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A new assay of bacterial selection with Pb reveals an unexpected effect of Pb on bacterial behavior: implications for remediation

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Abstract

Soil pollution by lead (Pb) is a major health concern due to Pb toxicity. Phytoextraction could remove Pb, but this technique is limited by the low Pb mobility in soils. Pb mobility can be increased by bioaugmentation, which consists in adding selected bacteria in soil to increase Pb bioavailability. Nonetheless, many bioaugmentation and phytoextraction experiments have failed because bacterial selections did not take into account the presence of metals. Therefore, we developed a microplate assay with Pb-enriched growth media for the rapid selection of bacterial strains. Selection criteria included the ability of bacteria to grow in soils, to promote plant growth and to increase Pb availability. Results show that 100–250 μM Pb induced a decrease of production of indole acetic acid (IAA), a plant growth promotor, by up to 49% for *Cupriavidus metallidurans*, compared to the control without Pb. This finding implies that application in real soil conditions with *C. metallidurans* would have probably failed, thus strengthening the value of our selection method in the presence of Pb. By contrast, 100–250 μM Pb induced a 8.5–11-fold higher degradation of aminocyclopropane carboxylic acid (ACC) by *C. metallidurans*. Surprisingly, *Pseudomonas putida* did not degrade ACC without Pb, but degraded ACC at 100–250 μM Pb. This observation means that Pb activates ACC degradation, which should reduce plant stress because ACC is the precursor of the ethylene phytohormone. Overall, our selection method in the presence of Pb allows to reveal new bacterial properties, which would not have been disclosed by current methods that do not take into account the effect of metals. Our method allows also to test simultaneously about 200 bacterial isolates. In addition, our findings show for the first time that Pb changes the production of IAA and the degradation of ACC.

Keywords Bacterial screening strategy · Bioaugmentation · Metal mobilization · Plant growth-promoting rhizobacteria

Introduction

Phytoextraction is a sub-process of phytoremediation by which plants are used to remove compounds from soil and water. Lead (Pb) phytoextraction is constrained because of the low mobility and bioavailability of Pb in soils (Van der Ent et al. 2013). Therefore, improving the efficiency of Pb phytoextraction requires to increase Pb availability, e.g., with the addition of chelating agents that form soluble complexes with metals (Shahid et al. 2012). Nonetheless, such chelating agents are usually toxic, e.g., ethylenediaminetetraacetic acid (EDTA), and thus forbidden in the natural environment. Alternatively, bioaugmentation, a gentler method involving the addition of microorganisms into soils, improves phytoextraction (Lebeau et al. 2008). Microorganisms are indeed able to increase the mobility of metals in soil and to promote plant growth.

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Plant growth-promoting rhizobacteria (PGPR) and endophytic bacteria are considered as the best options for bioaugmentation coupled to phytoextraction (Carlot et al. 2002, Rajkumar et al. 2009). Indeed, PGPR feed on root exudates and thus display a higher probability of root colonization and survival (Lebeau 2011). PGPR are also able to increase metal availability and plant biomass (Braud et al. 2006; Sessitsch et al. 2013; Chen et al. 2014; Braud et al. 2015).

Bacteria increase plant biomass using several mechanisms. For instance, plant nutrients availability in soils is increased by phosphate solubilization or by the production of chelating agents such as siderophores and weak organic acids (Weyens et al. 2014; Grobelak et al. 2018). Some bacteria produce also indole acetic acid (IAA), a phytohormone stimulating plant growth, or enzymes such as aminocyclopropane carboxylic acid (ACC) deaminase. Because ACC is the precursor of ethylene, a stress phytohormone, ACC degradation by this enzyme allows to decrease the plant stress (Arshad et al. 2007). Other beneficial properties have been sought such as protection against pathogens and N fixation (Khan et al. 2009).

The review of Sessitsch et al. (2013) on bioaugmentation-assisted phytoextraction shows that only 25.8% of the reports showed an increase in plant growth and Pb concentration.

41.2% showed higher plant biomass production but no increase in Pb concentration, and 19.6% did not show any change. This observation underlines that the previously selected microorganisms for bioaugmentation-phytoextraction do not behave in soil as expected in laboratory conditions. The current methods used to select bacteria with the potential ability to mobilize metals and to stimulate the plant growth are shown in Table 1. All methods are biochemical tests without plants, thus allowing a faster selection (Lebeau et al. 2008). The number of strains to be tested can indeed be high, up to hundreds. This is the reason why, owing to these time-consuming tests, scientists preselect bacteria based on one criterion, before testing other criteria on the remaining bacteria (Yahaghi et al. 2018; Kumar 2019). For example, Yahaghi et al. (2018) preselected bacterial isolates based on their ability to solubilize PbO and PbCO₃, before testing other criteria such as the production of IAA and siderophores. Noteworthy, the bacterial pretests were all realized in metal-free media. Metal effect on plant growth-promoting (PGP) properties is only considered once bacteria are preselected (Kumar 2019).

Yet, metals in soils are likely to have an effect on bacterial responses, as shown by Braud et al. (2015) and Arunakumara et al. (2015) for phosphate solubilization in the

Table 1 Tests used during the last 5 years to select bacteria with the potential ability to mobilize metals and to stimulate the plant growth

Bacteria type			Strains bank		Metal		Plant growth promoting properties						References
Autochthonous bacteria	Endophytic bacteria	Rhizobacteria	Isolation	Strains number	Types	Metal tolerance	Use of metal in the tests	Phosphate solubilization	Total siderophores	IAA production	ACC degradation	Others	
		X	X	23	Zn, Cu, As, Pb, Co, Ni, Cd	X	No	X		X	X	+	Andrades-Moreno et al. (2014)
		X	X	54	Cd, Zn	X	No		X	X	X	+	Weyens et al. (2014)
X			X	1	Co, Pb, Zn	X	200 mM	X					Arunakumara et al. (2015)
	X	X	X	45	Cd, Pb, Zn		No	X	X	X	X	++	Grobelak et al. (2015)
	X	X		2	Ni, Zn, Fe	X	No	X	X	X	X		Ma et al. (2015)
		X	X	15	Zn, Cu, As, Pb	X	Cu	X	X	X	X	+	Mesa et al. (2015)
	X		X		Se	X	No	X	X	X		++++	Sura-de Jong et al. (2015)
	X		X	1	Cd, Pb, Zn	X	No	X	X	X	X		Ma et al. (2016)
			X	110	Pb	X	No	X	X	X		+	Yahaghi et al. (2018)
	X		X		Cd, Zn	X	No	X	X	X	X	++++	Plociniczah et al. (2018)
		X	X	2	Cr	X	No	X	X	X		+	Gupta et al. (2019)
		X	X		Cr, Pb	X	Only for one bacteria	X	X	X	X	+	Kumar (2019)

Colors were added to highlight the fact that metals are hardly ever taken into account for the tests selection: red—no use of the metals; orange—limited use (only one bacteria or one metal); and yellow—use of the metal but under unrealistic conditions. IAA indole acetic acid, ACC aminocyclopropane carboxylic acid

presence or not of copper. Although Braud et al. (2015) have reduced the selection duration by using microplates and included metals (copper) in the whole selection process, they did not take into account criteria such as bacterial production of phytohormones and enzymes.

Here, we developed a microplate method for the simultaneous selection of a large number of bacterial isolates based on their ability to grow in soils, to promote plant growth and to increase Pb availability. Tests were performed at Pb concentrations usually encountered in the available fraction of urban soils. Pretests were realized with five strains from bacteriological collections, commonly found in polluted soils and displaying metal solubilization potential. Our objectives were to select the best conditions for the final tests, e.g., culture media and Pb concentrations; to evidence possible interactions between Pb and reagents used in tests; to determine whether the pretests give coherent responses according to the known PGP properties of the bacteria; and to study the effect of Pb on bacterial behavior.

Experimental

Bacterial pretests and tests

A series of biochemical tests were developed in microplates to quickly evaluate the potential of a large number of bacterial isolates. The following properties were tested:

- Bacterial growth in soil extracts, to evaluate their capacity to grow in the soils supposed to be bioaugmented,
- Production of total siderophores among which pyoverdine,
- Solubilization of Pb phosphates, the latter two tests making it possible to assess the ability of bacteria to mobilize Pb from some fractions of soils,
- Production of indole acetic acid (IAA) and
- Degradation of aminocyclopropane carboxylic acid (ACC)

Pb phosphate solubilization, IAA production and ACC degradation measure the bacterial capacity to promote the plant growth.

A pretest was performed for each property to choose the best conditions for subsequent tests. We compared several compounds or growth media at different Pb concentrations. We used five plant growth-promoting (PGP) bacteria from a bacteriological collection: *Bacillus subtilis* DSM 3256, *Pseudomonas fluorescens* ATCC 17400, *P. fluorescens* ATCC 50090, *Pseudomonas putida* ATCC 12633 and *Cupriavidus metallidurans* ATCC 43123 (van der Lelie et al. 2000; Braud et al. 2006, 2015).

The following paragraphs detail the experimental protocol for the pretests. The experimental objectives are

illustrated in Fig. 1. Before the first three pretests and final tests, bacteria were precultivated overnight with tryptic soy broth (TSB) 1:10 at 28 °C and 450 rpm. Then, the bacterial biomass from precultures was recovered after 20 min at 866 g, then washed twice with 9 g/L KCl and re-suspended in 9 g/L KCl in a way to set the final optical density (OD) to 1 at 620 nm. Media used for metal mobilization pretest and tests were the same as Braud et al. (2015). Each pretest and test was performed in triplicate for each bacterium. For each pretest and test, cross plots giving the relation between the optical density (OD) and the tested compound concentration were established with and without Pb. Results are presented based on specific production or degradation, i.e., tested compound concentration to the OD ratio, e.g., IAA production in $\mu\text{M}/\text{ODU}_{620\text{nm}}$. This original presentation, not used in other studies, allows to compare bacteria disregarding biomass production.

Bacterial growth in soil extract medium

Soil samples were collected from two urban gardens in Nantes, France, at the sites of *Les Eglantiers*, named E, located north from the city center, and *Les Oblates*, named O, located west. E soil is mainly fine silt, whereas O soil is sandy silt. The texture of soils E and O is, respectively, 3.6% and 1.5% of clay, 59.0% and 43.3% of silt, and 37.4% and 55.2% of sand. E and O soils have, respectively, 2.4% and 10.8% of organic matter (OM), a pH of 7.2 and 6.4 and a cation exchange capacity (CEC) of 80 and 139 cmol/kg of soil. Total Pb content in soils E and O is 208 ± 7 and 220 ± 16 mg/kg, respectively (see the other properties in SI—Table S1). Pb in soil E is geogenic from a Pb-rich source rock. Pb in soil O is anthropogenic from a nearby foundry.

The pretest was performed in soil extract medium by supplying Pb at increasing concentrations to select the appropriate Pb concentration for the selection tests. Soils were extracted using hot extraction according to Braud et al. (2015) without pH adjustment. Tested Pb concentrations were in the same order of magnitude as Pb concentrations found in soils extracted with DTPA (Lindsay and Norwell 1978) providing an estimation of the bioaccessible fraction of Pb. Pb amounts in those fractions were 7.5 mg/kg for E and 32.2 mg/kg for O (more details in SI—Table S2). Each microplate was prepared with 200 μL of soil extract medium and 25 μL of washed bacterial suspension with an optical density of 1 at 620 nm. Before inoculation, 25 μL of sterile solution of $\text{Pb}(\text{NO}_3)_2$ was added in each well of the microplate to reach a final concentration of 0, 10, 25, 50, 75, 100, 250 and 500 $\mu\text{mol/L}$ of Pb, respectively, 0.0, 3.3, 8.3, 16.6, 24.8, 33.1, 82.8 and 165.6 mg/L. Microplates were incubated at 28 °C and 450 rpm on a microplate shaker. Bacterial density was measured at 620 nm at 0, 24, 48 and 72 h with a microplate reader Multiskan FC from Thermo, USA.

Method
to evaluate
the capacity to:

Tested
parameters
per test #

PRETESTS	<div>Five strains : <i>Bacillus subtilis</i> DSM 3256, <i>Pseudomonas fluorescens</i> ATCC 17400, <i>Pseudomonas fluorescens</i> ATCC 50090, <i>Pseudomonas putida</i> ATCC 12633, <i>Cupriavidus metallidurans</i> ATCC 43123</div> <div>Objectives:</div> <div>(i) to select the best conditions for the final tests (media, Pb concentrations), (ii) to bring to light possible interactions between Pb and the reagents used in this method, (iii) to see whether the tests reveal coherent responses of the five used bacteria according to their PGP and if lead presence has a significant effect on bacteria responses.</div> <div>To see the effect of :</div> <div>① Pb on bacteria growth ② growth medium and Pb on siderophores production ③ phosphate nature on bacteria response ④ & ⑤ Pb on PGP properties</div> <div>Conditions to be selected</div> <div><div>- Pb concentrations: 0, 10, 25, 50, 75, 100, 250 and 500 µmol/L</div><div>- Medium CAA - Medium DF - Pb concentration</div><div>- Calcium phosphate - Lead phosphate</div><div>- Pb concentration</div></div> <div>Simplified protocol</div> <div><div><div>Microplate preparation</div><div><div>x 3</div></div><div>Per well of microplate:</div><div>- 200 µl of soil extract - 25 µl of sterile solution of Pb(NO₃)₂ with varying concentrations - 25 µl of bacterial suspension with an optical density of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm</div><div>Measure: Bacterial density at 620 nm at 0, 24, 48 and 72h</div></div><div><div>Microplate preparation</div><div><div>x 2 (CAA; modified DF)</div></div><div>Per well of microplate:</div><div>- 200 µl of CAA or modified DF - 25 µl of sterile solution of Pb(NO₃)₂ or not to reach 0, 100 and 250 µmol/L - 25 µl of bacterial suspension with an optical density (OD) of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 48h</div><div>Measure: OD at 620 nm Supernatant OD at 405 nm for pyoverdine production</div></div><div><div>Microplate preparation</div><div><div>Ca-P Pb-P</div><div>x 3</div></div><div>Per well of microplate:</div><div>- 200 µl of NBRIY medium - 25 µl of sterile Ca phosphate or Pb phosphate suspension to obtain a final concentration of 5 g/L - 25 µl of bacterial suspension with an OD of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 5d</div><div>Measure: Addition of 150 µl vanadomolybdate reactant to supernatant; OD at 450 nm after 2h</div></div><div><div>Microplate preparation</div><div><div>x 3</div></div><div>Per well of microplate:</div><div>- 200 µl of DF with glucose - 12.5 µl of sterile solution of Pb(NO₃)₂ - 12.5 µl of sterile 10 g/L tryptophane solution - 25 µl of bacterial suspension with an OD of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 48h</div><div>Measure: OD at 620 nm Addition of 200 µl 200 µl Salkowski reactant to 50 µl of supernatant OD at 541 nm after 2h</div></div><div><div>Microplate preparation</div><div><div>x 3</div></div><div>Per well of microplate:</div><div>- 200 µl of N-free DF medium - 12.5 µl of sterile solution of Pb(NO₃)₂ - 12.5 µl of sterile 60 mM ACC solution - 25 µl of bacterial suspension with an OD of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 48h</div><div>Measure: OD at 620 nm Addition of 30 µl of 0.2% 2,4-dinitrophenylhydrazine in 2 M HCl to 20 µl of supernatant Addition of 200 µl of NaOH 2M after 30min at 28°C OD at 541 nm</div></div></div> <div><div>Chosen conditions</div><div>① Pb 250 µmol/L</div><div>② - Medium CAA - Pb 250 µmol/L</div><div>③ - Lead phosphate</div><div>④ Pb 250 µmol/L</div><div>⑤ Pb 250 µmol/L</div><div>Objective:</div><div>evaluate bacterial isolates potential for bioaugmentation-phytoextraction coupling</div><div>Bank of <u>181 bacterial isolates</u></div><div>Simplified protocol</div><div><div>Microplate preparation per test</div><div><div>Microplate #1</div><div><div>x 3 replicates</div></div><div>Microplate #2</div><div><div>x 3 replicates</div></div></div><div>- 181 bacterial isolates; each one in one well - 3 control wells, without bacteria</div><div>Per well of microplate:</div><div>- 200 µl of soil extract - 25 µl of sterile Pb(NO₃)₂ solution to reach a final concentration of 250 µmol/L - 25 µl of bacterial suspension with an optical density (OD) of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm</div><div>Measure: Bacterial density at 620 nm at 0, 24, 48 and 72h</div><div>Per well of microplate:</div><div>- 200 µl of CAA - 25 µl of sterile solution of Pb(NO₃)₂ to reach 250 µmol/L - 25 µl of bacterial suspension with an OD of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 48h</div><div>Measure: OD at 620 nm Supernatant OD at 405 nm for pyoverdine production</div><div>Per well of microplate:</div><div>- 200 µl of NBRIY medium - 25 µl of sterile Pb phosphate suspension to obtain a final concentration of 5 g/L - 25 µl of bacterial suspension with an OD of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 5d</div><div>Measure: Addition of 150 µl vanadomolybdate reactant to supernatant; OD at 450 nm after 2h</div><div>Per well of microplate:</div><div>- 200 µl of DF with glucose - 12.5 µl of sterile solution of Pb(NO₃)₂ to reach 250 µmol/L - 12.5 µl of sterile 10 g/L tryptophane solution - 25 µl of bacterial suspension with an OD of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 48h</div><div>Measure: OD at 620 nm Addition of 200 µl 200 µl Salkowski reactant to 50 µl of supernatant OD at 541 nm after 2h</div><div>Per well of microplate:</div><div>- 200 µl of N-free DF medium - 12.5 µl of sterile solution of Pb(NO₃)₂ to reach 250 µmol/L - 12.5 µl of sterile 60 mM ACC solution - 25 µl of bacterial suspension with an OD of 1 at 620 nm</div><div>Incubation: 28°C; 450 rpm; 48h</div><div>Measure: OD at 620 nm Addition of 30 µl of DNPH (0.2% 2,4-dinitrophenylhydrazine in 2 M HCl) to 20 µl of supernatant Addition of 200 µl of NaOH 2M after 30min at 28°C OD at 541 nm</div></div><div>FINAL SELECTION METHOD</div></div>				
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◀**Fig. 1** Protocol used for bacterial screening. Bacterial isolates are evaluated for their ability to grow into the soils studied, to mobilize Pb and to promote plant growth. For that, five trials based on six tests were set up: (i) the bacterial growth in soil extracts, (ii) the production of siderophores including pyoverdine, (iii) the phosphate solubilization, (iv) the indole acetic acid (IAA) production and (v) the aminocyclopropane carboxylic acid (ACC) degradation. This study also presents the pretests used to develop the final method. This figure summarizes the final experimental conditions of the method

Following the results of this first pretest, two Pb concentrations, 100 and 250 $\mu\text{mol Pb/L}$, were used during the other selection pretests in order to adjust the Pb concentration for the final selection.

Siderophores production

The pretest was performed to compare the Casamino acid medium (CAA; Braud et al. 2015) and the modified Dworkin–Foster medium (DF; Dworkin and Foster 1958; Díaz de Villegas et al. 2002), two free-Fe media for siderophores production. The composition of the modified DF medium was: 4 g KH_2PO_4 , 6 g Na_2HPO_4 , 2 g $(\text{NH}_4)_2\text{SO}_4$, 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 $\mu\text{g H}_3\text{BO}_3$, 10 $\mu\text{g MnSO}_4$, 70 $\mu\text{g ZnSO}_4$, 50 $\mu\text{g CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10 $\mu\text{g MoO}_3$, in ultrapure water q.s.p. 1000 ml and pH adjusted to 7.2. 25 μL of the washed bacterial suspension from the above-mentioned preculture was inoculated into 200 μL of CAA medium or free-Fe Dworkin Foster medium with or without 25 μL of $\text{Pb}(\text{NO}_3)_2$ solution at 2 mM or 5 mM in order to get a final concentrations of 0, 100 or 250 $\mu\text{mol/L}$. After 48 h of incubation at 28 °C and 450 rpm, growth was determined by measuring $\text{OD}_{620\text{nm}}$, and microplates were centrifuged at 866 g for 20 min. Supernatant was collected and transferred into another microplate to measure pyoverdine production at 405 nm, using an extinction coefficient of 19600 $\text{mol}^{-1} \text{cm}^{-1}$ at pH 8. Total siderophores production was measured according to the Chrome Azurol S (CAS) universal method described by Schwyn and Neilands (1987).

Phosphate solubilization

This pretest aims to evaluate the possible effect of the phosphate nature (Pb or Ca) on bacteria phosphate solubilization capacity. The calcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ used in this pretest was a commercial product (VWR International). Pb orthophosphate (PbHPO_4) was synthesized from Na_2HPO_4 and $\text{Pb}(\text{NO}_3)_2$ according to Sauvé et al. (1998). Ca and Pb phosphate suspensions were sterilized for 20 min at 121 °C and washed with ultrapure water and then with the NBRIY culture media (Nautiyal 1999) and stored at 4 °C.

Each microplate well was filled with 200 μL of NBRIY medium, 25 μL of washed bacterial suspension with OD at 620 nm set to 1 and 25 μL of sterile Ca phosphate or

Pb phosphate suspension to obtain a final concentration of 5 g/L. Microplates were then incubated for 5 days at 28 °C and 450 rpm and then centrifuged for 20 min at 866 g. Due to opacity of the suspension, bacterial growth was not measured. Free phosphates in the supernatant were quantified by adding 150 μL of vanadomolybdate reactant commercial solution (VWR International) diluted to 1:2 into 150 μL of supernatant. After 2 h, a yellow complex developed and 450 nm optical density was measured.

Production of indole acetic acid

Bacterial isolates were incubated for 48 h in TSB 1:10 and then precultivated in Fe-containing Dworkin Foster medium with 10 g/L glucose for 24 h at 28 °C under 200 rpm. Bacteria were collected by centrifugation, washed with 9 g/L KCl and then re-suspended with an initial $\text{OD}_{620\text{nm}}$ set at 1. Microplates were prepared with 200 μL DF medium including Fe and glucose, 12.5 μL of sterile 10 g/L tryptophane solution, 12.5 μL of 2 mM or 5 mM $\text{Pb}(\text{NO}_3)_2$ —for a final concentration of 100 or 250 $\mu\text{mol/L}$ —and 25 μL of bacterial suspension with initial $\text{OD}_{620\text{nm}}$ of 1. Microplates were incubated for 48 h at 28 °C and 450 rpm. Growth was measured by measuring OD at 620 nm. Microplates were centrifuged for 20 min at 866 g; then 50 μL supernatant was sampled and mixed with 200 μL Salkowski reactant (Patten and Glick 2002). After 20 min, absorbance was measured at 541 nm, which corresponds to the pink color indicative of indole acetic acid.

Degradation of aminocyclopropane carboxylic acid

Bacterial strains were precultivated as in the previous section. Incubation was performed with 200 μL of N-free DF medium, 12.5 μL of sterile 60 mM aminocyclopropane carboxylic acid (ACC) solution, 12.5 μL of 2 mM or 5 mM $\text{Pb}(\text{NO}_3)_2$ —for a final concentration of 100 μM or 250 μM —and 25 μL of bacterial suspension to get a final optical density of 0.1. Microplates were incubated for 48 h at 28 °C and 450 rpm. Growth was measured at $\text{OD}_{620\text{nm}}$, and microplates were centrifuged for 20 min at 866 g. 20 μL of supernatant was sampled and mixed with 30 μL of 0.2% 2,4-dinitrophenylhydrazine in 2 M HCl and were then incubated for 30 min at 28 °C. This reaction produces the phenylhydrazone from α -cétobutyrate, an ACC degradation product. 200 μL of 2 M NaOH was added to reveal the brown color of phenylhydrazone at alkaline pH. The absorbance was measured at 541 nm.

Selection from the bacterial isolates bank

The final selection tests used the bank of 181 genetically different bacterial isolates recovered from sediments (Bois

et al. 2011). Final test protocols were the same as described above, except for some changes (Fig. 1). After pretests results, Pb concentration chosen for all five selection trials was 250 μ M. For the siderophore production, CAA medium has been chosen. Phosphate solubilization final test was performed only with Pb phosphate. No major interference has been detected between Pb and reagents, irrespective of the pretests.

Statistics were done on all data. Strains were evaluated with a mark from 0 to 2 depending on the quartile in which the strain value is found, i.e., 2 for the fourth quartile, 1 for the third quartile and 0 for the second and first quartiles. After having carried out the 5 tests giving access to the 6 parameters, marks were then added to give a final mark from 0 to 12 for each bacterial strain.

Results and discussion

A series of biochemical tests in microplates, based on bacterial capacities to grow in soils, to mobilize metals and to promote plant growth, were set up based on a first phase of pretests. Those pretests were done with five identified bacteria commonly found in polluted soils, known as plant growth-promoting (PGP) bacteria and displaying a metal solubilization potential. Levels of Pb in the culture medium were varied to study Pb effect on the bacteria responses.

Effect of Pb on bacterial production of indole acetic acid

Figure 2a shows the effect of Pb at 100–250 μ M on the specific production of indole acetic acid (IAA) by the five tested PGP bacterial strains. Specific production or degradation, which is the compound concentration to OD_{620nm} ratio, allows to compare bacteria response disregarding biomass production. Results show that Pb has no statistical effect on IAA specific production by *B. subtilis*, *P. fluorescens* and *P. putida*.

By contrast, Pb induced a high decrease in IAA production for *C. metallidurans*, from 45.9 mM/ODU_{620nm} for the control without Pb to 23.5 mM/ODU at 100 μ M Pb, i.e., a 48.8% decrease. These findings reveal that *C. metallidurans*, which appeared initially as an efficient potential candidate for phytoremediation due to its high IAA production, displays a different response under realistic conditions and is probably not well suited for bioaugmentation in Pb-polluted soils. As a consequence, current selection methods that do not take metal into account may select unsuitable strains. This result highlights the importance of proceeding with the studied metal at realistic concentrations in preliminary tests.

Effect of Pb on bacterial degradation of aminocyclopropane carboxylic acid

The effect of 100–250 μ M Pb on the degradation of aminocyclopropane carboxylic acid (ACC) is shown in Fig. 2b by analyzing α -ketobutyrate concentration, the compound resulting from ACC degradation. The results show that Pb has no statistically significant effect on ACC degradation by *B. subtilis* and *P. fluorescens*. However, Pb induced a very high increase in ACC degradation by *P. putida* and *C. metallidurans*. For *P. putida*, the absence of degradation in the control implies that Pb has activated the ACC degradation by this strain. Surprisingly, our findings show that adding Pb has activated plant growth-promoting properties for *P. putida*. As underlined in Sect. 3.1, this activation would not have been detected using current methods that do not take into account the metal during selection tests.

Our findings show that the presence of Pb modifies the bacteria influence on metal speciation and bioavailability. This phenomenon was already observed for the effect of Cu on phosphate solubilization (Braud et al. 2015). This work reveals for the first time the positive effect of Pb on bacteria responses. Strain selection in the literature is widely performed without taking into account metals (Plociniczak et al. 2018; Grobelak et al. 2018). Although bacteria responses under metal stress have been tested, this was done at very high concentrations, e.g., between 25 and 400 mg/L (Rizvi and Khan 2017) and 100 and 500 mg/L (Kumar 2019). Those concentrations are not in line with Pb amounts usually found in the available fraction of soils and are thus not representative of real conditions. Our results on the changes of bacterial behavior with the presence of metal could explain, at least partly, why some selected strains have not efficient during previous experiments of bioaugmentation and phytoextraction (Sessitsch et al. 2013). Overall, our findings imply that selection tests should be carried out with metal at concentrations similar to those found in soil water.

Bacterial strains selection in the presence of Pb for bioaugmentation and Pb phytoextraction

For further soil bioaugmentation-assisted Pb phytoextraction, we selected 7 out of 181 strains using the set of tests described above, in the presence of 250 μ mol Pb/L. Criteria were bacterial growth in studied soils, phosphate solubilization, pyoverdine and total siderophores production, indole acetic acid (IAA) production and aminocyclopropane carboxylic acid (ACC) degradation (Table 2). Those tests were used to rank the bacterial isolates. The best scores were obtained for isolates No. 308, 394, 419, 436, 473, 480 and 485. We selected as controls for upcoming bioaugmentation experiments the lowest ranked isolates, i.e., 30 and 177, which grew well in soils. These controls allow to have

Fig. 2 a Effect of Pb on the specific production of indole acetic acid (IAA), a plant growth promotor, by five bacterial strains: *Bacillus subtilis*, *P. fluorescens* ATCC 17400 and 50090, *P. putida* and *C. metallidurans*. Note the high decrease in acid production for *C. metallidurans* at 100 μ M Pb compared to the control. ODU_{620nm}: optical density units measured at 620 nm. **b** Effect of Pb on the specific degradation of aminocyclopropane carboxylic acid (ACC) by *Bacillus subtilis*, *P. fluorescens* ATCC 17400 and 50090, *P. putida* and *C. metallidurans*. Note the significant increase in ACC degradation in the presence of Pb for *P. putida* and *C. metallidurans*. This finding means that the presence of Pb activates ACC degradation, notably for *P. putida*. Such an effect would not have been revealed without metal. ODU_{620nm}: optical density units measured at 620 nm

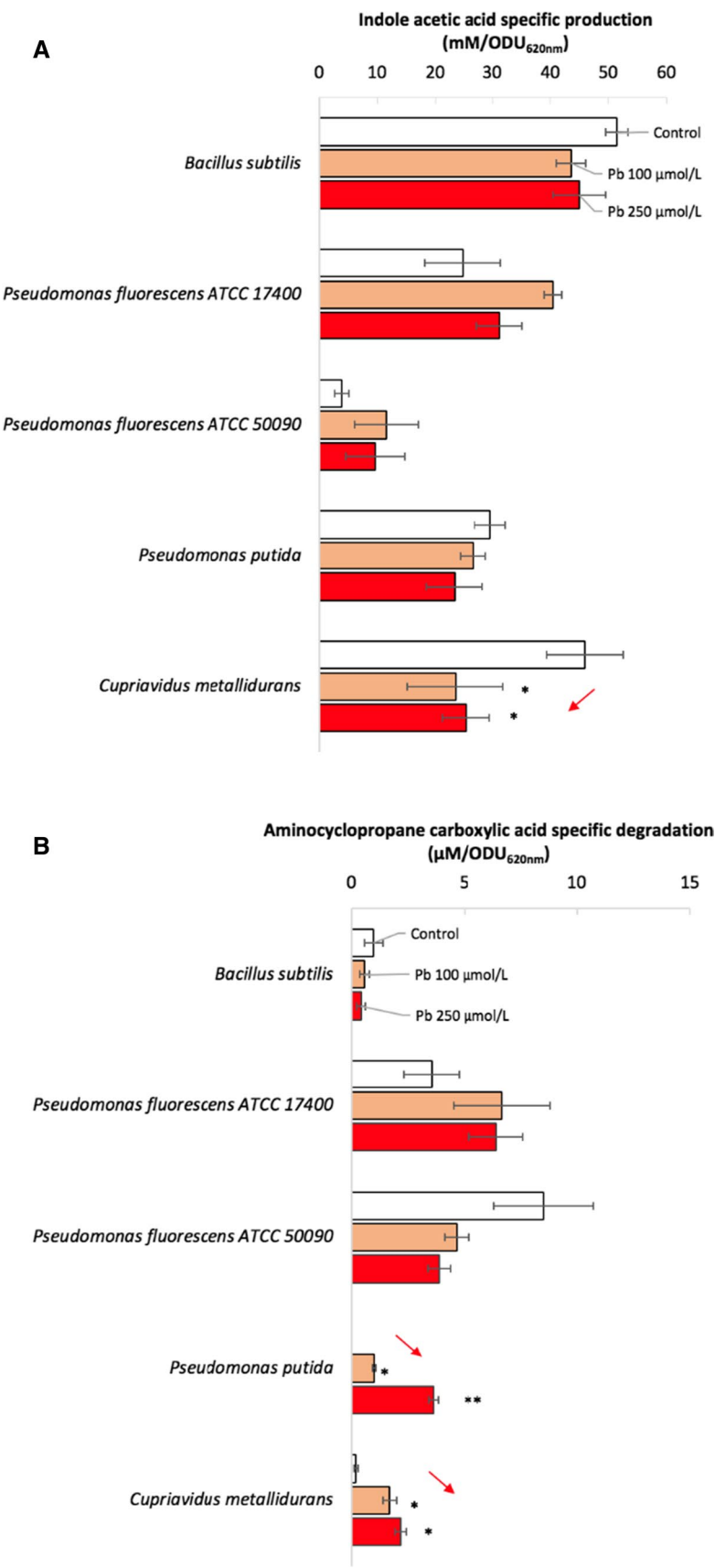


Table 2 Properties and marks, out of 2 (in brackets) for each test, of the best 7 top-rated bacterial isolates

Bacterial isolate number	Growth (%)	Phosphate solubilization (mg P/L)	Pyoverdine ($\mu\text{mol/L}$)	Total siderophores ($\mu\text{mol/L}$)	IAA (mmol/L)	ACC degradation ($\mu\text{mol/L}$)	Total score/12
<i>Preselected strains</i>							
308	42.7 (1)	24.9 (1)	550.5 (2)	1868.7 (2)	5.46 (2)	79.9 (2)	10
394	57.8 (2)	40.3 (2)	366.5 (2)	963.2 (2)	3.02 (0)	46.6 (2)	
419	50.4 (2)	30.6 (2)	339.0 (2)	912.0 (2)	3.22 (0)	52.0 (2)	
436	65.0 (2)	33.0 (2)	367.8 (2)	896.4 (2)	2.29 (0)	48.9 (2)	
473	63.2 (2)	34.1 (2)	446.8 (2)	1146.7 (2)	3.84 (1)	23.2 (1)	
480	51.8 (2)	33.6 (2)	334.3 (2)	932.7 (2)	2.46 (0)	86.9 (2)	
485	40.5 (1)	34.9 (2)	607.6 (2)	1495.4 (2)	4.38 (1)	73.0 (2)	
<i>Control bacterial isolates</i>							
30	50.3 (2)	15.4 (0)	ND	131.0 (0)	1.67 (0)	10.8 (0)	2
177	501.0 (2)	11.9 (0)	14.1 (0)	45.9 (0)	0.96 (0)	13.7 (0)	

Growth % corresponds to the bacterial isolate growth in soil extract to the one in TSB 1/10 ratio. Two isolates have been identified so far: No. 480 as *Comamonas testoteroni* and No. 485 as *Pseudomonas putida*. Control strains correspond to bacteria with good growth % in soil extract (mark of 2) but low performances for the other tests (mark of 0)

IAA indole acetic acid, ACC aminocyclopropane carboxylic acid, ND not detected

an insight of Pb biosorption on bacterial cells (Khan et al. 2009).

Results in Table 2 reveal that bacterial isolates No. 308 and 485 are the best options to stimulate plant growth due to their high production of siderophores and indole acetic acid (IAA) and high degradation aminocyclopropane carboxylic acid (ACC). Noteworthy, siderophores improve involved in the bioavailability of metals. On the contrary, the bacterial isolate No. 436 is the less effective for plant growth-promotion, as shown by a very low production of IAA and siderophores. Two bacterial isolates have been identified so far: 480 as *Comamonas testoteroni* and 485 as *P. putida*. Out of the 7 selected strains, strain 485 yielded the highest amount of pyoverdine, and strain 480 the lowest.

Our method designed for microplate analysis allows to characterize hundreds of bacterial isolates at the same time. The selection takes only 10–20 days considering incubation times. If this method is applied for another metal, the development stage takes approximately 20 days (1 month including incubation times). Besides, this method consumes less material and media. Indeed, based on triplicate and ninety strains to test, 273 Eppendorf tubes (3×91) would be necessary for each test with other methods, whereas the material with our method is reduced to three microplates, coupled with a medium consummation reduced by a factor of 7. This method is thus especially relevant for the screening of a large bank of bacterial isolates, taking into account the studied metals but also the studied soils.

Indeed, another constraint for bioaugmentation success is the colonization and establishment of the added bacterial isolates into soil. It is unlikely to be able to prepare in laboratory conditions a medium with the exact soil composition. The hot soil extraction method has the advantages to procure

a growth medium (1) not too rich in organic matter compared to other minimal media, as organic matter could have an impact on metal tolerance due to its possible complexation and (2) composed with nutrients that the studied soil could provide (Liebeke et al. 2009). Eventually it should be noted that the added Pb form used in the culture medium may have also an impact on the bacterial growth. $\text{Pb}(\text{NO}_3)_2$ has been chosen for its high solubility, but it may have a higher toxic effect than other Pb salts because of the NO_3^{2-} counterion effect (Bongers et al. 2004). Nonetheless, choosing a salt in this chemical form allows to accurately control the amount of available Pb and see its effect on the bacterial isolates.

Conclusion

We developed a rapid microplate assay for the selection of bacterial strains adapted to bioaugmentation–phytoextraction of soils contaminated by Pb. We highlighted unexpected effects of the presence of Pb on bacterial behavior, notably on the production of indole acetic acid (IAA) and on the degradation of aminocyclopropane carboxylic acid (ACC). In particular, the presence of Pb has activated and increased ACC degradation for some strains. These findings imply that the whole strain selection process should be performed in the presence of metals and more accurately under realistic conditions. Our results may also explain, at least partly, why previous remediation experiments using strains selected without metal have failed with potentially promising bacteria.

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Supplementary Material

Bouquet et al. (2020) A new assay of bacterial selection with Pb reveals an unexpected effect of Pb on bacterial behavior: implications for remediation. Environmental Chemistry Letters

Table S1. Characterization of the two studied soils: Eglantiers and Oblates. CEC: Cationic Exchange Capacity; DTPA: diethylenetriaminepentaacetic acid

SOILS	EGLANTIERS		OBLATES	
pH water	7.2		6.4	
CEC _{Metson} pH 7 (cmol/kg)	80		139	
CEC _{Metson} soil pH (cmol/kg)	103		195	
	Organic matter			
Organic matter (g/kg; <i>Optimum</i>)	24.4	(23)	107.8	(19)
Total organic Carbon (g/kg)	14.1		62.3	
Total N (g/kg)	1.29		5.99	
C/N	10.9		10.4	
	Clay-humic complex saturation			
Ca ²⁺ (%)	82.5		90	
K ⁺ (%)	8.2		1.5	
Mg ²⁺ (%)	8.7		7.9	
Na ⁺ (%)	0.6		0.6	
(<i>Optimum</i>)	Major elements			
P ₂ O ₄ Joret-Hebert (g/kg)	0.53	(0.16)	1.09	(0.25)
K ₂ O (g/kg)	0.31	(0.12)	0.1	(0.19)
MgO (g/kg)	0.14	(0.1)	0.22	(0.1)
CaO (g/kg)	0.42	(1.85)	3.66	(3.5)
Na ₂ O (g/kg)	0.01	-	0.02	-
K ₂ O/MgO	2.2	(1 à 2)	0.5	(1 à 2)
DTPA extraction (<i>Optimum</i>)	Trace elements			
Iron (mg/kg)	32.4	(20)	78.7	(20)
Zinc (mg/kg)	4	(8)	1.4	(8)
Manganese (mg/kg)	4.1	(1.1)	32.4	(0.9)
Copper (mg/kg)	3.9	(0.4)	4	(0.8)
Bore* (mg/kg)	0.48	(0.34)	0.36	(0.39)
*Boiling water extraction				

*Boiling water extraction

Table S2. Trace elements concentrations of the two soils (Eglantiers, E; Oblates, O) extracts with CaCl₂ 0.01M (μg/kg DM) and DTPA 0.005M (mg/kg DM; Lindsay & Norvell, 1978). DM: dry matter

SOILS	EGLANTIERS	OBLATES
CaCl ₂ extraction (μg/kg DM)		
Fe	33.1	126.6
Zn	nd	0.08
Mn	0.05	0.44
Cu	0.006	0.005
B	-	-
Pb	0.20	6.92
As	39.73	22.44
DTPA extraction (mg/kg DM)		
Fe	32.4	78.7
Zn	4.0	1.4
Mn	4.1	32.4
Cu	3.9	4.0
B	0.48	0.36
Pb	7.5	32.2
As	0.2	0.2