NB supplemented with 20% (vol/vol) glycerol. For each bacterial isolate, an identification number (from 1 to 115) was given.

Characterization of isolates by MALDI-TOF MS of whole-cell lysates. (i) Preparation of samples for MALDI-TOF MS analyses. A total of 115 Zn-solubilizing isolates were characterized by MALDI-TOF MS (Table 1, step 2) at Mabritec AG (Riehen, Switzerland) according to the procedure described by Ziegler et al. (41, 60). In brief, all the isolates were streaked on NA plates and grown for 24 h at 28°C. Each bacterial isolate was spotted in duplicates on MALDI target plates. Subsequently, spots were overlaid with 1 μl of 5.43 M formic acid, were air-dried, and were again overlaid with 1 μl of matrix solution consisting of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich, Switzerland) in 8.04 M acetonitrile (Sigma-Aldrich, Switzerland), 7.16 M ethanol, and supplemented with 263 μM trifluoroacetic acid (TFA). Spots were completely dried at room temperature before being introduced into the mass spectrometer.

(ii) MALDI-TOF fingerprinting, identification, and cluster analysis. Signatures of bacteria were obtained using a MALDI-TOF mass spectrometer (Axima Confidence; Shimadzu-Biotech Corp., Kyoto, Japan), with detection in the linear positive mode at a laser frequency of 50 Hz and within a mass range of 3 to 20 kDa. The acceleration voltage was 20 kV, and the extraction delay time was 200 ns. A minimum of 10 laser shots per sample was set to generate each of the protein fingerprints. For each spot, 50 protein mass fingerprints were averaged and processed using the Launchpad 2.8 software (Shimadzu-Biotech Corp., Kyoto, Japan). Each target plate was first externally calibrated using spectra of the reference strain *Escherichia coli* DH5α. The use of *Escherichia coli* DH5α enables proper calibration of the MALDI-TOF system, since its genome/proteome and hence its mass peaks are very well known. In this respect, *Escherichia coli* DH5α has been used for MALDI-TOF external calibration independently of the organism under study, such as yeast (61), fungi (62), and insects (63).

With the aim to assign taxonomic affiliations to the isolates (bacterial genera and/or species), the obtained mass fingerprints were analyzed and cross-matched with bacterial references in a proprietary and enlarged version of the database SARAMIS (AnagnosTec, Potsdam-Golm, Germany) at Mabritec AG, Switzerland. These references compile spectra obtained from several strains of the same species cultivated on different growth media for different incubation times (2 to 14 days) (41). Thus, the identification of our bacterial isolates was independent from the above-mentioned parameters.

The Launchpad 2.8 software was used to generate ASCII mass lists from the MALDI-TOF fingerprints. These were imported into SARAMIS, and a binary matrix was calculated using the SARAMIS SuperSpectrum tool with an error window of 800 ppm. A pairwise similarity matrix was calculated using Dice similarities (64). A cluster analysis was performed and a linear dendrogram was generated using the taxonomy tool of the SARAMIS software.

***In vitro* assessment of Zn solubilization efficiency.** We assessed solubilization efficiency by subjecting the strains to NA+ZnO and NA+$ZnCO_3$ media (cf. chemical compositions in "Media and chemicals") (Table 1, step 4). Fifty microliters of the bacterial suspensions was spread directly from the corresponding glycerol stocks on NA plates. After an overnight incubation at 28°C, NA plates were naturally covered with a thin layer of grown bacteria, and cores of 8 mm were transferred upside down onto the surface of NA+ZnO and NA+$ZnCO_3$ plates in four replicates per strain. The inoculated NA+ZnO and NA+$ZnCO_3$ plates were incubated in the dark for 7 days at 28°C. The calculation of Zn solubilization efficiency (SE) was adapted from that described by Nguyen et al. (65) and was defined as the average ratio from both ZnO and $ZnCO_3$

TABLE 5 Rhizosphere soils used in this study

Soil	Location (country)	GPS coordinate	Texture[a]	pH	Total Zn (mg kg^{-1})	DTPA Zn (mg kg^{-1})	Wheat variety (growing stages)[b]	Growth condition	References for other studies using these soils
Swiss soil no. 1	Oberembrach (Switzerland)	47°29′N, 8°39′E	Silty clay	7.4	Not available	Not available[c]	*Triticum aestivum* L. cv. Levis (22, 30)	Field parcel Sonnenbühl 2[d]	None
Swiss soil no. 2	Zurich-Reckenholz (Switzerland)	47°25′N, 8°31′E	Sandy loam	6.5	74.9	4.22	*Triticum aestivum* L. cv. Fiorina (22, 30)	Glass house[e]	67
Iranian soil	Rudasht research station (Iran)	32°29′N, 52°10′E	Silty clay loam	7.9	80.2	0.45	*Triticum aestivum* L. cv. Fiorina (77)		68

[a]IUSS Working Group WRB (69).
[b]BBCH development stage according to Lancashire et al. (70).
[c]Given the soil location and field-based observations, this soil was likely not below Zn deficiency threshold, corresponding to DTPA extractable Zn values lower than 0.5 mg · kg^{-1} (6).
[d]Rhizosphere soil samples were taken from the parcel Sonnenbühl 2 managed by Strickhof (Eschikon 21, CH-8315, Lindau, Switzerland).
[e]Wheat was grown in the same conditions as described by Aghili et al. (68).

solubilization assays: SE = ($[AS/AG]_{ZnO}$ + $[AS/AG]_{ZnCO3}$)/2, where AS is the area of solubilization and AG is the area of bacterial growth. These areas were measured with ImageJ software (version 1.49).

***In vitro* assessment of siderophore production.** For the determination of siderophore production ability (Table 1, step 4), each bacterial strain was pregrown in 10 ml NB with shaking for at least 24 h at 28°C. Fifty microliters from these cultures was transferred to 5 ml MM9 with no added Fe to induce siderophore production (cf. chemical compositions in "Media and chemicals"). To determine how many bacterial cells were initially transferred from NB to MM9, NB cultures were analyzed for bacterial abundance (CFU per ml) by inoculating NA plates with tenfold serial dilutions (10^{-3}, 10^{-4}, and 10^{-5}). MM9 cultures were incubated for 24 h on a rotary shaker at 28°C. Two-milliliter aliquots were sampled from these cultures and centrifuged at 2,500 × *g* for 10 min. Supernatants were filtered through 0.22-μm-pore-size filters (Millipore), and siderophore concentrations were measured in the sample filtrates with the chrome azurol S (CAS) microtiter method as described by Alexander and Zuberer (58). DFOM (Sigma-Aldrich, Buchs, Switzerland) was used as a standard siderophore, with standard curves ranging from 0 to 150 μM. The color change from blue to orange, indicating the presence of siderophores in solution, was monitored by measuring the absorbance (630 nm) with a microplate reader (Synergy HT; BioTek Instruments). The results were expressed as μmol of siderophore (DFOM equivalent) produced per CFU initially transferred per ml of MM9. *Bacillus cereus* ATCC 14579 (DSMZ no. 31) was used as a reference strain for siderophore production ability assays (42) and was obtained from the German Collection of Microorganisms and Cell Cultures.

16S rRNA gene sequencing. To identify selection 2 strains on the basis of 16S rRNA sequences (Table 1, step 4), all eight bacterial strains were incubated in NB on a rotary shaker for 24 h at 28°C, and genomic DNA was extracted using the Nucleospin microbial DNA isolation kit (Macherey-Nagel GmbH & Co. KG, Germany). Briefly, 16S rRNA was PCR amplified using primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT) under standard PCR conditions (66). After purification, the PCR products were Sanger sequenced at Microsynth (Balgach, Switzerland) using the two PCR primers as well as the internal primer 785F (GGA TTA GAT ACC CTG GTA). The sequence data for each strain were assembled and manually edited. Consensus sequences for each strain were then blasted against the NCBI nr database for species identification in September 2016.

***In vitro* liquid solubilization assays.** Bacterial mechanisms responsible for ZnO solubilization were investigated in SAM with glucose and SAM without glucose (cf. chemical compositions in "Media and chemicals") (Table 1, step 5). For each strain, 50 ml of sterile SAM was dispensed in 100-ml Erlenmeyer flasks and subsequently inoculated with 2.5×10^8 CFU per ml. The experiment was conducted with three independent replicates per strain. Eight-milliliter aliquots were sampled from the Erlenmeyer flasks initially and after 1, 2, 3, 4, and 6 days of shaking at 120 rpm (28°C). These samples were prepared for analyses by centrifugation at 2,500 × *g* for 10 min, and the supernatants were filtered through 0.22-μm-pore-size filters (Millipore). Dissolved ZnO in cultures (i.e., solubilized Zn) and pH values were measured at each time point with an inductively coupled plasma optical emission spectrometer (ICP-OES; Shimadzu ICPE-9820) and a pH meter (713 pH meter, electrode 6.0262.100; Metrohm), respectively. Three uninoculated Erlenmeyer flasks of SAM without glucose and SAM with glucose were incubated under the same conditions and used as the controls. In addition, siderophores and organic acids were measured in culture supernatants sampled after 4 days of incubation. Siderophores were quantified with the CAS microtiter method as described in "*In vitro* assessment of siderophore production." Organic acids in sample filtrates were analyzed by liquid chromatography (LC; NanoAcquity UPLC; Waters Corp.) coupled with a QTOF mass spectrometer (Synapt G2 HDMS; Waters Corp.). An ethylene-bridged hybrid (BEH) amide column (Acquity UPLC, column internal diameter, 200 μm; column length, 15 cm; particle size, 1.7 μm; Waters Corp.) was used for the separation of the organic acids. LC-MS was performed at the Functional Genomics Center Zurich (FGCZ; Zurich, Switzerland). Organic acids were also measured in an uninoculated SAM without glucose control to determine which compounds were originally present in the medium. To simplify the visualization of the organic acid data, the concentrations of each compound in SAMs after 4 days of incubation are shown semiquantitatively in Table 4, but complete data can be found in the supplemental material (Fig. S4).

Any graphics were constructed using GraphPad Prism, version 7.0a (GraphPad Software, La Jolla, CA).

Data availability. Selection 2 strains are stored at the Station for Plant Sciences Research of Eschikon (Lindau, Switzerland) (https://www.ethz.ch/en/campus/locations/zurich-region/lindau.html) and are available for noncommercial use upon request.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01715-17.

SUPPLEMENTAL FILE 1, PDF file, 5.1 MB.

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REFERENCES

1. Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007. Zinc in plants. New Phytol 173:677–702. https://doi.org/10.1111/j.1469-8137.2007.01996.x.
2. Blencowe DK, Morby AP. 2003. Zn(II) metabolism in prokaryotes. FEMS Microbiol Rev 27:291–311. https://doi.org/10.1016/S0168-6445(03)00041-X.
3. Alloway BJ. 2009. Soil factors associated with zinc deficiency in crops and humans. Environ Geochem Health 31:537–548. https://doi.org/10.1007/s10653-009-9255-4.
4. Frossard E, Bucher M, Machler F, Mozafar A, Hurrell R. 2000. Potential for increasing the content and bioavailability of Fe, Zn and Ca in plants for human nutrition. J Sci Food Agric 80:861–879. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<861::AID-JSFA601>3.0.CO;2-P.
5. Cakmak I, Yilmaz A, Kalayci M, Ekiz H, Torun B, Erenoglu B, Braun HJ. 1996. Zinc deficiency as a critical problem in wheat production in Central Anatolia. Plant Soil 180:165–172. https://doi.org/10.1007/BF00015299.
6. Lindsay WL, Norvell WA. 1978. Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci Soc Am J 42:421–428. https://doi.org/10.2136/sssaj1978.03615995004200030009x.
7. Cakmak I. 2008. Enrichment of cereal grains with zinc: agronomic or genetic biofortification? Plant Soil 302:1–17. https://doi.org/10.1007/s11104-007-9466-3.
8. Gadd GM. 2010. Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology 156:609–643. https://doi.org/10.1099/mic.0.037143-0.
9. Schippers A, Hedrich S, Vasters J, Drobe M, Sand W, Willscher S. 2014. Biomining: metal recovery from ores with microorganisms. Adv Biochem Eng Biotechnol 141:1–47. https://doi.org/10.1007/10_2013_216.
10. Ramesh A, Sharma SK, Sharma MP, Yadav N, Joshi OP. 2014. Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in vertisols of central India. Appl Soil Ecol 73:87–96. https://doi.org/10.1016/j.apsoil.2013.08.009.
11. Rana A, Joshi M, Prasanna R, Shivay YS, Nain L. 2012. Biofortification of wheat through inoculation of plant growth promoting rhizobacteria and cyanobacteria. Eur J Soil Biol 50:118–126. https://doi.org/10.1016/j.ejsobi.2012.01.005.
12. Ma Y, Rajkumar M, Luo YM, Freitas H. 2013. Phytoextraction of heavy metal polluted soils using *Sedum plumbizincicola* inoculated with metal mobilizing *Phyllobacterium myrsinacearum* RC6b. Chemosphere 93:1386–1392. https://doi.org/10.1016/j.chemosphere.2013.06.077.
13. Fang Q, Fan ZQ, Xie YJ, Wang XR, Li K, Liu YF. 2016. Screening and evaluation of the bioremediation potential of Cu/Zn-resistant, autochthonous *Acinetobacter* sp. FQ-44 from *Sonchus oleraceus L*. Front Plant Sci 7:1487. https://doi.org/10.3389/fpls.2016.01487.
14. Sessitsch A, Kuffner M, Kidd P, Vangronsveld J, Wenzel WW, Fallmann K, Puschenreiter M. 2013. The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. Soil Biol Biochem 60:182–194. https://doi.org/10.1016/j.soilbio.2013.01.012.
15. Sunithakumari K, Devi SNP, Vasandha S. 2016. Zinc solubilizing bacterial isolates from the agricultural fields of Coimbatore, Tamil Nadu, India. Curr Sci 110:196–205. https://doi.org/10.18520/cs/v110/i2/196-205.
16. Fasim F, Ahmed N, Parsons R, Gadd GM. 2002. Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. FEMS Microbiol Lett 213:1–6. https://doi.org/10.1111/j.1574-6968.2002.tb11277.x.
17. Di Simine CD, Sayer JA, Gadd GM. 1998. Solubilization of zinc phosphate by a strain of *Pseudomonas fluorescens* isolated from a forest soil. Biol Fertil Soils 28:87–94. https://doi.org/10.1007/s003740050467.
18. Saravanan VS, Madhaiyan M, Thangaraju M. 2007. Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconacetobacter diazotrophicus*. Chemosphere 66:1794–1798. https://doi.org/10.1016/j.chemosphere.2006.07.067.
19. Saravanan VS, Kumar MR, Sa TM. 2011. Microbial zinc solubilization and their role on plants, p 47–63. *In* Maheshwari DK (ed), Bacteria in agrobiology: plant nutrient management. Springer, Berlin, Germany.
20. de Werra P, Pechy-Tarr M, Keel C, Maurhofer M. 2009. Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. Appl Environ Microbiol 75:4162–4174. https://doi.org/10.1128/AEM.00295-09.
21. Intorne AC, de Oliveira MVV, Lima ML, da Silva JF, Olivares FL, de Souza GA. 2009. Identification and characterization of *Gluconacetobacter diazotrophicus* mutants defective in the solubilization of phosphorus and zinc. Arch Microbiol 191:477–483. https://doi.org/10.1007/s00203-009-0472-0.
22. Vaid SK, Kumar B, Sharma A, Shukla AK, Srivastava PC. 2014. Effect of zinc solubilizing bacteria on growth promotion and zinc nutrition of rice. J Soil Sci Plant Nutr 14:889–910.
23. Gadd GM. 2004. Microbial influence on metal mobility and application for bioremediation. Geoderma 122:109–119. https://doi.org/10.1016/j.geoderma.2004.01.002.
24. Li WC, Ye ZH, Wong MH. 2010. Metal mobilization and production of short-chain organic acids by rhizosphere bacteria associated with a Cd/Zn hyperaccumulating plant, *Sedum alfredii*. Plant Soil 326:453–467. https://doi.org/10.1007/s11104-009-0025-y.
25. Johnstone TC, Nolan EM. 2015. Beyond iron: non-classical biological functions of bacterial siderophores. Dalton Trans 44:6320–6339. https://doi.org/10.1039/C4DT03559C.
26. Brandel J, Humbert N, Elhabiri M, Schalk IJ, Mislin GLA, Albrecht-Gary A-M. 2012. Pyochelin, a siderophore of *Pseudomonas aeruginosa*: physicochemical characterization of the iron(III), copper(II) and zinc(II) complexes. Dalton Trans 41:2820–2834. https://doi.org/10.1039/c1dt11804h.
27. Ahmed E, Holmstrom SJM. 2014. Siderophores in environmental research: roles and applications. Microb Biotechnol 7:196–208. https://doi.org/10.1111/1751-7915.12117.
28. Hafeez FY, Abaid-Ullah M, Hassan MN. 2013. Plant growth-promoting rhizobacteria as zinc mobilizers: a promising approach for cereals biofortification, p 217–235. *In* Maheshwari DK, Saraf M, Aeron A (ed), Bacteria in agrobiology: crop productivity. Springer, Berlin, Germany.
29. Rais A, Shakeel M, Hafeez FY, Hassan MN. 2016. Plant growth promoting rhizobacteria suppress blast disease caused by *Pyricularia oryzae* and increase grain yield of rice. Biocontrol (Dordrecht) 61:769–780. https://doi.org/10.1007/s10526-016-9763-y.
30. Sharma SK, Sharma MP, Ramesh A, Joshi OP. 2012. Characterization of zinc-solubilizing *Bacillus* isolates and their potential to influence zinc assimilation in soybean seeds. J Microbiol Biotechnol 22:352–359. https://doi.org/10.4014/jmb.1106.05063.
31. Indiragandhi P, Anandham R, Madhaiyan M, Sa TM. 2008. Characterization of plant growth-promoting traits of bacteria isolated from larval guts of diamondback moth *Plutella xylostella* (*Lepidoptera*: *Plutellidae*). Curr Microbiol 56:327–333. https://doi.org/10.1007/s00284-007-9086-4.
32. Kisiel A, Kepczynska E. 2016. *Medicago truncatula* Gaertn. as a model for understanding the mechanism of growth promotion by bacteria from rhizosphere and nodules of alfalfa. Planta 243:1169–1189. https://doi.org/10.1007/s00425-016-2469-7.
33. Abbaszadeh-dahaji P, Savaghebi GR, Asadi-rahmani H, Rejali F, Farahbakhsh M, Moteshareh-zadeh B, Omidvari M, Lindstrom K. 2012. Symbiotic effectiveness and plant growh promoting traits in some *Rhizobium*

strains isolated from *Phaseolus vulgaris* L. Plant Growth Regul 68:361–370. https://doi.org/10.1007/s10725-012-9724-0.
34. Rokhbakhsh-Zamin F, Sachdev D, Kazemi-Pour N, Engineer A, Pardesi KR, Zinjarde S, Dhakephalkar PK, Chopade BA. 2011. Characterization of plant-growth-promoting traits of *Acinetobacter* species isolated from rhizosphere of *Pennisetum glaucum*. J Microbiol Biotechnol 21:556–566.
35. Wani PA, Khan MS, Zaidi A. 2007. Chromium reduction, plant growth-promoting potentials, and metal solubilization by *Bacillus* sp. isolated from alluvial soil. Curr Microbiol 54:237–243. https://doi.org/10.1007/s00284-006-0451-5.
36. Panneerselvam P, Saritha B, Mohandas S, Upreti KK, Poovarasan S, Sulladmath VV, Venugopalan R. 2013. Effect of mycorrhiza-associated bacteria on enhancing colonization and sporulation of *Glomus mosseae* and growth promotion in sapota (*Manilkara achras* [Mill] Forsberg) seedlings. Biol Agric Hortic 29:118–131. https://doi.org/10.1080/01448765.2013.779076.
37. Majeed A, Abbasi MK, Hameed S, Imran A, Rahim N. 2015. Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Front Microbiol 6:198. https://doi.org/10.3389/fmicb.2015.00198.
38. Yasmin S, Hafeez FY, Schmid M, Hartmann A. 2013. Plant-beneficial rhizobacteria for sustainable increased yield of cotton with reduced level of chemical fertilizers. Pak J Bot 45:655–662.
39. Desai S, Kumar GP, Sultana U, Pinisetty S, Ahmed S, Amalraj ELD, Reddy G. 2012. Potential microbial candidate strains for management of nutrient requirements of crops. Afr J Microbiol Res 6:3924–3931. https://doi.org/10.5897/AJMR12.224.
40. Shakeel M, Rais A, Hassan MN, Hafeez FY. 2015. Root associated *Bacillus* sp. improves growth, yield and zinc translocation for basmati rice (*Oryza sativa*) varieties. Front Microbiol 6:1286. https://doi.org/10.3389/fmicb.2015.01286.
41. Ziegler D, Pothier JF, Ardley J, Fossou RK, Pfluger V, de Meyer S, Vogel G, Tonolla M, Howieson J, Reeve W, Perret X. 2015. Ribosomal protein biomarkers provide root nodule bacterial identification by MALDI-TOF MS. Appl Microbiol Biotechnol 99:5547–5562. https://doi.org/10.1007/s00253-015-6515-3.
42. Wilson MK, Abergel RJ, Raymond KN, Arceneaux JEL, Byers BR. 2006. Siderophores of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. Biochem Biophys Res Commun 348:320–325. https://doi.org/10.1016/j.bbrc.2006.07.055.
43. Johnston HW. 1952. The solubilization of phosphate. I. The action of various organic compounds on dicalcium and tricalcium phosphates. N Z J Sci Technol Sect B 33:436–444. https://doi.org/10.7931/DL1-SBP-0038.
44. Gandhi A, Muralidharan G. 2016. Assessment of zinc solubilizing potentiality of *Acinetobacter* sp. isolated from rice rhizosphere. Eur J Soil Biol 76:1–8. https://doi.org/10.1016/j.ejsobi.2016.06.006.
45. Rahi P, Prakash O, Shouche YS. 2016. Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) based microbial identifications: challenges and scopes for microbial ecologists. Front Microbiol 7:1359. https://doi.org/10.3389/fmicb.2016.01359.
46. Ferreira L, Sanchez-Juanes F, Garcia-Fraile P, Rivas R, Mateos PF, Martinez-Molina E, Gonzalez-Buitrago JM, Velazquez E. 2011. MALDI-TOF mass spectrometry is a fast and reliable platform for identification and ecological studies of species from family *Rhizobiaceae*. PLoS One 6:e20223. https://doi.org/10.1371/journal.pone.0020223.
47. Sachdev D, Nema P, Dhakephalkar P, Zinjarde S, Chopade B. 2010. Assessment of 16S rRNA gene-based phylogenetic diversity and promising plant growth-promoting traits of *Acinetobacter* community from the rhizosphere of wheat. Microbiol Res 165:627–638. https://doi.org/10.1016/j.micres.2009.12.002.
48. Verma P, Yadav AN, Khannam KS, Kumar S, Saxena AK, Suman A. 2016. Molecular diversity and multifarious plant growth promoting attributes of *Bacilli* associated with wheat (*Triticum aestivum* L.) rhizosphere from six diverse agro-ecological zones of India. J Basic Microbiol 56:44–58. https://doi.org/10.1002/jobm.201500459.
49. Islam MR, Madhaiyan M, Boruah HPD, Yim W, Lee G, Saravanan VS, Fu QL, Hu HQ, Sa T. 2009. Characterization of plant growth-promoting traits of free-living diazotrophic bacteria and their inoculation effects on growth and nitrogen uptake of crop plants. J Microbiol Biotechnol 19:1213–1222. https://doi.org/10.4014/jmb.0903.3028.
50. Bobrov AG, Kirillina O, Fetherston JD, Miller MC, Burlison JA, Perry RD. 2014. The *Yersinia pestis* siderophore, yersiniabactin, and the ZnuABC system both contribute to zinc acquisition and the development of lethal septicaemic plague in mice. Mol Microbiol 93:759–775. https://doi.org/10.1111/mmi.12693.
51. Leach LH, Morris JC, Lewis TA. 2007. The role of the siderophore pyridine-2, 6-bis(thiocarboxylic acid) (PDTC) in zinc utilization by *Pseudomonas putida* DSM 3601. Biometals 20:717–726. https://doi.org/10.1007/s10534-006-9035-x.
52. Glasauer S, Beveridge T, Burford E, Harper F, Gadd G. 2004. Metals and metalloids, transformation by microorganisms. *In* Hillel D, Rosenzweig C, Powlson DS, Scow KM, Singer MJ, Sparks DL, Hatfield J (ed), Encyclopedia of soils in the environment. Elsevier, Amsterdam, Netherlands.
53. Ryan P, Delhaize E, Jones D. 2001. Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol 52:527–560. https://doi.org/10.1146/annurev.arplant.52.1.527.
54. Alden L, Demoling F, Baath E. 2001. Rapid method of determining factors limiting bacterial growth in soil. Appl Environ Microbiol 67:1830–1838. https://doi.org/10.1128/AEM.67.4.1830-1838.2001.
55. Illmer P, Schinner F. 1992. Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biol Biochem 24:389–395. https://doi.org/10.1016/0038-0717(92)90199-8.
56. Narsian V, Patel H. 2000. *Aspergillus aculeatus* as a rock phosphate solubilizer. Soil Biol Biochem 32:559–565. https://doi.org/10.1016/S0038-0717(99)00184-4.
57. Nautiyal CS. 1999. An efficient microbiological growth medium for screening phosphate-solubilizing microorganisms. FEMS Microbiol Lett 170:265–270. https://doi.org/10.1111/j.1574-6968.1999.tb13383.x.
58. Alexander DB, Zuberer DA. 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol Fertil Soils 12:39–45. https://doi.org/10.1007/BF00369386.
59. Kaiser K, Wemheuer B, Korolkow V, Wemheuer F, Nacke H, Schoning I, Schrumpf M, Daniel R. 2016. Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests. Sci Rep 6:33696. https://doi.org/10.1038/srep33696.
60. Ziegler D, Mariotti A, Pfluger V, Saad M, Vogel G, Tonolla M, Perret X. 2012. *In situ* identification of plant-invasive bacteria with MALDI-TOF mass spectrometry. PLoS One 7:e37189. https://doi.org/10.1371/journal.pone.0037189.
61. Kassim A, Pflüger V, Premji Z, Daubenberger C, Revathi G. 2017. Comparison of biomarker based matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and conventional methods in the identification of clinically relevant bacteria and yeast. BMC Microbiol 17:128. https://doi.org/10.1186/s12866-017-1037-z.
62. Chang S, Carneiro-Leão MP, de Oliveira BF, Souza-Motta C, Lima N, Santos C, de Oliveira NT. 2016. Polyphasic approach including MALDI-TOF MS/MS analysis for identification and characterisation of *Fusarium verticillioides* in Brazilian corn kernels. Toxins (Basel) 8:E54. https://doi.org/10.3390/toxins8030054.
63. Schaffner F, Kaufmann C, Pflüger V, Mathis A. 2014. Rapid protein profiling facilitates surveillance of invasive mosquito species. Parasit Vectors 7:142. https://doi.org/10.1186/1756-3305-7-142.
64. Dice LR. 1945. Measures of the amount of ecologic association between species. Ecology 26:297–302. https://doi.org/10.2307/1932409.
65. Nguyen C, Yan W, Letacon F, Lapeyrie F. 1992. Genetic variability of phosphate solubilizing activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus *Laccaria bicolor* (Maire) P.D. Orton. Plant Soil 143:193–199. https://doi.org/10.1007/BF00007873.
66. James G. 2010. Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene, p 209–214. *In* Schuller M, Theo Sloots P, James GS, Halliday CL, Carter IWJ (ed), PCR for clinical microbiology: an Australian and international perspective. Springer, New York, NY.
67. Oberholzer HR, Leifeld J, Mayer J. 2014. Changes in soil carbon and crop yield over 60 years in the Zurich organic fertilization experiment, following land-use change from grassland to cropland. J Plant Nutr Soil Sci 177:696–704. https://doi.org/10.1002/jpln.201300385.
68. Aghili F, Gamper HA, Eikenberg J, Khoshgoftarmanesh AH, Afyuni M, Schulin R, Jansa J, Frossard E. 2014. Green manure addition to soil increases grain zinc concentration in bread wheat. PLoS One 9:e101487. https://doi.org/10.1371/journal.pone.0101487.
69. IUSS Working Group. 2014. World reference base for soil resources. International soil classification system for naming soils and creating legends for soil maps, World soil resources reports, no 106. FAO, Rome, Italy.
70. Lancashire PD, Bleiholder H, Vandenboom T, Langeluddeke P, Stauss R, Weber E, Witzenberger A. 1991. A uniform decimal code for growth-stages of crops and weeds. Ann Appl Biol 199:561–601. https://doi.org/10.1111/j.1744-7348.1991.tb04895.x.