Phytoextraction process optimization : characterization of the soil bacteria flora associated to the hyperaccumulator Arabidopsis halleri

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Phytoextraction process optimization: characterization of the soil bacteria flora associated to the hyperaccumulator *Arabidopsis halleri*

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Abstract
Phytotechnologies are microbial-assisted techniques that use living plants for the treatment of contaminated sites. Among these, phytoextraction based on hyperaccumulator plants such as *Arabidopsis halleri*, may be an option to remove trace elements in soil. One method to optimize *Arabidopsis halleri's* phytoextraction is the inoculation of plant roots by plant growth promoting bacteria (PGPB). In this study, we analyzed the total bacterial diversity in a Zn and Cd contaminated industrial soil (Auby, France) with restriction fragment length polymorphism (RFLP). Preliminary results showed a large bacterial diversity in Auby's soil. Cultivable bacteria were isolated and characterized for their PGPB traits (ACC deaminase activity, siderophores and Indol-Acetic-Acid (IAA) productions). Bacteria that will answer positively to the three PGPB tests will be preferentially selected, and inoculated to *Arabidopsis halleri* roots. Bacteria effects on plant biomass and/or accumulating yield will be presented.

Introduction
Phytoextraction, a microbial-assisted plant technology usable for the treatment of contaminated sites, exploits natural physiological traits of certain plants able to accumulate high levels of metals in their aerial tissues.
The success of phytoextraction is strongly determined by the amount of plant biomass and the concentration of metals in plant tissues.
Arabidopsis halleri, a hyperaccumulator plant characterized by its exceptional Zn and Cd concentrations in its aerial parts, has a relatively small biomass, limiting its use for phytoextraction process.
However, some soil microorganisms described in literature, such as PGPB, are able to significantly increase plant biomass and/or metal uptake through the secretion of specific compounds such as organic acids, siderophores and/or phytohormones as well as through enzymatic activities such as ACC deaminase *(Zhuang et al., 2007; Lebeau et al., 2008)*. Therefore, rhizospheric microorganisms represent a potential valuable tool for the improvement of *A. halleri* mediated phytoextraction.
The aim of this work is to study the microbial diversity associated to the roots of *Arabidopsis halleri* growing on a Zn and Cd polluted soil by RFLP and to identify PGPB strains that may be potentially involved in plant biomass production and/or plant Zn and Cd accumulation. The effect on selected PGPB bacteria on the *A. halleri's* phytoextraction process will be studied.

Materials and methods
Three different soil samples (A, B and C) were collected from an industrial site contaminated with Zn and Cd located in Auby (59, France).
Each sample was chosen to reflect a different degree of soil contamination (ranging from 2700 ppm to 36 000 ppm Zn of FW polluted soil; Niton analyser). For each sample, three sub-fractions of soil were defined and collected independently relatively to their distance to *A. halleri*'s roots (global, rhizospheric and rhizoplan soils, respectively).
Total DNA extraction was performed for the nine bacterial samples using Power soil DNA kit (MoBio industries). A 1300 bp fragment of the 16S rDNA gene was amplified by PCR. Generated amplicons were ligated in pGEMt Easy cloning vector (Promega) to generate specific mini-libraries. For each sample, 100 clones were analysed by RFLP with Rsa I restriction enzyme (Invitrogen). Sequences generated were compared using the BLAST algorithm in Ribosomal Database Project.
Cultivable bacteria were spread on LB-agar plates to estimate global bacterial density. Individual clones were tested for the presence of PGPB traits (ACC deaminase activity, IAA and siderophore productions). Positive PGPB for the three criteria will be preferentially selected (in progress).
Arabidopsis halleri seedlings will be further inoculated with selected PGPB to see the potential effect on either biomass and/or metal accumulation. Plant growth as well as accumulation will be monitored regularly.

Results
RFLP profiles exhibited a large diversity in the nine soil samples analysed (Figure 1). No significant differences in visual profile distributions could be detected according to soil pollution level or root distance. In order to confirm this result, 50 clones issued from different samples and presenting a RFLP profile resemblance were sequenced, assuming that they may belong to the same OTU. Indeed, among 50 sequences, some

could be assigned to an identical genus: *Flavobacterium* (8), *Aquicella* (7), *Pseudomonas* (3), *Rhodoplanes* (3). However, sequence identities observed were 95% in average and none of the clone but two belonged to the same species.
A sample of each clone library is actually sequenced and analysed.

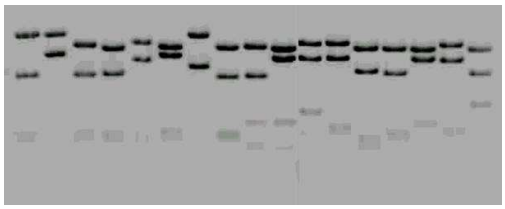

Figure 1: Seventeen RFLP profiles obtained with RSAI restriction enzyme for sample A, global fraction of soil (AG) and revealed with Sybr Green stain I (Sigma).

Bacterial densities were estimated after spreading on LB-agar medium 100 µl of diluted bacterial suspension and after 72 h of incubation at 25°C. The results are expressed in log CFU per gram of soil dry weight. For each soil sample a significant increased of bacterial density was observed when the root distance decreased: G< R< RP. The degree of pollution seemed also to influence significantly ($p<0.05$). The density of resident flora, following a gradient A>B>C (Figure 2).

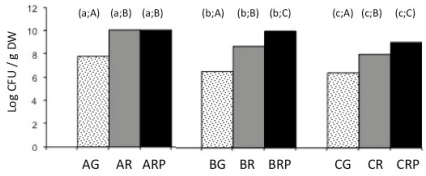

Figure 2: Estimation of bacterial densities for each soil A, B, and C split in global (G: white), rhizospheric (R: grey) and rhizoplan (RP: black) soils.

The number of CFU data were analysed by analysis of variance (two-ways ANOVA). For each sample, values with different small letters are significantly different ($p<0.05$). Values with different capital letters are significantly different ($p<0.05$) (means +/- SD).
The LB-agar plates allowed us to isolate 240 bacteria strains that presented different morphological aspects. PGPB tests such as activity ACC deaminase, siderophores and acid indol-acetic-3 productions, were performed.

Discussion

RFLP analysis showed a large diversity of the total flora present in Zn and Cd contaminated soils. RsaI restriction analysis alone was sufficient to efficiently distinguish the clones at species level. This was confirmed when sequencing the clones presenting the closest RFLP profile. The excellent discrimination power of RsaI on 16S amplicon is in good agreement with data published by Moyer et al. (1996), who recommanded RsaI as one of the best enzyme for RFLP analysis.
Considering the high diversity observed even on the most polluted soil, it would be tempting to conclude that pollution seems to have little impact on endogenous bacteria populations. However, since a clear restriction of cultivable flora diversity was observed when pollution increased, this may not be true. We were not able to identify predominant species, cultivable or not, in examinated soil. This can be explained by the fact that heavy metals are neither metabolized nor used by endogenous strains, as it is the case for organic pollutants (Taok et al., 2010). However, if no dominant species were found, some uncultivable genus such as the recently identified genus *Aquicella* (Santos et al., 2003) seems to be more present.
A higher cultivable bacterial density was also observed around the roots in all soils. This observation is in accordance with other studies and is generally explained by the root exudates that create a favorable microenvironment for bacterial survival and growth. On another hand, preliminary results obtained for classical PGPB tests allowed us to identify bacteria that are able to produce IAA, siderophores and/or presenting a significant ACC deaminase activity. Some of these bacteria will be tested in a bioaugmentation experiment to see their potential impact on phytoextraction. The presence of bacteria harboring PGPB traits, together with the higher cultivable density found in close vicinity of the roots is in favor of regular reciprocal exchanges between plants and microflora.

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