

Heavy Metal Resistance Patterns of *Frankia* Strains†

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The sensitivity of 12 *Frankia* strains to heavy metals was determined by a growth inhibition assay. In general, all of the strains were sensitive to low concentrations (<0.5 mM) of Ag^{1+} , AsO_2^{1-} , Cd^{2+} , SbO_2^{1-} , and Ni^{2+} , but most of the strains were less sensitive to Pb^{2+} (6 to 8 mM), CrO_4^{2-} (1.0 to 1.75 mM), AsO_4^{3-} (>50 mM), and SeO_2^{2-} (1.5 to 3.5 mM). While most strains were sensitive to 0.1 mM Cu^{2+} , four strains were resistant to elevated levels of Cu^{2+} (2 to 5 mM and concentrations as high as 20 mM). The mechanism of SeO_2^{2-} resistance seems to involve reduction of the selenite oxyanion to insoluble elemental selenium, whereas Pb^{2+} resistance and Cu^{2+} resistance may involve sequestration or binding mechanisms. Indications of the resistance mechanisms for the other heavy metals were not as clear.

Frankia, a member of the order *Actinomycetales*, forms a symbiotic nitrogen-fixing association with a variety of woody dicotyledonous plants (for reviews see references 3 and 28). The members of this bacterial genus are known to be associated with over 200 species of plants representing eight plant families. These bacteria fix N_2 from the atmosphere and produce a significant amount of the fixed nitrogen on the planet. Actinorhizal plants are ecologically important as pioneer community plants and have economic value in land reclamation, reforestation, and soil stabilization.

The lack of a well-established genetic system is a major obstacle in the elucidation of the mechanism of actinorhizal nitrogen fixation and plant-microbe interactions (for reviews see references 22, 23, and 25). There is a paucity of genetic markers for *Frankia*. Some of the most useful genetic markers include resistance to antibiotics, resistance to antimetabolites, and resistance to heavy metals. These directly selectable traits provide a mechanism for positive selection in genetic studies and are also useful in the development of cloning vectors. For example, metal resistance has been useful in the development of cloning vectors for *Rhodococcus*, another member of the *Actinomycetales* (8). Previously, we developed a growth inhibition assay that was used to screen several *Frankia* strains for resistance to antibiotics (27). Although several important antibiotic resistance markers were described in that study, we were interested in identifying other selectable genetic markers. Since actinorhizal plants have been used for land reclamation in strip-mined areas, we reasoned that it is possible that the bacteria are resistant to heavy metals. The purpose of this study was to extend the use of our growth inhibition assay to identify new selectable markers, resistance to heavy metals.

Frankia strains ACN1^{AG} (14), CcI3 (29), CcI.17 (18), CN3 (20), CpI1 succinate variant (CpI1-S) (5, 26), CpI1 propionate variant (CpI1-P) (5, 26), DC12 (1), EI5c (17), EAN1pec (16), EuI1c (2), EUN1f (14), and QA3 (12) were grown and maintained in basal growth medium with NH_4Cl as the nitrogen source, as described previously (26, 27). For the heavy metal sensitivity assays, the basal growth medium contained 20 mM glucose and 20 mM succinate as the carbon and energy sources (glucose-succinate medium). For strains EUN1f, CcI.17, CpI1-P, and QA3, the growth medium contained 5 mM propionate instead of glucose and succinate. For comparative purposes, *Escherichia coli* W3110, *Bacillus subtilis* 168, *Micromonospora echinospora* ATCC 15836, and *Streptomyces viridochromogenes* NRRL B-1511 were also used in this study.

The sensitivities of *Frankia* strains to heavy metals were determined by a growth inhibition plate assay that was developed initially to test for antibiotic sensitivity (27). For Cu^{2+} , AsO_2^{1-} , AsO_4^{3-} , and Pb^{2+} , the standard growth medium was replaced with a low-phosphate growth medium that contained 1 mM K_2HPO_4 . Metal ions, including AgNO_3 , Na_2HAsO_4 , NaAsO_2 , CdCl_2 , CoCl_2 , K_2CrO_4 , CuCl_2 , NiCl_2 , $\text{Pb}(\text{NO}_3)_2$, $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$, and Na_2SeO_2 , were added to the growth media at concentrations of 0.005, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 5.0, 6.0, 8.0, 10.0, and 20.0 mM; AsO_4^{3-} was also tested at concentrations of 25 and 50 mM. For these experiments, 14- to 21-day-old cultures were used as the inocula, and the hyphae were fragmented by using a tissue homogenizer. Each inoculum (0.5 ml) was added to a 10-ml sterile Falcon tube, and 3.0 ml of 0.8% Bacto Agar (Difco) at 45°C was also added to the tube. The contents of the tube were mixed by agitation with a vortex mixer and were poured onto the surface of a plate. Most plates were incubated at 30°C; the exceptions were the plates that contained strains DC12 and EAN1pec, which were incubated at 25°C, and the plates that contained strain CcI.17, which were incubated at 28°C.

Growth was scored after 2 weeks and was monitored for an additional 6 weeks. When this assay was used, growth could be easily scored visually. Growth was scored relative to the growth of the control by using a scale ranging from + to + + + +. With some *Frankia* strains, growth on the 10^0 control plates resulted

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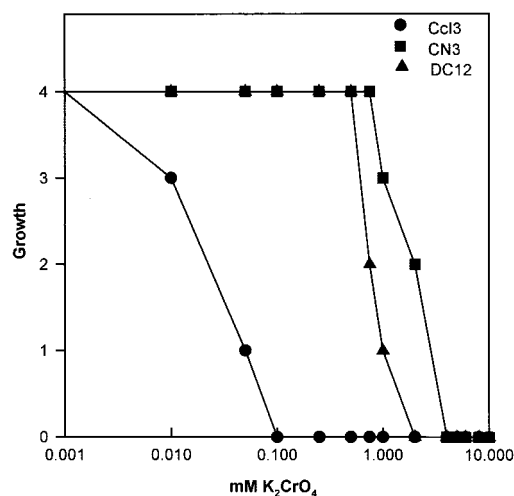


FIG. 1. *Frankia* growth as a function of heavy metal (chromate) concentration. Growth was scored on a scale from 0 to ++++ as described in the text.

in a thick bacterial lawn. After dilution and plating, the total number of hyphal fragments was also estimated. An average of about 10^7 fragments was plated for each assay. Plates that had the same amount of growth as the undiluted control were scored as +++, while plates that showed a 2-log decrease were scored as ++. Thus, the ++, ++, and + growth scores were equivalent to the growth on the 10^{-2} , 10^{-4} , and 10^{-6} control plates, respectively. A negative result indicated that there was no growth. For *E. coli* and *B. subtilis*, cells were incubated at 37°C and growth was scored on days 1 and 2. For *S. viridochromogenes* and *M. echinospora*, cells were incubated at 28°C and growth was scored on days 7 and 14.

Growth was quantified relative to the growth of a control containing no metals as described above. To evaluate the levels of resistance, the following two parameters were used: MIC and maximum tolerable concentration (MTC). The MTC is the highest concentration of metal which does not affect the viable count and is used with selective media designed to promote the

growth of a resistant bacterium by providing efficient counter-selection for undesired bacteria (19). The values were determined by inspection after the relative growth was plotted as a function of the log of the metal concentration. The MIC was determined by determining the intersection of the survival curve with the horizontal axis (Fig. 1). In each experiment each metal was tested in duplicate, and each metal was tested in three to seven replicate experiments. In all cases, similar results were obtained in the experiments performed.

Heavy metal sensitivity. Strains that showed resistance to metals as determined by the plate assay (Tables 1 and 2) were also resistant to the same metals in broth cultures (data not shown). All of the *Frankia* strains were sensitive to Ag^{1+} , Ni^{2+} , Cd^{2+} , SbO_2^{1-} , and AsO_3^{1-} . Compared to the control bacteria, a few *Frankia* strains had high MICs (≥ 0.4 mM) for Co^{2+} and were considered cobalt resistant. Most *Frankia* strains were resistant to AsO_4^{3-} , Pb^{2+} , SeO_2^{2-} , and CrO_4^{2-} . Several of these metal resistance properties and the effect of Cu^{2+} are described below.

CrO_4^{2-} resistance. Most of the isolates were resistant to elevated levels of CrO_4^{2-} (Table 1). The MICs for two isolates, CcI3 and EAN1pec, were <0.5 mM, and these isolates were considered chromate sensitive. For the chromate-resistant isolates the CrO_4^{2-} MICs ranged from 1.0 to 1.75 mM. The control bacteria *E. coli* and *B. subtilis* were chromate sensitive, while the actinomycetes *M. echinospora* and *S. viridochromogenes* were chromate resistant. When observed by phase-contrast microscopy, these bacteria exhibited no obvious morphological or structural changes (data not shown). As a mechanism of resistance to CrO_4^{2-} some organisms reduce the highly soluble oxyanion Cr(VI) to the less toxic cationic form Cr(III), which readily precipitates (6, 19, 24). No precipitate was observed with resistant strains that were grown in medium containing chromate. The amount of Cr(VI) in the spent growth medium was determined by measuring the absorbance at 380 nm. Cr(VI) absorbs light at 380 nm, while Cr(III) does not. The measurements showed that there was no change in the amount of Cr(VI) during growth. These results suggest that

TABLE 1. MICs of 10 heavy metals for 12 *Frankia* strains and other organisms

Strain or organism	MIC (mM) of ^a :									
	$AgNO_3$	Na_2HAsO_4	$NaAsO_2$	$CdCl_2$	$CoCl_2$	K_2CrO_4	$NiCl_2$	$Pb(NO_3)_2$	$K(SbO)C_4H_4O_6$	Na_2SeO_2
ACN1 ^{AG}	<0.05	>50	0.5	0.25	0.5	1.0	0.3	8.0	0.75	0.5
CcI17	<0.05	>50	0.2	0.1	1.0	1.0	0.5	6.0	0.25	2.0
CcI3	<0.05	50	0.1	0.3	0.3	0.1	0.3	6.0	0.25	0.3
CN3	<0.05	40	0.1	0.4	0.4	1.75	0.3	8.0	0.3	3.5
CpI1-P	<0.05	50	0.25	0.25	0.5	1.75	0.3	7.0	0.25	0.5
CpI1-S	<0.05	30	0.1	0.25	0.3	1.5	0.3	5.0	0.5	<0.5
DC12	<0.05	50	0.1	0.4	0.5	1.5	0.3	6.0	0.25	1.5
EAN1pec	<0.05	30	0.1	0.4	0.3	1.0	0.2	8.0	0.25	0.3
EI5c	<0.05	25	0.1	<0.1	0.3	1.0	0.3	5.0	0.25	2.5
EuI1c	<0.05	5	0.05	0.25	0.3	1.0	0.2	8.0	0.5	2.0
EUN1f	<0.05	>50	0.1	0.3	0.3	1.0	0.3	6.0	0.25	2.0
QA3	<0.05	>50	0.5	<0.1	0.1	0.5	0.3	8.0	0.25	0.3
<i>B. subtilis</i>	<0.05	30	3.0	<0.1	0.2	0.3	0.2	3.0	1.0	1.0
<i>E. coli</i>	<0.05	10	3.0	0.3	0.2	<0.05	0.2	2.0	2.0	0.5
<i>M. echinospora</i>	<0.05	10	0.5	0.1	0.2	1.0	0.2	3.0	0.25	1.5
<i>S. viridochromogenes</i>	<0.05	5	0.3	0.1	0.2	1.5	0.3	3.0	0.25	0.5

^a MICs were determined as described in the text.

TABLE 2. MTCs of nine heavy metals for 12 *Frankia* strains and other organisms

Strain or organism	MTC (mM) of ^a :								
	Na ₂ HAsO ₄	NaAsO ₂	CdCl ₂	CoCl ₂	K ₂ CrO ₄	NiCl ₂	Pb(NO ₃) ₂	K(SbO)C ₄ H ₄ O ₆	Na ₂ SeO ₂
ACN1 ^{AG}	0.5	0.1	0.1	0.1	0.5	0.1	6.0	0.1	0.1
Ccl17	25	0.05	0.05	0.1	0.5	0.1	4.0	0.1	0.1
Ccl3	10	<0.01	0.1	0.1	<0.01	0.1	4.0	0.1	<0.1
CN3	10	0.01	0.1	0.1	1.0	0.1	4.0	0.1	0.5
CpI1-P	10	0.05	<0.1	0.1	1.0	0.1	3.0	0.1	0.1
CpI1-S	1.0	0.01	<0.1	0.1	0.5	0.1	2.0	0.1	0.1
DC12	10.0	<0.01	<0.01	0.1	0.5	0.1	4.0	<0.1	0.5
EAN1pec	10.0	0.05	0.05	0.1	0.5	0.1	4.0	0.1	0.1
EI5c	5.0	0.05	<0.1	0.1	0.1	0.1	3.0	<0.1	0.5
EuI1c	<1.0	<0.01	0.1	<0.1	0.05	0.1	5.0	0.1	0.5
EUN1f	10.0	0.05	<0.1	0.1	0.1	0.1	4.0	0.1	0.1
QA3	1.0	0.1	<0.05	<0.05	0.25	<0.1	6.0	<0.01	0.1
<i>B. subtilis</i>	25.0	>1.0	<0.1	0.1	0.05	0.1	2.0	0.5	0.5
<i>E. coli</i>	5.0	>1.0	<0.1	0.1	<0.05	0.1	1.0	1.5	0.1
<i>M. echinospora</i>	5.0	0.1	<0.1	<0.1	0.5	<0.1	1.0	0.1	1.0
<i>S. viridochromogenes</i>	<1.0	0.1	<0.1	<0.1	0.5	0.1	1.0	0.1	0.1

^a MTCs were determined as described in the text.

CrO₄²⁻ resistance does not occur via a chromate reduction mechanism.

SeO₂²⁻ resistance. One-half of the *Frankia* isolates tested were resistant to elevated levels of SeO₂²⁻ (Table 2). Isolates for which this metalloid had an MIC of <0.5 mM were considered selenite sensitive, while the SeO₂²⁻ MICs for selenite-resistant isolates ranged from 1.5 to 3.5 mM. The control bacteria *E. coli* and *S. viridochromogenes* were selenite sensitive, while *B. subtilis* and *M. echinospora* were selenite resistant. *Frankia* isolates that were resistant to elevated levels of selenite formed red colonies on the surface of the growth medium. The red form is elemental selenium, an insoluble, generally less toxic state of the element. In liquid cultures, a red precipitate was found to be associated with cells (data not shown). *Frankia* strains resistant to SeO₂²⁻ formed globular structures associated with their hyphae. These results suggest that the selenium oxyanion is reduced to Se⁰, which remains associated with the cells. Selenite resistance is probably due to reduction of colorless soluble SeO₂²⁻ to an insoluble red form of Se⁰, which is much less toxic than selenite. This is a common mechanism of selenite resistance. In *B. subtilis*, an inducible detoxification system reduces selenite to elemental selenium (9). *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* exhibit intracellular oxyanion reduction. *R. sphaeroides* deposits the metal in the cytoplasmic membrane (21), while *R. rubrum* expels the elemental selenium across the cytoplasmic membrane and the cell wall (13). From our results, it is not clear whether selenite reduction occurs intracellularly, extracellularly, or at the *Frankia* membrane.

Cu²⁺ resistance. An unusual but consistent pattern of resistance was observed with Cu²⁺ (Fig 2). With strains DC12, EuI1c, and CN3, growth occurred in the presence of elevated levels of Cu²⁺ (2 to 5 mM and even up to 20 mM with strain CN3), but no growth was observed in the presence of moderate levels of Cu²⁺ (0.5 to 1.0 mM for EuI1c and CN3; 1.0 mM for DC12). None of the other *Frankia* strains tested grew in the presence of >0.1 mM CuCl₂ (data not shown). For *B. subtilis*, *S. viridochromogenes*, and *M. echinospora* the MIC of Cu²⁺ was 0.2 mM, while for *E. coli* the MIC was 0.3 mM. Similar patterns of sensitivity and resistance were observed with CuSO₄ (data

not shown). Blue colonies formed on the surface of medium containing Cu²⁺ (Fig. 2). In some cases, the growth medium surrounding the colonies appeared to be lighter blue or white. This observation suggests that colonies bound or absorbed the Cu²⁺ in the medium, possibly by producing a diffusible binding compound. Several bacteria have this type of Cu²⁺ resistance mechanism, and the genes encoding resistance are plasmid borne or located on the chromosome (4, 7, 10, 24). In broth medium, the Cu²⁺ was also found to be associated with *Frankia* cells. As determined by phase-contrast microscopy, Cu²⁺-resistant *Frankia* strains formed unusual globular structures that were associated with their hyphae (data not shown). All of the Cu²⁺-resistant strains either formed ineffective nodules (EuI1c) or were members of the atypical *Frankia* group (DC12 and CN3) containing organisms which are unable to reinfect host plants.

Pb²⁺ resistance. Several *Frankia* isolates were resistant to elevated levels of Pb(NO₃)₂, and the range of MICs for these strains was modest (Table 1). Compared to the four other bacteria tested, *Frankia* strains were more resistant to Pb²⁺. The MIC of Pb(NO₃)₂ for the most sensitive *Frankia* isolates was 5 mM, while for the most resistant isolates 8 mM Pb(NO₃)₂ was required to inhibit growth. Most of the *Frankia* lead-resistant strains had MTC values of ≥4 mM, while two strains (ACN1^{AG} and QA3) had a Pb²⁺ MTC of 6 mM. In broth media, Pb(NO₃)₂ forms a cloudy solution. Growth of the resistant isolates cleared the media. When Pb²⁺ was present in the growth medium, resistant *Frankia* isolates produced an amorphous matrix that was associated with the hyphae.

Our results suggest that Pb²⁺ resistance and Cu²⁺ resistance may result from a sequestration or binding mechanism. Cu²⁺ and Pb²⁺ appear to bind to material on the cell surface. Polysaccharides or proteins are potential binding sites. Pb²⁺-resistant strains of *Staphylococcus aureus* and *Citrobacter freundii* accumulate lead as Pb²⁺ phosphate (15, 16). Cells of *Streptomyces* sp. also accumulate Pb²⁺ (11). Cu²⁺ is accumulated in the periplasm of *Pseudomonas* by periplasmic CopA and CopB proteins, which also causes the bacterial colonies to turn blue (7, 24). Our results also suggest that Cu²⁺ is bound to the cells. Cu¹⁺ rapidly oxidizes to Cu²⁺ under aerobic conditions. With

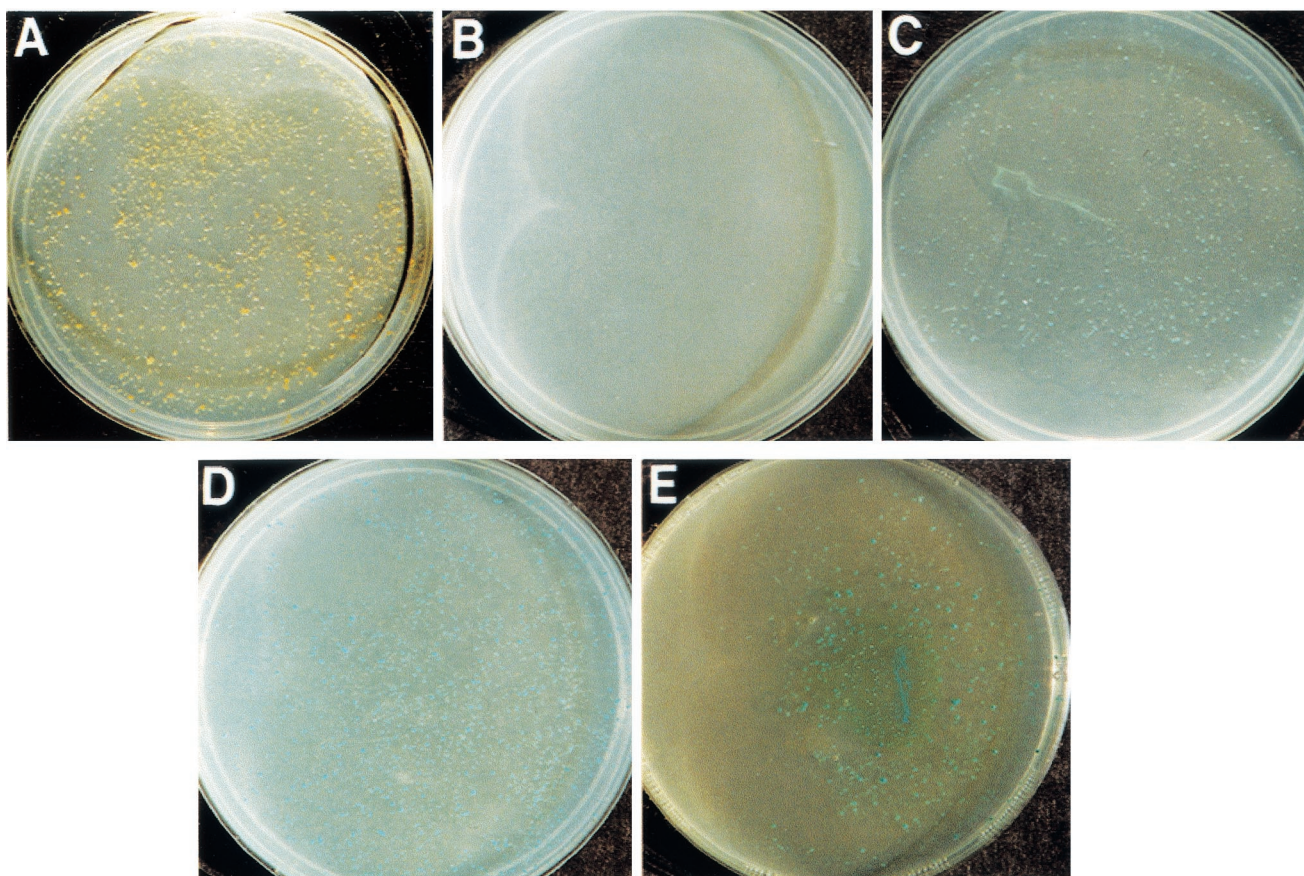


FIG. 2. *Frankia* strain CN3 colonies grown on succinate-glucose-NH₄Cl low-phosphate medium containing CuCl₂. Plates containing CuCl₂ at the following concentrations were incubated for 35 days at 30°C: no CuCl₂ (control) (A), 0.5 mM CuCl₂ (B), 2.0 mM CuCl₂ (C), 5.0 mM CuCl₂ (D), and 10.0 mM CuCl₂ (E).

some systems, Cu¹⁺ resistance is linked with Ag¹⁺ resistance (7, 24), but *Frankia* isolates were very sensitive to Ag¹⁺.

Most of the *Frankia* strains were resistant to elevated levels of several heavy metals. In genetic studies, these heavy metal resistance traits should be extremely valuable as positive selection markers. *Frankia* strains grow slowly and require long incubation times. Compared to antibiotics, heavy metals are more stable and do not degrade over long incubation times. The high MTCs of Pb²⁺, CrO₄²⁻, and SeO₂²⁻ (Table 2) suggest that these metals could be used as selective agents for some *Frankia* strains. We are currently testing the use of heavy metal resistance as a counterselection marker in conjugation experiments.

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