Soil antimony pollution and plant growth stage affect the biodiversity of auxin-producing bacteria isolated from the rhizosphere of *Achillea ageratum* L

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Abstract

A total of 4512 rhizobacteria were isolated at three stages of plant growth from *Achillea ageratum* colonizing a polluted site with an antimony concentration gradient. For 222 of these isolates auxin production (*aux*) was verified in vitro. The percentage of *aux*+ isolates increased with soil antimony concentration, as well as with plant growth stage. An amplified rDNA restriction analysis clustered the *aux*+ isolates into 51 clusters, one of which was numerically predominant and present throughout plant development and at all antimony concentrations. The *aux*+ population was genetically very diverse, and this diversity was related to both antimony concentration and plant growth stage.

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1. Introduction

Pollution of the biosphere by toxic metals has accelerated dramatically since the beginning of the industrial revolution. The sources of this pollution include mining and smelting of metallic ores, burning of fossil fuels, municipal wastes, fertilizers, pesticides and sewage. Toxic metal contamination of soil and groundwater poses a major environmental and human health problem [1]. Antimony is a toxic trace element of growing interest due to the increased anthropogenic input into the environment [2]. It is known to provoke DNA damage [3], to disturb the hematic and gastrointestinal systems and to cause other toxic effects [4].

Unlike organic pollutants, heavy metals cannot be degraded, but instead persist indefinitely in the environment, complicating their remediation. The techniques presently used for the clean-up of soils contaminated by heavy metals are mainly based on ex situ decontamination, using physico-chemical methods of extraction, which are very expensive. In addition, they destroy the soil structure and leave the soil biologically inactive [5].

Certain plants have the ability to grow on heavy-metal-contaminated soils, and some of them can accumulate heavy metals, which have no known biological function [6]. Such plants are investigated for their potential use in in situ phytoremediation, i.e. the use of plants to remove, contain or transform environmental contaminants, which is considered one of the most promising new technologies for heavy metal remediation [7]. Metals such as antimony, arsenic, cadmium, copper, mercury, nickel, selenium and zinc can be extracted from the soil by hyperaccumulator plants and concentrated into their roots and shoots. This process is called phytoextraction, as the metal-enriched plant biomass can be harvested using standard agricultural
methods and smelted to recover the metal [8]. However, most of the plants known to be metal hyperaccumulators emerged spontaneously, so their physiology, ecology and agronomic performances are often scarcely known.

In nature, the roots of plants interact with a large number of different microorganisms, and these interactions, together with soil and climatic conditions, are major determinants of the extent to which plants grow and spread [9]. Among soil microorganisms, several bacterial genera stimulate root proliferation and protect surrounding plants by producing auxins and/or other compounds [10–15]. They are called plant growth-promoting rhizobacteria (PGPR) [12]. Recent studies have shown that hyperaccumulator plants grown in heavy-metal-contaminated soils presented a more proliferated root system than in non-contaminated soils [16,17]. The phenomenon of PGPR has been investigated to clarify their potential role in the phytoremediation process. Indian mustard plants (Brassica juncea L.), when germinated in vitro on selenium-containing media [18] or in pot experiments with cadmium-contaminated soils [19], produced more root hairs when inoculated with PGPR strains. Furthermore, these inoculated plants accumulated more selenium than the non-inoculated ones [18]. Thus, PGPR are promising bacterial partners for the improvement of plant growth on heavy-metal-contaminated soil, and thus for phytoremediation.

In the case of antimony-polluted soils, plants such as Achillea ageratum L., Plantago lanceolata L. and Silene vulgaris (Moench) Garcke were recently investigated for their potential role in the phytoextraction process. Indian mustard plants (Brassica juncea L.), when germinated in vitro on selenium-containing media [18] or in pot experiments with cadmium-contaminated soils [19], produced more root hairs when inoculated with PGPR strains. Furthermore, these inoculated plants accumulated more selenium than the non-inoculated ones [18]. Thus, PGPR are promising bacterial partners for the improvement of plant growth on heavy-metal-contaminated soil, and thus for phytoremediation.

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The aim of this research was to characterize the diversity of a PGPR population in antimony-contaminated soils, and how this is influenced by metal and plant. In particular, we focused on PGPR genotypes present in antimony-contaminated soils regardless of the concentration of the pollutant, and the overall development of hyperaccumulator plants. For this purpose, thousands of rhizobacteria were isolated at three stages of plant growth from the rhizospheres of A. ageratum L. (Compositae), naturally colonizing a site contaminated by antimony 25 years ago.

2. Materials and methods

2.1. Bacterial strains

Four auxin-producing reference strains were used: Pseudomonas fluorescens M114 [13] and CHA0 [15], Pseudomonas putida M.3.1 (Digat, INRA, Angers, France) and Pseudomonas aureofaciens PGS12 [21]. Two non-auxin-producing reference strains were used: P. fluorescens Pf5 [11] and Pseudomonas corrugata 3-1 [22].

2.2. Characterization of the site

The model site was a 25-year-old abandoned open-sky antimony mine in Tuscany (Italy). This site is characterized by the existence of a gradient in the concentration of antimony. The description of the site contamination status was already available thanks to previous studies ([2,20], Boscagli, personal communication). Based on this knowledge, four soils were chosen for plant collection: soils with low (I), moderate (II), high (III) and very high (IV) antimony concentrations (Table 1).

2.3. Isolation and selection of bacteria able to produce auxins in vitro

The rhizosphere of four individual A. ageratum L. plants for each of the four soils was collected at three stages of plant growth, corresponding to (1) the emergence of new roots and shoots from the rhizome, (2) the beginning of flowering and (3) the death of the flower shoots and basal leaves. In this way, a total of 48 rhizospheric samples were collected (four plants×four soil antimony concentrations×three stages of plant growth). Immediately after each collection, plants with their rhizospheres were taken to the laboratory, and isolation of rhizobacteria was performed within 24 h.

For each sample, 10 g of roots were washed in 100 ml of sterile water, and then blended in a Waring blender with 100 ml of sterile water. Serial dilutions were plated onto solid tryptone soya medium (Oxoid, Basingstoke, Hampshire, UK). The plates were incubated at 27°C, and numbers of cfu were determined after 7 days.

For each sample, 94 randomly selected colonies were picked and each colony was separately transferred into a single well of a 96-well microplate containing 100 μl of liquid tryptone soya medium. In total, 4512 bacterial colonies were collected in this way (94 isolates×48 rhizospheres). After 3 days of growth at 27°C in the dark,
auxin-producing isolates (*aux*') were detected by a colorimetric technique derived from that of Salkowski [23–25], known to be specific for the detection of indolic compounds (IAA, IpyA and IAM) [24]. The Salkowski technique was adapted to the 96-well microplate format by adding 200 µl of a 35% perchloric acid–0.01 M FeCl₃ solution to each 100 µl culture. The specificity of the test was systematically verified by including the four *aux*’ reference strains, for which the addition of the Salkowski reagent should result in a pink coloration when auxin compounds are produced in the medium, and the two non-auxin-producing (*aux*⁻) reference strains, for which no visually detectable color reaction is produced. Among the 4512 isolates, only those presenting the diagnostic pink coloration were purified and multiplied on new solid tryptone soya medium. The test was repeated on pure cultures for confirmation of auxin production. Then, each purified *aux*’ isolate was preserved at −80°C in 40% glycerol.

### 2.4. Cell preparation prior to polymerase chain reaction (PCR) amplification

Bacterial isolates were grown overnight at 27°C on YPGA, which contained (per liter) 5 g of yeast extract, 5 g of peptone, 10 g of glucose and 15 g of agar. A single colony of each strain was suspended in 50 µl of distilled water and heated at 95°C for 10 min to lyse the cells. The lysate was then cooled in ice, briefly centrifuged in a microcentrifuge, and used for PCR amplification.

### 2.5. Amplified rDNA restriction analysis (ARDRA)

The DNA encoding the 16S rRNA of each isolate was amplified using the universal primers fD1 and rD1 [26], corresponding to positions 8–27 and 1524–1540 on the *Escherichia coli* rrs gene sequence. This primer pair is capable of amplifying nearly full-length 16S ribosomal DNA from a wide variety of bacterial taxa [26]. Amplification was performed as previously described [22]; each mixture contained 2 µl of lysed cell suspension in 25 µl of reaction buffer containing 1.5 mM MgCl₂, 150 ng of each primer, each deoxynucleoside triphosphate at a concentration of 250 mM, and 0.5 U of *Taq* DNA polymerase. The reaction mixtures were incubated in a thermocycler at 95°C for 1.5 min and then subjected to 35 cycles consisting of 95°C for 30 s, the annealing temperature for 30 s, and 72°C for 4 min. The annealing temperature was 60°C for the first five cycles, 55°C for the next five cycles, and 50°C for the last 25 cycles. Finally, the mixtures were incubated at 72°C for 10 min and then at 60°C for 10 min. Five µl of each amplification mixture was analyzed by agarose (1.2% w/v) gel electrophoresis in Tris-borate-EDTA (TBE) buffer.

Restriction analysis was performed with 5 µl of amplified product and 15 µl of restriction buffer containing 3 U of either *MspI*, *Sau96I*, *HinfI*, *RsaI*, *HhaI*, *TaqI* or *HindIII* (Roche Molecular Biochemicals). After a 3-h digestion at the appropriate temperature, the enzyme was inactivated by heating the preparations at 70°C for 15 min, and the restriction fragments were separated by gel electrophoresis (2% agarose) in TBE buffer. A 1-kb DNA ladder (Invitrogen) was used as molecular size marker. For each isolate, PCR amplification and restriction analysis were performed at least three times.

### 2.6. Statistical analysis

The AMOVA procedure was used to estimate the variance components for ARDRA patterns by partitioning the variations among plant growth stages and/or antimony
concentrations. The AMOVA technique is a method for analyzing molecular variance that produces estimates of variance components reflecting the correlation of haplotypic diversity at different levels of a hierarchical subdivision. The significance of the variance components is tested by a permutational approach [27]. The vectors for the presence of ARDRA markers (1 for the presence of each band on a gel; 0 for the absence of each band on a gel) for each strain were used to compute the genetic distance for each pair of strains. The parameter used was the Euclidean metric measurement \( E \) of Excofier et al. [27], as defined by Huff et al. [28] as follows: \( E = \sqrt{\sum_{xy} (n_{xy} - 2n_{xy})^2} \), where \( 2n_{xy} \) is the number of markers shared by two strains and \( n \) is the total number of polymorphic sites. All analyses were performed with the Arlequin program [29], which is used in several scientific fields (microbiology, medicine, population genetics) and is available at the following URL: http://anthropologie.unige.ch/arlequin/.

For each collection, the number of aux+ isolates is the mean of four replicates. Standard error between replicates was determined by analysis of variance followed by Tukey’s multiple comparison test (GraphPad PRISM Software, version 2.0).

2.7. Characterization of the isolates

Gram characteristics of the isolates were determined by the KOH test of Suslow et al. [30]. Isolates were phenotypically characterized using the Biolog system (Biolog, Hayward, CA, USA), which is based on the differential utilization of a large number of organic compounds. Biolog GN microplates were inoculated as recommended by the manufacturer and were incubated at 27°C for 24 h. Formazan accumulation in bacterial cells was measured by determining the optical density at 550 nm with an automatic microplate reader. The isolates were identified on the basis of their patterns of utilization of 95 substrates using the Biolog Microlog software. Efficiency of Biolog tests was checked by including reference strains 3-1 and M.3.1.

| Table 2 | Number of aux+ isolates from the rhizosphere of Achillea and their distribution among the six main ARDRA clusters |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Plant growth stagea | Soil | ARDRA cluster | 1 | 2 | 3 | 4 | 5 | 6 |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Em | I | 3 | 5 | 2 | 0 | 2 | 0 |
| II | 8 | 5 | 1 | 0 | 0 | 2 |
| III | 3 | 5 | 0 | 2 | 0 | 0 |
| IV | 8 | 0 | 1 | 0 | 0 | 0 |
| Fl | I | 2 | 0 | 0 | 2 | 0 | 0 |
| II | 1 | 0 | 1 | 0 | 2 | 0 |
| III | 3 | 1 | 1 | 1 | 0 | 2 |
| IV | 1 | 1 | 0 | 4 | 1 | 0 |
| De | I | 2 | 1 | 5 | 0 | 4 | 4 |
| II | 2 | 0 | 4 | 0 | 1 | 0 |
| III | 4 | 1 | 1 | 0 | 0 | 2 |
| IV | 4 | 2 | 2 | 1 | 0 | 0 |
| Total isolates | 42 | 21 | 18 | 10 | 10 | 10 |

*Em, emerging stage; Fl, flowering stage; De, death stage.

3. Results

3.1. Isolation of auxin-producing bacterial strains

Bacteria isolated on solid tryptone soya medium were enumerated 7 days after plating for each of the four soils examined. Stages of plant growth and antimony concentration in the soil had no effect on the densities of the total cultivable bacterial populations, since they ranged independently from \( 4 \times 10^7 \) to \( 2 \times 10^8 \) cfu g−1 of dry roots.

A total of 4512 colonies (94 isolates × 48 rhizospheres) were randomly picked and tested for auxin production. Auxin production was verified for 222 isolates, by comparison of their pink culture coloration with that of the four auxin-producing reference strains (below, these isolates are referred to as aux+ isolates). No color change was observed for the culture medium of the auxin-negative reference strains 3-1 and Pf5. The percentage of aux+ isolates increased with increasing concentration of antimony in the soil (Fig. 1A). This increase was quite low when bacteria were isolated from plants at the emerging stage, since the frequency of aux+ isolates varied from 4% of the total bacterial population isolated from soil I, to 5.7% from soil IV. For the other plant growth stages, the increase of the frequency of aux+ isolates with the increase of soil antimony concentration was more evident, i.e. from 6.5% and 4% (soil I, flowering and plant death stage, respectively) to 20% and 15% (soil IV, flowering and plant death stage, respectively). On the other hand, some fluctuation of the total number of aux+ isolates was observed with the stages of plant growth, since the frequency of aux+ isolates increased from the emerging stage (5%) to the flowering stage (11.73%), and decreased a little with the death of the flower shoots and basal leaves (8.25%) (Fig. 1B).

| Table 3 | Gel-detectable restriction fragment sizes (in bp) of amplified 16S rDNAs from main ARDRA clusters and from two reference strains |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Restriction endonuclease | HindI | MspI | RsaI |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| ARDRA cluster | 1 | 1000, 200 | 550, 460, 150 | 850, 350, 150 |
| 2 | 1000, 200 | 550, 350, 150 | 800, 350, 150 |
| 3 | 510, 360, 330 | 510, 290 | 510, 450 |
| 4 | 1200 | 700, 290 | 820, 450 |
| 5 | 1100 | 510, 350, 250 | 820, 450 |
| 6 | 1100 | 700, 290 | 820, 450 |
| Reference strains | 3-1 | 1000, 200 | 550, 460, 150 | 850, 350, 150 |
| CHAO | 1000, 200 | 550, 350, 150 | 800, 350, 150 |
3.2. ARDRA analysis

The 16S rDNAs of 214 out of 222 aux+ isolates were successfully amplified (data not shown). The remaining isolates were not investigated since they did not survive the cryoconservation in glycerol. The results of ARDRA with seven enzymes were subjected to cluster analysis. The different patterns obtained with each of the seven endonucleases were readily distinguishable from one another and highly reproducible from one experiment to the next. ARDRA with Hinfl, MspI, and RsaI revealed a significant level of genetic diversity between the isolates, since 51 clusters were found (designated ARDRA-1 to ARDRA-51). Isolates exhibiting the same Hinfl-MspI-RsaI cluster were indistinguishable when analysis was carried out with any of the four other endonucleases (data not shown).

In particular, one cluster (ARDRA-1) was predominantly represented, corresponding to 42 (19.6%) isolates. Furthermore, it was present in the Achillea rhizosphere for all the soil antimony concentrations and throughout the stages of plant growth (Table 2). Five other clusters (ARDRA-2–6) included 21 isolates (10%), 18 isolates (8.4%) (respectively for ARDRA-2 and -3) and 10 isolates (4.7%) (for ARDRA cluster 4–6). They were present throughout the stages of plant growth of Achillea, but in not more than three of the four soils (Table 2). Restriction patterns of these six main ARDRA clusters are presented in Table 3. Twenty-six clusters (ARDRA-26–51) were represented by only one isolate. The remaining clusters (ARDRA-7–25) included two to nine isolates that never originated from more than three soils and two stages of plant growth.

The number of diverse clusters isolated from each soil increased with its antimony concentration, from 18 for soil I to 21 for soils III and IV. Considering the stages of plant growth, the number of diverse clusters was lower at the emerging stage (21 cluster) than at the flowering and the plant death stages (28 and 26, respectively) (Table 4).

3.3. AMOVA analysis

Each ARDRA cluster was compared with the other clusters, and an Euclidean distance matrix (E) was calculated (data not shown). To analyze the ARDRA variation in the 214 isolates, we performed an AMOVA with the Euclidean distance matrix. Antimony concentrations in soil and stages of plant growth were considered two different groups, to evaluate independently their respective effect on the biodiversity of aux+ isolates. The AMOVA data revealed that 4.01% of the genetic diversity observed between the aux+ isolates was related to the soil antimony concentration, at a significant P value (P = 0.04). We also analyzed all of the possible combinations of two soils. The data obtained (Table 5) clearly show that a greater difference in antimony concentration between two soils (I vs. IV) corresponds to a higher genetic diversity between the aux+ isolates (7.95%, P = 0.04).

Similar analyses carried out on the stage of plant growth (Table 5) show that a part of the genetic variability between the aux+ isolates is due to this factor (7.74%, P = 0.04, and up to 10.76% between flowering and death stages, P = 0.07).

3.4. Identification of the most representative aux+ isolates

The 42 isolates belonging to the main ARDRA cluster

Table 4
Distribution of ARDRA clusters as affected by soil and plant parameters

<table>
<thead>
<tr>
<th>Soil</th>
<th>Plant growth stage</th>
<th>Total number of ARDRA clusters</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>II</td>
<td>III</td>
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<tr>
<td>I</td>
<td>II</td>
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*Em, emerging stage; Fl, flowering stage; De, death stage.

Table 5
Genetic diversity between the aux+ isolates determined by the AMOVA analysis on the ARDRA pattern variation

| Variance component | % of variance between sampling | % of variance within sampling | P*
|--------------------|--------------------------------|------------------------------|-----
| Soil               | I vs. II vs. III vs. IV       | 4.01                         | 95.99 | 0.04 |
|                    | (I+II) vs. (III+IV)           | 3.31                         | 96.69 | 0.03 |
|                    | I vs. II                      | 1.60                         | 98.40 | 0.03 |
|                    | II vs. III                    | 1.57                         | 98.43 | 0.01 |
|                    | III vs. IV                    | 1.73                         | 98.27 | 0.01 |
|                    | I vs. III                     | 4.99                         | 95.01 | 0.04 |
|                    | I vs. IV                      | 7.95                         | 92.05 | 0.04 |
|                    | II vs. IV                     | 2.67                         | 97.33 | 0.02 |
| Stage of plant growth | Em vs. Fl vs. De          | 7.74                         | 92.26 | 0.04 |
|                    | Em vs. Fl                     | 10.76                        | 89.24 | 0.07 |
|                    | Em vs. De                     | 7.82                         | 92.18 | 0.05 |
|                    | Fl vs. De                     | 5.77                         | 94.23 | 0.02 |

Values in boldface type are not significant.

*P* Probability of a more extreme variance distribution.

*Em, emerging stage; Fl, flowering stage; De, death stage.
(ARDRA-1) were all Gram-negative. Biological tests performed on 16 isolates belonging to ARDRA-1 and the two reference strains 3-1 and M.3.1 showed that nine of the isolates should be assigned to \textit{P. corrugata}, with similarity levels of at least 95\%, as well as strain 3-1. The seven other isolates were assigned to \textit{P. putida} biotype B (similarity levels of 94–98\%), as well as reference strain M.3.1.

4. Discussion

The effect of heavy metal contamination on soil microbial populations has often been analyzed by biochemical techniques, such as measurements of respiration and biomass. Less numerous are the studies that have analyzed the effect of heavy metals on soil microbial community structure or on bacterial diversity, and, to our knowledge, no data exist on the influence of long-term soil antimony contamination on the bacterial auxin-producing community present in the rhizosphere of hyperaccumulator plants. The design of heavy metal phytoextraction applications needs detailed data on both plant and plant growth-promoting bacterial genotypes for plant inoculations needs detailed data on both plant and plant growth-promoting bacterial genotypes for plant inoculation. Bearing that in mind, we showed in the present research that the auxin-producing population present in the \textit{Achillea} rhizosphere was genetically very diverse, and that this diversity was in part due to the antimony concentration in soil, and in part to the plant physiological stage. However, only one ARDRA cluster of aux+ was isolated throughout plant growth development, regardless of the antimony concentration in the soil. This highlights the importance of considering not only the heavy metal concentration in the soil but also the plant development stage, when screening bacterial strains for such applications.

Concerning the antimony concentration parameter, the total rhizospheric bacterial population did not change along the gradient, while the aux+ bacterial population fluctuated with the concentration of this pollutant. This is in apparent contradiction with numerous studies on other heavy metal soil contamination, showing a decrease of the total microbial population with increasing metal concentration [6,31,32]. Furthermore, we observed that a part of the genetic variability between aux+ isolates is associated with the antimony concentration in the soil, and that the number of diverse clusters isolated from each soil increases with the antimony concentration. These data are also in contradiction with numerous other studies that noted a lower diversity index between bacteria isolated from high metal-contaminated soils, compared to low metal-contaminated soils [33–36]. However, it is not possible to directly compare the results of the present study with those of the above cited works, as many of them investigated short-term effects of heavy metals in experimentally contaminated soils [34,35], or long-term contaminated soils, but without plants [33,36]. Fewer investigations address the effects of heavy metals on microorganisms in the rhizosphere of plants naturally colonizing long-term polluted soils. For nickel-rich soils, it has been shown that bacteria isolated from the rhizosphere of the hyperaccumulator plant \textit{Alyssum bertolonii} were genetically greatly diverse from those isolated from the non-rhizospheric soil [37]. Another study showed that the zinc concentration has no or only weak effects on the total number of bacteria isolated from the rhizosphere of \textit{Thlaspi caerulescens} or \textit{Trifolium pratense} [38], which is completely in accord with our data. Concerning specific bacterial populations such as PGPR, an investigation on \textit{Rhizobium leguminosarum} biovar trifolii in a range of heavy-metal-contaminated soils is partly in agreement with our data, since it observed that heavy metals have a quantitative effect on the population of rhizobia, but not on the genetic diversity [39].

Concerning the plant physiological stage parameter, the numerical fluctuation of the aux+ bacterial population could be explained by assuming that there was a selection for the auxin producers resulting from the composition of the root exudates, which are known to evolve with plant development [9]. Furthermore, our results showed that the plant stage not only affects the number of aux+ isolates, but also the diversity of their populations. The highest level of genetic diversity between aux+ isolates was found when the \textit{Achillea} roots were highly colonized by this type of bacteria (flowering and shoot death stages), suggesting that the selection of particular aux+ strains occurs when the roots are old. These results are consistent with published data for other plant species, which showed that the stage of plant growth significantly affects the level of genetic diversity of PGPR populations, and that this phenomenon is related to the quantity and quality of the root exudates [22].

The results of this research have practical importance in the context of using plant growth-promoting agents in heavy metal phytoextraction experiments. Indeed, we found that only the ARDRA-1 aux+ genotype is present in the \textit{Achillea} rhizosphere throughout plant growth development, regardless of the antimony concentration present in the soil. Thus, we hypothesize that if it were included in an inoculum, it would efficiently colonize the \textit{Achillea} rhizosphere. Furthermore, these strains were identified as \textit{P. corrugata} or \textit{P. putida} biotype B. Strains belonging to these species are widely distributed in nature and are often associated with a variety of plant species [19,22,40]. Some of them, isolated from seeds, hypocotyl tissue or the rhizosphere, are effective PGPR strains used as an inoculum to promote the root development of plants in agricultural systems [41], as well as in cadmium-contaminated environments [19]. Releasing such aux+ strains in the \textit{Achillea} rhizosphere could be of particular help in the phytoextraction of the antimony contaminant, especially in highly contaminated soils that naturally support a very poor flora.
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