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A potential role for bacterial endophytes in phytoremediation of heavy metal contaminated soils

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Though I do not believe
that a plant will spring up where no seed has been,
I have a great faith in a seed.
Convince me that you have a seed there,
and I am prepared to expect wonders.

Henry David Thoreau.

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Abbreviations

A.A.S.	Atomic Absorption Spectroscopy
ACC	1-amino-cyclopropane-1-carboxylate
ALF	Automated Laser Flurorescent
BICMER	Bacteria Immobilized Composite Membrane Reactor
BLAST	Sequence Similarity Searching
BMSR	Bio Metal Sludge Reactor
BSE	Back Scattered Electron
CAS	Chrome azurol S
CEC	Cation Exchange Capacity
CFU	Colony Forming Units
CDF	Cation Diffusion Facilitator
DW	Dry Weight
DTPA	Diethylenetriamine pentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy Dispersive X-ray
F	Fluorescein
FISH	Fluorescence *in situ* Hybridization
FEG	Field Emission Gun
FW	Fresh Weight
Gfp	Green fluorescent protein
ICP	Induced Coupled Plasma Spectroscopy
Kb	kilobases
Km	Kanamycin
K_{ow}	Octanol-water partitioning coefficient
LB	Luria Broth
MFP	Membrane Fusion Protein
MIC	Minimum Inhibitory Concentration
NCBI	National Centre for Biotechnology Information
NTA	Nitrilotriacetate
OD_{660}	Optical Density at 660 nm
OMF	Outer Membrane Factors
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
PGP	Plant Growth Promoting
RND	Resistance, Nodulation, Division
SEM	Scanning Electron Microscopy
SVR-medium	Minimal, low heavy metal complexing medium
TAR	Transporter assisting resistance
Tc	Tetracyclin
Tris	Tris(hydroxylmethyl)-aminoethane

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Summary

This thesis aims to study the potential contribution of heavy metal resistant endophytic bacteria on heavy metal uptake and translocation processes by their host plant. The extensive study of bacterial heavy metal resistance mechanisms showed that metal bioprecipitation processes near the bacterial cell wall are often found to be a consequence of efflux based systems that reduce the bioavailable metal concentration present. Heavy metal resistant endophytic bacteria with a similar behaviour would possibly result in a lowered heavy metal bioavailability that consequently would lead to a lowered heavy metal toxicity or an improved heavy metal accumulation within the host plant. Based on this concept, new strategies to improve phytoremediation would be developed.

In a first step the presence of heavy metal resistant bacteria inhabiting the Zn hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria* growing on a soil from an abandoned Zn and Pb mining and smelter site as well as the non-hyperaccumulator *Brassica napus* originating from a clean garden soil was demonstrated. The occurrence of such bacterial heavy metal resistance characteristics obviously is not restricted to the heavy metal polluted environments. An efficient re-colonization of the host plant by these plant associated bacteria is an important prerequisite to allow an evaluation of the potential effect on the metal household of the plant. Re-inoculation experiments revealed that this condition was not fulfilled. For this reason, the metal sensitive endophytic bacteria *Burkholderia cepacia* L.S.2.4 and *Herbaspirillum seropedicae* LMG2284 were subsequently selected as they proved to be efficient colonizers of their respective host plant *Lupinus luteus* and *Lolium perenne*.

By means of transposition, the Ni, Co, and Cd resistance mechanism *ncc-nre* of *Ralstonia metallidurans* 31A was introduced which resulted in stable *B. cepacia* L.S.2.4::*ncc-nre* and *H. seropedicae* LMG2284::*ncc-nre* transconjugants. An increased Ni resistance was observed accompanied by the ability to reduce bioavailable Ni levels in the growth medium up to 35% and 15% respectively. Re-colonization experiments with *B. cepacia* L.S.2.4::*ncc-nre* revealed that under the circumstances used, significant increased Ni levels (30%) actually were measured in the *lupinus* roots. In contrast, inoculation with the wild type strain L.S.2.4 didn't influence the concentration of Ni measured in the roots compared to the non-inoculated control plants. On the other hand, the presence of *H. seropedicae* LMG2284::*ncc-nre*, equipped with limited Ni removal capacities, didn't seem to affect the Ni uptake capacity of *Lolium perenne*. Efficient colonization as well as efficient Ni removal properties probably are essentially required in order to detect a clear effect.

To obtain a better understanding of the interactions between endophytic bacteria and their host plant, additional information concerning the mechanisms of plant colonization on one hand and the plants' metal household from uptake to translocation and storage on the other hand certainly would be necessary in order to interpret these results in a broader context. Additionally, *in situ* experiments remain to be performed before practical applications of these plant-bacterium interactions can be evaluated.

Samenvatting

Deze thesis heeft als doel de potentiële bijdrage van zware metalen resistente endofytische bacteriën op de zware metalen opname en translocatieprocessen van hun gastheerplant te bestuderen. De intensieve studie van de bacteriële zware metalen resistentiemechanismen toonde immers aan dat metaal bioprecipitatie processen ter hoogte van de bacteriële celwand dikwijls optreden als gevolg van efflux gebaseerde systemen die de aanwezige biologisch beschikbare zware metalen reduceren. In analogie hiermee zou de aanwezigheid van zware metaal resistente endofyten kunnen resulteren in een verlaagde biologische beschikbaarheid van de zware metalen in de plant wat tot een verminderde zware metaal toxiciteit of verbeterde zware metaalaccumulatie in de plant kan leiden. Hierop gebaseerd kunnen strategieën voor een verbeterde fytoremediatie van zware metalen worden ontwikkeld.

In een eerste stap werd aangetoond dat zware metalen resistente endofytische bacteriën effectief voorkomen in zowel hyperaccumulatoren afkomstig van een verlaten site van een Zn en Pb mijn en smelterij (zoals de Zn hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*) als in niet-hyperaccumulatoren gegroeid op een onvervuilde tuinbodem (zoals *Brassica napus*). De aanwezigheid van zware metalen resistentie eigenschappen bij bacteriën blijkt dus niet beperkt te zijn tot biotopen vervuild met zware metalen. Een efficiënte herkolonisatie van de gastheerplant door deze plantgeassocieerde bacteriën is een belangrijke vereiste om het mogelijk effect op de metaalhuishouding van de gastheerplant te evalueren. Gezien aan deze voorwaarde niet werd voldaan, werd een alternatieve strategie uitgewerkt. Hiertoe werden de metaalgevoelige endofytische bacteriën *Burkholderia cepacia* L.S.2.4 en *Herbaspirillum seropedicae* LMG2284 geselecteerd omwille van hun efficiënte herkolonisatie van respectievelijk *Lupinus luteus* en *Lolium perenne*. Door middel van transpositie werd het Ni, Co, Cd resistentiesysteem *ncc-nre* van *Ralstonia metallidurans* 31A in deze bacteriën geïntroduceerd wat resulteerde in de stabiele transconjuganten *B.cepacia* L.S.2.4::*ncc-nre* en *H. seropedicae* LMG2284::*ncc-nre*. Een verhoogde Ni resistentie werd waargenomen, welke gepaard ging met een verwijdering van respectievelijk 35% en 15% van de toegevoegde Ni uit het voedingsmedium. Herkolonisatie experimenten met *B. cepacia* L.S.2.4::*ncc-nre* toonden aan dat onder de gebruikte omstandigheden een significante verhoging van de Ni concentratie (30%) in de wortel van *Lupinus luteus* gemeten werd. Dit in tegenstelling tot de planten geïnoculeerd met de wildtype stam *B. cepacia* L.S.2.4 waar geen effect op de Ni verwijdering werd waargenomen. De aanwezigheid van *H. seropedicae* LMG2284::*ncc-nre*, welke slechts een beperkte Ni verwijderingscapaciteit heeft, bleek geen effect te hebben op de Ni opnamecapaciteit van *Lolium perenne*. Vermoedelijk zijn efficiënte Ni verwijderingscapaciteiten gecombineerd met efficiënte kolonisatie-eigenschappen essentiële vereisten waaraan voldaan moet worden opdat een duidelijk effect zou worden waargenomen.

Om deze resultaten in een bredere context te interpreteren is een meer uitgebreide kennis nodig van de bacteriële kolonisatiemechanismen alsook een dieper inzicht in de zware metaal opname-, translocatie- en de opslagmechanismen van de gastheerplanten. Verder zijn *in situ* experimenten onontbeerlijk om de praktische toepassingen van zulke plant-bacterie interacties te overwegen.

CHAPTER I
Introduction

I PHYTOREMEDIATION

I.1. Definition

The word phytoremediation consists of the Greek prefix *phyto* (plant) attached to the Latin root *remedium* (to correct or to remove an evil). Phytoremediation is defined as the use of green plants to remove, contain, or render harmless environmental contaminants. This definition applies to all plant-influenced biological, chemical, and physical processes that aid in remediation of contaminated substrates (Cunningham, S.D. & Berti, W.R. 1993). The concept of using plants to remove contaminants is already being used for many years in the treatment of some types of wastewater (Tchobanoglous, G. 1991; Green, M.B. & Upton, J. 1992). It was only more recently that research focussed on phytoremediation processes to treat contaminated soils and atmospheric pollutants.

Concerning phytoremediation techniques of soils, the contaminant will be either removed from the polluted soil in a process called decontamination, or sequestered via stabilisation. Phytodecontamination involves phytoextraction and phytodegradation strategies where the ability of plants to extract the contaminants on one hand and the degradation of the pollutant into non-toxic materials on the other hand is being studied. Phytostabilisation is defined as the process where pollutants precipitate from a solution or are absorbed or entrapped in either the plant tissue or the soil matrix. The most common stabilisation technique is through the addition of amendments to the soil, which prevents the contaminants to enter the groundwater and the food chain.

Potential benefits of phytoremediation are the minimal environmental impact and the low cost compared to more conventional techniques like incineration and soil washing, a chemically based, energy intensive approach. Another reason that makes phytoremediation an attractive strategy is the fact that it may have direct health benefits: the soil being covered by plants is a way to decrease exposure risk. Additionally, a vegetation cover is generally also considered to be an aesthetically pleasing means of remediating a contaminated site, which facilitates the public acceptance of such a new green remediation technology.

Phytoremediation has some limitations as well. Using plants requires oxygen for the roots and water and nutrients for their metabolism. Soil texture, pH, salinity, pollutant concentrations and the presence of other toxins must be within the limits of plant tolerance in order to guarantee its survival. As plants need time to grow, the phytoremediation process is slower than the more conventional physico-chemical techniques and should be considered as a long term remediation process.

I.2. Phytoremediation of organic contaminants

The choice of a remediation strategy depends on the nature of the contaminant. Soils contaminated with organic pollutants are usually treated by vapor stripping or thermal desorption, soil washing, incineration, and landfilling. Organic contamination in the water phase can be dealt with pump and treat, reactive zone, and barrier technologies. Certain organic contaminants, primarily petroleum hydrocarbons, are amenable to microbial treatment, although the volume of material currently treated in this manner is relatively small. Biological soil treatment systems include landfarming of some petroleum hydrocarbons, and *ex situ* techniques such as composting, biopiles and slurry reactors. The costs associated with soil remediation are highly variable and depend on the contaminant, soil properties, site conditions and the volume of the material to be treated. Techniques that remediate a soil *in situ* are generally less expensive than those that require excavation.

For sites with shallow contamination (<5m depth) caused by moderately hydrophobic pollutants such as BTEX compounds (benzene, toluene, ethylbenzene, and xylenes), chlorinated solvents, nitrotoluene ammunition wastes, or excess nutrients (nitrate, ammonium, and phosphate), phytoremediation could be an additional technique in

conjunction with other cleanup approaches for decontamination or stabilization. Although still in its developmental stage, different aspects concerning uptake and degradation by plants are intensively studied. Plants are able to remediate organic pollutants via three mechanisms (Schnoor *et al*., 1995):

- Direct uptake of contaminants and subsequent degradation, modification, and/or accumulation of non-phytotoxic metabolites into plant tissues or evaporation of contaminants (organic solvents).
- Release of exudates and enzymes that stimulate microbial activity and biochemical transformations.
- Enhancement of mineralisation in the rhizosphere, which is attributable to mycorrhizal fungi and/or the microbial consortia.

Direct uptake takes place at the roots, which absorb organics in nearly direct relationship to their relative lipophilicity. The most common parameter to predict plant uptake from the soil is the octanol-water partitioning coefficient (K_{ow}) (Cunningham & Berti, 1993). Contaminants with a log K_{ow} ($\leq$1) are considered as very water soluble and can be accumulated in plants where they are generally mobile in both plant xylem and phloem. Pollutants with intermediate log K_{ow} (1-4) are taken up by the roots and are considered xylem mobile but generally phloem immobile unless chemically modified by the plant. Compounds in this range would be expected to be good targets for phytoremediation. These include most BTEX chemicals, chlorinated solvents, and short-chain aliphatic chemicals. Compounds with log K_{ow}'s greater than 4 are greatly adsorbed to roots but are not substantially translocated to the shoot (Bromilow & Chamberlain, 1989).

Once absorbed, these compounds can have multiple fates: they can be stored in plant structures via lignification, volatilised, metabolised or mineralised all the way to carbon dioxide and water. The efficiency of these processes depends on the plant's uptake efficiency and transpiration rate as well as the physical-chemical properties and the concentration of the chemical in the soil.

The release of exudates and enzymes by plants in the soil environment may help in the degradation of organic components and these abilities can be further augmented by active microbial and fungal communities around their roots. In fact, plants provide a habitat of increased microbial populations due to supply of organic carbon from plant exudates and oxygen and this results in stimulated aerobic transformations near

the root. Other inhabitants of the rhizosphere are mycorrhizal fungi which help to metabolise organic pollutants through unique enzymatic pathways. These observations suggest a multiple approach of the phytoremediation concept.

Although many questions still have to be answered, in general phytoremediation of organic pollutants is considered as an additional clean-up technology next to the conventional methods. No data are available concerning the costs compared with those of the more standard practises of soil venting, soil washing, excavation, or bioremediation, but Schnoor *et al* (1995) predict that it should be very competitive with other technologies.

Glass (1999) estimated the 1998 U.S. market for phytoremediation to be $16.5 to 29.5 million with a growth from $55-103 million by 2000 to $214-370 million by 2005. The largest 1998 markets were attributed for the treatment of organic contaminants in groundwater ($5 to 10 million) and control of landfill leachate ($3 to 5 million) and this part was predicted to grow in a steady and strong way in the years to come while the markets involving heavy metals or radionuclides are only capable of dramatic growth as the technology efficiency becomes better established.

I.3. Phytoremediation of inorganic contaminants

Unlike organic compounds that can be mineralised, the remediation of inorganic contaminants must either physically remove the contaminant from the system or convert it into a biologically inert form. Due to the fact that inorganic pollutants are much easier to measure and no metabolites are formed, phytoremediation research, as far as the mechanisms are concerned, of inorganic contaminants is more advanced when compared to phytoremediation of organic pollutants. Heavy metals and radionuclides such as cesium, strontium, technetium, tritium, and uranium are considered to be pollutants when excess levels are abundant in soil or water. We will only focus here on phytoremediation processes for the decontamination of heavy metals.

Most heavy metals exist naturally in the earth's crust at trace concentrations (Bodek *et al.*, 1988), sufficient to provide local biota with trace nutrients, but too low to cause toxicity. Elevated levels of toxic heavy metals may occur in some natural environments such as serpentine soils, which result from the weathering of serpentine

rocks (Brooks, 1987). These soils, enriched with the heavy metals nickel, cobalt and chromium, can create ecological niches for heavy metal adapted species through natural selection processes. Since the industrial revolution, pollution by heavy metals has accelerated dramatically (Nriagu, 1979) through disposals of wastes from metal excavation and processing, through energy and fuel production, through intensive agriculture,..(Seaward & Richardson, 1990). As a consequence concentrated levels of heavy metals in soils and water became a threat to animal and human health and efforts are undertaken to detoxify these contaminated soils.

Traditional soil remediation techniques for toxic heavy metals can be grouped into three categories:

1. **Landfilling**: involves three stages of soil excavation, transport, and burial at a landfill site. This approach represents a rapid method of dealing with a contaminated site, but it has been criticised as it represents only a transfer of the contaminated material from one location to another rather than a final solution.
2. **Fixation**: the chemical processing of soils to immobilise heavy metals, usually followed by treatment of the soil surface to eliminate penetration by water.
3. **Leaching**: using acid solutions or proprietary leachants to desorb and leach heavy metals from soil followed by the return of clean soil residue to the site.

For the treatment of heavy metal contamination in water, precipitation or flocculation (followed by sedimentation and disposal of the resulting sludge), ion exchange, reverse osmosis, and micro-filtration are possible remediation methods.

Due to the fact that these conventional methods are often expensive and not sufficient, new plant based technologies are developed that seem to be a cost effective alternative. Table I.1 shows some cost prices of different techniques for remediation of heavy metal contaminated soils.

Remediation technologies	**Cost-price (10^6 Euro/ha)**
Excavation and landfilling	1.62
Chemical soil washing	1.5
Phytoextraction	0.26
Soil and asphalt capping	0.15
Immobilisation and phytostabilisation	0.01-0.05

Table I.1: Estimated cost of different techniques for remediation of heavy metal contaminated soils (J. Vangronsveld, personal communication).

I.3.1. General principles

Phytoremediation of heavy metals can be divided into phytoextraction, phytostabilisation, and rhizofiltration. In phytoextraction, metal accumulating plants are used to transport and concentrate heavy metals from the soil into the harvestable parts of roots and shoots. The process in which heavy metal tolerant plants are used to reduce the mobility of heavy metals is called phytostabilisation. The ability of plant roots to absorb, precipitate and concentrate toxic metals from polluted effluents is used in rhizofiltration processes.
It is clear that these plant based methods require fundamental knowledge concerning bioavailability, uptake, translocation, and heavy metal resistance in order to understand and eventually improve the conditions under which the processes will be carried out.

Bioavailability

Metal cations in soils may be present in various physicochemical forms: (1) as simple or complexed ions in the soil solution; (2) as easily exchangeable ions; (3) organically bound; (4) occluded by or co-precipitated with metal oxides, carbonates, or phosphates and other secondary minerals; or (5) as ions in crystal lattices of primary minerals (Viets, 1962; Mc Laren & Crawford, 1973; Soon & Bates, 1982). The first three forms are believed to be in equilibrium with one another and considered as the most "available" to plants while successive forms represent decreasing degrees of availability (Soon & Bates, 1982). The Tessier sequential extraction procedure (Tessier *et al*., 1979) allows to determine this partitioning of the heavy metals in different fractions using specific extraction solutions.
As plants need certain trace elements like metal cations in key metabolic events such as respiration, photosynthesis, fixation and assimilation of some major nutrients, inadequate supply causes disordered metabolic processes and abnormal or stunted growth. To guarantee that sufficient levels of these trace elements are being taken up, several plant based mechanisms aim to influence the availability of trace elements in the soil solution. Iron, although abundant in the earth's crust, predominates as insoluble Fe(III) precipitates and is largely unavailable to plants. Two distinct strategies are used by plants to assimilate Fe. The grasses release low-molecular-

weight, high affinity Fe(III)-chelating compounds called phytosiderophores, which solubilise ferric Fe in the rhizosphere and are recognised for uptake by specific membrane receptors (Römheld & Marschner, 1986; Chaney, 1987; Bienfait, 1988). Fe uptake in the dicotyledons and the non-grass monocotyledons is mediated by a plasmamembrane bound ferric reductase that transfers electrons from intracellular NADH (Buckhout *et al*., 1989) to Fe(II) chelates in the rhizosphere (Chaney *et al*., 1972). The ferrous ions (Fe^{2+}) released from the chelates by this process are subsequently transported into the cytoplasm via a separate transport protein (Kochian, 1991; Fox *et al*., 1996). In addition, tissue concentrations of other mineral elements also appear to be influenced by the plant's Fe status. Welch *et al*. (1993) demonstrated that the shoot concentration of many divalent cations, including Cu, Mn, and Mg increased in Fe deficient pea seedlings. Fe deficiency also elicits a large stimulation of Cd influx into roots of pea seedlings (Cohen *et al*., 1998) and it was speculated that the correlation found between enhanced Cd influx and induction of a high-affinity Fe transporter under Fe deficiency might suggest that the Fe transporter could be a relatively non-specific divalent cation transporter.

Plant roots also have been reported as being able to acidify their soil environment through extrusion of protons. However, experiments with *Alyssum murale* showed that reduction of the rhizosphere pH by increased proton release is probably not the mechanism by which this plant species solubilises heavy metals from soils (Bernal & McGrath, 1994). Acidification was not the mechanism either by which the Zn hyperaccumulator *Thlaspi caerulescens* mobilises Zn in the soil (McGrath *et al*., 1997). Other observations indicated that the high uptake of metal by this hyperaccumulator probably can be achieved by the effectiveness of root foraging and precision of root placement with respect to the sources of the metal (Whiting *et al*., 2000). The authors claimed that such root responses to localised Zn possibly are gene encoded.

It should be noted that these plant mediated processes should be considered as only part of the soil plant system responsible for the solubilisation of trace elements. Mycorrhizal fungi and root colonizing bacteria may also take part in these processes.

Uptake and translocation

Absorption by roots is the main access of trace elements to plants; however the ability of other tissues to readily adsorb some nutrients, including trace elements, also has been observed. Foliar adsorption for example is exploited in agriculture as a means of supplying plants with micronutrients, such as Fe, Mn, Cu and Zn but can also be a significant route for the entry of atmospheric pollutants, such as Cd, into the food chain (Hovmand *et al*., 1983). Foliar uptake is believed to consist of non-metabolic cuticular penetrations, which are generally considered to be the major route of entry and metabolic mechanisms which account for element accumulation against a concentration gradient. The second process is responsible for transporting ions across the plasma membrane and into the cell protoplast. Trace elements taken up by leaves can be translocated to other plant tissues, including roots where the excess of some elements seem to be stored. The rate of trace element movement among tissues varies greatly, depending on the plant organ, its age, and the element involved.

Non-metabolic and metabolic processes are also involved in root absorption. Passive uptake is the diffusion of ions from the external solution into the root endodermis, while active uptake requires metabolic energy and takes place against a chemical gradient. The ion activity in the solution is believed to be one of the significant factors that influence plant uptake of ions. Presumably this is an important factor when the uptake is active, but may not be important when the uptake is passive. Other important features that influence uptake include the surface area of the root, the root cation exchange capacity (CEC), the root exudates, and the rate of evapotranspiration of the plant.

Apoplastic (extracellular) and symplastic (intracellular) pathways can be considered as examples of non-metabolic and metabolic uptake processes. Apoplastic transport is only possible as far as the endodermis and is limited or retarded by the high cation exchange capacity of cell walls. Symplastic transport requires that trace elements move across the plasma membrane and this can be accomplished by energy dependent saturable processes via specific or generic ion carriers or channels (Clarkson & Lüttge, 1989). In the case of heavy metals, competition for the same transmembrane carrier between essential heavy metals and non-essential heavy metals has been observed. This relative lack of selectivity in transmembrane ion transport may partially explain why non-essential heavy metals can enter cells even against a

concentration gradient. In *Glycine max* seedlings, competition for root transport sites between Ni on one hand and Cu and Zn on the other hand was observed by Cataldo *et al.* (1978). Other data showed that essential Cu and Zn and non-essential Ni and Cd compete for the same transmembrane carrier (Clarkson & Lüttge, 1989).

The role of such transmembrane metal transporters in metal uptake was only recently highlighted in the study of hyperaccumulation mechanisms. Already 3 zinc transporter cDNAs were cloned and isolated from the Zn hyperaccumulator *T. caerulescens* (Assunção *et al*., 2001). Increased expression of these genes in *T. caerulescens* compared to the non-hyperaccumulator *Thlaspi arvense* suggests an important role for these transporters in Zn hyperaccumulation. Additionally, physiological data suggest the presence of a high-affinity and highly expressed Cd transporter in the root cell plasma membrane of a specific ecotype (Ganges) of *T. caerulescens* while another tested ecotype (Prayon) possibly seemed to mediate its Cd uptake by Zn transporters (Lombi *et al*., 2001).

Once the ions have entered the root, they can either be stored or exported to the shoot and this transport probably takes place in the xylem. To enter the xylem vessels, the ions have to cross the Casparian strip, which divides the central cylinder and the cortex. To cross this strip of water-impermeable cell walls, the ions must move symplastically, as apoplastic transport is blocked. This can be considered as a rate limiting step in translocation processes to the shoot. Xylem cell walls have a high cation exchange capacity which would be expected to also severely retard the movement to the shoots. In any way, it should be mentioned that in order to guarantee the availability and solubility of trace elements for their use in mineral nutrition, mechanisms should be present in order to prevent sorption, hydrolysis, and non-specific chemical reactions that could occur in the uptake and translocation processes. Similar processes must affect pollutant elements as well. For cations, this problem is most likely avoided by organic complexation. A substantial body of qualitative information indicates that the nutrient ions Ca, Co, Fe, Mn, and Zn exist in organically complexed forms in xylem exudates (Bradfield, 1976; Tiffin, 1977) but also non-essential trace elements have been shown to be complexed.

Plants are known to accumulate Ni readily and unlike most non-essential elements Ni is mobile in plants and accumulated in seeds (Mishra, 1974). In the Ni hyperaccumulator *Alyssum lesbiacum*, free histidine was produced as a specific and proportional response to Ni exposure (Krämer *et al.*, 1996). It was hypothesised that

histidine could be important both as a high-affinity ligand for nickel detoxification and in facilitating nickel transport out of the root in the xylem. There is also limited evidence that histidine may be involved in Ni transport in hyperaccumulator and non-hyperaccumulator *Thlaspi* species (Persans *et al*., 1999). Not only in Ni transport, but also in Zn transport in the hyperaccumulator *Thlaspi caerulescens*, histidine seems to play a role as was observed with X-ray absorption spectroscopy (Salt *et al.*, 1999b): the majority of intracellular Zn in the roots was found to be coordinated with histidine. Contrary to this observation, an amino acid analysis of the root exudates of *T. caerulescens* revealed that the concentration of histidine was found to be not dependent on the Zn concentration in the root (H. Schat, personal communication). The exact role of free histidine actually still is speculative and will remain so until more mechanistic data are available.

Heavy metal resistance

Metabolic disorders of plants are effected not only by micronutrient deficiencies, but also by their excesses. In general, plants are much more resistant to an increased concentration than to an insufficient content of a given element. Although many observations have been published on the harmful effects of trace element excesses, the nature of these processes is still poorly understood. Some basic reactions related to toxic effects of element excesses are the following: changes in permeability of the cell membrane (Ag, Au, Br, Cd, Cu, F, Hg, I, Pb, UO_2), reactions of thiol groups with cations (Ag, Cd, Cu, Hg, Pb) competition for sites with essential metabolites (As, Sb, Se, Te, W, F), affinity for reacting with phosphate groups and active groups of ADP or ATP (Al, Be, Sc, Y, Zr, lanthanides and possibly all heavy metals), replacement of essential ions (Cs, Li, Rb, Se, Sr, Zn) and occupation of sites for essential groups such as phosphate and nitrate (arsenate, fluorate, borate, bromate, selenate, tellurate, tungstate) (Kabata-Pendias & Pendias, 1992)

Among these elements, excess levels of heavy metals seem to be also responsible for many of these damaging processes. It can be stated that the most toxic heavy metals for higher plants are Hg, Cu, Ni, Pb, Co, Cd, Zn and possibly also Ag, Be, and Sn (Kabata-Pendias & Pendias, 1992). Especially redox-active heavy metals like Cu may also interact with cellular redox processes or may even directly generate reactive free radicals. By consequence, the toxicity of these metals can be ascribed to an increased

production of free radicals in cells, which results in oxidative stress (Sandmann & Böger, 1980; Freedmann *et al*., 1989; Gupta *et al*., 1999, Cuypers *et al*., 2000).

In order to protect itself, plants seem to have developed various mechanisms to overcome these toxic effects of heavy metals. Avoidance is a way to reduce the cellular uptake, but evidence for this method is very limited. In experiments with *Triticum aestivum* L. growing in solution culture, Zn efflux increased more than Zn influx in function of the external Zn concentration suggesting that the outward flux can act as a detoxification mechanism (Guillermo & Cogliatti, 1998). Alternatively, transgenic tobacco and papaya plants that overexpress a bacterial gene for citrate synthetase seem to be able to block Al(III) uptake through chelation outside the plasma membrane, preventing its uptake (de la Fuente *et al*., 1997).

Heavy metal resistant enzymes are reported to be present in some plant ecotypes endemic to heavy metal polluted soils (Thurman, 1981) but most attention is paid to the detoxification processes which are supposed to take place once the heavy metals accumulate within cells. Chelation, compartmentalization, or precipitation probably comprise the major resistance mechanisms.

Phytochelatines are a class of peptides with the molecular structures $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ that are known to play an important role in heavy metal chelation. A large variety of metals induce the synthesis of phytochelatins, but the formation of a phytochelatin complex has largely been examined with Cd and Cu. In the case of Cd, cadmium-sensitive mutants of *Arabidopsis thaliana* (*cad*1) seedlings proved to be deficient in phytochelatin synthase activity and this confirmed the essential role played by phytochelatins in Cd detoxification in this plant species (Howden *et al*., 1995). Salt *et al.* (1995) also reported that phytochelatins may be involved in binding a significant amount of Cd in the intact roots of *Brassica juncea*. This hypothesis might be confirmed by the experiments performed by Zhu *et al*. (1999): over-expression of the *Escherichia coli gshl* gene encoding gamma-glutamylcysteine synthetase (gamma-ECS) in *B. juncea* plants revealed increased biosynthesis of glutathione and phytochelatins which in turn enhanced Cd tolerance and accumulation.

Experiments with the fission yeast *Schizosaccharomyces pombe* revealed that a number of mutants impaired in metal detoxification were hypersensitive to Cd and failed to form wild-type levels of phytochelatin bound Cd complexes (Ow, 1996). Additionally arsenate sensitivity seemed to be the consequence of deleting the gene

required for phytochelatin synthesis in *S. pombe* (Ha *et al*., 1999). Also Cu tolerance appears to be attributable to phytochelatin formation in a naturally line of *Mimulus guttatis* (Salt *et al*., 1989). On the other hand, adaptive tolerance to Cd in *Silene vulgaris* cannot be explained by phytochelatins. It was shown that naturally selected Cd tolerant populations of *Silene vulgaris* contained lower concentrations of phytochelatins than Cd-sensitive populations of the same species (De Knecht *et al*., 1994). Other experiments with *cad*1 mutants of *A. thaliana* seedlings revealed that they were only slightly sensitive to Cu and Zn, indicating that phytochelatins only play a minor role in the detoxification of these heavy metals and suggests the presence of some other mechanism(s) for their detoxification (Howden *et al*., 1995). Transport of complexes of phytochelatins and heavy metals from the cytoplasm into the vacuoles might be an alternative detoxification mechanism. A similar process was observed in *S. pombe*: a correlation was revealed between heavy metal tolerance and expression of an ATP binding cassette ABC-type transporter in the vacuolar membrane which may carry complexes of phytochelatins and heavy metals into the vacuoles (Ortiz *et al*., 1992). Higher plants have also been found to transport a phytochelatin-Cd complex to the vacuole through what appears to be an ABC-type transporter (Salt & Rauser, 1995). More evidence that the vacuole plays a role in preventing the accumulation of possibly toxic levels in the cytoplasm was provided by the experiments performed with 10 days old *Hordeum vulgare* grown in hydroponic cultures. Analysis of the vacuolar pool revealed that at elevated Zn and Cd supply the Zn and Cd concentration increased. In contrast, only 23% of Ni was compartmentalised under Ni stress (Brune *et al*., 1995) which claims that compartmentalisation can again not be considered as a general detoxification mechanism for the tested cations.

Yet another way for plants to reduce toxic concentrations of heavy metals is precipitation. Also for this topic, mainly the fate of Zn and Cd have been studied extensively. Under certain conditions Zn can be precipitated as Zn phytate in *Lemna minor* (Van Steveninck *et al*., 1990) while *Brassica juncea* seems to be able to store Cd in external tissues of the leaves called trichomes (Salt *et al*., 1995) which also may be a possible detoxification mechanism.

If these mechanisms (which prevent the interaction between heavy metals and their site(s) of action) fail, a cell has to protect itself against the metal-induced damaging processes. The formation of free radical species for instance can cause severe damage

to different cell components. Cells are provided with an efficient anti-oxidative system, consisting of several non-enzymatic and enzymatic mechanisms (for a review, see Vangronsveld & Clijsters, 1994), that can interact with the reactive intermediary forms of oxygen (Cuypers, 2000).

I.3.2. Phytoextraction

As was mentioned before, phytoextraction is the process where metal accumulating plants are used to transport and concentrate heavy metals from the soil into the shoots in order to reduce soil concentrations of heavy metals to environmentally acceptable levels. Dried, ashed, or composted plant residues, highly enriched in heavy metals may be isolated as hazardous waste or recycled as biometal ore (phytomining). Although the most heavily contaminated soils do not support plant growth, sites with light to moderate toxic metal contamination might be suitable for growing metal accumulating plants for toxic metal cleanup.
It is evident that plants used in these processes need some characteristics in order to efficiently fulfil phytoextraction. The ideal plant should be able to tolerate and accumulate high levels of heavy metals but also have a rapid growth rate and the potential to produce a high biomass in the field.

Heavy metal hyperaccumulator plant species

Inventarisation of plants from areas where the soil is enriched with heavy metals due to geological factors or pollution, resulted in a wide range of different species, all having their own characteristics (Baker & Brooks, 1989; Reeves & Baker, 2000).
Three basic strategies are used by plants growing on metalliferous soils (Figure I.1) (Baker, 1981). Metal excluders effectively prevent heavy metals from entering their aerial parts over a broad range of metal concentrations in the soil; however they can still contain large amounts of metals in their roots. Metal non-excluders actively accumulate metals in their shoots and can be divided into two groups: indicators and hyperaccumulators. Heavy metal levels in the tissues of indicator species generally reflect metal levels in the soil while hyperaccumulators concentrate metals in their shoots to levels far exceeding those present in the soil or in the non-accumulating species growing nearby.

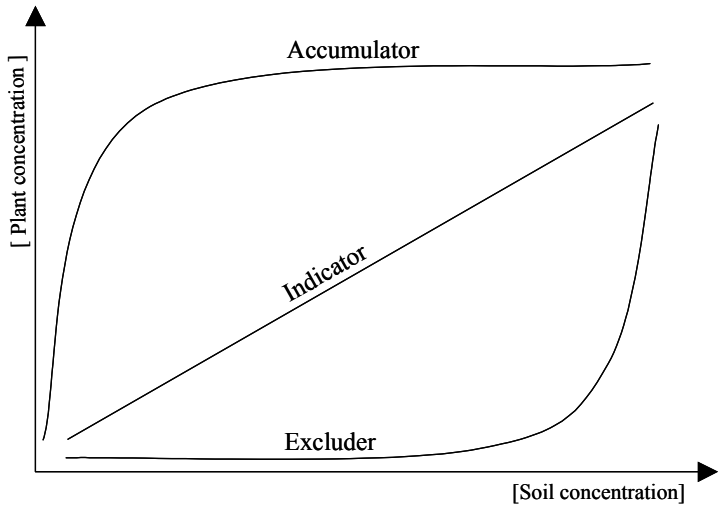

Figure I.1: Three ways in which the response of plants to increasing soil metal levels may be reflected by the metal concentrations in the aerial plant parts (Baker, 1981).

Among these plant types, hyperaccumulators seem to be the most interesting candidates for phytoextration processes because of the high accumulation rates of heavy metals in the harvestable parts. Generally a plant is considered as a hyperaccumulator if per kilogram dry weight more then 1000 mg cobalt, copper, chrome, lead, nickel or 10000 mg manganese or zinc is accumulated (Baker & Brooks, 1989). The list of hyperaccumulating plants is expanding rapidly and the largest numbers collected in the temperate zone belong to the *Brassicaceae* while in the tropic regions the *Euphorbiaceae* are the best represented (Baker & Brooks, 1989). Table I.2 shows some hyperaccumulators and their shoot metal concentration. For a complete overview, see (Baker & Brooks, 1989; Reeves & Baker, 2000).

Extensive research has been done on the mechanisms of hyperaccumulation within the family of the *Brassicaceae*. One member of this family that has been thoroughly studied for its Zn hyperaccumulation is *Thlaspi caerulescens* J&C Presl. As certain populations of this species have been shown to accumulate and tolerate up to 40000 µg/g Zn in their shoots (Chaney, 1993), it was considered as a very interesting experimental system for studying mechanisms of hyperaccumulation.

Heavy metal	Plant species	Concentration in shoots (per dry weight)
Cd	*Thlaspi caerulescens*	2130 mg/kg dry weight (0.2%)
Zn	*Thlaspi caerulescens*	43710 mg/kg dry weight (4.3%)
Cu	*Ipomoea alpina*	12300 mg/kg dry weight (1.23%)
Co	*Haumaniastrum robertii*	10232 mg/kg dry weight (1%)
Pb	*Thlaspi rotundifolium*	8200 mg/kg dry weight (0.8%)
Mn	*Vaccinium myrtillus*	5270 mg/kg dry weight (0.5%)
Ni	*Psychiotria douarrei*	19900 mg/kg dry weight (1.9%)
As*	*Pteris vittata*	4980 mg/kg dry weight (0.5%)

Table I.2: Hyperaccumulator species and their shoot metal concentrations (Reeves & Baker, 2000; Ma *et al*., 2001*).

Some research focussed on the root-soil interactions in the rhizosphere of *T. caerulescens* grown in contaminated soil as these might have an important influence on the availability or solubility of the metals taken up. McGrath *et al.*, (1997) correlated the accumulation capacity of Zn with the rhizosphere pH and concluded that acidification was not the mechanism used to mobilise Zn in the soil. Another interpretation of the relation availability-accumulation was suggested by Knight and co-workers (1997). They observed that on average only 0.8% of the Zn in plant tissue could be explained by a decrease of the Zn concentration of the soil solution, indicating that about 99% of the Zn taken up by the plant was from exchangeable and insoluble pools of Zn. A possible explanation might be that the soil solution is well buffered and therefore as free Zn is taken up by the plant, more free Zn dissociates into solution to maintain the initial free Zn concentration. Alternatively *T. caerulescens* might be able to mobilise insoluble Zn in soils through the production of root exudates. Once mobilised in the soil solution, radiotracer techniques revealed an enhanced Zn influx in the root symplasm of *T. caerulescens* when compared with the non-accumulator *T. arvense* (Lasat *et al*., 1996) suggesting that this also might contribute to hyperaccumulation. As was mentioned before, the recent discovery of transmembrane metal transporters might be involved in these root symplasm uptake processes. The ZTP1, ZNT1, and ZNT2 zinc transporter genes isolated and cloned from *T. caerulescens* showed increased expression in *T. caerulescens* compared with the non-hyperaccumulator *T. arvense* (Assunção *et al*., 2001). Additionally, ZNT1 and ZNT2 seemed to be exclusively expressed under Zn deficiency conditions in *T.*

arvense while the expression in *T. caerulescens* is barely Zn responsive. The authors suggested that Zn hyperaccumulation might rely on a deceased Zn induced transcriptional downregulation of these genes.

Not only enhanced influx of Zn might be important in uptake and translocation, but also plasma membrane and tonoplast Zn transport in leaf cells could be critical sites for Zn hyperaccumulation (Lasat *et al*., 1996). The observation that ZTP1 is mainly expressed in leaves indicates that similar Zn transporters as in the roots are also active in the leaves (Assunção *et al*., 2001). This brings us to the translocation of Zn from the root to the shoot. X-ray absorption spectroscopy was used to determine the ligand environment of Zn in different tissues of *T. caerulescens*. In the roots, the majority of the intracellular Zn was coordinated with histidine while transport appeared to take place in the xylem mainly as hydrated cations, with chelation by citrate only playing a small role. In the shoots, Zn coordination was dominated by citrate with smaller contributions from hydrated zinc, histidine, cell wall, and oxalate binding (Salt *et al*., 1999b).

Of particular interest are also the processes involved in internal detoxification. In the roots, Zn mainly accumulated in vacuoles and to a lesser extent in cell walls (Vazquez *et al*, 1992). Zn storage in the leaves may be accomplished by formation of Zn rich crystals in vacuoles of epidermal and sub-epidermal cells (Vazquez *et al*., 1994; Küpper *et al*., 1999). However, no such vacuolar Zn sequestration phenomena were observed by Frey *et al*. (2000) using energy-dispersive X-ray micro-analysis (EDXMA). These authors also showed that apoplastic compartmentation is another important mechanism involved in Zn tolerance in leaves of *T. caerulescens*.

Other data emphasised the importance of sequestration mechanisms: a strong constitutive sequestration mechanism was used as an explanation to the greater internal requirement for Zn in *T. caerulescens* compared to the closely related but non-accumulator *T. ochroleucum* (Shen *et al*., 1997). This would render a large proportion of intracellular Zn physiologically unavailable even when Zn supply is low.

Although intense studies have been done, many questions concerning the mobility, transport, requirement, tolerance, and detoxification of Zn within *T. caerulescens* still remain to be resolved.

As hyperaccumulators have the capacity to concentrate heavy metals far in excess of normal physiological concentrations in their upper plant parts, they could be

considered as useful in phytoextraction processes. But when other important factors are taken into account, there seem to be some limitations:

- Hyperaccumulators often accumulate only a specific element and have not been found for all elements of interest.
- Most hyperaccumulators grow slowly and produce small biomass
- Little is known about their agronomic characteristics, pest management, breeding potential and physiology.

Baker *et al*. (1994) evaluated the use of the Zn hyperaccumulator *T. caerulescens* in phytoextraction experiments. The dry weight yield of *T. caerulescens* growing over a 6 month period produced approximately 3-4 t/ha and they estimated that it would take 13 years to lower the Zn content of the soil from 400 ppm to 300 ppm Zn, which is an acceptable level of Zn in agricultural land. While the results of such studies were promising, some researchers have suggested that the small size and slow growth of this species may limit its utility for phytoremediation (Black, 1995; Brown *et al*., 1995). The same conclusion was drawn for the Pb hyperaccumulator plant *Thlaspi rotundifolium* (Huang *et al*., 1997). As a consequence, other plant species have been evaluated for their potential in phytoextraction experiments.

It should be mentioned that more recent opinions (Chaney *et al*., 2000) claim that the concentration is the most limiting factor rather than biomass for present phytoextraction technologies. In this context, McGrath *et al*. (2001) mentioned that the difference in concentrations between hyperaccumulators and high biomass crops is often greater than 50-fold whereas the difference in biomass is most likely to be less than 10 fold.

Non-hyperaccumulators with potential in phytoextraction

The primary objective of phytoextraction is to maximise the transfer of contaminant to the plant shoots so that the greatest total mass of contaminant is removed by each cropping. Moderate accumulating, high biomass species might possibly be more effective than hyperaccumulator species (Kumar *et al*., 1995; Salt *et al*., 1995c; Ebbs & Kochian, 1997). Particular emphasis was placed on the crop-related members of the *Brassicaceae* family that are related to many of the known heavy metal hyperaccumulators. *Brassica juncea* for example is a high biomass crop plant that

accumulates Pb and other heavy metals and may show promise for use in remediation of metal contaminated sites. Experiments performed by Ebbs and Kochian (1997) showed that growing *B. juncea* and *T. caerulescens* for 6 weeks in a contaminated soil, resulted in a 4-fold more Zn removal by *B. juncea* compared to *T. caerulescens*. They concluded that this was primarily due to the fact that *B. juncea* produced 10 times more biomass than *T. caerulescens* and this apparently can compensate the lower shoot metal concentration. These results suggested that a greater shoot biomass can more than compensate for a lower shoot metal concentration and that the plant species suitable for phytoremediation may not be limited to hyperaccumulators.
Other *Brassica* species have shown a similar tendency to accumulate moderate levels of heavy metals: *B. napus* and *B. rapa* appeared to be moderate accumulators of heavy metals, with root metal concentrations considerably higher than those in the shoot (Ebbs & Kochian, 1997). Not only *Brassica* species, but also grass species seem to be potential candidates in phytoextractionn processes. Hydroponic experiments with *Avena sativa* (oat), and *Hordeum vulgare* (barley) showed that these grass species tolerated the high Cu, Cd, and Zn concentrations present in the solution and also accumulated elevated concentrations of these metals in the plant shoots. Comparison with *B. juncea* revealed that the grasses were even more tolerant for Zn although the shoot Zn concentrations were greater for *B. juncea* (Ebbs & Kochian, 1998).
To validate these tests, field experiments and analyses should be the next step to take.

Possibilities to improve phytoextraction processes

It is obvious that for an effective development of phytoextraction processes, many factors have to be taken into account. In order to be able to improve these processes, additional understanding of the physiology, molecular biology, and chemistry of the heavy metal absorption, translocation and tolerance by plants is a first and important priority. Without this basic understanding, it will be difficult to exploit many of the recent advances in plant biology. The use of genetic engineering is one of such techniques that might be used to alter heavy metal tolerance or accumulation in plants through modification with foreign genes. The introduction of well studied bacterial heavy metal resistance genes into plants indicated for the first time that this might improve a plant's capacity to tolerate higher concentrations of heavy metals. A

mutagenised bacterial mercuric ion reductase gene (*merA*) was successfully transformed to *A. thaliana* seedlings and resulted in an increased tolerance for Hg (Rugh *et al*., 1996; 1998). Additional transformation with the bacterial gene *mer*B for organomercurial lyase resulted in *A. thaliana* plants that tolerated up to 10-fold higher methylmercury concentrations than plants that express *mer*B alone (Meagher *et al*., 2000) Although this might be a promising result, one must take into account that transfer of a bacterial gene into a plant is already complicated while often bacterial metal resistance is encoded on large plasmids containing operons with many genes involved in the resistance mechanism.

Genetic engineering of plant genes involved in heavy metal tolerance and accumulation might be another attractive possibility to transfer interesting traits for phytoextraction. Recently, a putative zinc transporter (ZAT1) from *Arabidopsis thaliana* was isolated, and overexpression of this gene resulted in an increased tolerance and accumulation of Zn in the roots of *A. thaliana* (van der Zaal *et al*., 1999). This result indicates that apparently only one gene might be responsible for Zn tolerance as well as Zn accumulation in transgenic *Arabidopsis* plants. Macnair and co-workers (1999) disputed this as they demonstrated that zinc tolerance and hyperaccumulation were independent characters. Crosses between the zinc hyperaccumulating and tolerant species *Arabidopsis halleri* and the non-hyperaccumulating, non-tolerant species *Arabidopsis petraea* revealed that the F_2 segregates for both characters and it appears that the two characters are genetically independent. These results have undoubtedly consequences for consideration of the mechanisms and applications of hyperaccumulation. If the characteristics are genetically independent, then it follows that different genes and mechanisms are responsible for the two characteristics. For phytoextraction strategies, both characteristics might not be necessary as only accumulation is the key to extracting metals from soils.

Another issue in this context concerns the regulation of potential important genes for hyperaccumulation. The detection of three Zn transporter genes in *T. caerulescens* and their expression characteristics under different Zn conditions compared with the non-hyperaccumulator *T. arvense* indicated the need to further investigate the transcriptional regulations involved (Pence *et al*., 2000). The fact that expression in *T. caerulescens* is barely Zn responsive might be attributed to a decreased Zn-induced transcriptional down-regulation of these genes (Lasat *et al*., 2000; Pence *et al*., 2000;

Assunção *et al*., 2001). This field of gene regulation certainly should be further explored in order to fully understand the processes involved.

As the number or types of genes responsible for metal tolerance and hyperaccumulation have not been completely elucidated so far, an alternative way to improve plants for phytoextraction processes might be somatic hybridisation. Somatic hybridisation is accomplished by hybrid regeneration from tissue culture after protoplast fusion and it encompasses species with complete sexual incompatibilities. The Zn hyperaccumulator *T. caerulescens*, belonging to the *Lepidiae* tribe of the *Brassicaceae* and the crop *Brassica napus*, belonging to the *Brassicaceae* tribe hybridised in this way and the resulting hybrids accumulated levels of zinc and cadmium that would have been toxic for *B. napus* (Brewer *et al*., 1999). This is the first known report of hybridisation between a heavy metal hyperaccumulator and a high biomass species, providing a potential mechanism for practical phytoremediation of a metal contaminated soil. However, it should be noted that the production of viable seeds from backcrosses to *B. napus* is still under investigation.

Another challenge in phytoextraction that should be mentioned is the improvement of the root structure. Root depth, structure, and density have not been altered for maximum efficiency and therefore established agronomic techniques like irrigation should be investigated for their contribution. Alternatively, the influence of bacteria should not be underestimated either. Rhizosphere bacteria might lower the level of stress ethylene in the plant thereby allowing the plant to develop longer roots and thus a better establishment in the soil during early stages of growth (Glick *et al*., 1998). Once established in the soil, the bacterium seems to help the plant to acquire sufficient iron for optimal plant growth, in the presence of levels of heavy metals that might otherwise make the acquisition of iron difficult (Burd *et al*., 2000).

The availability of heavy metals should also be considered as a feature that can influence the efficiency of phytoextraction. Robinson *et al*. (1999) described a best- and worst case scenario for successive phytoextraction processes in several ultramafic soils using Ni hyperaccumulator plants. In the best case, the available Ni in the soil will remain relatively constant in a scenario where an equilibrium situation between soluble and insoluble metal allows for full or partial replacements of the soluble fraction removed by the plant until all the metal in the soil has been removed; in a worst case scenario, most of the metal would be matrix-bound, and once the initial

metal harvest had been obtained, no further 'crops' could be grown without soil modification.

Modification may involve the addition of chemical chelates like EDTA, NTA, and DTPA to increase desorption of heavy metals from the soil. Addition of chelates to a Pb-contaminated soil increased shoot Pb concentrations of *Zea mays* (corn) and *Pisum sativum* (pea) from less than 500 mg/kg to more than 10000 mg/kg (Huang *et al*., 1997). As such chelates may pose a risk to the environment by increased heavy metal leaching, alternatives like a locally improved reductase activity or production of phytosiderophores (Briat & Lobréaux, 1997) should be investigated as well.

A range of different methods to improve phytoextraction processes are being studied and this should finally lead to better conditions to fulfil the remediation in an efficient way.

I.3.3. Phytovolatilisation

Another natural mechanism that offers exciting phytoremediation possibilities is the transformation of toxic elements into relatively harmless forms. Elements like selenium and mercury can exist in a variety of states, including different cationic and oxyanionic species and thio-and organometallics. These forms vary widely in their transport and accumulation in plants and in their toxicity to humans and other life forms.

Although Se is an essential trace element it is usually toxic at higher concentrations. Plants are able to detoxify selenate from contaminated substrates through transformation into the less toxic dimethylselenide which is volatilised from leaves and roots in a process called phytovolatilisation (Terry & Zayed, 1998). As anticipated for many processes of phytoremediation, Se volatilisation is enhanced by additional activities in the bacterial rhizosphere (de Souza *et al*., 1999). Such volatilisation prevents that Se enters the food-chain and also eliminates the need for disposal of hazardous plant waste. The chemical transformation of another toxic elemental pollutant, namely Hg, also leads to its removal from the soil. A bacterial mercuric ion reductase (m*erA*), able to convert toxic Hg^{2+} to the less toxic, gaseous and relatively inert metallic mercury (Hg^0), was mutated and efficiently expressed in *Arabidopsis thaliana* (Rugh *et al*., 1996) and *Liriodendron tulipifera* (Rugh *et al.*, 1998) generating transformants that were able to grow in the presence of normally

toxic levels of ionic mercury. These plants volatilise and possibly transpire Hg^0 from their tissues, and they accumulate far less Hg than control plants grown in low concentrations of Hg (Heaton *et al*., 1998).
However, biovolatilisation is not applicable for most inorganic ions, thus leaving biomass removal as the only alternative for the extraction of most of these contaminants.

I.3.4. Phytostabilisation

Soils that are highly polluted with heavy metals usually lack an established vegetation due to the toxic effects of the pollutants. As these barren soils are more prone to wind- and water-erosion and leaching, the pollutants can be spread easily in the environment. A simple solution to the stabilisation of these wastes is re-vegetation with metal-tolerant plant species. A good phytostabilising plant should tolerate high levels of heavy metals and immobilise these metals in the soil via root uptake, precipitation or reduction. In addition these plants should have low shoot accumulation of heavy metals to eliminate the necessity to treat harvested shoot residues as hazardous waste and reduce the risk of transfer to the food-chain.
Successful phytostabilisation was carried out on metalliferous mine wastes in the UK where local metal tolerant plant species were used to stabilise the site and establish an excellent vegetation cover (Smith & Bradshaw, 1979).
Alternatively, substances with strong metal immobilising capacities could be used to lower the toxicity of the soil and promote the re-vegetation. One has to take into account that total immobilisation of heavy metal cations like Zn and Cu must be avoided because they are both essential for plant growth. Vangronsveld *et al*. (1996) added beringite (cyclonic ashes) to a highly metal polluted acid sandy soil at the site of a former zinc smelter and after five years, a 70 times lower water extractable metal fraction of the treated soil compared to the non-treated soil was measured. The originally sown metal tolerant *Agrostis capillaris* and *Festuca rubra* were still healthy and regenerating. Compared to the untreated soil, several non metal-tolerant perennial forbs and their corresponding myorrhizal fungi had colonised the re-vegetated area suggesting the establishment of a functioning ecosystem. More recently, a test system for the evaluation and monitoring of the efficacy and durability of *in situ* inactivation of metal contaminated soils was described (Vangronsveld *et al*., 2001). This consisted

of sequential extraction procedures and microbial heavy metal biosensors for the physico-chemical evaluation and these results were complemented by the biological phytotoxicity tests and a zootoxicity test. A good conformity was found between these different evaluation criteria.

I.3.5. Rhizofiltration

The use of plant roots and submerged seedlings to absorb and adsorb heavy metals from aqueous waste streams is defined as rhizofiltration and can be considered as a subset of the phytoremediation technology (Dushenkov *et al*., 1995; Salt *et al*., 1997). An ideal plant for rhizofiltration should have rapidly growing roots with the ability to remove toxic heavy metals from solution over extended periods of time.

Roots of hydroponically grown terrestrial plants like *Brassica juncea* (Indian mustard) and *Helianthus annuus* (sunflower) effectively removed toxic heavy metals like Cu, Cd, Cr, Ni, Pb, and Zn from aqueous solutions. *B. juncea* roots concentrated these heavy metals from 131-563 fold above the initial solution concentrations (Dushenkov *et al*., 1995). In contrast to aquatic higher plants which usually have small size and slow growing roots, terrestrial plants seem to be better candidates in rhizofiltration because they develop much longer and fibrous root systems covered with root hairs which create an extremely high surface area.

Another way to improve rhizofiltration processes was proposed by Salt *et al*.(1999a). Hydroponically grown *B. juncea* inoculated with Cd tolerant rhizobacteria that proved to stimulate root growth, were found to increase the total amount of Cd removed from the solution by the roots compared to the non-inoculated control plants. This plant growth promoting effect may increase the root biomass available for metal accumulation.

II ENDOPHYTIC BACTERIA

II 1. Definition

Although the term endophyte is most commonly associated with fungal organisms, there is sufficient literature pertaining to bacteria as endophytes, some of which are regarded to have a beneficial effect, while others are regarded to have a neutral or detrimental effect on plants.

Bacteria have been proposed to exist inside plants without causing disease symptoms for over 50 years (Tervet & Hollis, 1948; Hollis, 1951). Various reports indicate that such bacteria exist in a variety of tissue types within numerous plant species suggesting an ubiquitous existence in most if not all higher plants. Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants that range from woody tree species such as oak (Brooks *et al*., 1994) and pear (Whitesides & Spotts, 1991), to herbaceous crop plants such as sugar beets (Jacobs *et al*., 1985) and maize (Fischer *et al*., 1992; Lalande *et al*., 1989; McInroy & Kloepper, 1995). Diversity associated with bacterial endophytes exists not only in the plant species colonised but also in bacterial taxa. Variation in bacteria that have been reported as endophytes span a significant range of both Gram-positive and Gram-negative species. As it remains difficult to compare earlier and more recent studies that identify bacteria, still certain trends are apparent with predominant bacterial types isolated as endophytes (Kobayashi & Palumbo, 2000).

A variety of definitions are given to the term endophyte and consideration of each leads to different interpretations**.** Kloepper *et al*. (1992b) simply called bacteria within tissues internal to the epidermis endophytes. Since quiescent endophytic bacteria can become pathogenic under certain conditions and or within different host genotypes (Misaghi & Donndelinger, 1990), James & Olivares (1997) adjusted the definition and stated that all bacteria that colonise the interior of plants, including active and latent pathogens can be considered as endophytes. Considering all bacteria that colonise the interior of plants, one should also take into account those bacteria that reside within living plant tissues without doing substantive harm or gaining benefit other than securing residency (Kado, 1992) and bacteria that establish endosymbiosis with the plant whereby the plant receives an ecological benefit from the presence of the symbiont (Quispel, 1992).

All these sub-definitions may give an overview of what is considered to be endophytic by the quoted authors and this might consequently be regarded as the most general definition of what an endophyte stands for.

II 2. Ecology

Plants can be considered as a complex micro-ecosystem where different habitats can be exploited by a wide variety of bacteria (McInroy & Kloepper, 1994). These habitats are not only represented by plant external surfaces, where epiphytic bacteria predominate, but even by internal tissues where many micro-organisms penetrate and survive. Inside the plant micro-ecosystem different microbial species, both bacteria and fungi (Fisher *et al*., 1992), are able to interact and to establish an equilibrium. Some of them can be considered as dominant species (Van Peer *et al*., 1990) and are represented by those micro-organisms most frequently isolated, and in large numbers, from the host plant. As well as the dominants, there is a large variety of species that cannot easily be isolated because of their low numerical consistence. These are considered as rare species. This distribution was observed by Gardner *et al*. (1982) who identified bacteria isolated from xylem fluid from the roots of rough lemon rootstock of Florida citrus trees. Among the thirteen genera found, the most frequently occurring genera being *Pseudomonas* (40%) and *Enterobacter* (18%) could be considered as the dominant species while the others might be the rare species. This biodiversity is considered as the most important condition in the establishment of any ecosystem.

It should be noted that attempts to evaluate total populations of bacteria in plants may produce varied results according to the growth medium used for isolation, variations in growth conditions of the host plant, and the state in which the plant tissue was used. Another important factor that could influence internal bacterial population numbers is the surface sterilisation method. No surface disinfection's protocol results in complete killing of surface bacteria on 100% of samples without penetrating interior tissues and thereby killing internal colonists. Therefore, comparisons between different studies should be carefully evaluated by the different methods and conditions. This is important when bacterial populations are counted. Generally, total colonisation by non-pathogenic endophytes rarely have been documented to reside at population

levels commonly observed for pathogenic bacteria in diseased tissue. Counted numbers of pathogenic bacteria can range from 1x 10^7 CFU/g fresh weight, e.g. for *Clavibacter michiganensis* subsp. *sepedonicus* on tomato (Tsiantos & Stevens, 1986), to 1 x 10^9- 1 x 10^{10} CFU/g fresh weight under severe disease pressure, as reported for *Ralstonia solanacearum* on tomato, and eggplant (Grimault & Prior, 1994). Contrary to these high concentrations of pathogenic bacteria, common population sizes of introduced endophytic bacteria vary between 1 x 10^3 and 1 x 10^5 CFU/g plant tissue for most investigated plant species (Dong *et al*., 1994, Frommel *et al*., 1991; Quadt-Hallmann & Kloepper, 1996; Lamb *et al*., 1996). Similar to indigenous endophytes, which are usually abundant in the same concentration range as the introduced endophytes, the highest bacterial densities are usually observed in the roots and decrease from the stem to the leaves (Quadt-Hallman & Kloepper, 1996; Lamb *et al*., 1996). Independent of the initial inoculum size, endophytic populations tend to approach optimal densities depending on the plant tissue. For potatoes inoculated with *Pseudomonas* sp., the root population increased to a final population density of 1 x 10^6 CFU/cm whereas the stem population decreased from an initial concentration of 3.3 x 10^5 to 1.9 x 10^3 CFU/cm (Frommel *et al*., 1991).

The fact that colonisation appears especially abundant in root tissue may reflect that the root is the primary site where endophytes gain entry into plants. With the exception of seed transmitted bacteria, which are already present in the plant, potential endophytes must first colonise the root surface prior to entering the plant. Potential internal colonists find their host by chemotaxis, electrotaxis, or accidental encounter. The main entry for endophytic bacteria appears to be through wounds that naturally occur as a result of plant growth, or through root hairs and at epidermal conjunctions (Sprent & de Faria, 1988). This was confirmed by several microscopic studies (Wiehe *et al*., 1994; Benhamou *et al*. 1996a, 1996b, Pan *et al*., 1997). Artificial wounding was shown to contribute to increased endophytic colonization in roots of plants compared to intact roots (Gagné *et al*., 1987). Besides providing entry avenues, wounds also create favourable conditions for the approaching bacteria by allowing leakage of plant exudates, which serve as a food source for the bacteria.

Wounds and lateral roots are not, however, absolutely required for entrance of endophytic bacteria. Seedlings grown with minimal disturbance in liquid media or on water agar were penetrated by endophytic bacteria long before lateral root emergence (Levanoy *et al*., 1989; Quadt-Hallmann *et al*., 1997). Since untreated control plants

were endophyte free, the observed bacterial behaviour might indicate active penetration. This hypothesis is supported by the presence of cellulytic and pectinolytic enzymes produced by numerous endophytic bacteria such as *Azoarcus* sp. (Hurek *et al*., 1994), *Azospirillum irakense* (Khammas & Kaiser, 1991), and *Pseudomonas fluorescens* (Benhamou *et al*., 1996a; Quadt-Hallmann *et al*., 1997). Enzymatic degradation of plant cell walls by these bacteria was only observed when they colonised the root epidermis but never after colonising intercellular spaces of the root cortex. These results suggest that the endophyte induced production of cellulase and pectinase only for penetration into the host plant. Although these observations demonstrate the possibility of active penetration mechanisms for some endophytic bacteria, very little is known about the origin and regulation of these enzymes. It is assumed that these bacteria must posses some regulatory mechanism to specifically regulate their enzyme production in terms of quantity and time of expression.

Although the root zone offers the most obvious site of entry for many endophytes, entry may also occur at sites on aerial portions of plants. Sharrock *et al*. (1991) suggested that in some cases endophytic populations within fruit may arise by entry through flowers. Penetration is also supposed to occur through natural openings on the leaves (e.g. stomata) or through stem lenticels (Kluepfel, 1993). A completely different way of penetration is described by Ashbolt & Inkerman (1990) on sugar cane via mealybug, and by Kluepfel (1993) via different insects.

Once inside plant tissue, endophytic bacteria either remain localised in a specific plant tissue like the root cortex or colonise the plant systematically by transport through the conducting elements or the apoplast (Hurek *et al*., 1994; James *et al*., 1994; Mahaffee *et al*., 1997; Quadt-Hallmann *et al*., 1997, Patriquin & Döbereinner, 1978). This difference in distribution might be due to interactions with other bacteria or to different requirements of each micro-organism that let them inhabit different niches, represented by a tissue and more specifically by intercellular spaces inside each tissue (Di Fiori & Del Gallo, 1995). There are a few studies that report on the intracellular presence of endophytes. This seems to be an occasional finding, except for particular cases such as *Rhizobium* or *Alcaligenes faecalis* (You *et al*., 1991) where both bacteria are enveloped by the plant in specialised structures.

The fact that bacteria seem to be capable of colonising the internal tissues of plants could confer an ecological advantage over bacteria that can only colonise plants epiphytically. The internal tissues of plants are thought to provide a more uniform and

protective environment for micro-organisms than plant surfaces, where exposure to extreme environmental conditions such as temperature, osmotic potentials, and ultraviolet radiation are major factors limiting long-term bacterial survival. However, there are probably other limiting factors that must be overcome when establishing populations in the internal tissues of plants. Thus, establishing and maintaining an introduced bacterial population would still be limited and influenced by the same factors that affect plant health.

II 3. Beneficial effects

The beneficial effects of bacterial endophytes appear to operate through similar mechanisms as described for plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1991a; Höflich *et al*., 1994). This makes sense because most of the bacterial endophytes isolated from trees, healthy crops and weeds can be considered as facultative endophytes because of their capability to live outside plant tissues as rhizospheric bacteria (Di Fiori & Del Gallo, 1995). In addition, many endophytic bacterial taxa from sweet corn or cotton were even reported to be common soil bacteria (McInroy & Kloepper, 1994).

Plant growth promoting bacteria can affect plant growth directly or indirectly. The direct promotion of plant growth by PGPR for the most part entails either providing the plant with a compound that is synthesised by the bacterium or facilitating the uptake of certain nutrients from the environment. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms.

II 3.1. Direct plant growth promoting activity

There are several ways in which different PGPR have been reported to directly facilitate the proliferation of their plant hosts: PGPR can fix atmospheric nitrogen and supply it to plants; they synthesise siderophores that can solubilise and sequester iron from the soil and provide it to the plant; they synthesise several different phytohormones that can act to enhance various stages of plant growth; they may have mechanisms for the solubilisation of minerals such as phosphorus that then become

more available for plant growth, and they may synthesise some less well characterised low molecular mass compounds or enzymes that can modulate plant growth and development (Brown, 1974; Kloepper *et al*., 1986, 1989; Davison, 1988; Lambert & Joos, 1989; Glick *et al*., 1994). A particular PGPR may affect plant growth and development by using any one, or more of these mechanisms. It is assumed that the same is true for endophytic bacteria.

Direct evidence for plant growth promoting activity by endophytic bacteria came from Sturz (1995). According to his study, approximately 10% of bacterial isolates recovered from within potato tubers were shown to promote plant growth. Other experiments with clover and potatoes in a crop rotation revealed that 21% of the isolated endophytic bacteria were plant growth promoting and this was reflected in increased shoot height (63%), shoot wet weight (66%), and increased root wet weight (55%) (Sturz *et al*., 1998).

Probably the most studied aspect of plant growth promotion in endophytic bacteria as well as in PGPR is nitrogen fixation. Diazotrophy is common in prokaryotes and the extensive biochemical and molecular biological studies of symbiotic diazotrophs, such as rhizobia, have served as a starting point for understanding the mechanisms of growth promotion involved. Since one of the major benefits that rhizobia provide to plants is fixed nitrogen in exchange for fixed carbon, it was initially thought that diazotrophic PGPR might also function in this way. However, not all PGPR are diazotrophic, and many of those that are diazotrophic fix only limited amounts of nitrogen and not nearly enough to provide for their own as well as the host plant's nitrogen requirements (Hong *et al*., 1991). The general lack of bacterial nitrogen release is thought to be the main reason why nitrogen fixation in the rhizosphere only poorly contributes to the nitrogen supply for the whole plant (Weniger & Van Veen, 1991).

Endophytic diazotrophs were supposed to have an advantage over root associated diazotrophs, such as *Azospirillum* and *Azotobacter*, in that they colonise the interior rather than the surface of the plants, and hence are better placed to exploit carbon substrates supplied by the plant (Döbereinner *et al*., 1995; McInroy & Kloepper, 1995, Boddey *et al*., 1995a, 1995b; Sprent & James, 1995; Triplett, 1996). Moreover, as they are often located within underground roots and/or dense plant tissue (stem nodes and xylem vessels), the bacteria are likely to be growing within a low pO_2

environment, which is necessary for the expression and operation of nitrogenase (Patriquin *et al*., 1983; Gallon, 1992; Baldani *et al*., 1997).

As these assumptions all seem to be beneficial for nitrogen fixation in endophytic bacteria, there is still no direct evidence that endophytic diazotrophs actually are the causative agents of biological nitrogen fixation. Although some agriculturally important grasses such as sugar cane (*Saccharum* sp.), rize (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*) contain numerous diazotrophic bacteria such as *Acetobacter diazotrophicus*, *Herbaspirillum* sp., and *Azospirillum* sp, there is little evidence that these bacteria actually fix N_2 in their host plant (James & Olivares, 1997). In the case of sugar cane, no correlation between bacterial numbers and biological nitrogen fixation have been demonstrated and the expression of nitrogenase by any bacterium has yet to be shown unambiguously (James & Olivares, 1997). The situation may even be complicated by the possibility that endophytic diazotrophs may affect plants in a manner other than via N_2 fixation. Inoculation of rice with the diazotrophic endophytic bacterium *Azoarcus* BH72 significantly promoted plant growth (Hurek *et al*., 1994). In this particular case, growth promotion also occurred with Nif^- mutants, indicating that N_2 fixation by *Azoarcus* sp. was apparently not involved in plant growth promotion. Therefore the authors speculated that the observed plant growth promotion might have been caused by enhanced plant mineral uptake and improved plant water relationships associated with the colonisation by *Azoarcus*.

Plant growth regulators such as ethylene, auxins, or cytokinins produced by some strains of *Pseudomonas*, *Enterobacter*, *Staphylococcus*, *Azotobacter*, and *Azospirillum* may also be considered as causal agents for altering plant growth and development (Arshad & Frankenberger, 1991; Bashan & Holguin, 1997; Leifert *et al*., 1994). *Azospirillum* for instance is generally regarded as being a rhizosphere bacterium that colonises mainly the elongation and root hair zones of roots (Okon & Kapulnik, 1986; Döbereinner et al., 1995, Vande Broek & Vanderleyden, 1995; Bashan & Holguin, 1995). Some *Azospirillum* strains actually can also be endophytic, being found within the roots of some *Gramineae* (Vande Broek & Vanderleyden, 1995). The observed plant growth promotion after inoculation of plant roots with *Azospirillum* is thought to be the consequence of bacterial auxin production and excretion (Barbieri & Galli, 1993).

Another way in which plant associated bacteria might influence the host plant was discussed by Glick *et al*. (1995;1998). They demonstrated that many plant growth promoting bacteria contain the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. It was hypothesised that this enzyme, which has no known function in bacteria, might be part in a mechanism used by certain bacteria to stimulate plant growth (Glick *et al*., 1998). This could occur by ACC deaminase modulating the level of ethylene in developing plants (Glick *et al*., 1994, Glick *et al*., 1997; Hall *et al*., 1996). It is also well documented that plants respond to a variety of different environmental stresses by synthesizing "stress" ethylene (Abeles *et al*., 1992; Hyodo 1991). In fact, a significant portion of the damage to plants from environmental stress, such as infection with fungal pathogens, may occur as a direct result of the response of the plant to the increased level of stress ethylene (Van Loon, 1984).

As ACC deaminase may act to insure that increased ethylene levels are lowered in a developing or stressed plant, it may enhance the plant's fitness and thus can be considered to act as a plant growth promoting characteristic.

II 3.2. Indirect plant growth promoting activity

PGPR as well as endophytic bacteria seem to be able to lessen or prevent the deleterious effects of one or more phytopathogenic organisms and this ability can be considered as an indirect way to promote plant growth.

A way in which rhizosphere inhabiting PGPR can prevent the proliferation of phytopathogens, and thereby facilitate plant growth, is through the production of siderophores with a very high affinity for iron (Castignetti & Smarrelli, 1986). The secreted siderophore molecules bind most of the Fe^{3+} that is available in the rhizosphere, and as a result effectively prevent any pathogens in this immediate vicinity from proliferating because of a lack of iron (O'Sullivan & O'Gara, 1992). The bacterium that originally synthesised the siderophore takes up the iron-siderophore complex by using a receptor that is specific for the complex and is located in the outer cell-membrane of the bacterium (O'Sullivan & O'Gara, 1992). Evidence for this mechanism comes from several studies including a report where a mutant strain of *Pseudomonas aeruginosa* deficient in siderophore production no longer protected tomato plants against *Pythium* damping-off (Buysens *et al*., 1994).

Generally, *Pseudomonas* sp. have been considered to play a major role in disease inhibition through the production of siderophores and thanks to this efficient iron uptake mechanism, they can quickly colonise the rhizosphere and thus physically displace deleterious organisms.

Unlike microbial or fungal phytopathogens, plants are generally not harmed by the localised depletion of iron caused by PGPR. Most plants can grow at much lower (about 1000 fold) iron concentrations than micro-organisms (O'Sullivan & O'Gara, 1992) and a number of plants even have mechanisms for binding the bacterial iron-siderophore complex, transporting it through the plant, and then reductively releasing the iron from the siderophore so that it can be used by the plant (Wang *et al*., 1993).

Another way that a PGPR can employ to prevent proliferation of phytopathogens is the synthesis of antibiotics. Genetically engineered *Pseudomonas* strains which produced enhanced amounts of additional antibiotic metabolites showed improved disease control (Fenton *et al*., 1992; Bangera & Thomashow, 1996). In contrast, non-antibiotic-producing mutants of several different disease suppressive bacterial strains were no longer able to prevent phytopathogen-caused damage to plants (Keel *et al*., 1992). These results confirm the hypothesis that antibiotics might contribute in the defence against phytopathogens.

Other ways for disease suppression have been reviewed by Glick (1995) and involve the production of several enzymes, competition for nutrients and suitable niches on the root surface and the induction of systemic resistance by PGPR.

In the case of endophytic bacteria, some studies reported the ability of fungal suppression. Several endophytic bacteria isolated from rice seeds exhibited strong anti-fungal activity against *Rhizoctonia solani*, *Pythium myriotylum*, *Gaeumannomyces graminis*, and *Heterobasidium* annosum (Mukhopadhyay *et al*., 1996). *Enterobacter cloaca*, an endophyte isolated from corn, seemed to be involved in antibiosis against *Fusarium moniliforme* (Hinton & Bacon, 1995) and this result could be added to the experiments performed by Chen *et al*. (1994) who showed that wilt disease symptoms (caused by a *Fusarium* sp.) could be successfully reduced when cotton plants were artificially inoculated with naturally occurring endophytes isolated from the same host plant.

Antagonistic activity of endophytic bacteria against bacterial pathogens has been reported for *Clavibacter michiganensis* subsp. *sepedonicum*, the causal agent for bacterial rot on tomato (Van Buren *et al*., 1993). In addition, *Pseudomonas*

fluorescens 89B-27 and *Serratia marcescens* 90-166 were observed to induce resistance in cucumber to *Pseudomonas syringae* pv. *lachrymans* (Liu *et al*., 1995).
There is also some evidence that endophytes may contribute to the control of plant-parasitic nematodes (Hallmann et al., 1995) and insects (Dimock et al., 1988). It has been demonstrated that rhizospheric and endophytic bacteria, associated with some crops with antagonistic properties to phytopathogenic nematodes, are involved in controlling these pests (Kloepper *et al*., 1991b). It should be mentioned that control of these parasites seems to be more complex and difficult than for fungal and bacterial pathogens, since damage from nematodes and insects occurs as a result of their feeding habit and migration, thus limiting the efficacy of bacterial antagonism.

II 4. Practical applications

The potential that endophytes have to offer for agricultural gains has been realised beyond their use as natural biocontrol agents. The inherent nature of certain endophytes to potentially colonise plants in a systemic manner provides a novel approach as a delivery system to plants for various beneficial traits. The use of genetically engineered strains of endophytic bacteria for enhanced pest control has been envisioned for some time (Dimock *et al*., 1988; Misaghi & Donndelinger, 1990). However several limitations hinder their rapid development. The selection of endophytes to construct genetically engineered organisms requires a variety of traits necessary for the construction of effective strains. First the endophyte must reside at or deliver the pest-controlling factor to a site within the plant that is accessible to the pest. Second, the endophyte must not have significant crop-threatening disease capabilities. Third, the endophyte must be readily amenable to genetic manipulation. Several additional factors must be considered in the construction of recombinant microbes, including the ability to readily express foreign genes and the long term maintenance of the gene(s) within the endophyte. This is most easily performed by stable integration into the chromosome of the endophyte, but it requires integration of a compatible form of the gene at a site that does not affect the fitness of the bacterium. Once incorporated, the gene product must be expressed in a form that is high enough in concentration and functionally accessible to the pest.

The most intensive study of a recombinant bacterial endophyte constructed for pest control purposes was performed with *Clavibacter xyli* subsp. *cynodontis* expressing the *cryIA* gene from *Bacillus thuringiensis* (Lampel *et al*., 1994; Tomasino *et al*., 1995). In this example, the resultant protein was an endotoxin that is active against the European corn borer. The recombinant bacterium was effective in reducing insect boring in corn in laboratory, greenhouse, and field studies. However, significant increases in overall yield were not detected compared to plants that were not treated with the endophyte.

Another issue that should be studied intensively is the inoculum delivery system. In order to guarantee practical economic production, reliable and practical methods of inoculum delivery should be developed. Intense testing of different delivery systems indicated that the application method for introducing endophytic bacteria into plant tissue is strain specific (Musson *et al*., 1995). Some methods that proved to be successful include the infusion of a bacterial suspension into imbibed seeds (Turner *et al*., 1993) or the bacterial application via alginate beads (Bashan 1986) which have the advantage of adding bacteria-specific nutrients to the alginate to improve bacterial survival rates. It should be noted that the development of successful application technologies will fully depend on improving our understanding of how bacterial endophytes enter and colonise plants. This remark could be applied to all aspects of the ecology of bacterial endophytes and only under those circumstances the potential use of bacterial endophytes for plant beneficial purposes can be fully evaluated.

III HEAVY METAL RESISTANCE IN BACTERIA

III 1. Possible effects of heavy metal resistant bacteria on heavy metal speciation

The resistance mechanisms of bacteria may influence the speciation of heavy metals in the biotope in which they reside. The well studied soil bacterium *R. metallidurans* CH34 affects the concentration of heavy metal ions present in a culture medium (Diels *et al*., 1995) and this phenomenon (due to efflux followed by complexation and precipitation processes near the bacterial cell wall) might be also responsible in

altering the speciation when present in the environment as well. This could have consequences on the bioavailability and toxicity of heavy metals for all the organisms sharing the same environment. This was illustrated by studies in which the composition of microbial communities of a contaminated soil were evaluated during a certain period. One example is the evaluation of the microbial diversity at the Maatheide site in Belgium. Due to the activity of a zinc smelter, the Maatheide area became contaminated with a mixture of heavy metals including zinc, aluminium, lead, cadmium and copper (Diels & Mergeay, 1990; Brim *et al*., 1999). In these soils, zinc was found to be highly abundant with concentrations up to 45000 ppm. It was also the most biologically available heavy metal and the main selection pressure on bacteria colonising this biotope (Vangronsveld *et al*., 2001). Over a 12 years period, samples were taken to evaluate the composition of the bacterial community. At the beginning of this monitoring campaign, the majority of bacteria (40%) were heavy metal resistant *R. metallidurans*-like strains. Samples taken 10 years later revealed that this subgroup made up only 1 to 4 % of the countable population, and had been replaced by other bacteria that showed reduced resistance to heavy metals, including zinc (Brim *et al*., 1999). The progressive decrease in the soil of Zn bioavailability and consequently its toxicity might be attributed to many factors such as natural weathering and attenuation processes including microbial interactions with the bioavailable fraction present in the soil.

Plant growth might also be affected when heavy metal resistant bacteria are abundant in the rhizosphere. Salt and co-workers (1999a) showed that under laboratory conditions cadmium resistant soil bacteria, able to precipitate cadmium at the root surface, reduced the amount of cadmium taken up into the roots of *Brassica juncea* L. thereby protecting the plants from the toxic effects of cadmium. This reduced cadmium toxicity allowed for the increased proliferation of roots with a healthier plant as a result. Similar observations were done when tobacco plants (*Nicotiana bentamiana*) were grown in Cd-polluted soils inoculated with *R. metallidurans* CH34 equipped with the mouse metallothionein I (MT) protein to the cell surface (Valls *et al*., 2000). The enhanced ability of these strains to immobilise Cd^{2+} ions from the external media probably decreased the toxic effects on the growth of the tobacco plants.

One might suspect that endophytic bacteria, equipped with similar heavy metal resistance properties could affect the metal speciation in the same way as the better

studied soil micro-organisms. The ability to colonise their host plant might imply that these endophytic bacteria, able to sequestrate heavy metals on their cell surface, stimulate the plant's uptake capacities as these 'immobilised' harmless metals do not interfere with the plant's metabolism and therefore cannot induce phytotoxic effects.
Different known bacterial heavy metal resistance mechanisms that are mentioned below will be discussed for their properties to test this hypothesis. Additionally a brief, general introduction concerning heavy metal enriched biotopes, bacterial diversity and heavy metal toxicity will explain the need for bacteria to protect themselves against the toxic effects heavy metals might have on their metabolism.

III 2. Habitat and diversity

Bacteria have been interacting with heavy metals since they originated and over geological timescales, they have evolved to occupy ecological niches containing high concentrations of heavy metals. In addition to natural heavy metal rich biotopes, human activities leading to increased atmospheric release as well as deposition into aquatic and terrestrial environments have generated new biotopes with high concentrations of heavy metals. Major sources of anthropogenic heavy metal pollution include mineral mining and processing, nuclear and other industrial effluents, the combustion of fossil fuels as well as the use of biocides and preservatives including the organometallic compounds (Gadd & White, 1993). Bacterial strains isolated from such natural and anthropogenic biotopes (soils from metallurgical industries in Belgium, copper belts in Congo, and serpentine soils in New Calidonia) have been assigned to the Gram negative β-Proteobacteria, which include the *Ralstonia eutropha* like strains (Brim *et al*., 1999; M. Mergeay, personal communication) and γ-Proteobacteria including many *Pseudomonas* strains. Taxonomic determination of these metal resistant *R. eutropha* like strains support the classification of the genus *Ralstonia* as new species *R*. *campinensis*, *R. basilensis*, and *R. metallidurans* (Goris *et al*., 2001).
Heavy metal resistance phenotypes exhibited by both β-and γ groups vary widely in the micromolar to millimolar range but the highest resistance levels appeared to be found in members of the β-group (Taghavi *et al*., 1997a; Wuertz & Mergeay, 1997). The other classes isolated from anthropogenic biotopes include low GC Gram positive

strains, which show heavy metal resistance in the micromolar concentration range, and some high GC content Gram positive bacteria (e.g. *Arthrobacter* (Margesin & Shinner, 1996)) which can exhibit resistance in the low millimolar range. However, the highest heavy metal resistance phenotypes are found in the Archaebacteria and in the *Thiobacillus* genus, which in general comprises acidophilic, obligate chemolithotrophic bacteria isolated from highly polluted sites, where metal concentrations can reach molar levels.

In the case of serpentine biotopes, bacterial inhabitants were able to grow on nickel concentrations up to 20 mM (Stoppel & Schlegel, 1995) and the majority of these Ni resistant strains belonged to the *Burkholderia* genus. Other isolates were *Hafnia*, *Pseudomonas*, *Acinetobacter*, *Comamonas* and *Agrobacterium*.

Heavy metal resistance characteristics mainly seem to be encountered with bacteria isolated from heavy metal enriched soils. Salt *et al* (1999a) conducted a study on the bacterial populations of rhizospheric contaminated (Cd, Pb) and non-contaminated soil and showed that on exposure to Cd, Pb, and Cr(VI), bacteria isolated from contaminated soil had a 12,5 % survival rate whereas only 0.05% of bacteria from the garden soil were found to survive.

The ability to resist high concentrations of bioavailable heavy metals clearly offers an advantage for surviving harsh environmental conditions caused by increased heavy metal concentrations. In these extreme environments, a selective advantage is conferred to those organisms that have adopted resistance mechanisms to withstand the toxic effects caused by high concentrations of bioavailable heavy metals.

III 3. Toxicity

Some heavy metals like Cr, Co, Cu, Mn, Mo, Ni, Zn, and Fe can be considered as micronutrients, which are required in nanomolar concentrations for normal bacterial growth. However, micro-or millimolar concentrations of these essential trace elements are toxic. Others like Al, Ag, Cd, Sn, Au, Sr, Hg, Tl, and Pb, have been reported to have no known essential biological function, but they can also induce toxic effects when present in high concentrations.

Transport of these essential and non-essential elements into the bacterial cell is the first aspect that should be considered. It is known that transport of macronutrients like

phosphate, potassium, and magnesium is chromosomally encoded and meticulously regulated. (for a review see Silver & Walderhaug, 1992). Except for the well studied chromosomally based transport system of Fe, little information is available concerning uptake of other micronutrients. Nies (1992) claimed that under normal conditions, a few constitutively expressed broad host range uptake systems with relative high rates satisfy the needs of bacterial cells for both macronutrients and trace elements. This was illustrated in *Ralstonia metallidurans* CH34 : Zn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} were transported into the cell by the Mg^{2+} uptake system and CrO_4^{2-} was accumulated via the SO_4^{2-} uptake system (Nies & Silver, 1989). Nies (1992) concluded that the uptake of a broad range of metal ions by a few high-capacity and relatively unspecific uptake systems could be considered as an economical solution for most cells and allows the accumulation of trace elements inside the cell for future needs. These uptake systems are constitutively expressed and cannot be shut down in times of heavy metal ion stress. Consequently, heavy metal resistance determinants had to evolve. Many of these resistance determinants seem to be encoded by extra-chromosomal elements, mainly plasmids or mobile genetic elements, and these are generally the best characterised. Extra-chromosomal elements have the advantage that mobilisation of the resistance determinants to other cells (or species) can occur, while additionally the cell is saved by the burden of carrying genes that are sometimes only occasionally needed. Nevertheless, chromosomally encoded resistance mechanisms have been described in literature as well (Rouch *et al*., 1995).

Resistance systems against heavy metals are needed because excess levels cause toxicity. This can be due to the fact that high concentrations of certain cations can competitively inhibit the normal functioning of analogous metals that are essential for cellular metabolism, for example as enzyme cofactors. Another group of metals are even not tolerated at low concentrations because they can interact with cellular compounds and damage them or they can form strong interactions with key proteins in the cell, thereby inhibiting or hyperstimulating their activity. On the level of DNA, DNA-metal complexes can damage the structural integrity which can induce mutations (Silver, 1992; 1996; 1998; Silver & Phung, 1996).

III 4. Heavy metal resistance

For protection against the toxic effects of heavy metals, bacteria can adapt in two strategies: they can survive by means of intrinsic properties including those related to their cell wall structure, extracellular polymeric substances (e.g. exopolysaccharides), and binding or precipitation of metals inside or outside the cell (Gadd, 1992). Alternatively they can develop specific resistance mechanisms for detoxification, which include active efflux mechanisms as well as reduction/oxidation, alkylation/dealkylation, and intracellular compartmentalisation/sequestration (Gadd, 1992). The regulation of these heavy metal mechanisms is extremely important because the homeostatic utilisation of essential heavy metals for normal growth must be ensured. This was confirmed in some regulatory mutant strains of *R. metallidurans* CH34, which constitutively expressed the *cnr* operon and as a result became nickel deficient (Collard *et al*., 1993).
Table I.3 reports on the main mechanisms described for resistance to heavy metals which will be discussed in more detail below.

III 4.1. Heavy metal resistance based on efflux

In order to prevent accumulation of toxic levels of heavy metals in the bacterial cell, mostly plasmid encoded efflux systems seem to be involved in detoxification. There are several types of efflux based mechanisms described. The most intensively studied mechanisms are the three-component cation/proton antiporter systems, which are members of the Major Facilitator Superfamily of transporters (Pao *et al*., 1998), and the P-type efflux ATPases.
The genetical, physiological, and biotechnological study of heavy metal resistance characteristics of the strain *R. metallidurans* CH34, formerly *Alcaligenes eutrophus* CH34 (Brim *et al*., 1999) has highly contributed to the understanding of these mechanisms. Strain CH34 was originally isolated from the metal-rich sediments of a decantation tank from a zinc factory in Belgium (Mergeay *et al*., 1985). In order to survive in metal rich biotopes, CH34 has evolved a number of genetic determinants which encode for resistance to many heavy metals and metal compounds (Mergeay *et al*., 1985, Taghavi *et al*., 1997; van der Lelie, 1998). Two endogenous megaplasmids

CATION	OPERON STRUCTURE	PROTON / CATION ANTIPORTER	ATPASES	CDF	ENZYMATIC REDUCTION	SEQUESTRATION	UNDEFINED EFFLUX	OTHERS	GENOMIC LOCALISATION	References
Ni	cnrYXHCBA	CnrCBA	X	X	X	?	X	X	P	Taghavi *et al*., 1997 Liesegang *et al*., 1993
	nccYXHCBAN	NccCBA	X	X	X	?	X	X	P	Taghavi *et al*., 1997; Schmidt & Schlegel, 1994
	nreAB	X	X	X	X	X	NreAB	X	P/C	Schmidt & Schlegel, 1994 Stoppel *et al*., 1995; Taghavi *et al*., 2001
Zn	czcNICBADRSE	CzcCBA	X	CzcD	X	CzcE	X	X	P	Collard *et al*., 1994;
	czrRSCBA	CzrCBA	X	X	X	X	X	X	C	Hassan *et al*., 1999
	zntRA	X	ZntA	X	X	X	X	X	C	Rensing *et al*., 1997b Brocklehurst *et al*., 1999
	ziaRA	X	ZiaA	X	X	X	X	X	C	Thelwel *et al*., 1998
	cadCA	X	CadA	X	X	X	X	X	P	Silver, 1996; Silver 1998
	smtBA	X	X	X	X	SmtA	X	X	C	Turner & Robinson, 1995
Cd	czcNICBADRSE	CzcCBA	X	CzcD	X	CzcE	X	X	P	Collard *et al*., 1994
	czrRSCBA	CzrCBA	X	X	X	X	X	X	C	Hassan *et al*., 1999
	cadCA	X	CadA	X	X	X	X	X	P	Silver, 1996; Silver 1998
	smtBA	X	X	X	X	SmtA	X	X		Turner & Robinson, 1995
Co	nccYXHCBA	NccCBA	X	X	X	X	X	X	P	Schmidt & Schlegel, 1994 Taghavi *et al*., 1997
	cnrYXHCBA	CnrCBA	X	X	X	?	X	X	P	Liesegang *et al*. 1993 Taghavi *et al*., 1997
	czcNICBADRSE	CzcCBA	X	CzcD	X	CzcE	X	CzcN, I	P	Collard *et al*., 1994
	czrRSCBA	CzrCBA	X	X	X	X	X	X	C	Hassan *et al*., 1999
	coaRA	X	CoaA	X	X	X	X	X	C	Parkhill *et al*., 1993
Ag	silPCBARSE	SilCBA	SilP	X	X	SilE	X	X	C	Gupta *et al*., 1999a
Cu	copSRABCDGFH	X	CopF	X	X	CopH	X	CopABCD, G	P	van der Lelie, 1998
	copABCDRS	X	X	X	X	X	X	CopABCD	P	Brown *et al*., 1993 Silver & Phung, 1996
	pcoABCDRS	X	X	X	X	PcoE	X	PcoABCD	P/C	Brown *et al*., 1993 Silver & Phung, 1996
	copYZAB	X	CopA, CopB	X	X	X	X	X	C	Odermatt *et al*., 1992 Brown *et al*., 1993
Hg	merRTPCAD	X	X	X	MerA	X	X	MerT, P, C	P/C	Hobman & Brown, 1997
	merRTPAD	X		X	MerA	X	X	MerT, P,	P/C	Hobman & Brown, 1997
	merRTPABD	X	X	X	MerA	X	X	MerT, P, B	P/C	Hobman & Brown, 1997
Pb	pbrTRABCD	X	PbrA	X	X	PbrD	X	PbrT, B, C	P	Borremans *et al.,* 2001
	cadCA	X	CadA	X	X	X	X	X	P	Silver, 1996; Silver 1998
Cr	chrDCAB	X	ChrD	X	X	X	ChrA	ChrB, C, D	P	Nies, 2000
	chrAB	X	X	X	?	X	ChrA	ChrB	P	Cervantes *et al*,, 1990
	chrAB	X	X	X	?	X	ChrA	ChrB	C	Wang *et al.*, 1990 Ohtake & Silver, 1994
As	arsRDABC	X	ArsA	X	ArsC	X	ArsB	X	P	Gladysheva et al., 1994 Silver, 1998
(Sb)	arsRBC	X	X	X	ArsC	X	X	X	P/C	Silver, 1998; Ji & Silver, 1992

Tabel I.3: Bacterial resistance mechanisms to heavy metals (P=plasmid, C=chromosome)

pMOL28 (180 kb) (Mergeay *et al*., 1985; Taghavi *et al*., 1997) and pMOL30 (240 kb) (Mergeay *et al*., 1985) contain resistance determinants to combinations of cobalt and nickel (*cnr*), cadmium, zinc and cobalt (*czc*), chromate (*chr*), mercury (*mer*), thallium (*tll*), copper (*cop*), and lead (*pbr*).

III 4.1.1. Three component cation/proton antiporter systems

The model system for three-component cation/proton antiporters is the *czc* system of *R. metallidurans* CH34, which confers resistance to cadmium, zinc and cobalt by pumping out these metals when their intracellular concentrations exceed a certain threshold (Collard *et al*., 1994; Rensing *et al*., 1997a; Nies & Silver, 1989; van der Lelie, 1998; Grosse *et al*., 1999) (Figure I.2). Other examples of 3-component cation/proton antiporters involved in heavy metal resistance include CnrCBA of CH34 (cobalt and nickel efflux) (Senfuss & Schlegel, 1988; Liesegang *et al*., 1993), NccCBA (nickel, cobalt and cadmium) *R. metallidurans* 31A (formerly called *Alcaligenes xylosoxidans* 31A) (Schmidt & Schlegel, 1994), CzrCBA (zinc and cadmium) *Pseudomonas aeruginosa* (Hassan *et al*., 1999), and SilCBA mediating silver resistance in *Salmonella typhimurium* (Gupta *et al*., 1999).

The $CzcAB_2C$ structural resistance proteins form an efflux pump that functions as a chemi-osmotic cation/proton antiporter that transports the divalent cations-zinc, cadmium, and cobalt- across the inner-membrane (Nies, 1995; Nies & Silver, 1995). CzcA, the central component of the system, functions as an inner-membrane transport protein and belongs to the RND (Resistance, Nodulation, Division) superfamily of transporter proteins (Nies & Silver, 1995; Nies, 1995; Goldberg *et al*., 1999). Such exporters transport various metabolites, antibiotics, or drugs to the extracellular space (Saier *et al*., 1994). The translocation of the metal cation is coupled with the concomitant uptake of protons from the cytoplasm into the periplasm (Nies & Silver, 1995; Rensing *et al*., 1997a; Silver, S., 1998; Goldberg *et al*., 1999). The uptake of protons results in an increase of the pH in the extracellular environment and this might contribute to alkalisation which can contribute to the extracellular immobilisation of the extruded metal cations. These phenomena together with the observation of the involvement of carbonates, extracellular polysaccharides and outer membrane proteins are considered to be important post efflux functions, which avoid re-entry of the metal ions in the cell (Diels *et al*., 1995). In this way, bioprecipitation

prevents the cell being energetically exhausted and in the other way is able to change the heavy metal speciation.

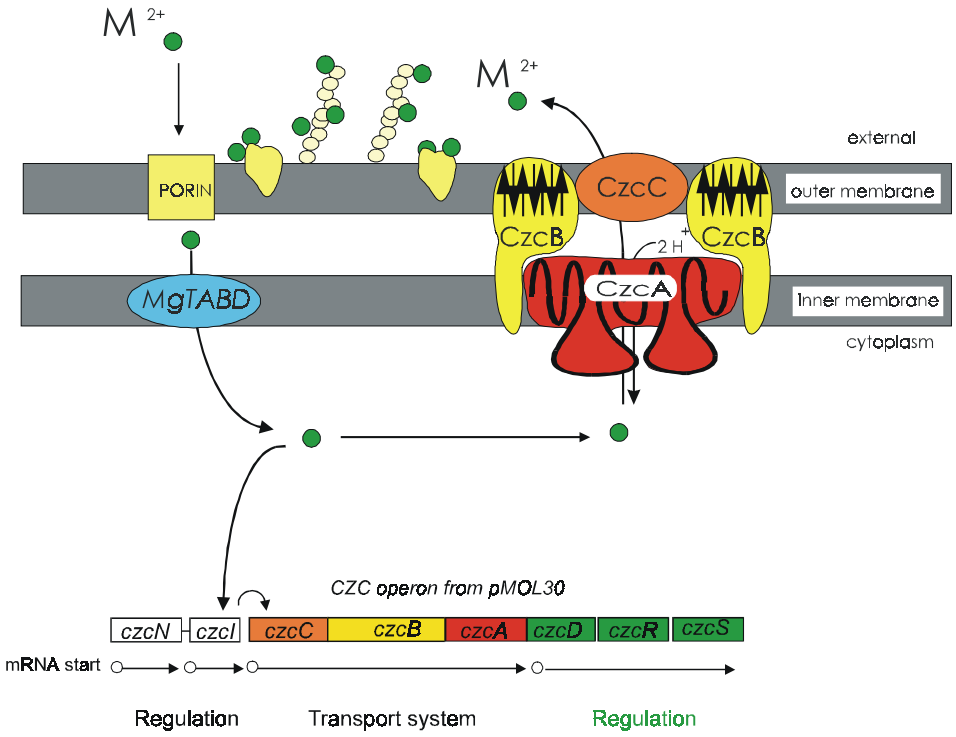

Figure I.2: Schematic presentation of the *czc* efflux system and a working model for heavy metal uptake, processing by efflux, and post efflux metal fixation on polysaccharides and proteins (adapted from Rensing *et al*., 1997b; Tibazarwa, 2000).

This three component cation/proton antiporter system of transport of a substrate differs from the ATP-driven mechanisms of ATP-binding cassettes where the driving force for metal cation transport comes from the translocation coupling of uptake of protons and expulsion of cations into the periplasm. The CzcA, CzrA, NccA, and CnrA proteins are highly homologous and their corresponding genes *czc*A, *czr*A, *ncc*A, and *cnr*A are considered to be the most conserved structural genes (Taghavi *et al*., 1997)

CzcC is thought to function as an outer membrane protein and is required in the *czc* efflux process to complete the efflux of ions in the extracellular medium (Rensing *et al*., 1997a). This protein, which is anchored to the outer membrane and protrudes into the periplasm (Rensing *et al*., 1997a), is a member of the OMF family of Outer Membrane Factors (Dong & Mergeay, 1994; Nies & Silver, 1995; Paulsen *et al*., 1997).

Finally, CzcB, functional as a dimer in the $CzcAB_2C$ complex, appears to function as a membrane fusion protein (MFP) that bridges the inner-and outer-cellmembrane, probably facilitating the export of ions across both membranes without release in the periplasm (Diels *et al*., 1995; Rensing *et al*., 1997a).

As already mentioned, regulation of such a resistance mechanism is necessary in order to guarantee bacterial homeostasis. In the case of the *czc* operon, van der Lelie *et al*. (1997) showed that it is inducible by zinc, cadmium, and to a lesser extent by cobalt. Tibazarwa & van der Lelie (2001) resumed that the main types of regulatory modules controlling cation/proton antiporter based resistance systems are the 2-component responder (R) /sensor (S) units. These are based on autophosphorylation of the sensor membrane spanning protein histidine kinase which activates the responder protein through allosteric conformational changes caused by the former. In the active state the DNA repression by the responder is relieved and transcription of the structural genes can occur. In the inactive state, the responder acts as a DNA-binding repressor preventing transcription. This mechanism allows a highly efficient on-and-off switch for expression of resistance, allowing a fine tuning of the level of induction in proportion to the metal concentration. The *czc* operon, regulated by such a 2 component CzcRS system allows the bacteria to respond optimally in a µM to mM concentration range of Zn^{2+} (Peitzsch *et al*., 1998) : the lower µM threshold allows CH34 to take up zinc freely for growth requirements and the effective working range of the regulatory unit allows the organism to modulate efflux of excess metal in proportion to the extracellular levels. CH34 also makes use of the additional functions of CzcD (and perhaps CzcN and CzcI), which are thought to control the upper limits of transcription of the resistance genes to prevent excessive metal efflux (van der Lelie, 1997; Peitzsch *et al*., 1998).

III 4.1.2. Heavy metal P-type efflux ATPases

ATPases are protein pumps that need the energy derived from ATP hydrolysis to actively transport substrates from one cellular compartment to another.

All known ATPases in heavy metal resistance are membrane spanning, typically integral proteins of the cytoplasmic membrane, and function in the outward (from the cell interior to the exterior) translocation of the metals. The fact that energy is required for the functioning of these systems could possibly explain why many of the

metal ATPases function in the elimination of highly toxic metal cations (Tibazarwa & van der Lelie, 2001).

The CadA of *Staphylococcus aureus* is considered to be the model system for the P-type ATP-ase family (Nucifora *et al*., 1989). Members of this family alter between two conformations called E1 (cation site outside) and E2 (cation side inside) during their catalytic cycle. All E1-E2 transporters are composed of a membrane tunnel and four interacting cytoplasmic domains: the ATP-binding site, the protein kinase site, a transduction domain, and the substrate binding site which is located near the N-terminus of the protein. When the co-substrate ATP is bound to the ATP-binding domain, a phosphate group can be transferred to the phosphate-binding domain and the protein changes into a conformation of high energy by hydrolysing the phosphate bond. This state of high energy is possibly relaxed again by transport of the cation through the membrane (Silver *et al*., 1989). A second gene present in the resistance operon of which *cad*A is a part, is *cad*C, and the CadC protein was identified as a DNA binding transcriptional regulatory protein (Endo & Silver, 1995)

Other members of this subfamily include PbrA (Borremans *et al*., 2001) and CopF (van der Lelie, 1998) from *R. metallidurans* CH34 which function for lead and copper efflux respectively, SilP for silver efflux in *S. typhimurium* (Gupta *et al*., 1999), and ZntA for zinc, cadmium and lead efflux in *E. coli* (Rensing *et al*., 1997b; Rensing *et al*., 1999). It should be mentioned that Gram positive ATPases are generally able to pump out the metal cations directly from the cytoplasm while Gram negative strains require additional protein functions to ensure that the cations are translocated across both the inner- and outer-membranes unidirectionally towards the outside, without leakage into the periplasmic space. Only recently a putative outer membrane lipoprotein PbrB belonging to a new family of transporter assisting resistance (TAR) proteins was described to assist in the detoxification of Pb^{2+} ions (Borremans *et al*., 2001). Additionally, Pb accumulation in the cytoplasm through binding of the PbrD protein, encoded by *pbr*D located downstream of *pbr*TRABC, was observed to take place as an alternative mechanism of efflux to overcome toxic Pb concentrations. This observation might possibly contribute to altering Pb speciation in the direct vicinity of the bacterial cell.

Three main families of regulators are described to take part in the regulation of the ATPase type efflux systems: the MerR family and ArsR subfamilies of transcriptional regulators and the 2-component responder/sensor units. Members of the MerR and the

ArsR protein family are characterised by their metal binding and DNA-binding capacities through which they repress gene transcription in the absence of inducer metal. This repression is abrogated by the specific binding of their cognate cation ligand (Tibazarwa & van der Lelie, 2001).

III 4.2. Enzymatic reduction

Another way to protect itself against the toxic effects of excess levels of heavy metals is through enzymatic conversion from a more toxic to a less toxic form. This mechanism is best known for the detoxification of inorganic and organomercurials but it may also be used for oxidation of As(III) (Santini *et al*., 2000) and reduction of Cr (VI) to less toxic forms (Peitzsch *et al*., 1998).

The best characterised is mercury resistance (*mer*), which is also the single most widespread resistance mechanism. All known bacterial mercury resistances are based on enzymatic reduction of Hg^{2+} to Hg^{0}, which is volatile and can escape from the immediate environment of the bacterial cell. The mechanism involves uptake of Hg^{2+} into the cell interior to avoid its deleterious interaction with cellular components. Consequently the enzymatic reduction takes place in the cytoplasm where the mercuric reductase protein, MerA, is localised. The organisation of the *mer* genes has shown to be well conserved in all Gram negative bacteria with the exception of *Thiobacillus ferrooxidans* (Silver & Walderhaug, 1992). It consists of two regulatory genes, *mer*R located upstream of the structural genes *mer*TPAB and *mer*D situated downstream these genes. The gene products of *mer*T and *mer*P are involved in transport of Hg^{2+} into the cytoplasm where it will be reduced subsequently by the MerA reductase protein (Misra, 1992; Silver & Walderhaug, 1992). The *mer*B gene product corresponds to an organomercurial lyase, which allows the cleavage of the C-Hg bond to release Hg^{2+}.

Regulation seems to be different when Gram negative and Gram positive *mer* resistance systems are compared. Both *mer* systems start with the regulatory gene, *mer*R, whose product is a unique positively-acting activator protein that twists and bends the operator DNA region, allowing RNA polymerase to synthesise mRNA (Misra, 1992; Summers, 1992; O'Halloran, 1993). In the *mer* systems of Gram negative bacteria, *mer*R is transcribed separately and in the opposite direction from the remaining *mer* genes, allowing tighter control than possible with Gram positive

bacteria, where *mer*R is transcriptionally linked to the structural *mer* resistance genes (Silver, 1996). MerD is considered to act as a DNA-binding repressor which sets the upper limits of expression of the *mer* operon (Mukhopadhyay *et al*., 1991).

III 4.3. Intra-and extracellular complexation, sequestration and precipitation

The intrinsic properties of the bacterial cell including those related to the cell wall structure, extracellular polymeric substances (e.g. exopolysaccharides), and binding or precipitation of metals inside or outside the cell (Gadd, 1992) provide alternative possibilities to exclude toxic heavy metals from their cellular metabolism.

An example of extracellular sequestration was observed in *R. metallidurans* CH34. This strain harbours mega-plasmids which encode efflux-based heavy metal resistance systems and extracellular sequestration of the metal cations at the cell surface is considered to be an important post-efflux mechanism which prevents re-entry of the heavy metal into the cell, particularly when the extracellular concentrations are high (Collard *et al*., 1994; Diels *et al*., 1993a; Diels *et al*., 1993b). In cultures of CH34, zinc and cadmium removal resulted in the immobilisation of the metal cations as complexes with carbonates, bicarbonates and hydroxides. These processes were found to be induced by carbon dioxide emitted by the metabolic activity as well as the alkalisation of the extracellular environment resulting from the cation/proton antiporter activity. These metal complexes are precipitated in the form of carbonate crystals at the cell surface (Diels *et al*., 1993a; Diels *et al*., 1993b; Collard *et al*, 1994; Taghavi *et al*., 1997a) as was observed by X-ray diffraction spectroscopy (Diels *et al*., 1995). It is estimated that extracellular polysaccharides and outer membrane proteins have important post efflux functions as well (Diels *et al*., 1995).

As these processes are quite complex and involve many different steps, the underlying mechanisms of control are not well understood.

The storage of excess copper in the periplasmic space has also been reported to be a mechanism to protect the cell from toxic copper. Strong copper resistance has been described with plasmids in *Pseudomonas* (Cooksey, 1994), *Xanthomonas* (Lee *et al*., 1994), and *E. coli* (Brown *et al*., 1994, 1995). The systems are highly homologous (Cooksey, 1993, 1994; Brown *et al*., 1994, 1995) and contains the same genes. For *Pseudomonas*, the two regulatory genes are *cop*R and *cop*S and the four structural genes *cop*ABCD whereas the comparable *E. coli* genes are named *pco*RS and

*pco*ABCD (Brown *et al*., 1994, 1995). Transcriptional regulation is based by a two component sensor/responder regulatory system and the structural genes determine the inner-membrane protein CopD, the outer-membrane protein CopB, and two periplasmic proteins CopA and CopC (Cooksey, 1993, 1994).

Not so abundant but still described for some prokaryotic organisms are the metal binding components in intracellular complexation processes. Cysteine rich proteins named metallothioneins are synthesised under heavy metal stress and have been found in a wide variety of animals and lower eukaryotes (Kojima & Kagi, 1978; Hamer, 1986) and also in plants (de Miranda *et al*., 1990). These proteins bind heavy metals with high affinity, thereby immobilising them within the cytoplasm (Silver, 1998). Plants produce generally also different cysteine rich polypeptide from gluthathione namely phytochelatins (Rauser, 1990).

Apparently some good examples of metallothioneins in bacteria have been described as well. The best characterised is probably the SmtA which affects zinc resistance in *Synechococcus* (Turner & Robinson, 1995). SmtA binds with high affinity to its substrates zinc and cadmium in metal-binding clusters which are organised at the N and C-termini of the protein (Turner & Robinson, 1995). Lead resistance in *Staphylococcus aureus* and *Citrobacter freundii* has also been shown to involve the detoxification of lead cations to a less reactive species and the subsequent intracellular immobilisation of the metal as lead-phosphate complexes (Levinson & Mahler 1998). This prevents the detoxified lead from participating in any cellular activity which could be potentially harmful to the organism and also permits the organism from wasting energy in pumping out the cation. The recently described lead resistance operon of *R. metallidurans* CH34 may also mediate sequestration of the metal, and PbrD, a presumed Pb-binding protein, is believed to be involved in this function (Borremans *et al*., 2001).

CHAPTER II

Aim and outline of the thesis

Phytoextraction is the process where metal accumulating plants are used to transport and concentrate heavy metals from the soil into the shoots in order to reduce soil concentrations of heavy metals to environmentally acceptable levels. It is evident that plants used in these processes need some characteristics in order to efficiently fulfil phytoextraction. The optimum plant should be able to tolerate and accumulate high levels of heavy metals but also have a rapid growth rate and the potential to produce a high biomass in the field. At the moment, extensive research is done at the molecular and physiological level in order to improve plants for phytoextraction processes. The potential influence in phytoextraction of plant associated bacteria, both rhizospheric and endophytic, is less well studied. It is known that some rhizobacteria as well as endophytes exert beneficial effects on their host plant (Kloepper *et al*., 1991a; Höflich *et al*., 1994): through direct plant growth promotion or through the suppression of phytopathogens. Those bacteria could be interesting tools because they live near or in plants and could possibly contribute to plant processes such as uptake and translocation of heavy metals.

In this context, the subjects mentioned below were investigated in order to gain a better understanding of the possible role of the microbial flora associated with potential interesting plants for phytoextraction processes involving heavy metals.

- Chapter III describes the characterisation and identification of the bacterial population isolated from the rhizosphere, the root and the aerial plant parts of the Zn hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*.
- Chapter IV gives a short overview of the endophytic population isolated from roots and shoots of *Brassica napus* growing in a clean garden soil.

- In chapter V, the potential use of a selected Zn resistant endophytic bacterium isolated from the shoots of *B. napus* is evaluated for its potential capacity in improving phytoremediation processes.
- Chapter VI focuses on the construction of mini-transposons with the nickel resistance determinants from *Ralstonia metallidurans* 31A (formerly *Alcaligenes xylosoxidans* 31A) in order to study more precisely the mechanism, the heterologous expression, and the effect of a known heavy metal resistance system in the selected endophytic strains *Burkholderia cepacia* and *Herbaspirillum seropedicae*.
- Chapter VII shows the results of the inoculation of the constructed Ni resistant *B. cepacia* and *H. seropedicae* in their respective host plant *Lupinus luteus* and *Lolium perenne*.
- In chapter VIII, a summarising discussion as well as the perspectives are provided.

CHAPTER III

Isolation, characterisation, and identification of endophytic bacteria from the hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*

To be submitted for publication

ABSTRACT

Bacterial populations associated with the well studied Zn hyperaccumulator Thlaspi caerulescens subsp. calaminaria grown in a soil collected from an abandoned Zn and Pb mining and smelter site in Plombières (Belgium) were investigated. The non-rhizospheric soil representing population consisted out of bacteria equipped with multiple heavy metal resistance characteristics that are often associated with polluted substrates: 7.8% and 4% of the bacterial population was able to survive elevated levels of the major pollutants Zn (1mM) and Cd (0.8mM) respectively when supplemented to the Tris buffered minimal medium. For the rhizosphere representing population, a survival of 88% and 78% on the respective selective heavy metals might imply a difference in metal availability in the vicinity of the root compared to the non-rhizospheric soil. Characterisation and identification of the inhabitants of the roots and shoots demonstrated that although similar species were isolated in both tissues, still some differences could be notified. In the presence of Zn and Cd, rhizoplane and root endophytic isolates showed to have a much lower survival rate under the same conditions and root endophytic bacteria even seemed to have different growth requirements. Additionally, only some bacteria residing in these plant root compartments showed to be able to produce siderophores under iron limiting conditions. Contrary to the root residing inhabitants, the shoot represented a niche rich in metal resistant bacteria and even seemed to contain species that were exclusively abundant in this environment. These differences in the characteristics of the bacterial microflora associated with T. caerulescens might possibly reflect among others altered metal speciation in the different compartments studied.

INTRODUCTION

The inventarisation of plant species from sites all over the world that are enriched with heavy metals (due to geological factors or antropogenic pollution) resulted in the identification of several hyperaccumulator species (Baker & Brooks, 1989; Reeves & Baker, 2000). A plant is considered to be a hyperaccumulator of Co, Cu, Cr, Pb and Ni if it contains more than 1000µg/g dry weight of any of these elements (Baker & Brooks, 1989). For Zn and Mn the concentration should be at least 10000µg/g dry matter in order to define the plant as a hyperaccumulator (Baker & Brooks, 1989). Hyperaccumulator plants have been intensively studied for their capacity of removing the available fraction of heavy metals in soils by concentrating them in roots and shoots. This process is called phytoextraction. However, the use of hyperaccumulators in phytoextraction processes seems to be limited. Although high concentrations of heavy metals can be accumulated in roots and shoots, the usually slow growth rate and low biomass production of naturally occurring hyperaccumulators are limiting factors for their practical applications. Probably the most extensively studied hyperaccumulator plant species in this context is *Thlaspi caerulescens* (*Brassicaceae*) which is able to hyperaccumulate Zn (up to 3%) (Brown *et al*., 1995) and up to 0.1% Cd without exhibiting symptoms of toxicity. However, due to its slow growth rates and limited biomass production, its potential use for large scale decontamination of field soils is not realistic (Black, 1995).

Despite many attempts to improve plants for phytoremediation, to our knowledge only few studies were performed addressing the potential contribution of plant associated bacteria in these processes. They mainly focus on the plant growth promoting effect of rhizospheric bacteria on their host plant. It was hypothesised that certain bacteria stimulate plant growth through the enzyme 1-amino-cyclopropane-1-carboxylate (ACC) deaminase (Glick *et al*., 1998). Since ACC deaminase is able to cleave the plant ethylene precursor ACC, the level of ethylene in a developing or stressed plant should be lowered resulting in improved root formation and reduced transport of ACC from stressed roots to the shoots. The bacterium *Kluyvera ascorbata* SUD165, isolated from a heavy metal contaminated wetland and able to express ACC deaminase, showed to be able to relieve a part of the growth inhibition of tomato, canola and Indian mustard plants caused by phytotoxic concentrations of Ni, Pb, or Zn

(Burd *et al*., 2000). In this respect, such plant growth promoting bacteria might be potential candidates to contribute in the improvement of plant biomass production in phytoremediation processes of heavy metal polluted soils. Another approach is based on bacterial heavy metal resistance mechanisms. Known bacterial heavy metal resistance systems like the *czc* system of *Ralstonia metallidurans* CH34 (previously *Alcaligenes eutrophus* CH34) were shown to be responsible for the alteration of the Zn and Cd speciation in growth cultures due to precipitation processes near the bacterial cell wall. (Diels *et al*., 1995). In addition, metal-chelating agents like siderophores also have been demonstrated to affect the bioavailability of Cd in *R. metallidurans* CH34 (Gilis *et al*., 1996). A reporter gene system designated to determine the level of biologically available Cd demonstrated that Cd availability decreased in the presence of the siderophore alcaligin E (Gilis *et al*., 1998). This might suggest that alcaligin E, besides its function as an iron transport vehicle for *R. metallidurans* CH34, could also provide protection against heavy metal toxicity (van der Lelie *et al*., 1999).

Not only the bacteria might be protected by these properties, but probably also the environment in which they reside. Observations done with Cd resistant rhizobacteria (Salt *et al*., 1999a) illustrate this hypothesis. Inoculation of Indian mustard plants with these rhizobacteria resulted in the precipitation of Cd on the root surface. This altered Cd speciation reduced the toxicity and allowed for an increased proliferation of the roots.

As hyperaccumulators such as *T. caerulescens* create specific plant associated environments due to their exceptional capacities to concentrate high levels of heavy metals in their shoots (McGrath *et al*., 1997; Lasat *et al*., 1996; 1998), the associated bacterial microflora might possess similar characteristics as observed for soil bacteria endogenous for heavy metal contaminated soils. The ability of bacterial populations associated with *T. caerulescens* to adapt to the existing conditions near and in this Zn hyperaccumulator were investigated in the roots as well as in the shoots. More precisely, the rhizosphere and phyllosphere populations as well as the endophytic root and shoot populations were characterised and identified. Emphasis was on heavy metal resistance and siderophore production since both characteristics have already been described to potentially affect heay metal bioavailability (Diels *et al*., 1995; van der Lelie *et al*., 1999). This information together with colonisation frequencies should

allow to determine to what extent these bacteria possibly interact with their host in altering heavy metal speciation.

MATERIALS AND METHODS

Plant growth conditions

Thlaspi caerulescens subsp. *calaminaria* seeds were collected on the site of an abandoned Zn and Pb mining and smelter site in Plombières (Belgium) in june 1997. The soil is severely contaminated with Zn and Pb but also Cd, Cu, and Ni levels are increased (table III.1). *T. caerulescens* is populating this area together with other metal tolerant plant species like *Viola calaminaria*, *Armeria maritima*, *Silene cucubalis*, *Agrostis capillaris* and *Festuca ovina*. Seeds as well as soil samples were transported to the laboratory in sterile plastic bags. After a cold treatment for 24 hours at –20°C to break dormancy and a subsequent imbibition in tap water, seeds were sown in polyethylene pots (400ml) filled with soil collected in Plombières, in an area were *T. caerulescens* grew spontaneously. During the 2 months growing period, the following greenhouse conditions were maintained: a 12 hours light and dark period, 65% relative humidity, and a constant temperature of 22°C. Regularly, the plants were watered with tap water.

Soil metal analysis

Total (aqua regia) and $Ca(NO_3)_2$ heavy metal extractions were performed on the soil at the start of the experiment and after harvest of the *T. caerulescens* plants. To do so, an aliquot of the soil was dried at 65°C for 24 hours and subsequently sieved prior to extraction. Total metal contents were determined by means of Induced coupled Plasma Spectroscopy (ICP) after a HCl/HNO_3 digestion. $Ca(NO_3)_2$ extractions were done by shaking the soil (10g) for 2 hours in a 0.1N $Ca(NO_3)_2$ solution followed by filtration over an ashfree filter (Whatmann nr 40) to release a filtrate analysed by means of ICP (Boisson *et al*., 1999)

Selection of plant associated bacteria

After 2 months growth of *T. caerulescens* subsp. *calaminaria* as mentioned above, roots and shoots were separated by means of a sterile pair of scissors and

subsequently the microbial microflora inhabiting the different plant niches were collected.

Selection of rhizosphere and phyllosphere bacteria

For the isolation of rhizosphere bacteria, the roots were washed in sterile H_2O for 5' in order to remove the bacteria associated with the rhizosphere. Dilutions were made in sterile H_2O and plated on Tris-buffered low-phosphate medium supplemented with a mixture of lactate, glucose, gluconate, fructose, and succinate (3mM each). For the selection of the Zn and Cd resistant populations, 1mM and 2mM of a sterile $Zn(SO_4)_2$ solution as well as 0.8mM and 1mM of a sterile $CdCl_2$ solution were added to the medium as described by Mergeay *et al* (1985). Ten times diluted Luria Broth (LB) medium was also used to compare the total amount of viable counts without any heavy metal resistance selection marker with the colony forming units growing on the minimal Tris buffered medium. The plates were incubated at 30°C for 7 days prior to the estimation of the viable counts.

Selection of endophytic bacteria

Endophytic bacteria were isolated from the roots and shoots by macerating the washed tissues in a 10^{-2}M $MgSO_4$ solution with an ultra-turrax mixer (Janke-Kunkel; IKA Labor technik) after a 5' surface sterilisation in a commercial NaOCl solution (Dumortier, Merksem) with 1% active chloride and one droplet (per 100 ml) of Triton X-100. Both roots and shoots were rinsed thoroughly in sterile H_2O for 5' and subsequently dilutions of the macerated suspension were plated on the same media as mentioned for the isolation of rhizosphere bacteria. After 7 days incubation at 30°C, colony forming units were determined and purifications of the different morphological types were performed three times before the strains were further characterised.

Selection of soil bacteria

From the non-rhizospheric soil, 2 g was added to a 18 ml sterile 10^{-2}M $MgSO_4$ solution and shaken for 2 hours at 250 rpm at 4°C. Subsequently, the solution was left for 1 hour in order to allow the soil particles to sink to the bottom before the upper phase was used for the determination of the soil bacterial population (Mergeay, 1995). The same growth media and incubation time were used as mentioned above.

Characterisation tests

Heavy metal resistance

The strains growing on $LB_{1/10}$ and the minimal Tris buffered medium were further characterised for their ability to resist elevated levels of Cd, Co, Cu, Ni, Pb, and Zn supplemented as sterile $CdCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$, $Zn(SO_4)_2$ and $Pb(NO_3)_2$ to the minimal Tris buffered medium containing the same carbon mixture as mentioned before. The Minimum Inhibitory Concentration (MIC) was determined and defined as the minimal concentration of the heavy metal inhibiting growth of the bacterial strains.

Siderophore assay

A chrome azurol S (CAS) shuttle solution (Schwyn & Neilands, 1987) was used for routine testing of siderophore production in liquid media.

16S rRNA amplification

A single purified colony from each of the strains to be identified was dissolved in 20µl of sterile H_2O and aliquots were used for 16S rRNA gene amplification from the genomic DNA by PCR, using primers designed to anneal to conserved positions in the 3' and 5' regions of bacterial 16S rDNA. The forward primer 27F (5'CTCGCTCGCCCAGAGTTTGATCMTGGCTCAG) and the reverse primer 1492R (5'CTGGTTCGGCCCACGGYTACCTTGTTACGACTT) (Weisburg *et al*., 1991) encompassing nearly the complete 16S rRNA gene sequence, were used to amplify the 16S rRNA genes.

The PCR reaction was completed in 100µl using Platinum *Taq* polymerase (Gibco BRL) according to the manufacturer's instructions. Platinum *Taq* polymerase was used to provide an automatic "hot start" for *Taq* DNA polymerase in order to reduce or eliminate non-specific amplification during the initial temperature cycle. Amplification was carried out as follows: a preliminary denaturation step was done at 95°C for 3min, followed by 35 cycli of 1min at 95°C, 1min at 60°C, and 3min at 72°C. Negative controls were systematically included and PCR products were checked by electrophoresis in 0.8% agarose gels.

Cycle sequencing.

The 1465 bp 16S rRNA PCR fragment was purified by means of the Concert Rapid PCR Purification System as described by the manufacturer (Gibco BRL-Life Technologies) and used as a template for cycle sequencing with a 685R fluorescein

(F) labeled primer (5'FTCTACGCATTTCACCGCTAC3') annealing to a conserved position of the 16S rRNA. The PCR reaction was performed in 12µl using Thermo Sequenase DNA polymerase as described by the manufacturer (Amersham Pharmacia Biotech). Thermo Sequenase is a new thermostable DNA polymerase specifically engineered for DNA sequencing. Amplification was as follows: a denaturation step was done for 5min at 95°C, followed by 30 cycli of 30 sec at 98°C, 1min at 50°C, and 30sec at 72°C.

Then, DNA was precipitated and washed with ethanol (70%). After drying, it was dissolved in 5µl of formamide loading buffer (formamide, EDTA, and fuchsin; Amersham Pharmacia Biotech), heated for 3 min at 90°C in order to have denaturation and loaded on the Automated Laser Fluorescent A.L.F. DNA sequencer (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The sequences were analysed using the BLAST (Sequence Similarity Searching) database of the NCBI (National Center for Biotechnology Information).

RESULTS

Figure III.1: *Thlaspi caerulescens* subsp. *calaminaria*

Thlaspi caerulescens.

Thlaspi caerulescens (Figure III.1) was grown for 2 months under controlled greenhouse conditions in soil that was taken from the site of Plombières. Total and $Ca(NO_3)_2$ extractions at the start of the experiment (T=0) and after the growth of the *Thlaspi caerulescens* plants (T=60 days) (Table III.1) revealed that the predominant pollutants appeared to be Zn, Cd and Pb which were deposited mainly as a consequence of the former mining and smelting industrial activities. Table III.1 shows that Zn is abundant in the highest concentration in both the total and the exchangeable soil fraction. High levels of total Pb were also measured but $Ca(NO_3)_2$ extractions revealed that only a small part (0.2%) of the total amount is exchangeable. In addition, Cu and Ni levels also seem to be mainly abundant in the biologically unavailable soil fractions. A different

situation was encountered for Cd: 10% of the total Cd concentration measured is from exchangeable pools.

After growth of *T. caerulescens*, the exchangeable fraction of mainly Zn was significantly decreased (Table III.1) which could be considered to be due to the capacity of this Zn hyperaccumulator to remove part of this metal from the soil. Indeed, with a measured Zn leaf accumulating capacity of 1.25% for these plants, the total shoot biomass produced under the conditions described is in fact able to store the amount of Zn that was observed to be removed from the exchangeable Zn pool of the Plombières soil. Limited reductions in the exchangeable fractions of Cd, Pb, and Ni were also found.

	Zn (mg/kg)	Cd (mg/kg)	Cu (mg/kg)	Pb (mg/kg)	Ni (mg/kg)
Total (aqua regia) extraction					
T=0	18450 ± 954	138 ± 6	211 ± 9	4451 ± 302	77 ± 1
T=60 days	17517 ± 1115	134 ± 7	194 ± 10	5240 ± 257	75 ± 3
$Ca(NO_3)_2$ extraction					
T=0	634 ± 12	13 ± 1	0.5 ± 0.1	7.8 ± 0.3	1.6 ± 0.1
T=60 days	466 ± 39	12 ± 0.8	0.5 + 0.1	6.03 ± 0.3	1.6 ± 0.1

Table III.1: Total (aqua regia) and $Ca(NO_3)_2$ concentrations ± SD of Zn, Cd, Cu, Pb, and Ni (mg/kg) before and after growth of *T. caerulescens* on soil from Plombières.

Bacterial extractions

To obtain an idea of the bacterial microflora associated with this Zn hyperaccumulator, root and shoot tissue were used to isolate endophytic bacteria that reside in the plant. In addition a comparison was made between the populations that could be recovered from macerated root and shoot tissue that was either surface sterilised or not. Additionally, the amount of bacteria in the non-rhizospheric soil and the distribution of their heavy metal resistance characteristics were also determined.

Table III.2 gives an overview of the bacterial countings (CFU/gFW) on the different media used. Similar amounts of bacteria were counted on the non-selective diluted rich medium LB and the Tris-buffered minimal 284 medium supplemented with a carbon mix, except when macerated surface sterilised root tissue was plated. Internal root colonists apparently have more specific requirements that allow them to grow on the diluted rich LB medium but abolish growth on the selected minimal medium. The

latter implies that these isolates could not be evaluated for their specific heavy metal resistance.

Surface sterilisation of roots and shoots eliminates a part of the bacterial population; this is reflected in the lower amounts of strains that were counted on the selected media. Focusing on the proportion of Zn and Cd resistant strains learns that the shoots contain a higher percentage of Zn resistant bacteria than the roots and even the non-rhizospheric soil. From the total population extracted from non-surface sterilised and surface sterilised shoot macerates, respectively 39% and 41% were shown to be Zn resistant whereas the non-surface sterilised roots only contained 0.6% of Zn resistant bacteria. The number of the Zn resistant population in non-rhizospheric soil was counted to be 7.8% of the total extractable population.

The amount of bacteria able to withstand 2 mM Zn is the highest in the surface sterilised shoot macerates (23.5%), followed by the non-rhizospheric soil isolates (3.4%) and the non-surface sterilised shoot extracts (0.45%).

Cd resistance seems to be less abundant in the shoot isolates when compared with the Zn resistance properties. Less then 1% of the bacteria isolated from the non-surface sterilised shoot macerates are able to survive Cd levels of 0.8 and 1 mM supplemented to the Tris buffered minimal medium while respectively 9 and 8 % internal shoot colonists resist these concentrations. The root population contains only 0.03% survivors on 0.8 mM Cd and no survivors if Cd 1 mM is used as a selective metal. Non-rhizospheric soil harbours 4% and 2 % Cd resistant inhabitants when plated on the respective growth media.

Media	Shoot (no surface sterilisation)	Shoot (surface sterilisation)	Root (no surface sterilisation)	Root (surface sterilisation)	soil
LB	9.4×10^6	5.9×10^3	1.6×10^7	7.5×10^4	5.6×10^5
284 C-mix	6.9×10^6	1.7×10^4	9×10^6	0	4.1×10^5
284 C-mix + Zn 1mM	2.7×10^6	7×10^3	5×10^4	0	3.2×10^4
284 C-mix + Zn 2mM	3.1×10^4	4×10^3	0	0	1.4×10^4
284 C-mix + Cd 0.8mM	2.1×10^4	1.3×10^3	2.6×10^3	0	1.8×10^4
284 C-mix + Cd 1mM	2.8×10^4	1.5×10^3	0	0	9×10^3

Table III.2: Colony Forming Units (CFU)/gFW on the different selected growth media of the Plombières soil and the macerated root and shoot tissue of *T. caerulescens* that were either or not surface sterilised.

Additional information concerning the distribution of these resistance characteristics is supplied by the results presented in table III.3. Prior to extracting the root and shoot tissue, a washing procedure in sterile H_2O was performed. Plating this wash medium on the selected growth media resulted in the growth of the bacterial population that is loosely attached to the *T. caerulescens* root (= rhizosphere bacteria) and shoot tissue (= phyllosphere bacteria). The proportion of Zn resistant bacteria of the phyllosphere population (40%) is in agreement with the value obtained from the non-surface sterilised shoot macerates except when the survivors on the minimal growth medium supplemented with 2 mM Zn are counted. No bacteria isolated from the phyllosphere were able to grow on this Zn concentration. Cd resistance of phyllosphere bacteria seems to be more pronounced since 15% and 3% could grow on the media containing 0.8 mM and 1 mM Cd respectively.

In contrast to the low percentage of Zn and Cd resistant representative strains isolated from the roots of *T. caerulescens*, a high amount of the bacteria recovered from the rhizosphere were found to be able to grow on the selected Zn and Cd concentrations added to the growth medium. More then 88% of this rhizosphere population is equipped with Zn resistance mechanisms while 77% also survived the toxic Cd concentrations used.

Media	Shoot wash medium: phyllosphere bacteria	Root wash medium: rhizosphere bacteria
LB	3×10^5	5.2×10^5
284 C-mix	5×10^4	2.4×10^5
284 C-mix + Zn 1 mM	2×10^4	2.1×10^5
284 C-mix + Zn 2 mM	0	2×10^5
284 C-mix + Cd 0.8 mM	7.6×10^3	1.7×10^5
284 C-mix + Cd 1 mM	1.3×10^3	4×10^4

Table III.3: Colony Forming Units (CFU)/ml recovered from the wash medium in which roots and shoots of *T. caerulescens* were washed prior to extraction and surface sterilisation procedures.

Subpopulations

From the bacterial populations associated with *T. caerulescens* growing on a contaminated soil, a first rough estimate of the different subpopulations was performed using the morphological colour as a selection criterion. Figure III.2 shows the % levels of the yellow, orange, pink, and white Colony Forming Units that were

observed in the different niches studied. As these different morphological colours absolutely do not give any information concerning the bacterial species, they just provide some interesting preliminary indications of their distribution in the different plant compartments. At first glance, the shoot and root isolates can be differentiated by their bacterial content. The shoot seems to contain morphological species that have not been detected in the root, nor in the non-rhizospheric soil population. Pink colonies were only recovered from the non-surface and surface sterilised shoot macerates suggesting that they only reside in close association with the shoots of *T. caerulescens*. Yellow and white morphological species were detected in every niche of the plant that was investigated in this study. Identifications by means of 16S rRNA cycle sequencing must allow to verify if these represent similar bacterial populations.

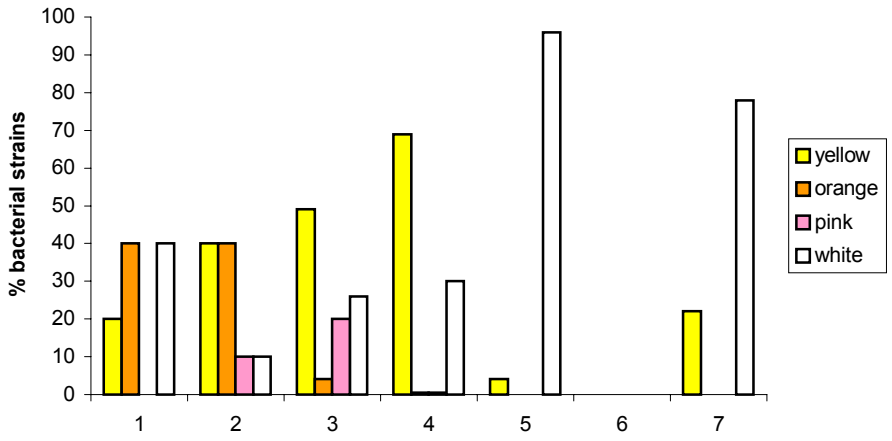

Figure III.2: % levels of the different subpopulations counted on the (Tris buffered) minimal medium (without addition of Zn or Cd) and grouped by means of their colour. (1) the population recovered from the shoot wash medium; (2) the population recovered from the non-surface sterilised shoot macerates; (3) the population recovered from the surface sterilised shoot macerates; (4) the population recovered from the root wash medium; (5) the population recovered from the non-surface sterilised root macerates; (6) the population recovered from the surface sterilised root macerates; (7) the population recovered from the non-rhizospheric soil.

Identification

The bacteria, which were recovered from the non-selective diluted rich medium LB and the Tris buffered minimal medium supplemented with a selected carbon mixture, were purified and subsequently tested for their ability to grow in the presence of the metal cations Zn, Cd, Co, Ni, Pb, and Cu supplemented to the minimal growth medium. MIC values were determined by growing the bacteria in the presence of these increasing metal concentrations. Subsequently, identification of these strains

was done by means of a cycle sequencing procedure in which conserved regions of the 16S rRNA were sequenced and analysed. Table III.4 summarises the results obtained from representative bacteria inhabiting the shoots, the roots and the non-rhizospheric soil. It should be mentioned that it was not always possible to determine the MIC for the selected metals due to the inability of some strains to grow on the minimal medium selected for this purpose. Although the diluted rich medium LB actually enabled most of these strains to grow, it was not further used for determination of metal resistance since the different composition would influence the final MIC values (due to a higher heavy metal complexing activity) and consequently would interfere with the interpretations.

From those bacteria that could grow on minimal medium, MIC values revealed the high frequency of Zn resistance among the isolates from the non-rhizospheric soil as well as those isolated from the roots and shoots of *T. caerulescens*. At the same time, MIC values for Cd indicated that Zn resistant bacteria often are also capable of tolerating elevated levels of Cd, notwithstanding the fact that these characteristics are not exclusively coupled. Especially for the shoot isolates, the abundance of the combined high Zn resistance levels and low Cd resistance levels appeared to be as frequent as multiple heavy metal resistance within the species characterised.

Co and Ni resistance also occurred among the populations isolated. Except for one isolate, increased levels of resistance against these metals were always linked to increased Zn resistance. As for Zn and Cd resistance, Co and Ni resistance were not exclusively linked, but were often found to be increased within the same strain. Resistance to Pb and Cu doesn't seem to be an essential prerequisite for these populations to survive. Only two shoot isolates were able to tolerate up to 1 mM of Cu added to the growth medium while the majority of strains had MIC values below 0.6 mM Cu, as was also the case for Pb.

Apart from the heavy metal resistance characteristics, the production of siderophores is another important feature to be considered. These metal chelating agents have affinity to bivalent heavy metal ions and might affect the bioavailability. The ability to produce siderophores was investigated and the results shown in table III.4 indicate that the majority of the isolated bacterial strains was unable to grow in the absence of iron. In the case growth was observed, the CAS solution was added and revealed that only some bacteria isolated from the non-surface sterilised root suspension and the

non-rhizospheric soil gave a positive result. No endophytic root nor shoot isolates apparently were equipped with this characteristic.

Table III.4 also gives an overview of the isolated species and their corresponding identities. Obviously, inhabitants of *T. caerulescens* and the non-rhizospheric soil in which the seeds germinated, mainly belong to the Alfa and Beta Proteobacteria. Although similar species could be identified in the different niches studied, a clear distinction could be made between the non-rhizospheric soil isolates and those able to colonise *T. caerulescens* roots and shoots. *Sphingomonas* species were observed to be able to colonise soil as well as the roots and shoots. For the majority of these strains, we were unable to define the MIC of the selected heavy metals suggesting specific growth requirements. Verification of the morphological colour proved those strains to be yellow and this observation confirmed the presumption that these species are quite common in every niche studied (Figure III.2). Common soil bacteria like *Arthrobacter* and *Acidovorax* were repeatedly identified in the non-rhizospheric soil sample but were not found in the shoot and root associated species representing the population associated with *T. caerulescens*. Although the root system can be considered to be in close contact with its surrounding soil, the bacterial population associated with it was found to harbour bacteria that were not found in the soil. Known plant associated bacteria like *Afipia* and *Rhodococcus* also seem to be abundant in the vicinity of the Zn hyperaccumulator growing in a highly contaminated soil. It is impossible to estimate to which extent they colonise their host but their presence might indicate that even in such highly contaminated environments, plant associated bacteria seem to survive in some way.

All the inhabitants of the shoot, except *Sphingobacterium multivorum* and *Nocardioides* sp., belong to the Alfa group of Proteobacteria. Among these, *Afipia* species (Rhizobiaceae), which was also traced in the root system, seemed to be also able to reach the shoot and establish itself there. Other species like the pink *Methylobacterium* were found to be exclusively shoot colonisers since they were only present in the shoot macerates, either or not surface sterilised. These results are in agreement with figure III.2 where pink Colony Forming Units were exclusively isolated from the shoot. Each *Methylobacterium* species identified proved to possess high resistance against the tested heavy metals Zn, Cd, Co, and Ni. Those strains that were isolated from the surface sterilised shoot tissue were also able to tolerate Cu levels up to 1 mM.

It should be mentioned that in some cases, the bacterial species couldn't be identified and this was mainly due to their inability to grow in liquid culture media.

	Siderophore assay	**Heavy metal resistance**						**Strain Identification (16S rRNA)**
		Zn (mM)	**Cd (mM)**	**Co (mM)**	**Ni (mM)**	**Pb (mM)**	**Cu (mM)**	
Shoot isolates (no surface sterilisation)								
13	ND	ND	ND	ND	ND	ND	ND	*Sphingomonas asaccharolytica*
14	-	ND	ND	ND	ND	ND	ND	*Afipia* sp.
17	ND	>5	<0.6	2	1	<0.6	0.8-1	*Sphingomonas* sp.
18	ND	>5	>2	>2	2	<0.6	2	*Methylobacterium* sp.
19	ND	>5	2	>2	2	0.8	<0.6	*Aureobacterium esteraromaticum*
20	ND	ND	ND	ND	ND	ND	ND	*Sphingomonas* sp.
21	-	2	<0.6	<0.6	<0.6	<0.6	<0.6	*Nocardioides*
22	-	>5	>2	>2	2	0.8	2	*Methylobacterium radiotolerans*
23	-	5	<0.6	<0.6	<0.6	<0.6	<0.	*Sphingomonas* sp.
24	-	5	<0.6	>2	<0.6	<0.6	1	*Sphingomonas adhaesiva*
Shoot isolates (surface sterilisation)								
25	ND	2	<0.6	<0.6	<0.6	<0.6	<0.6	*Sphingomonas* sp.
26	-	>5	2	>2	<0.6	<0.6	<0.6	*Methylobacterium* sp.
27	ND	2	<0.6	<0.6	1	<0.6	<0.6	*Afipia*
28	ND	<0.8	<0.6	<0.6	0.8	<0.6	<0.6	*Sphingomonas* sp.
29	-	5	2	>2	>2	0.8	<0.6	*Methylobacterium* sp.
30	ND	ND	ND	ND	ND	ND	ND	*Sphingomonas* sp.
31	ND	ND	ND	ND	ND	ND	ND	*Sphingobacterium multivorum*
Root isolates (no surface sterilisation)								
82	-	2	<0.6	<0.6	<0.6	<0.6	<0.6	*Sphingomonas* sp.
83	+/-	<0.8	<0.6	<0.6	<0.6	<0.6	<0.6	*Matsuebacter chitosanotabidus*
87	+	<0.8	<0.6	<0.6	<0.6	<0.6	<0.6	*Matsuebacter chitosanotabidus*
88	-	5	2	<0.6	0.8	<0.6	<0.6	*Variovorax*
89	-	>5	2	<0.6	1	0.8	<0.6	*Variovorax*
90	+	2	2	<0.6	0.8	<0.6	<0.6	ND
Root isolates (surface sterilisation)								
106	ND	ND	ND	ND	ND	ND	ND	ND
107	ND	<0.8	0.8	<0.6	0.8	0.8	<0.6	*Phyllobacterium* sp.
108	ND	ND	ND	ND	ND	ND	ND	New species, related or belonging to the genus *Devosia*
109	ND	ND	ND	ND	ND	ND	ND	New species, related or belonging to the genus *Devosia*
110	ND	ND	ND	ND	ND	ND	ND	*Afipia* sp.
113	-	>5	2	<0.6	<0.6	<0.6	<0.6	*Sphingomonas* sp.
114	-	ND	ND	ND	ND	ND	ND	*Sphingomonas* sp.
115	-	<0.8	<0.6	0.8	>2	<0.6	<0.6	*Rhodococcus* sp.

	Siderophore assay	Heavy metal resistance						Strain Identification (16S rRNA)
		Zn (mM)	Cd (mM)	Co (mM)	Ni (mM)	Pb (mM)	Cu (mM)	
Soil isolates								
41	ND	5	2	>2	2	0.8	<0.6	*Arthrobacter ramosus*
42	ND	5	2	>2	1	0.8	<0.6	*Aquaspirillum delicatum*
44	+	2	<0.6	<0.6	>2	<0.6	<0.6	*Renibacterium salmoninarum*
45	ND	5	2	>2	2	0.8	<0.6	*Acidovorax* sp.
46	ND	ND	ND	ND	ND	ND	ND	*Sphingomonas* sp.
64	ND	<0.8	1	<0.6	0.8	0.8	<0.6	ND
65	ND	1	<0.6	<0.6	<0.6	<0.6	<0.6	ND
67	-	2	<0.6	0.8	>2	<0.6	<0.6	*Rhodococcus* sp.
69	-	>5	2	<0.6	1	<0.6	<0.6	ND
70	-	ND	ND	ND	ND	ND	ND	*Sphingomonas* sp.
126	-	>5	2	<0.6	>2	<0.6	<0.6	*Arthrobacter ramosus*
128	-	ND	ND	ND	ND	ND	ND	*Acidovorax* sp.
130	ND	ND	ND	ND	ND	ND	ND	*Acidovorax* sp.
131	ND	<0.8	<0.6	<0.6	<0.6	<0.6	<0.6	ND
132	ND	<0.8	<0.6	<0.6	<0.6	<0.6	<0.6	ND
133	ND	2	<0.6	<0.6	0.8	<0.6	<0.6	*Sphingomonas terrae*
138	ND	ND	ND	ND	ND	ND	ND	*Sphingomonas asaccharolytica*
139	-	<0.8	<0.6	<0.6	<0.6	<0.6	<0.6	*Acidovorax* sp.
141	ND	ND	ND	ND	ND	ND	ND	*Sphingomonas* sp

Table III.4: Minimum Inhibitory Concentrations of Zn, Cd, Co, Ni, Pb, and Cu (in mM) supplemented to a Tris minimal growth medium of the purified bacteria isolated from the either or not surface sterilised roots and shoots of *T. caerulescens* and those isolated from the non-rhizospheric soil. Siderophore production was determined by means of a CAS solution added to a well grown liquid bacterial culture in the absence of iron. Strain identifications using 16S rRNA cycle sequencing provide the closest match by comparison with the BLAST database of the NCBI. ND: not determined

DISCUSSION

The phenomenon of hyperaccumulation of heavy metals by some terrestrial plants has already intrigued many researchers. Although heavy metal mobility, transport, requirement, tolerance and detoxification in plants were intensively studied, fundamental aspects and answers regarding the hyperaccumulation mechanisms still remain to be elucidated. As hyperaccumulators are able to concentrate heavy metals in their shoots to levels far exceeding those present in the surrounding soil or in non-accumulating plant species nearby, they create specific plant associated environments correlated with these exceptional capacities. These environments might include the

rhizosphere, the roots as well as the shoots which may represent a habitat for fungal and bacterial organisms. The bacterial population associated with the well studied hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria* was investigated in order to learn about the possible interactions between those micro-organisms and their host plant. For this reason *T. caerulescens* was grown under controlled greenhouse conditions on a contaminated soil of an abandoned Zn and Pb mining and smelter site in Plombières (Belgium). Specific attention was given to the endophytic population inhabiting the plant's internal root and shoot tissues since it is generally known that healthy plants have a well-regulated associative endophytic microflora (Di Fiori & Del Gallo, 1995) that might exert beneficial effects on its host plant.

Plants were grown for 2 months on a metal contaminated soil originating from Plombières. At the end of this growth period, $Ca(NO_3)_2$ extractable Zn levels in the soil were reduced suggesting the interaction of the plant's Zn uptake capacities with the exchangeable fraction of the metal (Table III.1). Although this reduction might contribute to the soils' detoxification, it should be noticed that a decrease of the exchangeable Zn in the soil might induce more Zn from less accessible pools to dissociate.

Bacterial countings of the soil after harvest of the *T. caerulescens* plants showed the soil to harbour a typical bacterial population of a contaminated soil (Brim *et al*., 1999; Salt *et al*. 1999a). The amount of Zn resistant isolates was comparable to that counted by Brim *et al*. (1999) in a soil with Zn being the predominant pollutant; identification revealed these bacteria to be soil bacteria often associated with polluted substrates. Resistance to Cd, Co, and Ni were also frequently detected and could be the result of multiple metal resistance mechanisms, such as the plasmid encoded *czc* (Zn^r, Cd^r, Co^r) or *cnr* (Co^r, Ni^r) system of *Ralstonia metallidurans* CH34 isolated from an industrial biotope (Taghavi *et al*., 1997; Mergeay, 2000) or the *czr* (Zn^r, Cd^r) resistance system of *Pseudomonas aeruginosa* CMG103 isolated from a Pakistan river polluted by heavy metals (Hassan *et al*., 1999).

In contrast to the soil habitat, which is not directly influenced by exudates or other substances produced by living plant roots, the rhizosphere might be considered to have altered physicochemical characteristics due to rhizodeposition of for instance feed cap cells, polysaccharides, soluble secretions and lysates released by the living roots in their direct vicinity (Whipps, 1990). The *T. caerulescens* rhizosphere contained extremely high percentages of Zn (88%) and Cd (71%) resistant isolates as

compared to the non-rhizospheric soil populations. A possible explanation might be found in the results of Lasat and co-workers (1996) with *T. caerulescens* and the non-hyperaccumulator *Thlaspi arvense*. They suggested that hyperaccumulation required the mobilisation of Zn through the production of root exudates. This assumption was confirmed by McGrath *et al*. (1997) who claimed that rhizosphere soils in which *T. caerulescens* was grown, tended to have higher concentrations of mobile Zn than the non-rhizosphere soils. This might explain the high amount of Zn and Cd resistant bacteria surviving in the direct environment of the roots of *T. caerulescens* (Table III.3). In addition, this bacterial rhizosphere population might also contribute to the bioavailability of Zn in the vicinity of the plant's root system. Since bacterial siderophore production can be stimulated by the presence of heavy metals (van der Lelie, 1999) and since most siderophores show affinity to bivalent heavy metal ions (Neilands, 1981), they possibly affect the bioavailability as well. In addition, experiments performed with plants and siderophore producing bacteria showed that microbial ferric iron-siderophore complexes were also assimilated by the plants (Cline *et al*., 1984; Bar-Ness *et al*., 1992) suggesting a possible interaction between both bacteria and plants concerning the bioavailability of bivalent heavy metal ions. A preliminary indication that this hypothesis might be applied to the firmly attached rhizoplane and root endophytic representing bacterial population of *T. caerulescens* was supplied by the results of the siderophore assay performed on the bacterial population isolated (Table III.4). Mainly inhabitants of this niche seemed to possess the capacity to produce siderophores in the absence of iron. In contrast to the rhizosphere population, these bacteria seemed to have different Zn and Cd resistance features. Less than 1 % of this isolated population was able to resist to Zn and Cd levels of 1 mM supplemented to the minimal growth medium. Due to the fact that the rhizosphere population was not identified at the species level, it was impossible to verify if these community structures were completely different at the level of the bacterial genera or only in their abundance. An indication supplied by the estimated amount of the different subpopulations based on the colony colour, favours the hypothesis of a shift in the high abundance of highly resistant bacteria in the rhizosphere to a high abundance of sensitive rhizoplane and endophytic strains. Indeed 96% of the rhizoplane and root endophytic representing population was characterised as white colony forming units sensitive to the heavy metals tested. Similar conclusions were drawn by Mahaffee and Kloepper (1997) studying the

bacterial communities of soil, rhizosphere, and endorhizospheric inhabitants of field grown cucumber: the relative abundance of particular bacterial genera differed among the different habitats while a common membership was shared.

The root endophytic bacterial population of *T. caerulescens* had different physiological characteristics compared to the bacteria isolated from the other niches studied (Table III.2). No estimation of the amount of Zn and Cd resistant populations could be made since they could not be grown on the selected minimal medium. This might possibly be due to the very low abundance of such bacteria in the root interior. One way to explain these results is that heavy metal resistance is not essential for survival in an environment that is not in direct contact with toxic heavy metal concentrations as encountered in the rhizospheric and non-rhizospheric soil. On the other hand, the fact that the majority of isolated bacteria have different growth requirements might not imply that they could be defined as heavy metal sensitive.

Contrary to the root residing inhabitants, the shoot seemed to represent a niche rich in metal resistant bacteria. Among these isolates, 40% was found to survive Zn concentrations of 1 mM; a similar distribution occurred in the wash medium, in the non-surface sterilised shoot macerates and in the surface sterilised shoot macerates suggesting the inhabitants of the phyllosphere as well as those of the internal shoot tissues to be equipped with Zn resistance capacities (Table III.2 & III.3). 23.5% of the shoot endophytic population also survived Zn levels of 2 mM compared to less than 1% for the phyllosphere population; Cd survival on 1 mM of the metal added to the minimal growth medium was estimated to be 9% for internal shoot compared to 3% for the phyllosphere population. The fact that shoot endophytic bacteria are equipped with these Zn and Cd resistance capacities could be interpreted in different ways: as hyperaccumulation of mainly Zn involves high levels of Zn in the shoots, the shoot inhabiting endophytic bacteria able to survive these harsh conditions should be equipped with efficient resistance mechanisms. This interpretation assumes Zn to be physiologically available and thus toxic. Previous studies performed with X-ray absorption spectroscopy (XAS) suggested that Zn coordination in the shoots of *T. caerulescens* mainly occurs via organic acids like citrate (38%) and histidine (16%) with a proportion (12%) complexed to the cell wall (Salt *et al*., 1999b). The same study also mentioned Zn to be present as hydrated cations in the shoot (26%) and in the xylem sap (79%). This high percentage measured in the xylem would possibly require endophytic inhabitants to be equipped with proper resistance mechanisms. The

organic acids mentioned might possibly be used by the endophytic bacteria as a carbon and/or nitrogen source. This would imply that the Zn is released and consequently requires resistance mechanisms for the bacteria in order to survive .

Alternatively, characterisation of the bacteria representing the endophytic shoot population revealed not only Zn and Cd but also resistance to Co and Ni to be abundant. These multiple resistance capacities were also observed in the non-rhizospheric soil representing population and in general this might be a common feature among bacteria of heavy metal contaminated environments including the typical hyperaccumulator flora. As most endophytic bacteria might be considered to be also able to live outside plant tissues as rhizospheric bacteria (Di Fiori & Del Gallo, 1995) or sometimes even as common soil bacteria (McInroy & Kloepper, 1994), their occurrence in every niche studied in this study was not surprising. Obviously, the endophytic population also contained species that were not isolated from the roots nor the non-rhizospheric soil and therefore can be considered to preferably colonise shoots. *Methylobacterium* species were identified as being typically pink forming colonies with high resistance capacities to Zn, Cd, Co, and Ni that comprised 20 % of the shoot endophytic population isolated. In fact, previous studies revealed an intimite relationship of this species with plants (Holland & Polacco, 1994; Holland, 1997). The presence of Zn, Cd, as well as Co resistance possibly might be encoded by a resistance mechanism similar to the *czc* resistance system of *R. metallidurans* CH34 which was shown to alter Zn and Cd speciation due to precipitation processes near the bacterial cell wall (Diels *et al*., 1995). This observation leads to another possible interpretation of the presence of resistant endophytic bacteria colonising the shoots of the hyperaccumulator *T. caerulescens*. In case precipitation processes similar to *R. metallidurans* CH34 should occur, Zn and Cd should be immobilised near the bacterial cell wall as a consequence of the resistance mechanism resulting in a decreased toxicity for the host plant. Future research concerning the bacterial ecology within hyperaccumulators as well as fundamental knowledge of the mechanisms of metal translocation and hyperaccumulation should provide a broader context to test this hypothesis.

CHAPTER IV

Isolation, characterisation, and identification of endophytic bacteria from *Brassica napus*

ABSTRACT

Endophytic bacteria isolated from surface sterilised roots and shoots of Brassica napus were investigated for their heavy metal resistance properties. Although harvested from a non-polluted garden soil, some endophytic bacteria from both roots and shoots showed to be equipped with heavy metal resistance properties. Minimum Inhibitory Concentrations up to 5 mM Zn were measured for Pseudomonas sp. VM422 and VM431 and Comamonas acidovorans VM418 and VM427. Additionally, elevated tolerance against Co and Ni always seemed to accompany this characteristic.

INTRODUCTION

Members of the *Brassicaceae* family, which are crop plants, already have been evaluated for their potential use in phytoextraction processes. It is presumed that moderately accumulating, high biomass producing species might be even more effective than hyperaccumulator species (Kumar *et al*., 1995; Salt *et al*., 1995c, Ebbs & Kochian, 1997). *Brassica napus* for example was shown to be a moderate accumulator of heavy metals with root metal concentrations considerably higher than those in the shoot (Ebbs & Kochian, 1997). Being a potential candidate for phytoextraction studies, we were particularly interested in the endophytic microflora harboured in both roots and shoots. Endophytic bacteria were isolated and examined for their heavy metal resistance properties. The most promising strains were subsequently used to test the hypothesis that endophytic bacteria, efficiently colonizing their

host plant, and equipped with heavy metal removal capacities, might possibly contribute to altering uptake, translocation, and/or accumulation of heavy metals by the host plant.

MATERIALS AND METHODS

Bacterial isolation and characterisation

Brassica napus plants growing on a clean garden soil in Zonhoven (Belgium) were used to isolate endophytic bacteria both from roots and shoots. The sandy soil contained total concentrations of Zn (61 mg/kg DW), Cd (1.1 mg/kg DW), Ni (8.9 mg/kg DW), and Co (<0.5 mg/kg DW) determined by aqua regia extractions followed by Atomic Absorption Spectroscopy (A.A.S.). Endophytic bacteria were selected on Luria Broth (LB) medium after a surface sterilisation procedure which consisted of a 5' washing step in a commercial NaOCl solution (Dumortier, Merksem) with 1% active Cl and one droplet (per 100ml) of Triton X-100 followed by a 5' washing step in sterile H_2O. From the population able to grow, one representative from each colony type observed was selected and purified on LB medium. Consequently their heavy metal resistance capacities were tested on Tris buffered minimal growth media. A chrome azurol S (CAS) shuttle solution (Schwyn & Neilands, 1987) was used for routine testing of siderophore production in liquid media.

Further identification was done by means of 16S rRNA amplification using primers 27F (5'CTCGCTCGCCCAGAGTTTGATCMTGGCTCAG) and 1492R (5'CTGGTTCGGCCCAC GGYTACCTTGTTACGACTT) (Weisburg *et al*., 1991**)** annealing to conserved positions in the 3' and 5' regions of bacterial 16S rRNA. Subsequently cycle sequencing with a 685R fluorescein (F) labeled primer (5'FTCTACGCATTTCACCGCTAC3') was performed using a cycle sequencing kit (Amersham Pharmacia Biotech). The sequence was analysed using the BLAST (Sequence Similarity Searching) database of the NCBI (National Center for Biotechnology Information).

RESULTS

Characterisation and identification

The characterisation and identification tests performed on the endophytic bacterial populations associated with the roots and shoots of *B. napus* are presented in table IV.1.

At first glance, only few different species predominantly belonging to the Beta and Gamma Proteobacteria were collected from the different plant parts. Obviously each root colonising bacterial species identified could also be detected in the shoots suggesting a common presence within the whole plant. Characterising their heavy metal resistance properties revealed that *Pseudomonas* sp. VM422 and VM431 and *Comamonas acidovorans* sp. VM418 and VM427 were able to survive Zn concentrations up to 5 mM supplemented to the Tris minimal growth medium. Additionally, elevated tolerance against Co and Ni always seemed to accompany this characteristic. The other bacterial inhabitants seemed to be able to survive moderate to low concentrations of the selected metals except in the case of Ni. MIC values of 5 mM were observed in 40-60% of the whole population considered. The ability to produce siderophores was frequently observed to be present.

	Siderophore assay	**Heavy metal resistance**						**Strain Identification (16S rRNA)**
		Zn (mM)	**Cd (mM)**	**Co (mM)**	**Ni (mM)**	**Pb (mM)**	**Cu (mM)**	
Root								
VM415	ND	<0.4	<0.2	0.8	0.6	0.4	0.1	*Exiguobacterium acetylicum*
VM416	+	1	0.6	0.2	0.1	0.6	0.1	*Pseudomonas putida*
VM417	+/-	1	0.6	<0.2	0.1	0.6	0.1	*Pseudomonas putida*
VM418	+	5	0.1	3	5	0.6	0.4	*Comamonas acidovorans*
VM419	+	2	0.1	3	5	0.8	0.4	*Delftia* sp.
Shoot								
Stem								
VM420	+	3	0.4	3	5	0.8	0.4	*Pseudomonas* sp.
VM421	+	2	0.6	3	5	0.6	0.4	*Delftia* sp.
VM422	-	5	0.1	2	5	0.8	0.1	*Pseudomonas* sp.
VM423	+	2	0.6	3	5	0.8	0.4	*Comamonas acidovorans*
VM424	ND	0.4	0.1	0.2	1	0.4	0.1	*Exiguobacterium* sp.
VM425	+	0.4	0.1	0.2	0.1	0.4	0.6	*Acinetobacter* sp.
VM426	+	1.5	0.6	0.6	0.4	0.6	0.4	*Pseudomonas* sp.
VM427	ND	5	0.4	3	5	0.8	0.4	*Comamonas acidovorans*
Leaf								
VM428	+/-	1.5	0.6	0.2	5	0.6	0.8	*Pseudomonas* sp.
VM429	-	0.4	0.1	0.2	0.4	0.6	0.1	*Enterobacter asburiae*
VM430	+	0.8	0.6	0.6	0.4	0.6	0.4	*Delftia* sp.
VM431	-	5	0.4	3	5	0.8	0.4	*Pseudomonas* sp.
VM432	-	2	0.4	3	5	0.6	0.4	*Comamonas acidovorans*
VM433	+/-	1	0.6	0.2	0.1	0.8	0.1	*Pseudomonas putida*

Table IV.1: Minimum Inhibitory Concentrations of Zn, Cd, Co, Ni, Pb, and Cu (in mM) supplemented to a Tris minimal growth medium of the purified bacteria isolated from surface sterilised roots and shoots of *B. napus* growing on a clean garden soil. Siderophore production was determined by means of a CAS solution added to a well grown liquid bacterial culture in the absence of iron. Strain identifications using 16S rRNA cycle sequencing provide the closest match by comparison with the BLAST database of the NCBI. ND: not determined.

DISCUSSION

The endophytic bacterial population isolated from the surface sterilised roots and shoots of a *B. napus* species supplied surprising information concerning heavy metal resistance characteristics. Although growing on a non-polluted garden soil, a significant number of bacterial root and shoot inhabitants seemed to express heavy metal resistance characteristics against Zn, Ni, and Co, and were able to survive elevated concentrations of the respective heavy metals when supplemented to the growth medium. This is a striking result because earlier studies often show that heavy metal resistance characteristics are mainly observed in bacteria isolated from contaminated soils or waste waters where they are essential to guarantee survival. Schmidt & Schlegel (1989) were only able to isolate bacteria with high Ni resistance from waste waters and special tanks of the metal-processing industry and they claimed that attempts to isolate bacteria from garden and field soils, which tolerated nickel concentrations higher than 1 mM, were unsuccessful. On the other hand, bacterial extractions performed with clean residential garden soil revealed 0.05 % survivors when grown in the presence of Cd, Cr(VI), and Pb (Salt *et al*., 1999) while a 12.5 % survival rate of bacteria isolated from a contaminated soil was observed. Our results however indicate that the presence of heavy metal resistance characteristics among the bacterial populations is not restricted to contaminated biotopes. A careful estimation of total amounts of extractable bacteria from both non-surface sterilised and surface sterilised roots and shoots is needed in order to determine precisely the actual proportion of heavy metal resistance characteristics present in this population of *B. napus*.

In comparison to the endophytic bacterial population isolated from the Zn hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria* growing on an abandoned Zn and Pb mine and smelter site (Plombières-Belgium) (Chapter III), some interesting observations were made. Like for the *B. napus* population, high Zn resistance characteristics were often accompanied by increased tolerance against Ni and Co in the endophytic population of the Zn hyperaccumulator. The presence of such multiple heavy metal resistance mechanisms might possibly be a widespread characteristic among bacteria from different environments. On the other hand, the bacterial species from both plants seemed to be quite different. This might possibly be due to the different environments in which these plants were grown. The presence of several *Sphingomonas* species in *T. caerulescens* for instance might be linked with the harsh conditions of a contaminated area.

In conclusion, naturally occurring endophytic bacteria from a high biomass producing crop plant apparently are equipped with efficient heavy metal resistance systems that make them interesting candidates in the frame of this work.

CHAPTER V

The potential use of naturally occurring heavy metal resistant endophytic bacteria for improving phytoremediation processes

Abstract

Plant associated bacteria, able to change heavy metal speciation, were studied for their capacity to alter the heavy metal assimilation of their host plant for improving the phytoextraction process. For this reason, a heavy metal resistant Pseudomonas species VM422, originally isolated as an endophytic inhabitant of a Brassica napus plant, was investigated for its properties to alter Zn speciation in growth cultures as well as for its effects on re-inoculated 21 days old B. napus sp. oleifera var. Lirajet seedlings. Growth experiments in the presence of 0.8 mM Zn showed a fast adaptation of strain VM422 to the toxic conditions. A drastic decrease (up to 99%) of the Zn concentration measured in the cell free supernatant as a consequence of mainly Zn phosphate deposition indicated efficient Zn 'immobilising' processes to be abundant. Despite this interesting characteristic together with the capacity to promote root growth, no significant difference in Zn uptake and accumulation could be detected in inoculated B. napus seedlings compared to non-inoculated controls. The fact that mainly the rhizosphere was efficiently re-colonised would imply that the rhizobacterial influence on Zn speciation is of minor importance in the global Zn management of its host plant.

INTRODUCTION

The interaction between plants and their associated microbial flora has been the subject of many studies. Various groups of bacteria have been demonstrated to be beneficial to plants. They can be divided in two main types: those that form a symbiotic relationship with the plant and those that are free living in the soil, but are often found near to, or even within the roots of plants. In the case they are able to enter the interior of plants, they are considered to be endophytic. Many of these endophytic bacteria belong to species previously identified as plant-associated bacteria (Kobayashi & Palumbo, 2000) and their beneficial effects appear to operate through similar mechanisms as described for plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1991a; Höflich *et al*., 1994). These may include nitrogen fixation, synthesis of siderophores which can mobilise and sequester iron from the soil, production of phytohormones such as auxins and cytokinins which can enhance plant growth, development of ACC deaminase activity able to cleave the plant ethylene precursor ACC, and solubilisation of minerals such as phosphorus. Common rhizosphere colonising bacteria such as *Bacillus* sp., *Enterobacter* sp., fluorescent pseudomonads, *Serratia* sp., and *Stenotrophomonas* sp. are examples of bacteria that have been demonstrated to colonise plant root tissues and thus can be considered to be endophytic as well (Lalande *et al*., 1989; Kloepper *et al*., 1992b; Benhamou *et al*., 1996a; 1996b; Duijff *et al*., 1997; Pan *et al*., 1997). This endophytic character might raise the question to what extent these beneficial effects are also exerted within the plant, which can be considered to be a protective environment compared to the rhizosphere in which these effects are predominantly studied.

Plant growth promotion by such rhizospheric and/or endophytic bacteria is one of the interesting characteristics that might be useful in phytoremediation processes. As phytoremediation is defined as the processes in which plants are used to remove, destroy, or sequester hazardous substances from the environment (Cunningham & Berti, 1993; Salt *et al*., 1995c), it is obvious that plants should be able to withstand the toxic effects caused by the pollutant in one way and alternatively should be capable to efficiently deal with the contaminant trough uptake, degradation or sequestration.

Promotion of plant growth, and more specifically promotion of root growth would increase the root surface and consequently the uptake capacities of the pollutant by the plant. In this context, Höflich & Metz (1997) observed that both root and shoot growth of maize and consequently the uptake of Cd, Cu, Pb, Ni, Cr, and Zn was stimulated in weakly polluted soils

when phyto-effective bacteria like *Pseudomonas* sp., *Agrobacterium* sp., *Rhizobium* sp., and *Stenotrophomonas* sp. were inoculated. Stimulation of root growth was also observed in hydroponically grown Indian mustard (*Brassica juncea* L.) inoculated with a *Pseudomonas* species (Salt *et al*., 1999a). In this case the root growth stimulation was attributed to the ability of the bacteria to reduce Cd toxicity through precipitation of the metal near the root and to the subsequent decrease of the toxic effects of Cd on the plant and its root system. Consequently, these *Pseudomonas* species are considered to exert their beneficial effects through precipitation mechanisms that alter the Cd bioavailability near the root system. We already mentioned that such rhizosphere colonising bacteria could possibly also colonise the internal plant parts. Supposing that the observed precipitation processes, associated with the bacterial cell wall of efficiently colonizing endophytic bacteria, alter the heavy metal speciation, this might have consequences for the host plant's metal uptake, translocation and/or accumulation and toxicity. Metals immobilised at the cell wall of endophytic bacteria actually can be considered as harmless; this could allow the colonised plant to accumulate much higher amounts of metals than would normally be possible without affecting its metabolic processes.

The aim of this study was to test this hypothesis. The influence of *Pseudomonas* species VM422, a Zn resistant endophyte isolated from *Brassica napus* was investigated for its Zn precipitating abilities followed by its potential role in accumulation of Zn by its host plant. Inoculated and non-inoculated seedlings were grown for 21 days under controlled laboratory conditions in the presence and absence of a toxic but sublethal amount of Zn. Morphological parameters as well as the Zn concentration in both roots and shoots were compared in order to trace any effect of the bacterial inoculum on these plant characteristics. Recovery of VM422 from its host plant was carried out at harvest in order to evaluate its distribution and to correlate it with the measured plant parameters.

MATERIALS AND METHODS

Zn removal

Pseudomonas sp. VM422 and the control strain *R. metallidurans* CH34 were grown for 67 hours in SVR medium (per litre 294 mg Na-β-glycerol-phosphate, 1 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$, 20 g $C_6H_{12}O_7$) supplemented with 0.8 mM $ZnSO_4$ until the stationary growth phase. Regularly, the OD_{660} was determined and samples were taken from the culture, centrifuged (6000 rpm for 10'; Sorvall RC5C) and the

supernatant was filtered through a 0.45 µm filter unit (Gelman sciences). Subsequently, the Zn concentrations in the supernatant were determined using A.A.S.. The filters were frozen at –20°C until the end of the experiment. Subsequently they were vacuum dried for 24 hours before Energy Dispersive X-ray (EDX) analysis

Preparation of samples for Scanning Electron Microscopy

The vacuum dried filters used in the Zn removal experiment were evaporated with carbon (20-30 nm) to avoid charging up. The SEM-EDX study was performed using a Philips XL30 FEG-SEM (field emission gun - Scanning Electron Microscope) equipped with an EDX detection system containing a super ultra thin window. The microscope was operated at an acceleration voltage of 15 kV. Backscattered electron (BSE) images, containing Z (atomic number) contrast, are used to visualise locations with a different atomic number. In general locations with a higher Z number generate more backscattered electrons and appear brighter in the BSE images. At those locations single-spot analyses are carried out to determine the local chemical composition and the distribution of Zn.

Plant inoculation experiments

Surface sterilisation of seeds

Seeds of *Brassica napus* sp *oleifera* var. Lirajet were surface sterilised by shaking for 30 minutes in a commercial NaOCl solution supplemented with 1% active chloride (Dumortier, Merksem) and one droplet (per 100ml) of Triton X-100, rinsed thoroughly in sterile water for 30 minutes, and incubated during three days at 30°C on LB medium in order to test sterility.

Preparation of bacterial inocula

An overnight culture of VM422 was grown in LB medium supplemented with 5mM $ZnSO_4$ at 30°C on a rotary shaker, centrifugated at 6000 rpm during 15' (Sorvall, RC5C), washed twice in a 0.85% NaCl solution, diluted and plated on LB medium with and without 5mM $ZnSO_4$ in order to test the purity of the solution and the presence of the Zn resistance characteristic.

Inoculation and plant growth conditions

Surface sterilised *B. napus* sp *oleifera* var Lirajet seeds were grown in sterile plastic jars (800 ml) filled with sterilised perlite (200 ml) and saturated with 1/2 sterile Hoagland's solution. Perlite was chosen as plant growth substrate because it can easily be sterilised and provides the roots with moisture, nutrients and a good aeration due to the large surface area and the physical shape of each particle. The bacterial inoculum was added at a concentration of 10^8 Colony Forming Units (CFU) per ml (in NaCl 0.85%), whereas for the non-inoculated control

plants the same amount of NaCl 0.85% was added. Twenty seeds were planted in each jar; they were kept closed for 7 days in order to allow a good bacterial colonisation and to decrease the possible interference of other contaminating species. After 7 days, the lids were removed and the plants could develop further under the following conditions: a 12 hours light and dark cycle, 65% relative humidity, and a constant temperature of 22°C. Every four days sterile water was added in order to keep the perlite moistened.

Harvest of plant material and Zn determination

After 21 days, the *Brassica napus* plants were harvested. Root and shoot length were measured as well as the produced biomass. For the extraction of Zn from the tissue, the fresh root and shoot material was vigorously washed with distilled water in order to remove all trace amounts of Zn that could be present on the surface of the plant material. After drying the root and shoot biomass for 24 hours at 65°C, it was macerated with a mortar and pestle and microwave digested in supra pure HNO_3 (70%); Zn concentration was determined by means of A.A.S.

Recovery of bacteria

To determine the amount of bacteria that colonised the 21 days old *B. napus* plants, roots and shoots were separated, washed 5' in sterile H_2O, macerated by means of a mixer (ultra turrax) in a sterile 5 ml $MgSO_4$ (10^{-2} M) solution and immediately plated on Tris buffered minimal media containing 0.2% gluconate (gluc) as well as 2mM $ZnSO_4$ in order to count for the Zn resistance trait of strain VM422. For the enumeration of endophytic colonists, root and shoot tissue were surface sterilised prior to maceration in a commercial NaOCl solution supplemented with 1% active Cl (Dumortier, Merksem) and one droplet of Triton X-100 per 100 ml for 5' by shaking, followed by a washing step in sterile H_2O for 5'. From the perlite growth substrate, 5 g was shaken for 30' in a 25 ml $MgSO_4$ (10^{-2} M) solution and subsequently plated on the same growth media as mentioned before. Incubation for 3 days at 30°C preceded the bacterial countings.

Statistical analysis

The morphological data were analysed by a one way analysis of variance (ANOVA). The different treatments were compared by means of a Tukey honest significant difference (HSD) test. Significance was tested at the level of P=0.05. The Zn concentrations in roots and shoots of *Brassica napus* were analysed by means of a t-Test. Significance was tested at the level of P=0.05.

RESULTS

Characteristics of isolate VM422

The 16S rRNA gene sequence of the VM422 strain, isolated as an endophytic bacterium of the shoot of a *Brassica napus* species (Chapter IV), showed 96% similarity to that of *Pseudomonas* sp. FSL D1-45. The next closest neighbours were *Pseudomonas putida* and *Pseudomonas* sp. FSL D1-024 with 93% similarity.

Table V.1 shows the Minimal Inhibitory Concentrations (MIC) of strain VM422 for Cd, Zn, Co, Ni, Cu, and Pb in a Tris buffered minimal medium. Additionally MIC values of *Ralstonia metallidurans* strain CH34 harbouring plasmid borne multiple resistance to heavy metals and its plasmid cured derivative AE104 provide a context in which these results might be interpreted. Compared to the heavy metal sensitive AE104 strain, strain VM422 clearly showed to resist significantly higher levels of Zn, Co, and Ni added to the Tris buffered minimal growth medium while comparison with strain CH34 indicates that isolates of highly contaminated industrial biotopes tolerate much higher levels of mainly Cd, Zn, and Co.

	Minimum Inhibitory Concentration (mM)						**References**
	Cd	**Zn**	**Co**	**Ni**	**Cu**	**Pb**	
Pseudomonas sp. VM422	0.1	5	2	5	<0.2	0.8	This study
Ralstonia metallidurans CH34	2.5	12	20	5	ND	ND	Mergeay *et al*., 1985
Ralstonia metallidurans AE104	0.6	0.2	0.2	0.6	ND	ND	Mergeay *et al*., 1985

Table V.1: Minimal Inhibiting Concentrations in mM of Cd, Zn, Co, Ni, Cu, and Pb in a Tris buffered minimal medium for strains *Pseudomonas* sp VM422, *Ralstonia metallidurans* CH34 and its plasmid cured heavy metal sensitive derivative AE104.

Zn removal experiments

The ability of strain VM422 to remove the Zn ions from a liquid culture medium was studied in function of time. This phenomenon, previously observed in liquid cultures of *R. metallidurans* CH34 and attributed to precipitation and sequestration of the metal ions as a consequence of the *czc* (Cd, Zn, Co) resistance mechanism (Diels *et al*., 1995), would allow us to decide whether it could be used to test its impact on accumulation of Zn by its host plant after colonisation. For this reason, VM422 cultures were grown until the late stationary phase and samples were taken at the start of the experiment (T=0h), the early stationary phase

(T=43h) and the late stationary phase (T=67h) (Table V.2). *R. metallidurans* CH34 was selected as a control strain and grown under the same conditions (Table V.2). Both bacterial cultures reached their maximum density after 40 hours of growth, indicating that a fast adaptation to the Zn ions in the medium took place. The pH increase observed is also an indication of well grown cultures since CO_2 released by the bacteria as a consequence of their metabolism is supposed to contribute to the alkalisation of the growth medium. *R. metallidurans* CH34 as well as *Pseudomonas* sp. VM422 showed to be able to reduce the Zn concentration in the cell-free supernatant up to 95 % and 98 % respectively after 67 hours of growth. Apparently, *Pseudomonas* sp. VM422 has similar Zn removal capacities as *R. metallidurans* CH34 under the conditions used. This ability provides this endophytic strain with the potential properties to alter Zn speciation.

		T= 0	**T= 43**	**T= 67**
R. metallidurans CH34	OD_{660}	0	0.9	0.3
	pH	6.3	7.5	8.3
	[Zn] (mg/l)	61	30	3
Pseudomonas sp. VM422	OD_{660}	0	1.1	0.7
	pH	6.4	7.4	8.8
	[Zn] (mg/l)	60	39	1

Table V.2: OD_{660}, pH, and Zn concentration (mg/l) of the supernatant of VM422 and CH34 cultures grown in SVR minimal medium supplemented with 0.8 mM $ZnSO_4$ at different time intervals.

Scanning Electronic Microscopy

Backscattered electron (BSE) images, containing Z (atomic number) contrast, were used to visualise locations with a different atomic number on the vacuum dried filters used in the Zn removal experiments of *Pseudomonas* sp. VM422 and *R. metallidurans* CH34 respectively (Figure V.1.A & B). Locations with a higher Z number generate more backscattered electrons and appear brighter in the BSE images. At those locations single-spot analyses were carried out to determine the local chemical composition and the distribution of Zn.

Areas with Zn deposits were detected in both samples. An EDX analysis for both bacterial strains showed that Zn predominantly is present as Zn phosphate in the bright areas in the BSE images (higher mean Z value) (Figure V.2.B & V.3.B). The darker areas in the same BSE images (lower mean Z value) contain hardly any Zn (Figure V.2.A; V.3.A). These results

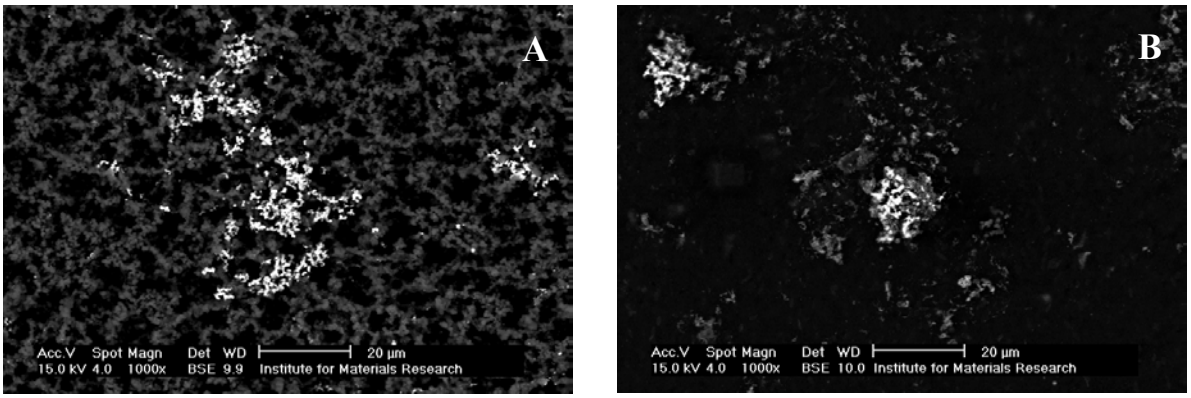

Figure V.1.A & B: Backscattered electron (BSE) images, containing Z contrast, that visualise locations with a different atomic number on the vacuum dried filters used in Zn removal experiments of *R. metallidurans* CH34 (A) and *Pseudomonas* sp. VM422 (B) respectively.

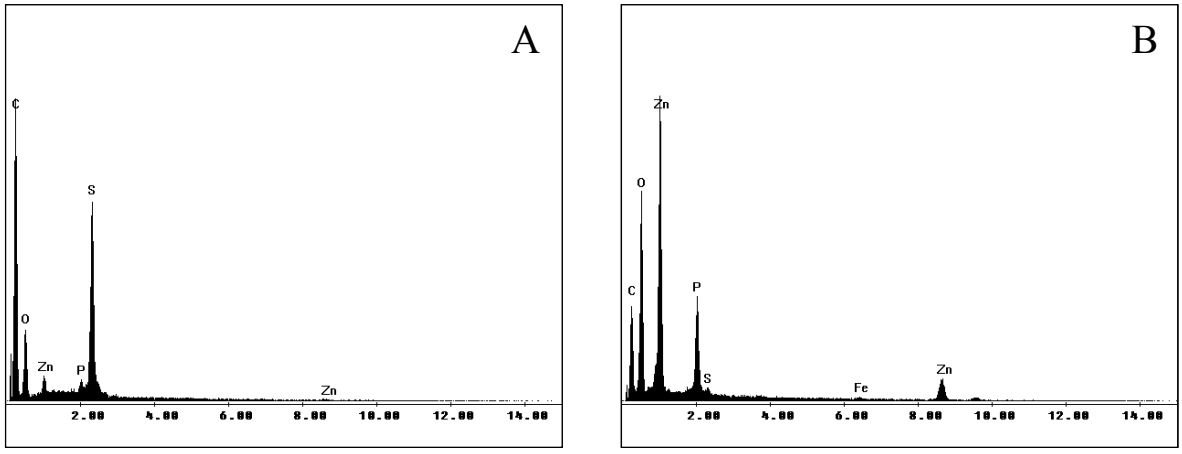

Figure V.2.A & B: SEM-EDX analysis of the chemical composition and Zn distribution on vacuum dried filters used in Zn removal experiments with *R. metallidurans* CH34. The chemical composition in the darker areas of the BSE images is shown in A while B represents the composition in the bright areas.

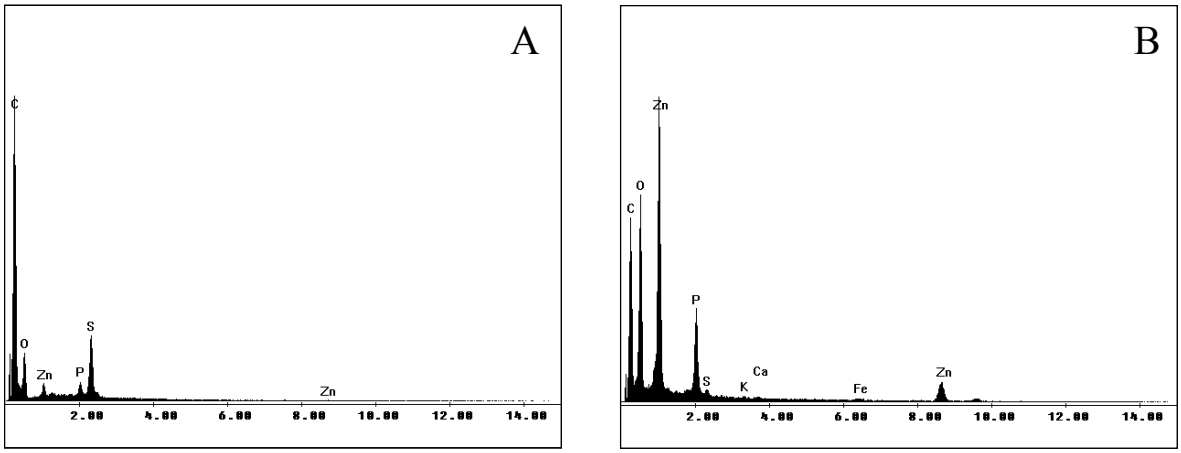

Figure V.3.A & B: SEM-EDX analysis of the chemical composition and Zn distribution on vacuum dried filters used in Zn removal experiments with *Pseudomonas* sp. VM422. The chemical composition in the darker areas of the BSE images is shown in A while B represents the composition in the bright areas.

indicate that the precipitation of Zn phosphate is probably involved in altering Zn speciation in both bacterial growth cultures.

Plant inoculation experiments

The endophytic isolate *Pseudomonas* strain VM422, able to alter Zn speciation in liquid culture media, was re-inoculated with a high inoculum (10^8 CFU/ml) in *B. napus* sp. *oleifera* var. Lirajet and evaluated for its capacity to influence uptake of Zn in both roots and shoots after 21 days of growth under controlled conditions.

Figures V.4 and V.5 show the results of the measured morphological parameters. The seedlings were grown in the absence or presence of 0.8 mM Zn and with or without an inoculum of 10^8 CFU/ml of the bacterial VM422 strains. Inoculation of high concentrations of the bacterial inoculum resulted in a significantly higher root weight (35 %) after 21 days of growth in the absence of 0.8 mM Zn compared to the non-inoculated control plants (Figure V.5). On the other hand, inoculation didn't seem to influence root length (Figure V.4) nor the shoot parameters (Figure V.4 & V.5). The addition of 0.8 mM Zn abolished this stimulatory root effect, most probably due to the phytotoxic concentration of the metal (Figure V.5). In fact, its phytotoxic impact is clearly seen in the significant decreases of the morphological parameters measured (Figure V.4 & V.5) compared to those of the *B. napus* plants growing in the absence of Zn.

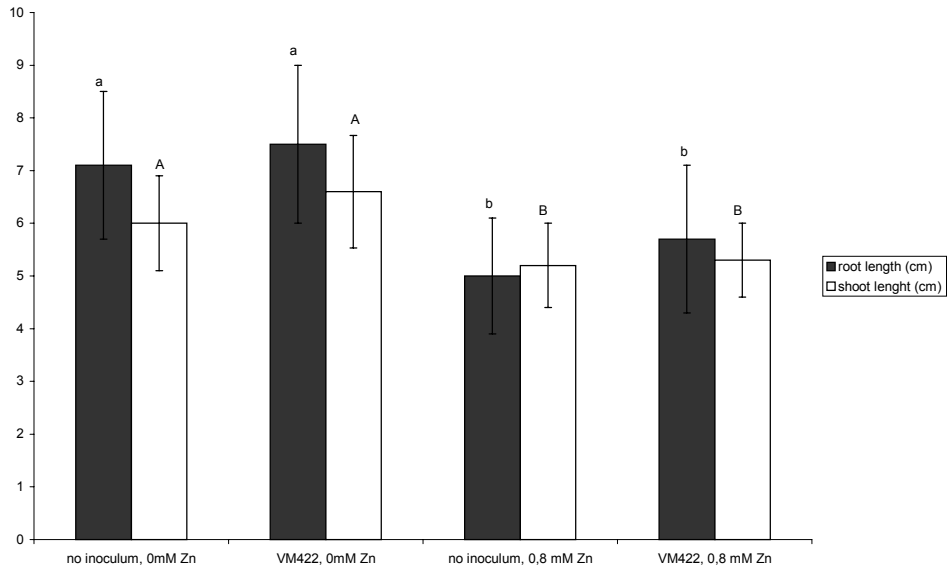

Figure V.4 : Root and shoot length of 21 days old *B. napus* sp *oleifera* in non-inoculated control plants and plants inoculated with VM422 in the absence and presence of $ZnSO_4$. Data are mean values of 30 replicate samples. The letters indicate values for the different morphological parameters that either are not (when the letters are the same) or are (when the letters are different) statistically significant ($P< 0.05$).

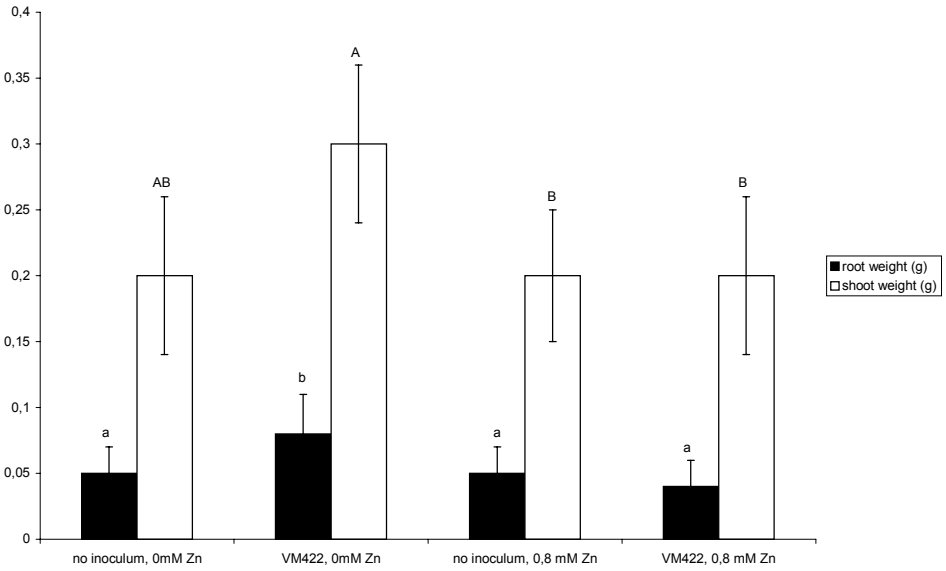

Figure V.5: Root and shoot weight of 21 days old *B. napus* sp *oleifera* in non-inoculated control plants and plants inoculated with VM422 in the absence and presence of $ZnSO_4$. Data are mean values of 30 replicate samples. The letters indicate values for the different morphological parameters that either are not (when the letters are the same) or are (when the letters are different) statistically significant ($P< 0.05$).

Table V.3 shows the Zn concentrations in roots and shoots of the 21 day old *B. napus* plants grown in the presence of 0.8 mM Zn. There seems to be a tendency to lower the Zn uptake in the presence of the bacterial inoculum, but this difference proved not to be significant compared to the non-inoculated controls. No effect on metal translocation within the plant was observed either. The root Zn content proved to be 3 times higher as the shoot content; this was the case in the non-inoculated control plants as well as in the plants inoculated with VM422. Therefore the translocation process doesn't seem to be affected by the presence of strain VM422.

	Zn concentration (mg/kg DW)	
	No inoculum	**VM422**
Root	$6172.3^{a} \pm 935$	$4705^{a} \pm 719$
Shoot	$1721.7^{b} \pm 242.6$	$1476.7^{b} \pm 126$

Table V.3: Zn concentrations (mg/kgDW) in the roots and shoots of *B. napus* sp *oleifera* in non-inoculated control plants and plants inoculated with VM422 treated with 0.8 mM $ZnSO_4$. Data are mean values of 3 replicate samples ± S.D. The letters indicate values for the different treatments in each tissue that either are not (when the letters are the same) or are (when the letters are different) statistically significant ($P< 0.05$).

These results show that with the Zn concentration used in these experiments, no positive effect of the endophytic Zn resistant strain VM422 on the Zn uptake and translocation capacities of its host plant *B. napus* could be detected.

Recovery of bacteria

In order to estimate the amount of bacteria that could be recovered from the 21 days old *B. napus* seedlings, root and shoot tissue were macerated, the suspension was plated on non-selective and selective media, and after incubation at 30°C for 3 days, colony forming units were counted (Table V.4). A surface sterilization step was done to differentiate between internal and external colonists. The amount of bacteria from the suspensions of the perlite growth substrate aimed to deliver information concerning the survival rate of the inoculum under the conditions used.

	Non-selective medium (284 gluc) (CFU/gFW)	**Selective medium (284gluc +Zn 2mM) (CFU/gFW)**
No surface sterilisation		
Root	5.9×10^7	1.2×10^7
Shoot	9.5×10^4	0
Surface sterilisation		
Root	5.9×10^2	0
Shoot	2.1×10^3	0
Perlite		
T=0	6.5×10^8	1.5×10^8
T=21 days	1.5×10^7	3.3×10^6

Table V.4: Colony Forming Units (CFU) per g/FW on non selective and selective media of the perlite growth substrate suspension and the macerated 21 days old root and shoot *B. napus* tissues preceded or not by a surface sterilization step.

In the perlite, a small reduction of the total amount of bacteria was observed after 21 days compared to the inoculum concentration at the start of the experiment; this was the case for both the non-selective media as well as on the selective plates containing Zn. Additionally, the fact that similar morphological colony types as VM422 were found, convinced us that these bacteria were no contaminants. A high amount of identical colony forming units were counted on the media containing the macerated root suspension which could suggest an efficient colonisation. Surface sterilisation of these roots reduced the amount of bacteria by a

factor 10^5 indicating that only a minor part of the bacteria should be located inside the root. Moreover, the fact that those, presumably endophytic bacteria, were not able to grow in the presence of Zn, indicated that they were not the bacteria that were added as the initial Zn resistant inoculum. The macerated shoot suspension had a lower bacterial yield from which the bacteria didn't seem to possess Zn resistance either. Therefore, the bacteria that could be recovered from the shoot could be considered as "contaminants" as well, rather than originating from the initial inoculum of the Zn resistant *Pseudomonas* species. Similar results were repeatedly found in independent experiments, confirming the inability of *Pseudomonas* sp. VM422 to re-colonise its host plant under the conditions used.

DISCUSSION

Pseudomonas species are considered to be by far the most common rhizobacteria (Kloepper *et al*., 1993) and they even seem to be isolated most commonly from the internal plant tissue of healthy plants, suggesting their potential endophytic character as well (Hallmann *et al*., 1997). This statement was confirmed by previous experiments where the endophytic population of a *Brassica napus* species growing in an unpolluted garden soil was investigated (Chapter IV); the results revealed *Pseudomonas* species to be abundant in both surface sterilised roots and shoots. Among this isolated bacterial population, *Pseudomonas* strain VM422 was selected for a more detailed study since growth experiments on Tris buffered minimal medium revealed its capacity to grow on relatively high levels of Zn, Co, and Ni (Table V.1.). Since Zn might be considered as an important pollutant, this metal was selected for further investigations. The Zn resistance mechanism of strain VM422 was firstly examined for the involvement of Zn sequestration and/or bioprecipitation processes in the metal detoxification of the bacterial cell. Diels *et al*. (1995) demonstrated that *R. metallidurans* CH34, harbouring multiple resistance to heavy metals on its megaplasmids pMOL28 and pMOL30, was able to immobilise Zn, Co, and Cd at its bacterial cell wall as a consequence of the *czc* (Cd, Zn, Co) resistance mechanism. This phenomenon was demonstrated by means of X-ray diffraction spectroscopy and transmission electron microscopy. Another convincing argument to prove this hypothesis resulted from the study of the cell-free supernatant of a growth culture of CH34 in the presence of high concentrations of Cd (2mM) or Zn (5mM): a drastic decrease (up to 99%) of the metal concentration in the late log phase was measured (Diels *et al*., 1995). Similar Zn removal properties were observed when *R. metallidurans* CH34 and *Pseudomonas* sp. VM422 were grown under the conditions used in this study. Additional EDX analysis of

the Zn deposits seen on the filters, used to separate the bacterial cells from the supernatant, revealed Zn to be predominantly present as Zn-phosphate. These results suggest that both bacterial species apparently induce similar processes that lead to a removal of the Zn ions present in the growth medium. Therefore, one could postulate that a similar resistance mechanism for Zn, based on *czc*-like efflux functions accompanied by post-efflux metal sequestration, should operate in the endophytic strain. A possible explanation for the predominant precipitation of Zn as Zn-phosphate might be found in the presence of Beta-glycerophosphate in the growth medium. Transmission Electron Microscopy should definitively be performed in a next step in order to verify if this precipitation is linked with the bacterial cellwall.

The fact that Zn is almost completely removed from the growth medium, provides *Pseudomonas* strain VM422 with an interesting characteristic towards its host. A resistance mechanism that is able to efficiently immobilise Zn will in the first place avoid re-entry of the metal ions in the bacterial cell, but secondly would possibly also immobilise potentially toxic Zn ions in the host plant. To potentially affect the fate of Zn in the plant, a first prerequisite would be an efficient colonisation by strain VM422 and secondly Zn should be present in a form, bioavailable for VM422 and inducing the Zn resistance mechanism. This could result in a reduction of metal biotoxicity and subsequently in enhanced metal accumulation and translocation in the host plant.

To test this hypothesis, *B. napus* plants were grown in the presence of a high inoculum of this Zn 'immobilising' strain VM422. After 21 days, the colonizing capacity of *Pseudomonas* strain VM422 was determined as well as some morphological growth parameters and the Zn content in the root and shoot. Recovery of bacteria from the root and shoot tissue showed that only the rhizosphere was efficiently colonised by Zn resistant strains, since after surface sterilisation no Zn resistant bacteria were found neither in the internal root nor in the shoot tissue. This means, that although initially isolated from surface sterilised *B. napus* shoots, *Pseudomonas* VM422 is unable to re-colonise this species endophytically under the conditions used in this experiment. Nevertheless, figure V.5 shows that *Pseudomonas* sp. VM422 exerted a significant growth promoting effect on root development by increasing its biomass with 35 %, compared to the non-inoculated control plants. This observation is quite common for plant associated *Pseudomonas* species. Among the bacterial endophytes isolated from clover and potatoes growing in a crop rotation, *Pseudomonas* was one of the genera most frequently isolated and potato plant growth assays revealed that plant growth promoting effects included increased shoot weight, length and root weight (Sturz *et al*., 1998). Frommel

et al. (1991) even reported a 44-201% increase of the root dry weight when the non-fluorescent *Pseudomonas* sp. strain PsJN colonised potato. Possible explanations for this growth promotion might be found in the alteration of the hormonal balance within the affected plant, the synthesis of siderophores, or the solubilisation of minerals (Glick, 1995).

Addition of Zn to the *B. napus* seedlings abolished this plant growth promoting effect of the VM422 strain. At least at the concentration used in these experiments, Zn caused a growth reduction and this phytotoxic effect dominated the stimulatory effect of the bacteria. In weakly polluted soils, maize was found to be stimulated both in its root and shoot development when inoculated with plant growth promoting strains like *P. fluorescens*, *P. putida*, *Agrobacterium rhizogenes* and *Stenotrophomonas maltophilia*, indicating that at low metal levels, the stimulatory effect should not be neglected (Höflich & Metz, 1997). In these conditions, these maize plants were able to increase their heavy metal uptake capacity. Zn uptake by the roots of *B. napus* seedlings appeared to be 3 times higher than by the shoots (Table V.3). It is generally observed that when plants are grown in Zn contaminated soils, a great proportion of the metal is accumulated in the roots while in ecosystems where Zn is an airborne pollutant, the tops of plants are likely to concentrate the metal (Kabata-Pendias, 1992). Additional information provided by Ebbs & Kochian (1997) indicated that three *Brassica* species, *B. juncea*, *B. rapa*, and *B. napus*, appeared to concentrate 5 to 8 times higher amounts of Zn in the roots than in the shoots, which is in agreement with our results. Compared to the non-inoculated control plants, inoculation with *Pseudomonas* VM422 resulted in a slightly decreased (statistically not significant) Zn level in the roots (24%) and in the shoots (14%) of 21 days old *B. napus* plants. Similar results were obtained in roots and shoots of maize inoculated with *P. fluorescens* soil isolates and growth on highly polluted soil (Höflich & Metz, 1997). A possible hypothesis to explain the results of this study might follow from the behaviour of *Pseudomonas* strain VM422 in the presence of Zn. If the Zn resistance mechanism is induced and functions in the same way as observed in the Zn removal experiments (Table V.2), the predominant rhizosphere colonisation with *Pseudomonas* strain VM422 might alter Zn speciation in the rhizosphere by bioprecipitation and/or sequestration processes near the bacterial cell wall. This would explain the lower levels of Zn taken up by the inoculated *B. napus* plants compared to the non-inoculated controls.

In conclusion, as *Pseudomonas* strain VM422 predominantly colonises the rhizosphere of *B. napus*, it can not be used to increase Zn levels in its host plant in order to improve phytoextraction processes.

CHAPTER VI

Construction of minitransposons expressing broad host nickel resistance and evaluation of their use in endophytic bacteria

Part of this work was published by Taghavi S., Delanghe, H., Lodewyckx, C., Mergeay, M., van der Lelie, D. 2001. Appl. And Environ. Microbiol. 67: 1015-1019.

ABSTRACT

The ncc and nre nickel resistance determinants of Ralstonia metallidurans 31A were used to study broad host nickel resistance. Minitransposons containing ncc, nre, as well as both ncc and nre were constructed in order to guarantee a stable insertion into the hosts' chromosome. The plasmid free and metal sensitive derivative of R. metallidurans CH34, named AE104, turned out to have the highest frequency of transfer. Homologous expression of ncc, nre, as well as ncc-nre was observed in the transconjugants and Minimum Inhibitory Concentrations (MIC) together with the data of the Ni removal experiments confirmed the function of both Ni resistance determinants as observed in the native R. metallidurans 31A strain: ncc is responsible for a high Ni resistance and Ni removal from the growth medium while the presence of nre causes only a low Ni resistance that is not coupled with the capacity to remove Ni from the growth medium.

Introduction of these Ni resistance determinants in plant associated Beta- and Gamma Proteobacteria showed that generally broad-host-range Ni resistance is encoded by nre while the ncc determinant is expressed only in R. metallidurans like strains. As in some cases Ni removal was observed when both the Ni resistance determinants ncc and nre were present, ncc might have a role in altering Ni speciation without influencing the MIC values. This Ni removal capacity might be a promising tool for altering Ni availability in plant environments in which these bacteria reside.

INTRODUCTION

The heterologous expression of heavy metal resistance determinants might influence the speciation of the heavy metals, as was concluded from the growth experiments described by Diels *et al*. (1995) with the soil bacterium *Ralstonia metallidurans* CH34 (formerly *Alcaligenes eutrophus* CH34) in the occurrence of Zn and Cd. The *czc* resistance system encoding Cd, Zn, and Co resistance is situated on the 240 kb large megaplasmid of *R. metallidurans* and is amongst the most completely studied heavy metal resistances of this strain. Resistance is based on a three component cation/proton anti-porter system which pumps out the metals and prevents them to re-enter by means of post efflux functions. These post efflux events are still not completely understood but bioprecipitation and sequestration seem to be major events which showed to be responsible to reduce the amount of Zn and Cd in growth cultures of CH34 in function of time (Diels *et al*., 1995). Since these post efflux systems seem to be able to contribute to the efficiency of the resistance mechanism and the subsequent 'immobilisation' of the metals, some practical applications like the BICMER reactor (Bacteria Immobilised Composite Membrane Reactor) (Diels *et al*., 1993a) and the BMSR (Bio Metal Sludge Reactor) (Diels *et al*., 1999) reactor were developed based on these bacterial characteristics.

Unfortunately, the *czc* operon has never been expressed outside *R. metallidurans* (van der Lelie *et al*., 1999). Similar operons to *czc* might be useful to verify if the phenomena observed with *R. metallidurans* also accompany other bacterial strains able to express these resistance determinants. The *ncc* operon on pTOM8 and pTOM9 of *R. metallidurans* 31A (formerly *Alcaligenes xylosoxidans* 31A) encoding Ni, Cd and Co resistance was presumed to act in the same way as the *czc* system (Nies, 1992). Downstream of *ncc*, the *nre* nickel resistant determinant was identified, which was responsible for a lower, additional Ni resistance phenotype (Schmidt & Schlegel, 1994).

Despite the lack of heterologous expression of *czc*, heterologous expression of the *ncc-nre* cluster was indeed observed. The *ncc-nre* cluster could be expressed in *Escherichia coli*, *Sphingobacterium heparinum*, and *Pseudomonas putida* (Dong *et al*., 1998). Additionally, plant associated bacteria like *Pseudomonas stutzeri*, *Azospirillum irakense*, *Burkholderia cepacia* and *Herbaspirillum seropedicae* were also able to express this Ni resistance determinant (Chapter VII; Y. Wang, personal communication). A possible explanation for this difference in heterologous patterns between *ncc-nre* and *czc* might be the dissimilarity

between the regulatory loci associated with the structural resistance genes (van der Lelie *et al*., 1999)

In this chapter, we describe the construction of miniTn*5*-Km1 based transposons (de Lorenzo *et al*., 1990) containing *ncc* and *nre* in order to have the Ni resistant determinants stably inserted in the chromosome. Under these circumstances, heterologous expression of *ncc*, *nre*, as well as both *ncc* and *nre* will be studied. By means of growth experiments, the existence of post efflux mechanisms will be examined, and this information will allow us to predict the possible effects on Ni speciation these recombinant strains might have in their host plant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are mentioned in table VI.1. *B. cepacia* L.S.2.4 was originally isolated from surface sterilised stems of lupin, while *B. cepacia* W1.2 was an isolate of wheat roots. Both plant species were growing on a wheat-pasture rotation soil from Kapunda, South Australia. *H. seropedicae* LMG2284 was obtained from the Laboratory of Microbiology in Gent, Belgium and *Pseudomonas stutzeri* A15 was a kind gift from the FAJanssens laboratory in Leuven, Belgium. *Pseudomonas putida* VM433 was from our own collection. All strains were growing at 30°C in a Tris buffered 284 minimal medium (Mergeay *et al.,* 1985) supplemented with 0.2% gluconate as a carbon source. *E. coli* strains were growing at 37°C in Luria Broth (LB) medium (Sambrook *et al*., 1989). Resistance to heavy metal salts was tested in Tris-minimal medium (Mergeay *et al*., 1985) in the case of the *Ralstonia metallidurans* and endophytic strains. In order to test heavy metal resistance in the auxotrophic *E. coli* strains, the Tris-minimal medium was supplemented with 40 µg/ml of the appropriate amino acid in order to guarantee growth.

Construction of miniTn5 transposon vectors with Ni resistance markers

The Ni resistance markers *ncc* and *nre* of *R. metallidurans* 31A were introduced in miniTn5 transposon vectors in order to study the expression range and the possible resistance mechanisms.

The *ncc* operon of pTOM9 was cloned in pUC18/*Not*I as a 8.1-kb *Bam*HI-*Pst*I fragment resulting in pMOL1522 (*E. coli* CM2395). Plasmid pMOL1522 was digested with *Not*I, and the *ncc*-containing *Not*I fragment was subsequently cloned in the unique *Not*I site of

Strain or plasmid	Relevant characteristics	Source/ Reference
Strains		
Ralstonia metallidurans		
31A	Wild type, pTOM8, pTOM9, *ncc*, *nre*	Schmidt & Schlegel, 1989
AE104	Plasmid free derivative of CH34, metal sensitive	Mergeay *et al*., 1985
Burkholderia cepacia		
W.1.2	Wild type, endophyte of wheat	K. Ophel-Keller (personal gift)
L.S.2.4.	Wild type, endophyte of lupin	K. Ophel-Keller (personal gift)
Herbaspirillum seropedicae		
LMG2284	Wild type, endophyte of mainly tropical grasses	Baldani *et al*., 1996
Pseudomonas stutzeri		
A15	Wild type, diazotrophic endophyte of rice	Qiu *et al*., 1981; You *et al*., 1983
Pseudomonas putida		
VM433	Wild type, endophyte of rape	Vito collection
Escherichia coli		
DH10B	Plasmid free, λ^-, Δ(*ara*, *leu*)7697, Δ*lac*X74, ϕ80*lac*ZΔM15, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC), *deo*R, *rec*A1, *end*A1, *ara*D139, *gal*U, *gal*K, *rps*L, *nup*G, F^-	Gibco BRL
CC18 *λpir*	Plasmid free, *λpir*, Δ(*ara*, *leu*), Δ*lac*X74, *ara*D, *arg*E (Am), *gal*E, *gal*K, *pho*A20, *rec*A1, *rpo*B, *rps*E, thi-1; renamed CM0983	Herrero *et al*., 1990
S17-1 *λpir*	Plasmid free, λpir, *hsd*M^+, *hsd*R^-, pro, RP42-Tc::Mu-Km::-Tn7-Ap::ISR1, renamed CM0985	de Lorenzo & Timmis, 1994
CM1962	CM1120 containing pMOL222	Taghavi *et al*., 2001
CM2395	CM1120 containing pMOL1522	"
CM2428	CM0983 containing pMOL1524	"
CM2438	CM1120 containing pMOL1525	"
CM2440	CM1120 containing pMOL1526	"
CM2442	CM0983 containing pMOL1527	"
CM2444	CM0983 containing pMOL1528	"
CM2446	CM0983 containing pMOL1529	"
CM2500	CM1120 containing pMOL1548	"
CM2520	CM0985 containing pMOL1554	"
CM2676	CM0985 containing pMOL1528	"
CM2677	CM0985 containing pMOL1529	"
Plasmids		
pUC18(*Not*I)	Ap^r, lac^+	Herrero *et al*., 1990
pUTminiTn5-Km1	Ap^r, Km^r	De Lorenzo *et al*., 1990
PMOL222	pKT240-*ncc-nre;* Ap^r, Km^r, Ni^r, Cd^r, Co^r in *R. metallidurans*	Dong *et al*., 1998
PMOL1522	PUC18 (*Not*I)::*ncc* (*Bam*HI-*Pst*I); Ap^r	
PMOL1524	pUTminiTn5-Km1::*ncc* (*Not*I); Ap^r, Km^r	Taghavi *et al*., 2001
PMOL1525	pUC18(*Not*I)::*nre* (*Pst*I-*Eco*RI); Ap^r, Ni^r	"
PMOL1526	pUC18(*Sfi*I)::*nre* (*Pst*I-*Eco*RI); Ap^r, Ni^r	"
PMOL1527	pUTminiTn*5*-Km1::*nre* (*Not*I); Ap^r, Km^r, Ni^r	"
PMOL1528	pUTminiTn*5*::*nre* (*Sfi*I) Ap^r, Km^r, Ni^r, Tra^+	"
PMOL1529	pUTminiTn*5*-Km1::*nre* (*Sfi*I) Ap^r, Ni^r, Tra^+	"
PMOL1548	pUC18(*Not*I)::*ncc-nre* (*Pst*I); Ap^r, Km^r, Ni^r	"
PMOL1554	pUTminiTn*5*-Km1::*ncc-nre*; Ap^r, Km^r, Ni^r, Tra^+	"

Table VI.1: Bacterial strains and plasmids used.

pUTminiTn*5*-Km1::*ncc*. This resulted in plasmid pUTminiTn*5*-Km1/*ncc* (pMOL1524 in *E. coli* CM2428).

In order to construct an *nre*-based miniTn*5* transposon vector, it was necessary to first inactivate the *Not*I site in *nre*B. Appropriate primers, shown in table VI.2 were designed based on the *nre* sequence (Gene bank accession number L31491; Lemke 1994). PCR mutagenesis performed with primers *nre*-*Pst*I (sense) and *nre*-*Not*I (anti-sense) and *nre*-*Not*I (sense) and *nre*-*Eco*RI (anti-sense) was used to change the *Not*I site with the sequence GCGGCCGC into GCGGCAGC. This resulted in two PCR fragments that were approximately 1.6 and 1.1 kb long respectively. Subsequently, these fragments were mixed and joined by using a PCR-based ligation strategy and were amplified with primers *nre*-*Pst*I (sense) and *nre*-*Eco*RI (antisense). This resulted in a 2.7-kb *Pst*I-*Eco*RI fragment with the mutated *nre* operon. The mutation did not affect the amino acid sequence of the NreB protein, since GCC and GCA both encode alanine. The 2.7-kb *Pst*I-*Eco*RI fragment was subsequently cloned into pUC18/*Not*I, resulting in plasmid pMOL1525 (*E.coli* CM2438). Plasmid pMOL1525 was digested with *Not*I, and the *nre*-containing *Not*I fragment was cloned in the unique *Not*I site of pUTminiTn*5*-Km1. This resulted in plasmid pUTminiTn*5*-Km1::*nre* (*Not*I) (pMOL 1527 in *E. coli* CM2442).

The region with the mutated nreB gene was also amplified as a 2.7-kb *Pst*I fragment by using primers *nre*-*Pst*I (sense) and *nre*-*Pst*I (anti-sense). Subsequently, this fragment was cloned in the unique *Pst*I site of pMOL1522. This resulted in a 10.8-kb *ncc*-*nre* fragment flanked by two *Not*I sites (plasmid pMOL1548 in *E. coli* CM2500). This fragment was subsequently cloned in the unique *Not*I site of pUTminiTn*5*-Km1, resulting in plasmid pUTminiTn*5*-Km1::*ncc*-*nre* (*Not*I) (pMOL 1554 in *E. coli* CM2520).

To inactivate the *Sfi*I site in *nre*A, we used a strategy similar to that used for mutation of the *Not*I site, except that primers *nre*-*Pst*I (sense) and *nre*-*Pst*I (anti-sense) and *nre*-*Sfi*I (anti-sense) and primers *nre*-*Sfi*I (sense) and *nre*-*Eco*RI (anti-sense) were used. The mutations did not affect the amino acid sequence of the NreA protein, since GCC and GCG both encode for alanine. A 2.7-kb *Pst*I-*Eco*RI fragment with the mutated *nre* operon (*Sfi*I site) was subsequently cloned in pUC18/*Sfi*I (Herrero *et al*., 1990), resulting in plasmid pMOL1526 (*E. coli* CM2440). Plasmid pMOL1526 was digested with *Sfi*I, and the *nre*-containing *Sfi*I fragment was cloned in *Sfi*I-digested pUTminiTn*5*-Km1. This resulted in plasmids pUTminiTn*5*-Km1::*nre* (*Sfi*I) (pMOL 1529 in *E. coli* CM2446) and pUTminiTn*5*::*nre* (*Sfi*I) (pMOL1528 in *E. coli* CM2444), in which the Kanamycin resistance gene was replaced by

nre. The restriction maps of the miniTn*5* Ni resistance transposons are presented in figure VI.1.

The range of expression of Ni resistance was examined in the Ni sensitive plasmid free derivative of *R. metallidurans* AE104, *E. coli* DH10B, the endophytic strains *B. cepacia* W1.2 and L.S.2.4, *Herbaspirillum seropedicae* LMG2284, *Pseudomonas stutzeri* A15, and *Pseudomonas putida* VM433. For this purpose, the miniTn*5* transposons pUTminiTn*5*-Km1::*ncc* (*Not*I), pUTminiTn*5*-Km1::*ncc-nre* (*Not*I), pUTminiTn*5*-Km1::*nre* (*Sfi*I) and pUTminiTn*5*::*nre* (*Sfi*I) were selected. These pUT-based constructs were introduced into *E. coli* S17-1 (*λpir*) and consequently transferred by biparental conjugation experiments to their hosts. Selection and purification of transconjugants was done on minimal medium supplemented with Ni and Km.

Primer	Start position	End position	Sequence (5'-3')	Comment
*nre-Pst*I (sense)	1477	1510	**CGC CTG CAG CGC AGA CCG TGG CGG CAG CGG CGC C**	-
*nre-Pst*I (antisense)	4183	4160	**AAA CTG CAG CCC GGA TTG AAA ATG CGA CTC ATG**	**AAA CTG CAG** was added at the 5' end
*nre-Eco*RI (antisense)	4189	4160	**AAA GAA TTC CCC GGA TTG AAA ATG CGA CTC ATG**	**AAA** was added at the 5' end
*nre-Sfi*I (sense)	2234	2286	**GGA GAG CGC CGT GAC CCA GGC gAA GAA GGC gCT GGT GCA TGA CCA TAT CGA CC**	Mutations (C to G) are indicated by lowercase **g**
*nre-Sfi*I (antisense)	2286	2234	**GGT CGA TAT GGT CAT GCA CCA GcG CCT TCT TcG CCT GGG TCA CGG CGC TCT CC**	mutations (G to C) are indicated by lowercase **c**
*nre-Not*I (sense)	3107	3164	**GAA GGG ACT GCT GGC GCT GAA TCT TGC CGC GGC aGC AGC CAG CGC CAT GGT GAT CGT G**	Mutation (C to A) is indicated by lowercase **a**
*nre-Not*I (antisense)	3164	3107	**CAC GAT CAC CAT GGC GCT GGC TGC tGC CGC GGC AAG ATT CAG CGC CAG CAG TCC CTTC**	Mutation (G to T) is indicated by lowercase **t**

Table VI.2: The positions of the relevant restriction sites used for cloning of the amplified PCR fragments are underlined in the primer sequences. Sequence positions are based on the *nre* sequence (Gen-bank accession number L31491; Lemke 1994).

Additionally, the *ncc-nre* cluster was also introduced in the receptor strains by means of pMOL222 in order to study its resistance mechanism, but due to the instability of the plasmid the work was further focused on the minitransposons.

Determination of mutation and transconjugation frequencies

The frequencies of conjugation mediated transconjugants and spontaneous mutants were determined by counting colony forming units (CFU) on minimal Tris buffered medium supplemented with the appropriate concentration of the selective marker. In the case of Ni, selection was done at 1mM $NiCl_2$ for all the strains mentioned, while the concentration of Km as an antibiotic selective marker varied (100-or 1000 µg/ml) for the different strains.

Donor and receptor strains were grown overnight in LB medium, washed in $MgSO_4$ (10^{-2} M) and aliquots of 5 µl were added to a sterile filter (0.45 µm) and incubated overnight at 30°C on solid LB medium. Aliquots of the undiluted washed receptors were streaked homogenously over the selective media in order to determine the frequency of mutants. The filter containing both donor, receptor, and transconjugants was washed in a 10^{-2}M $MgSO_4$ solution in order to release the bacteria. Transconjugants were selected by means of their acquired Ni and Km resistance while the *E.coli* auxotrophic donor strains were counter-selected by their inability to grow on the minimal medium used.

PCR analysis with nre primers

PCR using *nre* primers was used to verify the presence in the transconjugants of the minitransposon, pUTminiTn*5*-Km1::*nre* (*Sfi*I) and pUTminiTn*5*::*nre* (*Sfi*I). One freshly grown colony was picked up from a selective medium (in the case of transconjugants) and dissolved in 20 µl of a $MgSO_4$ (10^{-2} M) solution. From this solution 5 µl was subjected to a 100 µl reaction mixture using Takara Ex Taq polymerase according to the manufacturers instructions (Takara). The forward primer being *nre* (*Sfi*I) and the reverse primer *nre* (*Not*I) (Table VI.2) were used to amplify a 930 bp fragment. Amplification was carried out as follows: a preliminary denaturation step was done at 95°C for 10 min, followed by 35 cycles of 2 min at 95°C, 1 min at 55°C, 2 min at 72°C, and 8 min at 72°C. Negative controls were systematically included and PCR products were checked by electrophoresis in 0.8% agarose gels.

Ni removal

Strains *R. metallidurans* 31A, *R. metallidurans* AE104::*ncc*, AE104::*nre*, AE104::*ncc-nre* and the endophytic strains *B. cepacia* L.S.2.4::*nre*, L.S.2.4::*ncc-nre*, *H. seropedicae* LMG2284::*nre*, LMG2284::*ncc-nre* were grown at 30°C for 7 days in SVR medium (per litre 294 mg Na-β-glycerol-phosphate, 1 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$, 20 g $C_6H_{12}O_7$) supplemented with 0.4mM $NiCl_2$ until the stationary growth

phase. Regularly, the absorbance (660nm) was determined and samples were taken from the culture, centrifuged (6000 rpm for 10'; Sorvall RC5C) and the supernatant was filtered through a 0.45 µm filter unit (Gelman sciences). Subsequently, the Ni concentrations were determined using Atomic Absorption Spectroscopy (A.A.S.).

RESULTS

MiniTn5 transposon vectors with Ni resistance markers

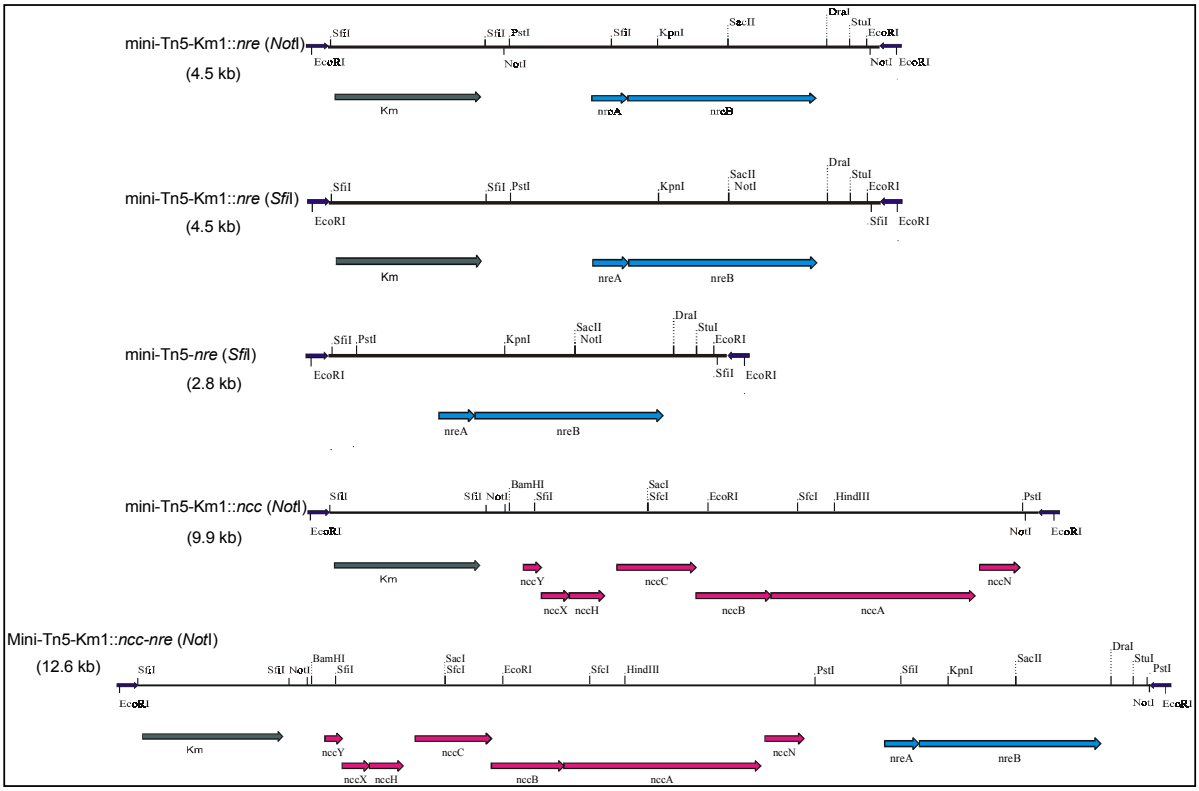

Figure VI.1: Schematic representation of different miniTn*5* Ni resistance transposons. The positions of the Kanamycin marker (Km), the Ni resistance determinants *ncc* and *nre*, the inverted repeats and the extremities of the minitransposons, as well as the positions of important restriction sites are indicated. The sizes of the minitransposons are marked between brackets.

Figure VI.1 gives a schematic representation of the different miniTn*5* Ni resistance transposons that have been constructed as mentioned before.

Frequency of transfer of the Ni resistance marker versus frequency of mutation on the selective medium

In order to verify if elevated resistance to Ni was due to a successful transfer and heterologous expression of the *ncc*, *nre*, and *ncc-nre* determinants, CFUs of transconjugants as well as

wild-type strains were counted on selective minimal media containing Ni levels that would normally inactivate the wild-type strains. Table VI.3 shows the frequencies of transconjugants and mutants on selective Ni minimal media. Each wild type strain used in this study was unable to grow on the Ni selective medium (1mM) suggesting the toxic effect of the metal

Donor	Recipient	Frequency of transconjugation		Frequency of mutation	
		Ni marker	Kanamycin marker	Ni marker	Kanamycin marker
CM2676(*nre*, Km^r)	AE104	9.2×10^{-5}		$<7.1 \times 10^{-10}$	2.4×10^{-8}
	DH10B	4.6×10^{-6}		$<4.2 \times 10^{-10}$	$<4.2 \times 10^{-10}$
	LMG2284	9.7×10^{-8}		$<1.8 \times 10^{-9}$	1.3×10^{-8}
	W1.2	7.1×10^{-10}		$<4.5 \times 10^{-10}$	$<4.5 \times 10^{-10}$
	L.S.2.4	5.8×10^{-8}		$<8 \times 10^{-10}$	5.7×10^{-8}
	A15	1.7×10^{-9}		$<1.3 \times 10^{-9}$	$<1.3 \times 10^{-10}$
	VM433	3.9×10^{-6}		$<9.1 \times 10^{-10}$	3.5×10^{-8}
CM2677(*nre*)	AE104	9.5×10^{-5}			
	DH10B	9×10^{-6}			
	LMG2284	1.1×10^{-7}			
	W1.2	7.1×10^{-10}			
	L.S.2.4	2.7×10^{-8}			
	A15	9.6×10^{-9}			
	VM433	3.7×10^{-6}			
CM2536(*ncc* Km^r)	AE104	1.5×10^{-5}	8.2×10^{-6}		
	DH10B	$<4.2 \times 10^{-10}$	2.8×10^{-7}		
	LMG2284	$<1.8 \times 10^{-9}$	1.2×10^{-7}		
	W1.2	$<4.5 \times 10^{-10}$	1.3×10^{-9}		
	L.S.2.4	$<8 \times 10^{-10}$	2.3×10^{-7}		
	A15	$<1.3 \times 10^{-9}$	3.3×10^{-9}		
	VM433	$<2.5 \times 10^{-10}$	1.4×10^{-7}		
CM2520(*ncc-nre*,Km^r)	AE104	8.6×10^{-7}			
	DH10B	1.8×10^{-7}			
	LMG2284	1.4×10^{-8}			
	W1.2	1.1×10^{-9}			
	L.S.2.4	2×10^{-9}			
	A15	1.3×10^{-9}			
	VM433	1.5×10^{-7}			

Table VI.3: Mutation and transconjugation frequencies of the strains used to test transfer and heterologous expression of *ncc*, *nre*, and *ncc* plus *nre*. The strains used as recipients in the matings were *R. metallidurans* AE104, *E. coli* DH10B (Gibco BRL), *H. seropedicae* LMG2284, *B. cepacia* W1.2 and L.S.2.4, *P. stutzeri* A15, and *P. putida* VM433. *E. coli* S17-1(*λpir*) (de Lorenzo & Timmis, 1994) containing pUTminiTn*5*-Km1::*ncc-nre* (strain CM2520), pUTminiTn5-Km1::*ncc* (strain CM2536), pUTminiTn5-Km1::*nre* (strain CM2676), and pUTminiTn5::*nre* (strain CM2677) were used as donors. Ni resistant transconjugants were selected on 284 minimal medium containing 1 mM $NiCl_2$. Most of the Kanamycin-resistant transconjugants were selected on 284 minimal medium (Mergeay *et al*., 1985) containing 100 mg of Kanamycin per litre; the only exception was strain AE104, whose transconjugants were selected on 284 minimal medium containing 1000 mg of kanamycin per litre.

and the inability of the strains to resist it. On the other hand, spontaneous mutants resistant to the Ni concentration used are not observed and therefore Ni can be considered as a reliable

selective agent. The use of Km as a selective antibiotic seemed to induce mutations in the strains AE104, LMG2284, L.S.2.4, and VM433 and was not further used to select transconjugants. But still, in order to verify the occurrence of the minitransposon inserted in the bacterial chromosome, the Ni marker as well as the Km marker (except in the case of transfer of miniTn*5*::*nre* (*Sfi*I)) were always used to double check the transconjugants.

The frequency of transconjugation varied between 10^{-5} and 10^{-10} with the highest transfer observed with AE104. The fact that comparable frequencies of transconjugants were found for each strain when mated with the donor strains containing different constructs of Ni resistance determinants, suggests that the efficiency of transfer is not affected by this variable.

The ability to stably insert the transferred Ni resistance markers in the chromosome, was verified by performing stability tests. Growing the transconjugants for 100 generations under non-selective conditions (in the absence of Ni and Km) revealed that no loss of any marker took place (data not shown) and therefore a stable insertion and transfer from one generation to the next could be concluded from this experiment.

Heterologous expression of the Ni resistance markers

Purified transconjugants were tested for their MIC value for Ni. Table VI.4 shows the MIC values for the wild type strains, the transconjugants harbouring the pMOL222 plasmid carrying the *ncc-nre* cluster, and the transconjugants containing *ncc* (MiniTn*5*-Km1::*ncc* (*Not*I)), *nre* ((MiniTn*5*-Km1::*nre* (*Sfi*I)) and (MiniTn*5*::*nre* (*Sfi*I)), and *ncc* plus *nre* (MiniTn*5*-Km1::*ncc-nre* (*Not*I)) in their genome. Strain AE104 reflected the function of both Ni resistance determinants as observed in the native *R. metallidurans* 31A strain: a high level of Ni resistance was observed in the presence of *ncc*, and a lower level of Ni resistance was observed when *nre* was present (Schmidt & Schlegel, 1994). The presence of both *ncc* and *nre* seems to be responsible for an even higher final MIC value in the case of insertion through the miniTn*5* transposon but this was not observed in the presence of the pMOL222 encoded *ncc* and *nre*. This might possibly be explained by the involvement of an extra chromosomal promotor.

The endophytic strains *H. seropedicae* LMG2284, *B. cepacia* L.S.2.4 and *P. putida* VM433 responded similarly after introduction of the different Ni resistance determinants. The MIC value in the presence of plasmid pMOL222 encoded *ncc-nre* was in the same range as when *ncc-nre* or *nre* alone was inserted in the chromosome through transposition. This observed increased resistance was comparable with the observed *nre* mediated Ni resistance in the plasmid free derivative of *R. metallidurans* AE104. Indeed, *ncc* was not expressed in either of

these endophytic strains and it is presumed that only *nre* might be responsible for the Ni resistance observed. Despite this similarity, two exceptions were encountered: *P. stutzeri* A15 was able to express *ncc* slightly and *B. cepacia* W1.2. was only able to grow on higher Ni levels when both *ncc* and *nre* were present. This latter phenomenon might imply that *ncc* as well as *nre* contribute in Ni resistance, but it is not clear in what way.

Strain	**Wild type**	**pMOL222 (*ncc-nre*)**	**MiniTn*5*-Km1::*ncc* (*Not*I)**	**MiniTn*5*-Km1::*nre* (*Sfi*I)**	**MiniTn5 ::*nre* (*Sfi*I)**	**MiniTn*5*-Km1::*ncc-nre* (*Not*I)**
Ralstonia metallidurans AE104	0.6 mM	20-40 mM	20-40 mM	3-4 mM	3-4mM	> 40 mM
Herbaspirillum seropedicae LMG2284	0.4 mM	3 mM	0.4 mM	3-4 mM	3-4 mM	2mM
Burkholderia cepacia W1.2.	0.4 mM	3 mM	0.4 mM	0.4 mM	0.4 mM	2 mM
LS 2.4.	0.6 mM	unstable	0.6 mM	2 mM	2 mM	2 mM
Pseudomonas putida VM433	0.6 mM	ND	0.6 mMD	4 mM	3 mM	3 mM
Pseudomonas stutzeri A15	0.6 mM	3 mM	1 mM	4mM	2 mM	3mM
Escherichia coli DH10B	≤ 0.6 mM	2-3 mM	≤ 0.6 mM	3 mM	2 mM	2-3 mM

Table VI.4: Minimum Inhibitory Concentrations (MIC) of Ni in Tris buffered minimal medium of wild type strains and their respective Ni resistance determinants. The ranges of the MICs were determined with four individual transconjugants. ND, not determined.

The behaviour of *E. coli* DH10B cells in the presence of the mentioned Ni resistance genes is in agreement with the results obtained by Schmidt & Schlegel (1994): no expression of *ncc* and a low level Ni resistance mediated by the *nre* locus.

Resistance to Cd and Co is also encoded by *ncc* in *R. metallidurans* 31A (Schmidt & Schlegel, 1994), and this was also observed for strain AE104 after transfer of the plasmid vector pTV1 containing the *ncc* 14.5 kB BamHI fragment (Schmidt & Schlegel, 1994). This result was confirmed by our experiments with the minitransposons: resistance to these metals

was observed in strain AE104; the other strains used as mentioned in table VI.4 were not able to grow on higher Cd and Co concentrations as their respective wild types (data not shown).

PCR analysis with nre primers

Except for *B. cepacia* W1.2, all tested bacterial strains were able to express Ni resistance encoded by *nre*. It was for this reason that the presence of *nre* was verified by PCR using *nre* primers. Amplification using the forward *nre*(*Sfi*I) and the reverse *nre*(*Not*I) primers resulted in a 930 bp fragment in the positive control strains CM2676 and CM2677 as well as in the transconjugants confirming the presence of the *nre* resistance determinant (Figure VI.2).

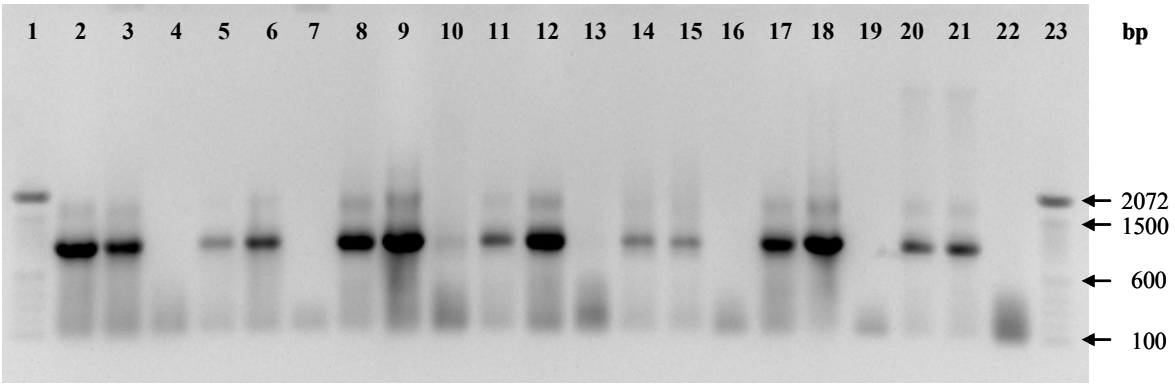

Figure VI.2: 930 bp PCR fragment using the forward primer *nre*(*Sfi*I) and the reverse primer *nre*(*Not*I).

1. 100 bp marker
2. CM2676
3. CM2677
4. AE104
5. AE104 X CM2676
6. AE104 X CM2677
7. DH10B
8. DH10B X CM2676
9. DH10B X CM2677
10. LMG2284
11. LMG2284 X CM2676
12. LMG2284 X CM2677
13. VM433
14. VM433 X CM2676
15. VM433 X CM2677
16. A15
17. A15 X CM2676
18. PST3 X CM2677
19. L.S.2.4
20. L.S.2.4 X CM2676
21. L.S.2.4 X CM2677
22. negative control
23. 100 bp marker

Ni removal

Ni resistant transconjugants of the strains *R. metallidurans* AE104, *B. cepacia* L.S.2.4, and *H. seropedicae* LMG2284 were selected to perform Ni removal experiments. Strain *R. metallidurans* 31A was used as a positive control. As negative controls the wild-type strains, sensitive to Ni, were used. For this reason, they were grown under lower Ni concentration conditions (0.2 mM $NiCl_2$) in order to allow growth and to exclude any removal effect caused by these wild type strains. In table VI.5, the optical density, the pH of the growth medium as well as the Ni concentrations in the cell free supernatant at the start of the experiment (T_0) and after 180 hours of growth (T_{180}) at 30°C are presented. *R. metallidurans* 31A cultures reached

the highest bacterial density compared to the other strains tested. A pH increase from 6.6 to 9.2 was measured in the growth medium but this alkalisation was also measured when 31A cells were growing under non-selective conditions (data not shown) and can therefore be considered as a consequence of the bacterial metabolism rather then being the result of proton influx processes as observed during *czc* mediated proton anti-porter efflux of cations (Diels *et al*., 1995).

The Ni concentration in the medium was reduced by 74 % when compared to the initial concentration at the start of the experiment. This Ni removal is likely to be attributed to the Ni resistance mechanism and more precisely the *ncc* system. Evidence for this presumption is supplied by the Ni removal observed in the plasmid free derivative of CH34, namely AE104::*ncc* and the inability of reducing the Ni concentration if *nre* is present. A striking result was obtained in the case AE104::*ncc-nre* was grown under the same conditions. No reduction in the Ni concentration of the growth medium was seen and as a consequence a lower final OD was reached indicating less efficient growth. Previous studies done with *R. metallidurans* CH34 showed that cobalt and nickel resistance encoded by *cnr* required at least 0.6 mM Ni^{2+} in poor medium in order to fully express this operon (Tibazarwa, 2000). In the context of these results, a similar threshold concentration might be needed in the case of *ncc.* As both resistance characteristics are present, expression of *nre* might be preferred in low Ni environments. Growing these bacteria, harbouring both *ncc* and *nre*, under higher Ni stress (0.6 and 1 mM) revealed that similar levels of Ni removal were observed as seen in the supernatant of growth cultures of AE104::*ncc* (Taghavi, personal communication) indicating that the former strain might be more effective in Ni removal at higher Ni concentrations.

A different situation was encountered with the endophytic strains. Both *B. cepacia* and *H. seropedicae* were not able to express Ni resistance encoded by *ncc*. The occurrence of *ncc* as well as *nre*, allowed the bacteria to proliferate to a higher final optical density when compared to the strains only harbouring *nre*. Apparently, the presence of *ncc* enhances the bacterial growth capacity in a Ni containing growth medium. Additionally, 35 % of the initial Ni concentration in the growth medium of *B. cepacia* L.S.2.4::*ncc-nre* and 15 % in the growth medium of the *H. seropedicae* LMG2284::*ncc-nre* were removed after 180 hours of growth. These phenomena might be explained by the fact that in these tested endophytic strains, *ncc* is expressed only in the presence of *nre* resulting in a better tolerance for Ni. On the other hand table VI.4 shows that MIC values obtained for Ni of *B. cepacia* L.S.2.4::*ncc-nre* and *H. seropedicae* LMG2284::*ncc-nre* are in the same range as their corresponding strains harbouring only *nre*. The same observation was done for *P. stutzeri* A15 (Y. Wang, personal

communication). This bacterium actually also proved to be able to slightly express *ncc*, but the final MIC observed for the strains harbouring both *ncc-nre* was in the same range as for the strains that only had *nre* inserted in their chromosome (table VI.4). Consequently, growing these bacteria for 7 days in the presence of Ni showed that *ncc-nre* equipped the strains with better tools to survive the nickel stress as compared to the strains only possessing *ncc* or *nre*. Additionally, the highest Ni removal was measured in the cell-free supernatant of A15::*ncc-nre* (73%) while A15::*ncc* only was responsible for a decrease of 4% of the Ni concentration in the growth medium (data not shown).

Bacterial strains	OD_0	OD_{180}	pH_0	PH_{180}	Ni_0	Ni_{180}	% Ni removal
Ralstonia metallidurans							
31A	0.001	1.1	7.1	9.2	25.2	6.5	74%
Ralstonia metallidurans							
AE104*	0.001	0.2	6.9	7.5	12	12.3	0
AE104::*ncc*(*Not*I)	0.001	0.9	6.7	9.1	23	12.1	47%
AE104::*nre* (*Sfi*I)	0.001	0.3	6.6	9.1	23	23	0
AE104::*ncc-nre* (*Not*I)	0.001	0.3	6.6	8.4	23	23	0
Burkholderia cepacia							
L.S.2.4*	0.001	0.03	7.1	7.1	12.2	12.3	0
L.S.2.4::*nre* (*Not*I)	0.001	0.3	7.1	7.6	22.6	23.2	0
L.S.2.4::*ncc-nre* (*Not*I)	0.001	0.6	7	8.3	24.5	16	35%
Herbaspirillum seropedicae							
LMG2284*	0.001	0.02	7.3	6.8	12.6	12.9	0
LMG2284::*nre* (*Not*I)	0.001	0.09	6.5	7.6	23	24	0
LMG2284::*ncc-nre* (*Not*I)	0.001	0.5	7.3	9	24.8	21	15%

Table VI.5: The optical density (OD_{660}), pH, and Ni concentration (mg/l) of the cell-free supernatant of cultures of *R. metallidurans* 31A, *R. metallidurans* AE104, *B. cepacia* L.S.2.4., and *H. seropedicae* LMG2284 with the *ncc*, *nre*, and *ncc-nre* resistance operons of *R. metallidurans* inserted through transposition. These parameters were determined at the start of the experiment (T=0) and after 180 hours of growth (T=180) in a SVR medium containing 0.2 mM $NiCl_2$ for all the wild type strains mentioned (*) and 0.4 mM $NiCl_2$ for the *R. metallidurans* AE104, *B. cepacia* L.S.2.4 and *H. seropedicae* LMG2284 constructs.

DISCUSSION

Ralstonia metallidurans 31A, isolated from a copper galvanization tank in Germany and characterised by its high tolerance for Ni (40 mM), Co (20mM), Zn (10mM), Cd (1mM) and Cu (1mM) (Schmidt & Schlegel, 1989), supplied us with the genetic tools of the *ncc-nre* Ni, Cd, and Co resistance system able to be expressed in other heterologous organisms like *E. Coli* (Schmidt *et al*., 1991), *S. heparinum* and *P. putida* (Dong *et al*., 1998). The use of such heavy metal resistance markers with a broad host expression range was already shown in the genetic manipulation of *Pseudomonas* strains potentially designated for environmental release (Sanchez-Romero *et al*., 1998) and as selection marker for the follow up of soil bacteria with the capacity to degrade organic pollutants (D. Springael, personal communication).

Previous information concerning the potential mechanism of Ni, Cd, and Co resistance encoded by *ncc* suggested that it functions in the same way as the *czc* system of *R. metallidurans* CH34 which codes for an efflux pump based on a chemi-osmotic cation/proton anti-porter (Nies, 1995; Nies & Silver, 1995). The second nickel resistant determinant, *nre* is responsible for a low additional Ni resistance (Schmidt & Schlegel, 1994) with an unknown underlying mechanism.

In order to study these resistance determinants in more detail, minitransposons containing *ncc*, *nre*, and *ncc-nre* were constructed based on the knowledge that once minitransposons insert in a target sequence, they are inherited in a stable fashion and, unlike natural transposons, do not provoke DNA rearrangements or other forms of genetic instability because they lack the cognate transposase gene and the greater parts of the IS elements present in the wild-type transposons (de Lorenzo & Timmis, 1994). The fact that introduction of plasmid encoded *ncc-nre* resistance genes into the selected hosts sometimes resulted in the loss of the plasmid due to instability under non-selective conditions, convinced us to clone *ncc* and *nre* in these miniTn*5* transposons. Indeed, stability tests performed on these constructs showed the stable inheritance of the Ni resistance characteristics from one generation to the next.

The metal sensitive strain AE104 turned out to have the highest frequency of transfer (table VI.3) and was able to express *ncc*, *nre*, as well as *ncc-nre* in a homologous way in the same range as in the native strain 31A (Table VI.4). Ni removal studies suggested that the ability to remove Ni from the growth medium is correlated with the presence of *ncc* although a threshold Ni concentration might be necessary for an optimal action. A similar observation was done with cobalt and nickel resistance encoded by *cnr* in *R. metallidurans* CH34 where at least 0.6 mM Ni was required in poor medium in order to fully express this operon (Tibazarwa, 2000).

Transfer and heterologous expression in the selected endophytic strains revealed that *H. seropedicae* LMG2284, *B. cepacia* L.S.2.4, *P. putida* VM433, and *P. stutzeri* A15 were able to obtain an increased Ni resistance in the case *nre* and *ncc-nre* were inserted in their chromosome (Table VI.4). The presence of *ncc* induced only in *P. stutzeri* A15 an increase in the Ni resistance but this in a far lower extent than observed in *Ralstonia* strains. The fact that insertion of *nre* caused comparable MIC values in all the tested strains, except for *B. cepacia* W.1.2 where it was not expressed, convinced us to conclude that *nre* is responsible for a low Ni resistance in our tested strains and can be considered to have a broad-host-range. Acquiring this Ni resistance determinant doesn't imply Ni precipitation processes and the fact that poor growth is observed in Ni containing medium could mean that the cell is under a continuous nickel stress and consequently requires a high amount of metabolic energy in order to survive (table VI.5).

More interesting features were observed in the endophytic strains harbouring *ncc* as well as *nre*. Ni removal linked with a better growth was observed; indeed, this might imply that the addition of *ncc* could be responsible in some way for this phenomenon. It is not clear why this capacity doesn't increase the MIC values for Ni measured on Tris minimal solid growth medium while in liquid medium a better growth is seen compared to the strains only equipped with *nre*. In any way, this Ni removal capacity equips the endophytic strains *B. cepacia* L.S.2.4::*ncc-nre* and *H. seropedicae* LMG2284::*ncc-nre* with the potential ability to change Ni speciation. In a next step, these strains will be inoculated in their respective host plant growing in the presence of Ni, and the extent in which the Ni removal capacity changes the speciation of the metal near or in the plant will allow us to evaluate their practical application in improving phytoremediation processes.

CHAPTER VII

The effect of recombinant heavy metal resistant endophytic bacteria on heavy metal uptake by their host plant

Lodewyckx, C., Taghavi, S., Mergeay, M., Vangronsveld, J., Clijsters, H., van der Lelie, D. 2001. Int. J. of Phytorem. (in press)

ABSTRACT

The ncc-nre Ni resistance system of Ralstonia metallidurans 31A was efficiently expressed in Burkholderia cepacia L.S.2.4 and Herbaspirillum seropedicae LMG2284. The heterologous expression of ncc-nre encoded Ni resistance was accompanied by nickel removal from the culture medium. B. cepacia L.S.2.4::ncc-nre and H. seropedicae LMG2284::ncc-nre were able to remove 35% and 15% Ni, respectively. The capacity to remove Ni possibly through sequestration or bioprecipitation processes and consequently lowering the free Ni concentration, could offer interesting benefits for these endophytic bacteria and their host plants. Once inoculated in their host plant, they could possibly alter the Ni speciation and therefore decrease the free ions and thus toxic concentration for the plant metabolism. Lupinus luteus L, when grown on a nickel enriched substrate and inoculated with B. cepacia L.S.2.4::ncc-nre, showed a significant increase (30%) of Ni concentration in the roots, whereas the Ni concentration in the shoots remained comparable with that of the control plants. The inoculation of Lolium perenne (cv Atlas) with the Ni resistance derivative of H. seropedicae LMG2284::ncc-nre resulted in a significant decrease of the Ni concentration in the roots (11%) as well as in the shoots (14%). As this phenomenon was also observed in the Lolium perenne plants inoculated with the wild type strain LMG2284, the Ni resistance characteristics probably are not responsible for the altered Ni uptake observed.

INTRODUCTION

Living plants have the ability to easily assimilate heavy metals that are essential in trace concentrations for their growth and development and this has been used to develop phytoextraction as a remediation technique (Baker & Walker, 1990; Mehra & Farago, 1994). Although offering some interesting benefits compared to the traditional remediation techniques, phytoextraction still has its limitations. A suitable plant used in extraction of heavy metals should possess several characteristics which are rarely found within one plant species. For this reason, different strategies are currently being investigated in order to improve crops for phytoextraction processes (Cunningham & Berti, 1993; Cunningham & Ow, 1996; Burd *et al*., 1998; Arazi *et al*., 1999; Brewer *et al*., 1999). It was in this context that we investigated the possible interaction between plant associated endophytic bacteria and their host plant with respect to the uptake and accumulation of heavy metals. Endophytic bacteria are defined as those micro-organisms that are able to enter plant tissues and to establish themselves inter-and intracellularly (Di Fiori & Del Gallo, 1995). They have the ability to establish an active relationship with their hosts and can be defined as colonists (Kloepper & Beauchamp, 1992; Kloepper *et al*., 1992a). Introduction and heterologous expression of known heavy metal resistance genes in these endophytic bacteria might have an effect on the uptake capacities of heavy metals by their host plant. In fact, Salt *et al*. (1999a) showed that Cd tolerant rhizobacteria were able to promote Cd precipitation processes near the root surface of Indian mustards plants and consequently decreased the toxic effects of the metal cation for the roots. Previous studies have shown that several mechanisms can be responsible for bacterial heavy metal resistance e.g. blocking the entry of toxic ions in the cells, intracellular sequestration of the metals by metal binding proteins, enzymatic conversion of the metal to a less toxic form and energy driven efflux systems for cations and anions encoded by resistance genes, such as the *czc*, *cnr*, *ncc*, *cad*, and *ars* operons (Mergeay, 1997). Bioprecipitation and sequestration processes also seem to take place when bacteria are equipped with efflux mechanisms. This phenomenon was observed in cultures of *Ralstonia metallidurans* CH34 (previous *Alcaligenes eutrophus* CH34) when grown in the presence of high concentrations of Cd or Zn and attributed to the action of the *czc* resistance operon on the pMOL30 plasmid (Diels *et al*., 1995). Such

bio-precipitation and sequestration characteristics could offer interesting benefits for the bacteria, and in the case of endophytic bacteria the speciation of the heavy metals might be altered in the host plant from a free to a less available form. This could lead to a reduced toxicity of the heavy metals on plant metabolism. In order to test this hypothesis, *Burkholderia cepacia* and *Herbaspirillum seropedicae* were selected as endophytic bacteria. Some *B. cepacia* strains have been reported as facultative endophytes of lupin plants (K. Ophel Keller, personal communication) and are able to colonise roots of various maize cultivars (Hebbar *et al*., 1992a; Hebbar *et al*., 1992b). *H. seropedicae* strains were described as obligate endophytes of many genera of the *Gramineae* and do not seem to survive in uncropped soils (Baldani *et al*., 1992; 1996; Olivares *et al*., 1996). Both species belong to the Beta-Proteobacteria. In these strains, we investigated the heterologous expression of plasmid encoded heavy metal efflux systems related to the *czc* operon of *R. metallidurans* CH34. The *ncc* system encodes Ni, Co, and Cd resistance in *R. metallidurans* 31A (previous *Alcaligenes xylosoxidans* 31A) (Schmidt & Schlegel, 1994), and *czr* is involved in Zn and Cd resistance in *Pseudomonas aeruginosa* CMG103 (Hassan *et al*., 1999). Other plasmid encoded heavy metal resistance operons from pMOL30, such as the *pbr* (Pb^r) and *cop* (Cu^r) determinant (Taghavi *et al*., 1997) were also tested for their heterologous expression in the endophytic strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in table VII.1. *B. cepacia* L.S.2.4 was originally isolated from surface sterilised stems of lupin growing on a wheat-pasture rotation soil from Kapunda, South Australia. The endophytic character of this strain was confirmed by growing *Lupinus luteus* L. seedlings in the presence of 10^8 CFU/ml of the Ni resistant variant (L.S.2.4::*ncc-nre*) added to the perlite growth medium. Surface sterilised and macerated root and shoot tissue of 14 days old plants harboured $1.4x10^6$ CFU/gFW and $4.3x10^3$ CFU/gFW respectively when plated on selective media containing Ni.

H. seropedicae LMG2284 was obtained from the Laboratory of Microbiology in Gent, Belgium. *Lolium perenne* cv Atlas, selected to check the ability of this strain to

establish itself endophytically, also proved to function as a host plant. After growth under similar conditions as mentioned for *Lupinus luteus* L., 6.8 x10^6 CFU/gFW and 1.1 x10^5 CFU/gFW of the Ni resistant variants (LMG2284::*ncc-nre*) were recovered from surface sterilised and macerated root and shoot tissue respectively when plated on Ni containing selective media.

Strain or plasmid	Relevant characteristics	Source/ Reference
Strains		
Ralstonia metallidurans		
CH34	Wild type, pMOL28, pMOL30	Mergeay *et al*., 1985
31A	Wild type, pTOM8, pTOM9	Schmidt & Schlegel, 1989
Burkholderia cepacia		
L.S.2.4.	Wild type, endophyte of lupin	K. Ophel-Keller (personal gift)
Herbaspirillum seropedicae		
LMG2284	Wild type, endophyte of *Gramineae*	Baldani *et al*., 1996
Escherichia coli		
CM0404	pRK2013	
CM1366	pMOL864	
CM1666	pMOL1027	
CM1684	pMOL1024	
CM1962	pMOL222	
CM2520	pMOL1554	
Plasmids		
pRK2013	Km^r, Tra^+	Figursky *et al*., 1982
pMOL222	(pKT240-*ncc-nre*) Ap^r, Km^r, Ni^r, Cd^r, Co^r	Mergeay & Dong (unpublished results)
pMOL864	(pLAFR3-*czr*) Tc^r, Cd^r, Zn^r	Hassan, *et al*., 1999
pMOL1024	(pLAFR3-*cop)*, Tc^r, Cu^r	Borremans & van der lelie, (in preparation)
pMOL1027	(pLAFR3-*pbr*), Tc^r, Pb^r	Borremans & van der Lelie, (submitted)
pMOL1554	(pUTminiTn*5*-Km1::*ncc-nre*) Ap^r, Km^r, Ni^r, Tra^+	Taghavi *et al*., 2001

Table VII.1: Bacterial strains and plasmids used.

All strains were growing at 30°C in a 284 minimal medium (Mergeay *et al.,* 1985) supplemented with 0.2% gluconate as a carbon source. *Escherichia coli* strains were grown at 37°C in Luria Broth (LB) medium (Sambrook, *et al*., 1989). Resistance to

heavy metal salts was tested in Tris-minimal medium (Mergeay *et al*., 1985). Minimum Inhibitory Concentrations were determined by growing the strains in these media supplied with increasing concentrations (0.1-5 mM) of the metal to be tested. At least 4 replications were done for each strain.

Heterologous expression of Cu, Cd, Co, Ni, Pb, and Zn resistance

For the introduction of plasmids in *B*. *cepacia* L.S.2.4 and *H*. *seropedicae* LMG2284, triparental matings were performed using L.S.2.4 and LMG2284 as receptor strains, *E. coli* CM404 (pRK2013) (Figursky *et al*., 1982) as a helper strain for the mobilisation, and the *E. coli* strains CM1366, CM1623, CM1666 and CM1684 as donors. LMG2284 transconjugants were selected on 284 minimal medium supplemented with 20 µg/ml Tetracyclin, while L.S.2.4 transconjugants were selected on 284 minimal medium supplemented with 150 µg/ml Tetracyclin.

Due to the instability of the introduced plasmids in *B*. *cepacia* and *H*. *seropedicae*, miniTn*5*-Km1 based transposons (de Lorenzo *et al*., 1990) containing *ncc* and *nre* were constructed in order to have the expressed Ni resistant determinants inserted in the chromosome stably (Taghavi *et al*., 2001). This resulted in plasmid pMOL1554, which was used as donor for the mobilisation of the pUTminiTn*5*-Km1::*ncc-nre* to *B. cepacia* and *H. seropedicae*. Transconjugants were selected on 284 minimal medium containing either Km or Ni.

Ni removal

Strains *B*. *cepacia* L.S.2.4::*ncc-nre*, *H*. *seropedicae* LMG2284::*ncc-nre* and *R*. *metallidurans* 31A were grown at 30°C for 7 days in SVR medium (per litre 294 mg Na-β-glycerol-phosphate, 1 g NH_4NO_3, 0.2 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$, 20 g $C_6H_{12}O_7$) supplemented with 0.4mM $NiCl_2$ until the stationary growth phase. Absorbance at 660 nm (A_{660}) was determined regularly and samples were taken from the culture, centrifuged (6000 rpm for 10'; Sorvall RC5C) and the supernatant filtered through a 0.45 µm filter unit (Gelman sciences). Subsequently, the Ni concentrations were determined using Atomic Absorption Spectroscopy (A.A.S.).

Plant inoculation experiments

Surface sterilisation of seeds

Seeds of *Lupinus luteus L.* were surface sterilised by shaking for 30 minutes in a commercial NaOCl solution supplemented with 1% active chloride (Dumortier, Merksem) and one droplet (per 100ml) of Triton X-100, rinsed thoroughly in sterile water for 30 minutes, and incubated during three days at 30°C on LB medium in order to test sterility.

The same procedure was followed for the surface sterilisation of the *Lolium perenne* seeds, except that the NaOCl solution contained 3% active chloride and the seeds were shaken for 60 minutes.

Preparation of bacterial inocula

Overnight cultures of L.S.2.4::*ncc-nre* and LMG2284::*ncc-nre* were grown in LB medium supplemented with 4mM $NiCl_2$ at 30°C on a rotary shaker, centrifuged at 6000 rpm during 15' (Sorvall, RC5C), washed twice in a 0.85% NaCl solution, diluted and plated on LB medium with and without 4 mM $NiCl_2$ in order to test the purity of the solution and the presence of the Ni resistance. Wild type strains L.S.2.4 and LMG2284 were grown under the same conditions without addition of $NiCl_2$.

Inoculation and plant growth conditions

Surface sterilised *Lupinus luteus L.* seeds were grown in sterile plastic jars (800 ml) completely filled with perlite and saturated with 1/2 sterile Hoagland's solution. Perlite was chosen as plant growth substrate because it can be easily sterilised and provides the roots with moisture, nutrients and good aeration due to the large surface area and the physical shape of each particle. The bacterial inoculum was added at a concentration of 10^8 Colony Forming Units (CFU) per ml (in NaCl 0.85%), whereas for the non-inoculated control plants the same amount of NaCl 0.85% was added. Five seeds were planted in each jar and than covered with tinfoil in order to prevent contamination and dispersion of the inoculated bacteria through the air. Once the germinated seeds were able to grow through the holes that were made in the tinfoil, they were grown with a 12 hour light and dark cycle, 65% relative humidity and at a constant temperature of 22°C. Sterile water was added every four days in order to keep the perlite moistened.

The surface sterilised *Lolium perenne* seeds were grown under the same conditions as the *Lupinus* seeds except that the plastic jars were filled with 200 ml perlite. Seeds

were randomly spread over the available area in order to guarantee a similar biomass production as for the *Lupinus* seedlings.

Harvest of plant material and Ni determination

After 21 days, the *Lupinus luteus* and *Lolium perenne* plants were harvested. For 30 plants, the length of roots and shoots was measured separately as well as the produced biomass. Fresh root and shoot material was vigorously washed with distilled water in order to remove all trace amounts of Ni that could be present on the surface of the plant material. After drying the root and shoot biomass for 24 hours at 65°C, it was macerated with a mortar and pestle and microwave digested in supra pure HNO_3 (70%) in order to release all Ni, whose concentration was determined by means of A.A.S.

Statistical analysis

The morphological data as well as the Ni concentrations in roots and shoots of *Lupinus luteus* and *Lolium perenne* were analysed by a one way analysis of variance (ANOVA). The different treatments were compared by means of a Tukey honest significant difference (HSD) test . Significance was tested at the level of P=0.05.

RESULTS

Heterologous expression of Cu, Cd, Co, Ni, Pb, and Zn resistance

The *cop*, *pbr*, *ncc-nre*, and *czr* heavy metal resistance operons were cloned in broad host range Inc Q (pKT240) or Inc P (pLAFR3) plasmids and transferred to the endophytic strains by triparental matings. After selection for antibiotic resistance, heterologous expression of heavy metal resistance was examined. The best expressed marker for both *B. cepacia* and *H. seropedicae* was the nickel resistance of *ncc-nre* whose expression resulted in an increase of the MIC value for Ni from 0.4 or 0.6 mM to 2 mM for *H. seropedicae* LMG2284 and *B. cepacia* L.S.2.4 respectively. Low expression of *cop* encoded Cu resistance was seen in *H. seropedicae* while no heterologous expression of the *pbr* and *czr* operons was observed. Because the *ncc-nre* Ni resistance was the best marker in both strains, we decided to study its heterologous expression in more detail. Due to the instability of the pMOL222 plasmid, a miniTn*5* transposon containing *ncc-nre* was used to obtain a stable

insertion of the Ni resistance determinant into the chromosome. For both *B. cepacia* and *H. seropedicae* the minitransposon carrying *ncc-nre* proved to be stable when inserted in the chromosome (no loss of Ni resistance after growth for 100 generations under non selective conditions) and gave a MIC for Ni of 2 mM on 284 minimal medium in both strains.

Ni removal

The Ni resistant endophytic strains L.S.2.4::*ncc-nre* and LMG2284::*ncc-nre* were tested for their ability to remove Ni from the growth medium. As a positive control, the wild type strain *R. metallidurans* 31A was used, which contains copies of both *ncc* and *nre* on its endogenous megaplasmids pTOM8 and pTOM9. Growing the strains for 180 hours in minimal SVR medium supplemented with 0.4 mM $NiCl_2$ revealed that the *R. metallidurans* 31A strain was able to grow at a higher rate than the endophytic strains (Figure VII.1). This was probably due to the multiple copies of the Ni resistance determinants and perhaps other important functions of Ni resistance on pTOM8 and pTOM9. These features are also important when the Ni removal capacity is considered (Figure VII.2). *R. metallidurans* 31A strain removed 74% of the Ni present in the growth medium, while *B. cepacia* L.S.2.4::*ncc-nre* and *H. seropedicae* LMG2284::*ncc-nre* only removed 35% and 15% of Ni, respectively. The pH of the

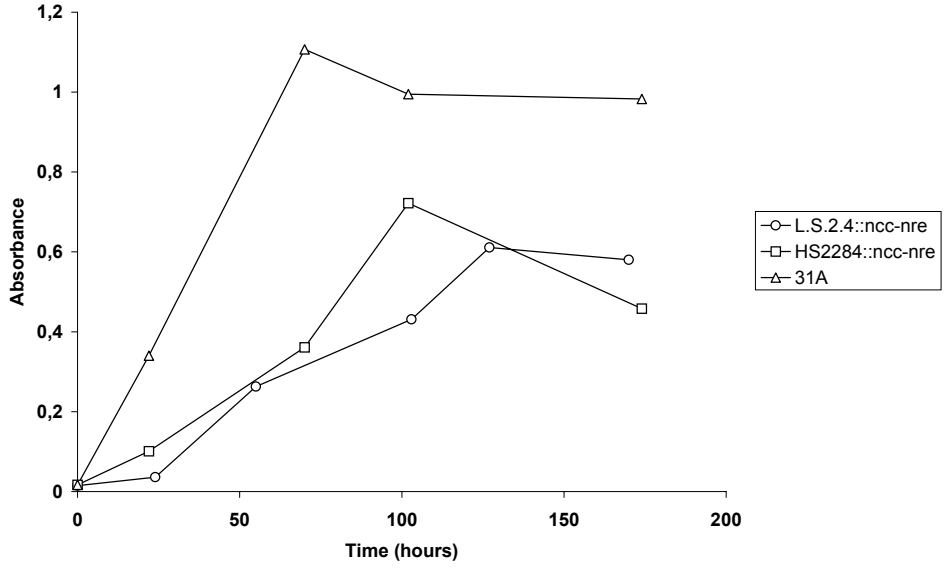

Figure VII.1: Optical Density (OD_{660}) in function of time for *R. metallidurans* 31A, *B. cepacia* L.S.2.4::*ncc-nre*, and *H. seropediacae* LMG2284::*ncc-nre* cultured in SVR medium supplemented with 0.4 mM $NiCl_2$. The data are representative for 3 separate and independent experiments.

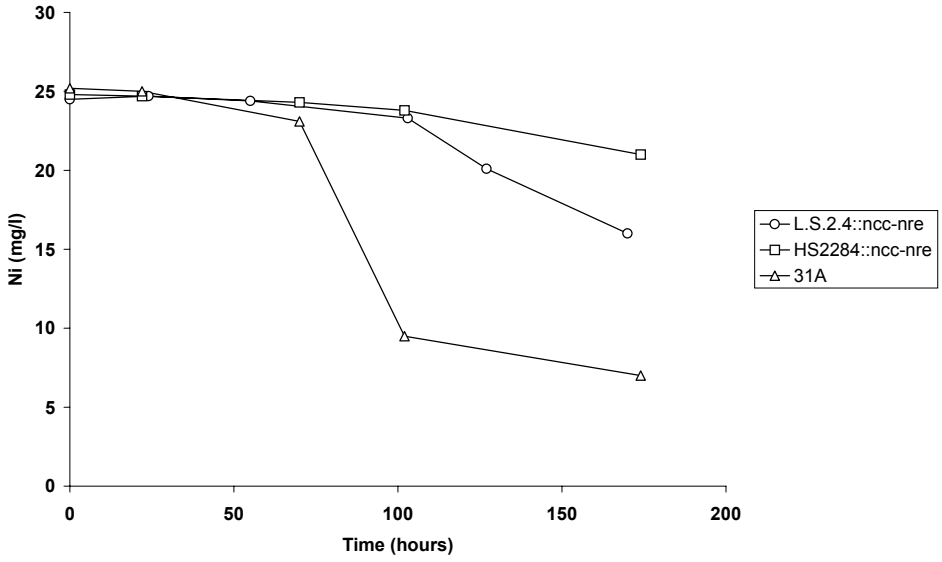

Figure VII.2: Concentration of Ni concentration (mg/l) in the culture supernatant over time for *R. metallidurans* 31A, *B. cepacia* L.S.2.4::*ncc-nre*, and *H. seropediacae* LMG2284::*ncc-nre,* grown in SVR medium supplemented with 0.4 mM $NiCl_2$. The data are representative for 3 separate and independent experiments.

growth medium also increased over time. The pH of the *R. metallidurans* 31A culture was increased from pH 7 to pH 9 while the pH of the L.S.2.4::*ncc-nre* and LMG2284::*ncc-nre* changed from 7 to 8 (data not shown).

Ni toxicity for Lupinus luteus L. and Lolium perenne (cv. Atlas)

Preliminary experiments were performed to test the sensitivity of *Lupinus luteus L.* seedlings for Ni in order to decide which concentration would be suitable for the inoculation experiments. From these experiments it was decided to test the effect of 0.25 mM $NiCl_2$ because this concentration had a visible but non-lethal effect on the growth response of the *Lupinus* seedlings. Root and shoot biomass and length were significantly reduced when Ni was added to the perlite (data not shown).

As was observed for the *Lupinus luteus L.* seedlings, the growth of *Lolium perenne* seedlings was also reduced when treated with increasing $NiCl_2$ concentrations. Root and shoot biomass and length significantly decreased with increasing Ni concentrations added to the growth substrate (data not shown). In order to compare the effects in both plant species, we decided to use the same concentration (0.25 mM $NiCl_2$) as for the experiments with the lupin plants.

Inoculation of Lupinus luteus L. with B. cepacia L.S.2.4 and its Ni-resistant derivative L.S.2.4::ncc-nre

Wild type strain L.S.2.4 and its Ni resistant derivative L.S.2.4::*ncc-nre* were inoculated in perlite and the *Lupinus* seedlings were grown on this substrate for 21 days under controlled environmental conditions. Non-inoculated sterile plants were used as controls. In the absence of $NiCl_2$, no difference in growth response was observed between the 21 days-old non-inoculated control plants and the inoculated lupin plants when the shoot biomass and length were considered (Table VII.2). The roots seemed to be slightly but significantly affected in their growth when *B. cepacia* was added as a wild type strain or as the Ni resistant derivative, indicating that the presence of *B. cepacia* L.S.2.4 has a minor effect on the root development in the absence of Ni (Table VII.2). The Ni concentration in both roots and shoots was measured with A.A.S but was below the detection limit (<2.5 mg/kg DW).

Addition of 0.25 mM $NiCl_2$ to the perlite resulted in a decrease of the growth parameters when compared to the treatment without $NiCl_2$, suggesting a toxic effect of the Ni cations. Table VII.2 shows the morphological parameters of the lupin plants

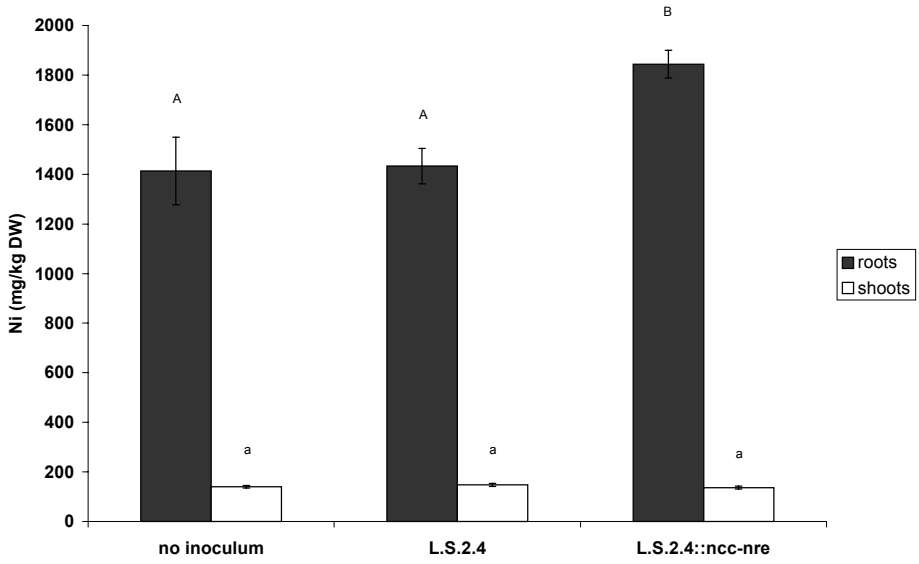

Figure VII.3: Ni concentration (mg/kgDW) in roots and shoots of *Lupinus luteus L.* plants for the different inocula and the 0.25mM $NiCl_2$ treatment. L.S.2.4 was inoculated as the wild type strain and L.S.2.4::*ncc-nre* as its Ni resistant derivative. Data are mean values of 3 replicate samples ± S.D. Different letters indicate values that are statistically significant (P< 0.05)

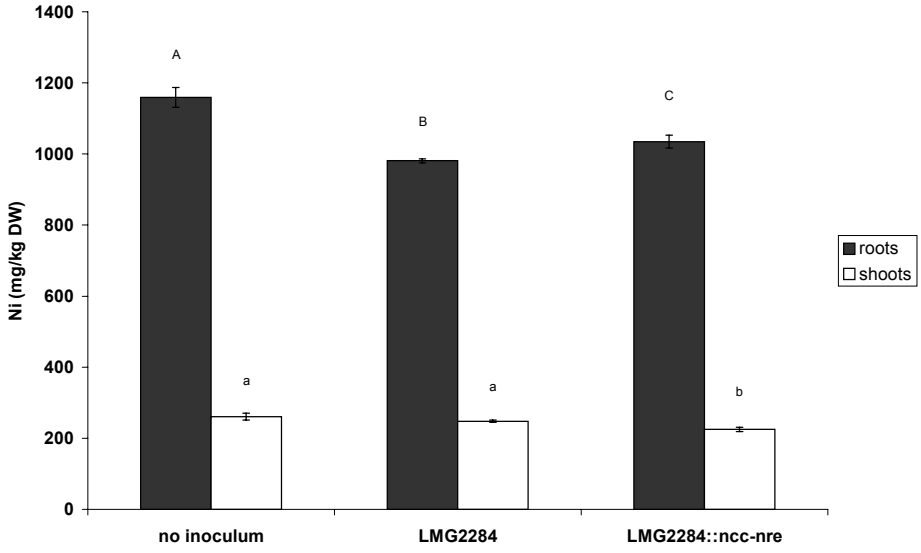

Figure VII.4: Ni concentration (mg/kgDW) in roots and shoots of *Lolium perenne.* plants for the different inocula and the 0.25mM $NiCl_2$ treatment. LMG2284 was inoculated as the wild type strain and LMG2284::*ncc-nre* as its Ni resistant derivative. Data are mean values of 3 replicate samples ± S.D. Different letters indicate values that are statistically significant ($P< 0.05$)

with the different inocula in the presence of 0.25 mM $NiCl_2$. The presence of *B. cepacia*, both wild type or its Ni resistant derivative, did not influence the growth of the plants. No significant differences were observed when root and shoot biomass and length were measured. However, a different response in Ni accumulation was observed when the Ni concentration in the roots was compared for the different treatments (Figure VII.3). A significantly higher total Ni concentration was measured in the lupin roots inoculated with the Ni resistant *B. cepacia* L.S.2.4::*ncc-nre*, while the non-inoculated control plants and the plants inoculated with the wild type strain L.S.2.4 had similar but lower Ni contents. In contrast to the roots, the Ni concentration in the shoots was comparable for the different treatments (Figure VII.3).

Inoculation of Lolium perenne with H. seropedicae LMG2284 and its Ni resistant derivative LMG2284::ncc-nre

In the absence of Ni, the 21 days-old *Lolium perenne* plants did not show significant differences in their shoot growth parameters when the different treatments were compared (Table VII.2). The presence of *H. seropedicae* LMG2284 did not influence the growth conditions of the plant except for the root development (Table VII.2). Slight but significantly lower root lengths were measured when the plants were

inoculated with the wild type and the Ni resistant derivatives of LMG2284. The Ni concentration in both roots and shoots was below the detection limit of 2.5 mg/kg DW.

	Root biomass (g)	Root length (cm)	Shoot biomass (g)	Shoot length (cm)
Lupinus luteus L.				
0 mM $NiCl_2$ treatment				
No inoculum	1.3 ± 0.2^a	13.1 ± 2^a	2.6 ± 0.3^a	14.5 ± 0.8^a
L.S.2.4	1.5 ± 0.2^a	10.6 ± 1.2^b	3 ± 0.5^a	14.6 ± 0.7^a
L.S.2.4::*ncc-nre*	1 ± 0.1^b	9.5 ± 0.8^c	2.7 ± 0.3^a	14.9 ± 0.7^a
0,25 mM $NiCl_2$ treatment				
No inoculum	0.3 ± 0.1^a	4.9 ± 1.6^a	1.3 ± 0.2^a	10.3 ± 1.1^a
L.S.2.4	0.4 ± 0.1^a	5 ± 1.4^a	1.4 ± 0.3^a	11.3 ± 0.7^a
L.S.2.4::*ncc-nre*	0.4 ± 0.1^a	4.8 ± 1.9^a	1.3 ± 0.3^a	10.7 ± 0.9^a
Lolium perenne				
0 mM $NiCl_2$ treatment				
No inoculum	0.1 ± 0.02^a	5.3 ± 1.5^a	0.4 ± 0.1^a	11 ± 1.8^a
LMG2284	0.1 ± 0.02^a	3.6 ± 1^b	0.5 ± 0.02^a	10.7 ± 2^a
LMG2284::*ncc-nre*	0.1 ± 0.004^a	4.4 ± 1.6^{ac}	0.4 ± 0.07^a	9.2 ± 2.4^b
0,25 mM $NiCl_2$ treatment				
No inoculum	0.1 ± 0.03^a	1 ± 0.5^a	0.3 ± 0.05^a	6.2 ± 1.2^a
LMG2284	0.1 ± 0.04^a	1.6 ± 1.1^b	0.3 ± 0.06^a	6.3 ± 1.6^a
LMG2284::*ncc-nre*	0.1 ± 0.01^a	1.2 ± 0.6^{ab}	0.4 ± 0.03^a	5.7 ± 1.3^a

Table VII.2: Morphological parameters of *Lupinus luteus* L. and *Lolium perenne* plants for the different inocula and the different Ni treatments. L.S.2.4 and LMG2284 were inoculated as wild type strains whereas L.S.2.4::*ncc-nre* and LMG2284::*ncc-nre* were inoculated as its Ni resistant derivatives in *Lupinus* and *Lolium perenne*, respectively. Data are mean values of 30 replicate seedlings ± S.D. Different superscripted letters indicate values that are statistically different ($P<0.05$).

The *Lolium perenne* plants did suffer from the 0.25 mM $NiCl_2$ treatment. Root and shoot biomass and length strongly decreased compared to the untreated control (Table VII.2). As was observed for the 0 mM $NiCl_2$ treatment, the different inocula actually did not seem to have major effects on plant growth (Table VII.2). In contrast, analysis of the Ni content in the roots and shoots revealed that the non-inoculated *Lolium perenne* plants accumulated more Ni as compared to the inoculated plants (Figure

VII.4). Addition of the wild type LMG2284 and its Ni resistant derivative leads to a significantly lower Ni content in the roots as compared to the non-inoculated plants and this was even more pronounced for the plants inoculated with the Ni resistant LMG284::*ncc-nre*. The same phenomenon was observed in the shoots except that the Ni content was not significantly different for both wild type LMG2284 and Ni resistance derivative LMG2284::*ncc-nre* inoculation treatments.

Discussion

The introduction of heavy metal resistance systems from bacteria of polluted environments in the selected endophytic strains *B. cepacia* L.S.2.4 and *H. seropedicae* LMG2284 was performed in order to test the effect of such bacteria in uptake and tolerance for heavy metals in their host plants. Among the heavy metal resistance systems used in this study, only the *ncc-nre* system encoding for Ni, Cd, and Co resistance in *R. metallidurans* 31A was clearly expressed in the endophytic hosts. In contrast to the observation of Schmidt & Schlegel (1994) that transfer of pTOM8 and pTOM9 confers an increased resistance of Ni, Cd, and Co in the metal sensitive cured mutant of *R. metallidurans* CH34 (AE104), our study shows that the endophytic strains only proved to have an increased Ni resistance compared to the wild type. Previous reports already mentioned the broad host range of this resistance system: heterologous expression was observed in *Escherichia coli*, *Sphingobacterium heparinum*, and *Pseudomonas putida* (Dong *et al*., 1998) and in the plant associated strains *Pseudomonas stutzeri* A15 and *Azospirillum irakense* KBC1 (Y.Wang, personal communication). Taghavi *et al.* (2001) concluded that broad host Ni resistance is encoded by *nre* and that the *ncc* determinant is only expressed in *R. metallidurans*-like strains. This could explain the fact that only an increase in Ni resistance was observed in the transconjugants of *B. cepacia* L.S.2.4 and *H. seropedicae* LMG2284.

Due to the instability of the pMOL222 plasmid, we introduced the *ncc-nre* genes in the endophytic strains by means of miniTn*5* transposons. This approach resulted in stable Ni resistant strains that were used in the subsequent Ni removal and inoculation experiments.

The removal of Ni and alkalisation of the growth medium as observed for the *R. metallidurans* 31A culture and to a lesser extent for the endophytic strains was similar

to the removal of Zn and Cd observed with the *czc* system of *R. metallidurans* CH34 (Diels *et al*., 1995). In the case of Cd removal by *czc*, alkalisation of the medium, the formation of metal carbonates from CO_2 produced by the cell metabolism, and precipitation of the metal carbonates on the cell surface are thought to be the main factors (Diels *et al*., 1993a). The mechanism of Ni removal in our study is unknown but Taghavi *et al.* (2001) demonstrated that *R. metallidurans* cells are able to sequestrate Ni during the beginning of the stationary growth phase due to the expression of *ncc*, which was postulated to act as a cation-proton anti-porter system (Nies, 1992).

Table VII.2. shows that the measured morphological parameters from 21 days-old *Lupinus* and *Lolium perenne* plants whether or not inoculated with their respective endophytic bacteria were the same except for root length. This parameter was slightly but significantly lower for the inoculated than for the control plants. This suggests that the endophytic strains demonstrated neither the positive effect on growth like Plant Growth Promoting Bacteria (PGP) do, nor the negative growth effect or the development of disease symptoms as is observed for pathogens. This agrees with Bevivino *et al* (1998) who showed that bacterisation of *Zea mays* L. seeds with *B. cepacia* isolates had no significant influence on plant development. *Herbaspirillum* sp. were reported to colonise Graminaceous plants efficiently, without causing disease symptoms (Reinhold-Hurek & Hurek, 1998).

When the Ni content in the roots and shoots is considered, *Lolium perenne* and *Lupinus* plants responded differently. Figure VII.3 shows that *Lupinus* was able to accumulate higher levels of Ni in its roots while *Lolium perenne* seemed to be a relatively better translocator of Ni to the shoots (Figure VII.4). The effect of the inoculation on the Ni content in the roots and shoots was different for the selected *B. cepacia* and *H. seropedicae* strains. Colonisation by the Ni-resistant *B. cepacia* L.S.2.4::*ncc-nre* caused a significant increase in the Ni concentration of the roots. This phenomenon could be attributed to the heterologous expression of the *ncc-nre* system in the *B. cepacia* L.S.2.4::*ncc-nre* strain and to the subsequent sequestration processes, which are presumed to occur and which could affect the free and therefore toxic Ni fraction in the roots. In contrast to the root, the Ni concentration in the shoots was not affected by the presence of the Ni resistant *B. cepacia* L.S.2.4::*ncc-nre*. A possible explanation is the preferential colonization of the *Lupinus* roots. Another

reason might be that the Ni ions are complexed in the roots, and consequently their transfer to the shoots is blocked.

The presence of Ni resistant *H. seropedicae* in *Lolium perenne* seems to have the same effect on the Ni concentration in both the roots and shoots. Compared to the non-inoculated control plants, the Ni content decreased slightly in the 21 days old *Lolium perenne* roots and shoots when inoculated with the wild-type as well as the Ni resistant derivative of *H. seropedicae*. This suggests that the occurrence of these strains, both Ni sensitive and Ni resistant, could be responsible for a kind of protection, which results in a decreased Ni uptake by the host plant. The fact that only minor Ni complexation was observed in growth cultures of LMG2284::*ncc-nre* might imply that even a successful inoculation in its host plant would not change Ni uptake and accumulation significantly.

The experiments with inoculated plants reported here suggest that *B. cepacia* and *H. seropedicae* as endophytic wild-type and Ni resistant bacteria affect Ni uptake in their respective host plant. However, their presence does not contribute to an improvement of the Ni translocation to the shoots which would have been an important feature to improve the efficiency of phytoextraction processes focussing the aboveground plant tissues. It should also be mentioned that these experiments were performed under laboratory conditions and in a sterile environment without any interference of competing micro-organisms that are present in soil. *In situ* conditions should certainly be studied as well in order to evaluate the behaviour of these endophytic bacteria and their potential contribution to the heavy metal uptake and translocation capacities of their host plant.

CHAPTER VIII

General conclusions and perspectives

The close association between endophytic bacteria and their host plant together with the knowledge on the functioning of bacterial heavy metal resistance systems were presumed to be important aspects to be explored to improve phytoextraction of heavy metals from contaminated soils. Therefore, naturally occurring endophytic bacteria with heavy metal resistance characteristics as well as genetically engineered endophytic bacteria were studied to examine their effect on heavy metal speciation and on the heavy metal uptake and translocation characteristics of their host plant.

The bacterial populations associated with the well studied Zn hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*, growing on a soil from an abandoned Zn and Pb mining and smelter site, were investigated in order to obtain more information about their heavy metal resistance characteristics and the possible interactions with their host plant concerning metal speciation. It was clearly shown that not only rhizosphere and soil isolates but also the endophytic population residing in the shoots possess increased resistance properties against the main pollutants Zn and Cd. In addition, also Co and Ni resistance characteristics were frequently increased. These results might indicate that the presence of bacteria equipped with resistance mechanisms against heavy metals is a common phenomenon in such polluted environments including the plants' internal tissues. However, one should take into account that the isolation and sterilisation procedures probably eliminated part of the full bacterial population that could therefore not be evaluated in these experiments. The isolated populations in fact seemed to reflect the bioavailability of Zn in the different plant compartments. The rhizosphere was populated with more then 88% Zn resistant micro-organisms (see Table III.3) which confirmed the results of McGrath *et al*. (1997) claiming that rhizosphere soils in which *T. caerulescens* was grown tended to have higher concentrations of mobile and thus bioavailable Zn than non-rhizospheric soils. The resistance

properties of the endophytic population indicated that mainly the shoot is inhabited by bacteria with high Zn resistance characteristics while the root seemed to harbour bacteria with low heavy metal resistance and bacteria unable to grow on the media selected to test heavy metal resistance (Table III.2). This clear difference between the root and shoot endophytic populations might reflect different physiological conditions in these plant compartments. The fact that in *T. caerulescens* Zn seems to be mainly transported to the shoots as hydrated cations (Salt *et al*., 1999) requires xylem residing bacteria to be equipped with efficient mechanisms to protect themselves against the toxic effects of the metal. The same study also reported that intracellular Zn in the roots mainly is associated with histidine or complexed with the cell wall while in the shoots Zn coordination mainly seems to occur through organic acids. This may result in a difference in zinc bioavailability, which according to our study is reflected by the resistance properties of the different endophytic communities. Detailed localisation studies of the endophytic bacteria within *T. caerulescens*, using advanced molecular techniques such as Fluorescence *in situ* Hybridization (FISH), will certainly contribute to linking the physiological data on in planta heavy metal availability with the heavy metal resistance properties of the endophytic bacterial communities.

Identification of the individual endophytic community members by means of 16S rRNA revealed that part of them can be considered to be facultative endophytic while others might be obligate endophytic since their occurrence was found to be exclusively plant associated. *Sphingomonas* sp. for instance were found in every niche studied and may therefore have a facultative endophytic character. The shoot colonising *Methylobacterium* sp. on the other hand most probably belong to the obligate endophytic population that only survives within its host plant. Although its relationship to plants is relatively unknown, it already has been isolated from more than 70 species of plants including monocotyledons, dicotyledones, angiosperms, gymnosperms, and vascular and non-vascular plants (Corpe & Basile, 1982; Corpe, 1985; Dunleavy, 1988).

As facultative endophytic bacteria also might be colonisers of soil or rhizosphere environments, the presence of the observed resistance capacities might be acquired in order to survive the harsh conditions that are found outside the plant. Obligate endophytic inhabitants apparently have similar characteristics. As mentioned earlier, not only resistance against the main pollutants Zn and Cd was observed, but in some cases the bacteria also survived increased concentrations of Co and Ni. This could be the result of multiple heavy metal resistance mechanisms. Multiple resistance against heavy metals, encoded by one resistance operon, is often found, and well-studied examples include the plasmid encoded *czc* (Zn^r, Co^r,

Cd^r) and *cnr* (Co^r, Ni^r) operons from *R. metallidurans* CH34 isolated from an industrial biotope (Taghavi *et al*., 1997; Mergeay, 2000), the *czr* (Zn^r, Cd^r) resistance system of *P. aeruginosa* from a Pakistan river polluted by heavy metals (Hassan *et al*., 1999) and the *ncc-nre* (Ni^r, Co^r, Cd^r) operon of *R. metallidurans* 31A, a strain isolated from a Zn decantation tank (Schmidt *et al*., 1991). Future research should focus on the characterisation of the metal resistances observed in the endophytic bacteria. Using probes of known heavy metal resistance mechanisms might already give a first indication concerning the identity of the mechanisms involved in the observed heavy metal resistance phenotypes. Further, metal removal experiments, as realised for *Pseudomonas* sp. VM422, should certainly be performed in order to reveal the ability of the endophytic bacteria to change metal speciation and to select potentially interesting strains for further detailed investigations. One might expect that endophytic bacteria, when equipped with similar heavy metal resistance properties as observed in the more extensively studied soil micro-organisms, could possibly affect heavy metal speciation in the same way. Precipitation and sequestration processes near the bacterial cell wall were found to be responsible for the removal of the Zn and Cd ions from cultures of the soil bacterium *R. metallidurans* CH34 (Diels *et al*., 1995). The metals loaded on these bacteria are supposed to reduce metal interference with the plants' metabolism and therefore reduce toxic effects of assimilated metals.

To test this hypothesis, Zn, Co, and Ni resistant *Pseudomonas* species that fulfilled these requirements were available. Since they were isolated from surface sterilised roots and shoots of *Brassica napus* plants growing in a non-polluted garden soil, they were expected to colonise their host plant endophytically. Further, the capacity to efficiently remove and precipitate Zn from solutions (up to 98 %) was also observed for *Pseudomonas* sp. VM422 indicating the presence of a similar Zn resistance and sequestration mechanism as observed for *R. metallidurans* CH34. Obviously, the presence of heavy metal pollution is not a prerequisite for the bacterial population to be equipped with resistance mechanisms. Re-inoculation studies (Chapter V) actually suggested that the endophytic character of *Pseudomonas* sp. VM422 might only be facultative. For this reason the observed results concerning Zn uptake and accumulation could not be linked to the presence or absence of the *Pseudomonas* sp. VM422 in the internal tissues. Despite the failure to efficiently colonise its host plant endophytically, the root growth stimulation and the high amount of Zn resistant colony forming units recovered from the non-surface sterilised root indicated that the plant associated character of *Pseudomonas* sp.VM422 is mainly linked to the root environment. Although not statistically significant, the lower amount of Zn measured in the inoculated *B.*

napus plants compared to the non-inoculated control plants might be attributed to the ability of *Pseudomonas* sp. VM422 to reduce the bioavailable Zn levels present in the perlite soil.

Endophytic bacteria isolated from our *T. caerulescens* and *B. napus* plants turned out to provide interesting information concerning heavy metal resistance characteristics but it was difficult to evaluate their plant colonising properties under the conditions used. Due to the sterilisation and isolation procedures applied, it is impossible to determine total population levels and to distinguish between obligate endophytic bacteria and facultative inhabitants. Molecular techniques like PCR, using appropriate primers, could possibly supply more detailed information concerning such populations since non-cultivable bacteria would be detected as well. On the other hand, it is unclear whether such heavy metal resistant bacteria, facultatively or obligately endophytic, actually are able to affect the heavy metal bioavailability in their host plant. To gain more information on this topic, some other bacteria like *Comamonas acidovorans* VM418 and VM427 with an endophytic character and high resistance levels against Zn (Chapter IV) could be alternative candidates for re-inoculation studies in *B. napus*. The obligate endophytic Zn resistant *Methylobacterium* species isolated from the shoots of *T. caerulescens* is another promising candidate. As for the endophytic populations of the hyperaccumulator *T. caerulescens*, localisation studies in *B. napus* would be a prerequisite to better evaluate colonisation capacities and to gain more information concerning the settlement of the bacteria within the different plant compartments. A close examination of the bacterial cell within the host plant, growing in the presence of Zn, might provide information about the ability to bio-precipitate or sequestrate Zn, as was already postulated for the Zn resistant *Pseudomonas* sp. VM422 when grown in solutions under laboratory conditions.

In this study on heavy metal resistant endophytic bacteria and their potential contribution to heavy metal phytoextraction, an alternative strategy using well characterised metal sensitive facultative and obligate endophytic strains was followed to better control the colonizing capacities (Chapter VII). Additionally, the introduction of known heavy metal resistance systems in these bacteria demonstrated that heterologous expression was only efficient for the *ncc-nre* resistance determinants from *R. metallidurans* 31A. More specifically, *nre* was found to be responsible for the broad-host-range and for the increased Ni resistance observed in the bacterial transconjugants, while *ncc* only generated a combined high resistance to Ni, Cd, and Co in *R. metallidurans* (Chapter VI). Ni removal experiments confirmed the presumption that the *ncc* operon functions in the same way as the *czc* operon (Nies, 1992) possibly generating similar bioprecipitation processes near the bacterial cell wall as seen for *czc*. Although *ncc*

alone is not expressed in the endophytic strains used, still its presence together with *nre* supplied *Burkholderia cepacia* L.S.2.4 and *Herbaspirillum seropedicae* LMG2284 strains with the capacity to reduce the initial Ni concentration in the growth medium by 35% and 15% respectively (Figure VII.3 & VII.4). For this reason these strains were considered to fulfil the requirement to test the above mentioned hypothesis. The involvement of *ncc* is not clear and should be studied in more detail in order to understand its contribution in the capacity of these strains to change Ni speciation under the experimental conditions applied. The Ni resistant derivative of *B. cepacia* L.S.2.4, defined as a facultative endophytic coloniser of lupin, showed that re-colonisation might influence the hosts' Ni metabolism to some extent. Only in the root tissue of 21 days old *Lupinus luteus* L plants, a significant increase of the Ni concentration was observed compared to the non-inoculated control plants (Figure VII.3). The predominant root colonising capacity of this strain could imply that a certain bacterial cell density is absolutely required in order to interfere with the plant's Ni metabolism. It would be interesting to link these results to microscopic studies to fully relate the bacterial bio-precipitation and sequestration processes with the increased Ni concentrations observed in the root. These experiments were performed under controlled laboratory conditions where the exclusive micro-organism–plant interactions were studied without considering multiple factors interfering in field situations. For this reason, field experiments are essential to evaluate to what extent these laboratory results are representative for practical applications.

Metal loading of roots might be useful for phytostabilisation and phytoextraction. Plants which reduce the bioavailable heavy metal fraction in the soil, improve the diversification of the vegetation. Root colonisation of plants, which develop a high root or tuber biomass, with metal resistant endophytic bacteria with properties analogous to those of *B. cepacia* L.S.2.4::*ncc-nre* might provide a strategy for improving phytoextraction.

The inoculation experiments performed with *Lolium perenne* (cv Atlas) (Chapter VII) suggest that besides the plant colonising capacity the ability of bacteria to efficiently reduce bioavailable Ni levels is probably a further important parameter. In contrast to *B. cepacia* L.S.2.4, Ni resistant derivatives of the obligate endophytic strain *H. seropedicae* LMG2284 probably did not meet this criterion. Although high numbers of Ni resistant colony forming units were recovered both in roots and shoots of *L. perenne*, they did not contribute to a change of the Ni speciation. The Ni removal capacity of LMG2284::*ncc-nre* probably was too low to generate a significant effect on the plant's Ni uptake and translocation capacity. Therefore, bacteria able to colonise roots and shoots to the same extent as *H. seropedicae*

LMG2284 combined with at least similar but preferably even better Ni removal capacities as observed for *B. cepacia* L.S.2.4::*ncc-nre* would certainly provide more information concerning the use of such bacteria in phytoextraction.

In conclusion, the interaction between plants and heavy metal resistant endophytic bacteria, able to efficiently colonise their host plant and to alter heavy metal speciation, is an important issue not to be neglected for improving phytoremediation strategies.

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APPENDIX

Transfer of heavy metal resistance determinants via exogenous isolation

An alternative way for introducing heavy metal resistance systems in bacteria is through exogenous isolation. This procedure has been successfully applied before to isolated environmental traits (Bale *et al*., 1987, 1988; Top *et al*., 1994) and is mainly based on the direct transfer of potential interesting resistance or degradation traits from a bacterial consortium into a host strain. It also provides a tool to screen communities for the presence of self-transferable plasmids that potentially play a role in the distribution of traits in the population. The fact that this technique can be considered as a relatively spontaneous and natural way to transfer genetic material from a bacterial population, without the need of isolating and cultivating individual strains, offers some benefits against the more standard techniques of transfer. In order to perform an exogenous isolation, a well marked recipient strain is required in order to distinguish transconjugants from the bulk population growing on the selection media. A metal-sensitive, auxotrophic plasmid-free derivative of *R. metallidurans* CH34, possessing a *gfp* marker as well as resistance against Naladixic acid (strain AE2547) was selected to function as a host strain (Table A.1) to isolate Zn resistance properties from an environmental population. Rhizosphere samples of the Zn hyperaccumulator *Thlaspi caerulescens,* residing in a contaminated soil of an abandoned Zn and Pb smelter in Plombières (Belgium), were used as the donor population. Additionally, pULB113 containing *E. coli* strains were added to function as helper strain to facilitate the transfer of Mob^+ Tra^- plasmids.

Strain or plasmid	Relevant characteristics	Source/reference
Ralstonia metallidurans		
AE0229	*aut*, *dca*, *nal*-110, *ntr*, *phe*-42	Sadouk & Mergeay, 1993
AE0593	*aut*, *dca*, *ntr*, *tim*, *trp*-593	Sadouk & Mergeay, unpublished
AE2547	*aut*, *dca*, *nal*-110, *ntr*, *phe*-42; gfp^+, Tet^+	This study
AE2558	Zn^r, Co^r, Cd^r and Cu^r derivative of AE2547	This study, obtained after exogenous isolation
AE2559	Zn^r, Co^r, Cd^r and Cu^r derivative of AE2547	This study, obtained after exogenous isolation
Escherichia coli		
CM0214	Plasmid RP4::Mu3A (=pULB113), Δ(*lac-pro*), F^-, galE, *rec*A, *thi*A	Van Gijsegem & Toussaint, 1982

Table A.1: Strains and plasmids used.

The exogenous isolation was performed under standard conditions used for conjugation experiments except that the donor population was a non-cultivated extract from the rhizosphere of *T. caerulescens*. The mating was carried out on 10 times diluted LB medium for 4 days at 30°C. Transconjugants were selected at 30°C for their ability to grow on Tris buffered minimal medium supplemented with 100 µg/ml Naladixic acid, 30µg/ml Tetracyclin, 40µg/ml Phenylalanine, and 0.8 mM Zn. Additionally *gfp* expression was verified. The efficiency of transfer was found to be 8 x 10^{-8} transconjugants per recipient in the presence of the helper strain CM0214. In the absence of this helper strain, no transconjugants were found. Purification of these transconjugants revealed that transfer of Zn resistance characteristics indeed took place under the conditions used. Apparently, two different phenotypes were observed: those with high combined Zn, Cd, and Co resistance characteristics (AE2559) and those with lower resistance capacities for these heavy metals (AE2558) (Table A.2) High levels of Cu resistance were also observed in both types. These results indicate that transfer of multiple heavy metal resistance characteristics from the rhizosphere population to the recipient strain AE2547 is possible under the conditions used in these experiments.

	AE2547	**AE2558**	**AE2559**
Zn	< 1 mM	2-10 mM	10-20 mM
Cd	< 0.8 mM	1 mM	2-5 mM
Co	< 1 mM	0.8-5 mM	5-10 mM
Cu	1 mM	1 mM	2 mM
Pb	< 1 mM	< 1 mM	< 1 mM
Ni	< 0.6 mM	< 0.6 mM	< 0.6 mM

Table A.2. Minimum Inhibitory Concentrations of Zn, Cd, Co, Cu, Ni, and Pb added to the Tris buffered minimal medium for the recipient strain AE2547 and two representatives (AE2558 and AE2559) of the purified transconjugants with different heavy metal resistance phenotypes.

Using strain AE2559 as a donor for Zn resistance, the frequency of transfer to a related heavy metal sensitive, auxotrophic *R. metallidurans* species AE0593 was 10^{-4} transconjugants per recipient, indicating a very efficient transfer under laboratory conditions between related species. Again, the second generation transconjugants showed similar resistance properties compared to donor strain AE2559 (data not shown). Plasmid extractions on AE2547 and AE2558 revealed the presence of mega-plasmids (> 200 kb) in both strains (data not shown). Combined with the high transfer frequencies of the heavy metal resistance phenotypes between the *R. metallidurans* strains, it is hypothesised that the observed heavy metal resistances are encoded by these mega-plasmids.

Attempts to transfer these Zn resistances to the endophytic strains *H. seropedicae* and *B. cepacia* by means of biparental and triparental matings failed. Since no suitable marked derivatives of these endophytic strains were available in order to explore the possibilities of a direct transfer from the rhizosphere population, we were unable to apply the strategy of exogenous isolation to improve the heavy metal resistance properties of these endophytic bacteria.

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Nickel-Resistance-Based Minitransposons: New Tools for Genetic Manipulation of Environmental Bacteria

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The *ncc* and *nre* nickel resistance determinants from *Ralstonia eutropha*-like strain 31A were used to construct mini-Tn*5* transposons. Broad host expression of nickel resistance was observed for the *nre* mini-transposons in members of the α, β, and γ subclasses of the *Proteobacteria*, while the *ncc* minitransposons expressed nickel resistance only in *R. eutropha*-like strains.

Several nickel resistance determinants have been identified in *Ralstonia eutropha (Alcaligenes eutrophus*) (24) strains isolated from different biotopes heavily polluted with heavy metals. The *cnr*YXHCBA operon of *R. eutropha* CH34 plasmid pMOL28 (12), which mediates medium levels of nickel resistance (up to 10 mM) and cobalt resistance, is the most thoroughly studied determinant (3, 11, 17, 18, 20). The resistance mechanism mediated by *cnr* is inducible and is due to an energy-dependent efflux system driven by a chemo-osmotic proton-antiporter system (6, 18, 22, 23). A 14.5-kb *Bam*HI fragment of plasmid pTOM9 from *R. eutropha*-like strain 31A (*Alcaligenes xylosoxidans* 31A) (10) and a similar *Bam*HI fragment of plasmid pGOE2 from *R. eutropha*-like strain KTO2 were also found to encode Ni resistance. On both fragments a locus mediating high-level nickel resistance (up to 20 to 50 mM) and a locus mediating low-level nickel resistance (3 mM) were identified and designated *ncc* and *nre*, respectively (15, 16). The *ncc*YXHCBAN determinant, which except for the *ncc*N gene is very similar to *cnr*, causes high levels of nickel and cobalt resistance and a low level of cadmium resistance in *R. eutropha*. Neither *cnr* nor *ncc* is expressed in *Escherichia coli*. On the other hand, the 1.8-kb *nre* locus causes low levels of nickel resistance in both *Ralstonia* and *E. coli* (16). An *nre*-like determinant, which could be expressed in *E. coli* and *Citrobacter freundii*, was also found in *Klebsiella oxytoca* CCUG15788 (19, 20).

Recently, amplified ribosomal DNA restriction analysis was used to determine the phylogenetic position of zinc- and nickel-resistant *Ralstonia*-like strains (2). The *ncc* operon was found in many nickel-resistant *R. eutropha*-like strains and in environmental strains in the direct vicinity of the genus *Burkholderia* (2), a member of the β subclass of the class *Proteobacteria* like the genus *Ralstonia*. This might indicate that *ncc* has range of expression broader than the genus *Ralstonia*.

Heavy metal resistance markers with broad host expression ranges have been shown to be useful for genetic manipulation of *Pseudomonas* strains potentially designated for environmental release (14). Broad-host-range expression of *ncc-nre* was recently confirmed by Dong et al. (7), who found *ncc-nre*-based Ni resistance in *Comamonas, Sphingobacterium heparinum*, flavobacteria, and even gram-positive bacteria related to *Arthrobacter*. However, it was not clear from this study which of the Ni resistance determinants was responsible for the broad-host-range Ni resistance. In addition, plasmid instability problems were encountered with some of the transconjugants. In order to study the range of expression of *ncc* and *nre* and to develop new tools for genetic manipulation of environmental bacteria, which are not based on antibiotic resistance markers, the Ni resistance markers were introduced into mini-Tn*5* transposon vectors. The new *nre*-based minitransposons were found to have a broad expression range and were successfully used for constructing Ni-resistant transconjugants of plant-associated bacteria belonging to families of the α, β, and γ subclasses of the class *Proteobacteria*, including plant-associated endophytic bacteria with potential to improve phytoremediation strategies (C. Lodewyckx, S. Taghavi, M. Mergeay, J. Vangronsveld, H. Clijsters, and D. van der Lelie, submitted for publication).

Construction of Ni resistance minitransposons. The *ncc* operon of pTOM9 was cloned in pUC18/*Not*I as a 8.1-kb *Bam*HI-*Pst*I fragment, resulting in pMOL1522 (*E. coli* CM2395). Plasmid pMOL1522 was digested with *Not*I, and the *ncc*-containing *Not*I fragment was subsequently cloned in the unique *Not*I site of pUTmini-Tn*5*-Km1 (4). This resulted in plasmid pUTminiTn*5*-Km1/*ncc* (pMOL1524 in *E. coli* CM2428).

In order to construct an *nre*-based mini-Tn*5* transposon vector, it was necessary to inactivate the *Not*I site in *nre*B. PCR mutagenesis performed with primers *nre-Pst*I (sense) and *nre-Not*I (antisense) and with primers *nre-Not*I (sense) and *nre-Eco*RI (antisense) (Table 1) was used to change the *Not*I site with the sequence GCGGCCGC into GCGGCAGC. This resulted in two PCR fragments that were approximately 1.6 and 1.1 kb long, respectively. Subsequently, these fragments were mixed and joined by using a PCR-based ligation strategy and were amplified with primers *nre-Pst*I (sense) and *nre-Eco*RI (antisense). This resulted in a 2.7-kb *Pst*I-*Eco*RI fragment with the mutated *nre* operon. The mutation did not affect the amino acid sequence of the NreB protein, since GCC and GCA both

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TABLE 1. Primers used for PCR mutagenesis and amplification of the *nre* region[a]

Primer	Start position	End position	Sequence (5′-3′)	Comment
*nre-Pst*I (sense)	1477	1510	CGC CTG CAG CGC AGA CCG TGG CGG CAG CGG CGC C	
*nre-Pst*I (antisense)	4183	4160	AAA CTG CAG CCC GGA TTG AAA ATG CGA CTC ATG	AAA CTG CAG was added at the 5′ end
*nre-Eco*RI (antisense)	4189	4160	AAA GAA TTC CCC GGA TTG AAA ATG CGA CTC ATG	AAA was added at the 5′ end
*nre-Sfi*I (sense)	2234	2286	GGA GAG CGC CGT GAC CCA GGC gAA GAA GGC gCT GGT GCA TGA CCA TAT CGA CC	Mutations (C to G) are indicated by lowercase g
*nre-Sfi*I (antisense)	2286	2234	GGT CGA TAT GGT CAT GCA CCA GcG CCT TCT TcG CCT GGG TCA CGG CGC TCT CC	Mutations (G to C) are indicated by lowercase c
*nre-Not*I (sense)	3107	3164	GAA GGG ACT GCT GGC GCT GAA TCT TGC CGC GGC aGC AGC CAG CGC CAT GGT GAT CGT G	Mutation (C to A) is indicated by lowercase a
*nre-Not*I (antisense)	3164	3107	CAC GAT CAC CAT GGC GCT GGC TGC tGC CGC GGC AAG ATT CAG CGC CAG CAG TCC CTTC	Mutation (G to T) is indicated by lowercase t

[a] The positions of the relevant restriction sites used for cloning of the amplified PCR fragments are underlined in the primer sequences. Sequence positions are based on the *nre* sequence (GenBank accession number L31491 [10]).

encode alanine. The 2.7-kb *Pst*I-*Eco*RI fragment was subsequently cloned into pUC18/*Not*I, resulting in plasmid pMOL1525 (*E. coli* CM2438). Plasmid pMOL1525 was digested with *Not*I, and the *nre*-containing *Not*I fragment was cloned in the unique *Not*I site of pUTmini-Tn*5*-Km1. This resulted in plasmid pUTminiTn*5*-Km1/*nre*(*Not*I) (pMOL1527 in *E. coli* CM2442).

The region with the mutated *nre*B gene was also amplified as a 2.7-kb *Pst*I fragment by using primers *nre-Pst*I (sense) and

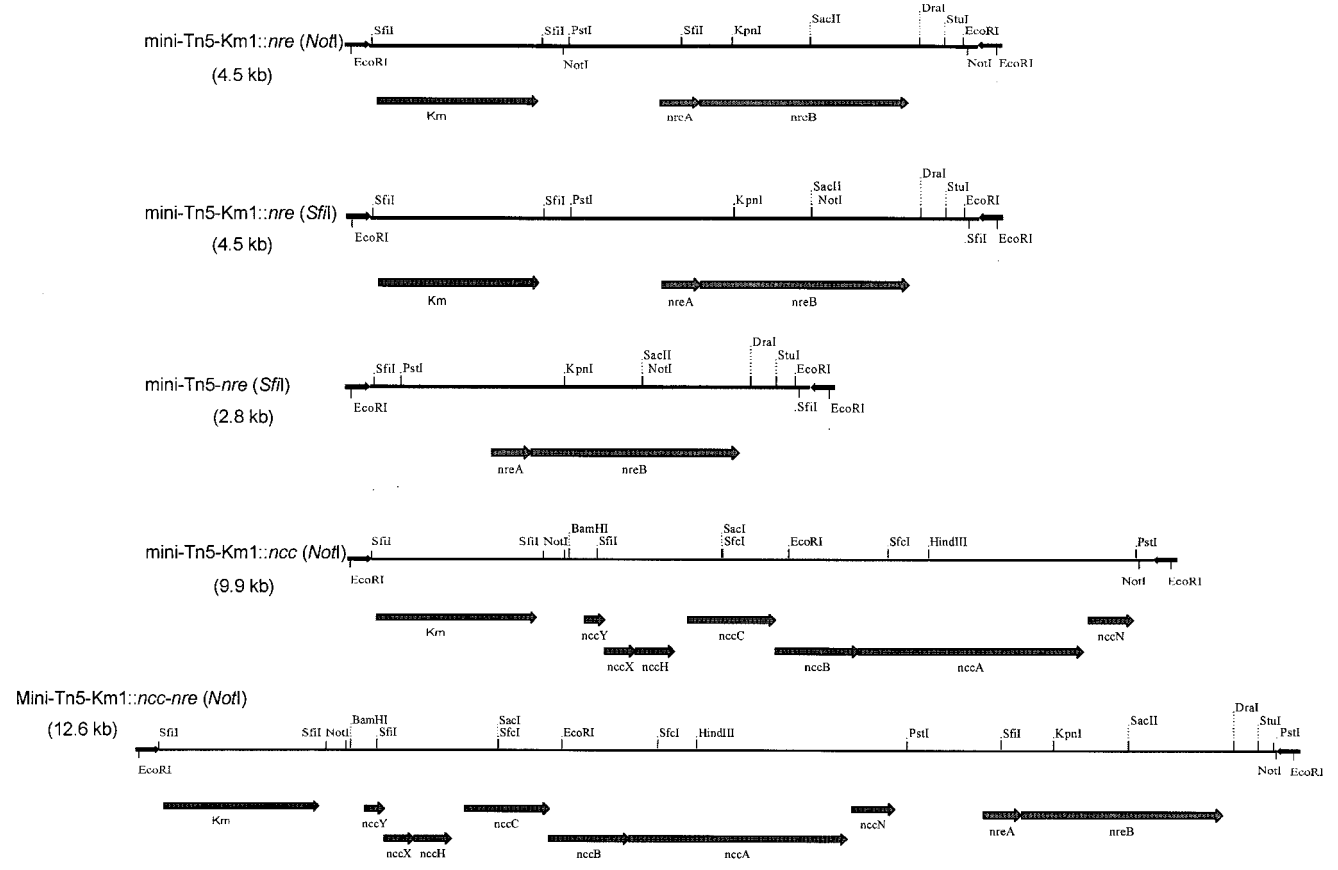

FIG. 1. Schematic representation of different mini-Tn*5* Ni resistance transposons. The positions of the kanamycin resistance marker (Km), the Ni resistance determinants *ncc* and *nre*, the inverted repeats at the extremities of the minitransposons, and important restriction sites are indicated. The sizes of the minitransposons are given in parentheses.

TABLE 2. Mutation and transconjugation frequencies of the strains used to test transfer and heterologous expression of *ncc, nre*, and *ncc* plus *nre*[a]

Donor	Recipient	Frequency of transconjugation		Frequency of mutation	
		Ni resistant	Kanamycin resistant	Ni resistant	Kanamycin resistant
CM2676 (*nre* Km^r)	AE104	9.2×10^{-5}		$<7.1 \times 10^{-10}$	2.4×10^{-8}
	DH10B	4.6×10^{-6}		$<4.2 \times 10^{-10}$	$<4.2 \times 10^{-10}$
	LMG2284	9.7×10^{-8}		$<1.8 \times 10^{-9}$	1.3×10^{-8}
	W1.2	7.1×10^{-10}		$<4.5 \times 10^{-10}$	$<4.5 \times 10^{-10}$
	LS2.4	5.8×10^{-8}		$<0.8 \times 10^{-9}$	5.7×10^{-8}
	A15	1.7×10^{-9}		$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$
	VM0433	3.9×10^{-6}		$<2.5 \times 10^{-10}$	3.5×10^{-8}
CM2677 (*nre*)	AE104	9.5×10^{-5}			
	DH10B	9×10^{-6}			
	LMG2284	1.1×10^{-7}			
	W1.2	$<4.5 \times 10^{-10}$			
	LS2.4	2.7×10^{-8}			
	A15	9.6×10^{-9}			
	VM0433	3.7×10^{-6}			
CM2536 (*ncc* Km^r)	AE104	1.5×10^{-5}	8.2×10^{-6}		
	DH10B	$<4.2 \times 10^{-10}$	2.8×10^{-7}		
	LMG2284	$<1.8 \times 10^{-9}$	1.2×10^{-7}		
	W1.2	$<4.5 \times 10^{-10}$	1.3×10^{-9}		
	LS2.4	$<0.8 \times 10^{-9}$	2.3×10^{-7}		
	A15	$<1.3 \times 10^{-10}$	3.3×10^{-9}		
	VM0433	$<2.5 \times 10^{-10}$	1.4×10^{-7}		
CM2520 (*ncc nre* Km^r)	AE104	8.6×10^{-7}			
	DH10B	1.8×10^{-7}			
	LMG2284	1.4×10^{-8}			
	W1.2	1.1×10^{-9}			
	LS2.4	2×10^{-9}			
	A15	1.3×10^{-9}			
	VM0433	1.5×10^{-7}			

[a] The strains used as recipients in the matings were *R. eutropha* AE104, *E. coli* DH10B (Gibco BRL), *H. seropedicae* LMG2284, *B. cepacia* W1.2 and LS2.4, *P. stutzeri* A15, and *P. putida* VMO433. *E. coli* S17-1 (λ*pir*) (5) containing pUTminiTn*5*-Km1::*ncc-nre* (strain CM2520), pUTminiTn*5*-Km1::*ncc* (strain CM2536), pUTminiTn*5*-Km1::*nre* (strain CM2676), and pUTminiTn*5*-*nre* (strain CM2677) were used as donors. Ni-resistant transconjugants were selected on 284 minimal medium containing 1 mM $NiCl_2$. Most of the kanamycin-resistant transconjugants were selected on 284 minimal medium (12) containing 100 mg of kanamycin per liter; the only exception was strain AE104, whose transconjugants were selected on 284 minimal medium containing 1,000 mg of kanamycin per liter.

*nre-Pst*I (antisense). Subsequently, this fragment was cloned in the unique *Pst*I site of pMOL1522. This resulted in a 10.8-kb *ncc-nre* fragment flanked by two *Not*I sites (plasmid pMOL1548 in *E. coli* CM2500). This fragment was subsequently cloned in the unique *Not*I site of pUTmini-Tn*5*-Km1, resulting in plasmid pUTminiTn*5*-Km1/*ncc-nre (Not*I) (pMOL1554 in *E. coli* CM2520).

To inactivate the *Sfi*I site in *nre*A, we used a strategy similar to that used for mutation of the *Not*I site, except that primers *nre-Pst*I (sense) and *nre-Sfi*I (antisense) and primers *nre-Sfi*I (sense) and *nre-Eco*RI (antisense) were used. The mutations did not affect the amino acid sequence of the NreA protein. A 2.7-kb *Pst*I-*Eco*RI fragment with the mutated *nre* operon (*Sfi*I site) was subsequently cloned in pUC18/*Sfi*I (8), resulting in plasmid pMOL1526 (*E. coli* CM2440). Plasmid pMOL1526 was digested with *Sfi*I, and the *nre*-containing *Sfi*I fragment was cloned in *Sfi*I-digested pUTmini-Tn*5*-Km1. This resulted in plasmids pUTminiTn*5*-Km1/*nre(Sfi*I) (pMOL1529 in *E. coli* CM2446) and pUTminiTn*5*-*nre(Sfi*I) (pMOL1528 in *E. coli* CM2444), in which the kanamycin resistance gene was replaced by *nre*. The restriction maps of the mini-Tn*5* Ni resistance transposons are presented in Fig. 1.

Range of expression of Ni resistance. The range of expression of Ni resistance was examined for all mini-Tn*5* Ni resistance transposons. To do this, the pUT-based constructs were introduced into *E. coli* S17-1 (λ*pir*) (5) and subsequently transferred by conjugation into the nickel-sensitive strains *R. eutropha* AE104 (12), *E. coli* DH10B, *Burkholderia cepacia* W1.2 (isolated from wheat) and LS2.4 (isolated from lupine shoots) (a gift from K. Ophel-Keller), *Herbaspirillum seropedicae* LMG2284 (associated with rye grass) (1), *Pseudomonas stutzeri* A15 (associated with rice roots) (13, 25), *Azospirillum irakense* KBC1 (a rice endophyte) (9), and *Pseudomonas putida* VMO433. The last strain was isolated as an endophytic bacterium after surface sterilization of *Brassica napus* plants (Lodewyckx and van der Lelie, unpublished data). Transfer frequencies, as well as the appearance of nickel- and kanamycin-resistant mutants, were examined. The results are presented in Table 2.

Comparing the efficiencies of transfer of the minitransposons, we observed that *R. eutropha* AE104, *E. coli* DH10B, and *P. putida* VM0433 showed the highest transfer frequencies. The lowest transfer frequencies were observed for *B. cepacia* W1.2 and *P. stutzeri* A15; this might have been due to the presence of efficient restriction-modification systems present in these two strains.

No spontaneous Ni-resistant mutants were found for the strains used in the experiments; this is in contrast to the kanamycin-resistant mutants that were observed at low frequencies ($\sim 10^{-8}$) for most of the strains tested. This indicates that

TABLE 3. MICs of Ni in 284 minimal medium for wild-type strains and their Ni resistance transconjugants

Strain	MICs (mM)[a]				
	Wild type	MiniTn*5*-Km1::*ncc* (*Not*I)	MiniTn*5*-Km1::*nre* (*Sfi*I)	MiniTn*5*::*nre* (*Sfi*I)	MiniTn*5*-Km1::*ncc-nre* (*Not*I)
Ralstonia metallidurans AE104	0.6	20–40	3–4	3–4	>40
Herbaspirillum seropedicae LMG2284	0.4	0.4	3–4	3–4	2
Burkholderia cepacia					
W1.2	0.4	0.4	0.4	ND[b]	2
LS2.4	0.6	0.6	2	2	2
Pseudomonas putida VM0433	0.6	0.6	3–4	3	3
Pseudomonas stutzeri A15	0.6	1	3–4	2–3	3
Escherichia coli DH10B	≤0.6	≤0.6	3	2	2–3

[a] The ranges of the MICs were determined with four individual transconjugants.
[b] ND, not determined (no transconjugants available).

nickel resistance is a more reliable marker for selecting transconjugants than kanamycin.

Transconjugants were selected for kanamycin or nickel resistance (Table 2). The stabilities of the transconjugants were confirmed by growing them for more than 100 generations under nonselective conditions. Subsequently, the Ni resistance of these organisms was compared to that of the wild-type strains. As expected, both *ncc*- and *nre*-containing mini-Tn*5* transposons gave Ni resistance in *R. eutropha* AE104, and the MICs on 284 gluconate medium (Lodewyckx et al., submitted) were 3 and 40 mM Ni for *nre* and *ncc*, respectively (Table 3).

For all of the other strains tested except *B. cepacia* W1.2, Ni resistance was observed when the *nre* determinant was present. For these strains miniTn*5*-Km1/*ncc*-containing transconjugants had to be selected for kanamycin resistance. The presence of *nre* resulted in MICs of Ni for Ni resistance on 284 minimal medium with an appropriate C source that varied from 2 to 3 mM depending on the bacterial species (Table 3). In all cases the presence of *nre* was confirmed by PCR (results not shown). No Ni resistance was observed for transconjugants containing *ncc*, and the presence of both *ncc* and *nre* in general did not increase the MIC for Ni resistance, as determined for *nre*. However, two exceptions were found: in *P. stutzeri* A15 the presence of *ncc* resulted in an increase in Ni resistance (MIC) from 0.6 to 1.0 mM, while *B. cepacia* W1.2 transconjugants showed Ni resistance only when both *ncc* and *nre* were present. The latter phenomenon might imply that both *ncc* and *nre* contribute to Ni resistance, but it is not clear in what way. Therefore, it can be concluded that in general broad-host-range Ni resistance is encoded by *nre* and that the *ncc* determinant is expressed only in *R. eutropha*-like strains. This implies that only the *nre*-based nickel resistance minitransposons, such as miniTn*5*-*nre*(*Sfi*I), are suitable as broad-host-range selection markers for construction of antibiotic resistance-free but selectable strains belonging to the families of the α, β, and γ subclasses of the class *Proteobacteria*.

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