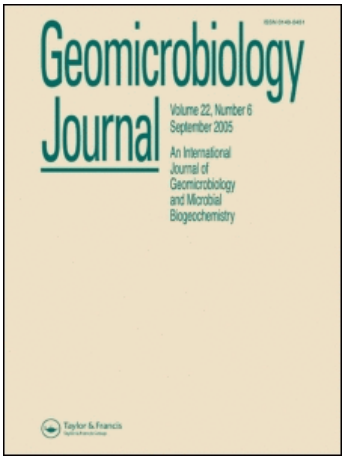

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Bacterial Activity in Heavy Metals Polluted Soils: Metal Efflux Systems in Native Rhizobial Strains

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Bacterial Activity in Heavy Metals Polluted Soils: Metal Efflux Systems in Native Rhizobial Strains

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The negative effect of high levels of heavy metals on the activity of soil microorganisms is well-known. However, some microorganisms survive even in high levels of heavy metals, and the microbial activity can therefore, help to recover these polluted soils. Microbial metal uptake in contaminated soils has to be tightly regulated to avoid toxic effects for the cells. These mechanisms of metal resistance are frequently associated to transport-related membrane proteins that mediate bacterium's direct metabolic interactions with the complex soil and aquatic harsh environments. This study reports the identification of gene clusters in rhizobial strains that are regulated by heavy metals, particularly chromium. A DNA fragment was amplified from *R. leguminosarum*, and *in silico* analysis of the sequence obtained revealed a putative protein homologue to a cation/multidrug efflux pump component (GenBank DQ398937). Another amplified DNA fragment, with 960 bp, has strong homology with anion ABC transporters (GenBank ZP_002212691) and a peptide ABC transporter (GenBank NP_766950), was identified in *Mesorhizobium loti* (GenBank DQ398941) and Sinorhizobium *meliloti*. Using Chromosome Walking technique, a single product from *Sinorhizobium meliloti* was cloned and sequenced. This new fragment enlarged more 302 bp to the initial sequence corresponding to the ABC transporter, confirming homology with an ATPase from PP superfamily (GenBank ZP_00197146.1).

Keywords ABC transporter, chr, heavy-metal resistance, metal efflux system, multidrug transporter, Rhizobia

INTRODUCTION

Estarreja is one of the most important industrial areas in Portugal. Intensive industrial activity along with direct discharge of the effluents from chemical industry in high concentrations of toxic pollutants, mainly heavy metals (namely As, Hg, Pb, and Cr), lead to extremely contaminated soils (Costa and Jesus-Rydin 2001). Legumes, and their association with rhizobial bacteria, are important components of the N cycle in agricultural and in natural ecosystems, being an important high-protein feed for humans and animals (Reichman 2007). Thus, legumes and rhizobia are often desirable species during, and after, the remediation of heavy metals contaminated soils, namely As, Cr, Hg.

Sinorhizobium meliloti (formerly *Rhizobium meliloti,* de Lajudie et al. 1994), is a Gram-negative nitrogen-fixing α-proteobacterium, and is able to establish a symbiosis with host plants from the genera *Melilotus* and *Trigonella* leading to the formation of nodules on roots.

In order to propagate in soils and for successful symbiotic interaction, the bacterium needs to sequester transition metals like iron and manganese, from the environment. A number of these micronutrients are known to be essential for bacterial metabolism (Johnston et al. 2001). The metal uptake in contaminated soils has to be tightly regulated to avoid toxic effects (Derek and Sharon 2002). However, the mechanisms of metal import and resistance to metal toxic concentrations, used in common cell processes in rhizobial species, are largely unknown.

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Microorganisms have developed, for each metal, a specific or a set of resistance mechanisms. The efficiency of these mechanisms depends on many parameters, among which the metal itself, the species studied, time, temperature, pH, presence of plants communities near the microfauna, interactions of the metal with other compounds. Isolation and identification of some Rhizobiales in highly contaminated soils, presume that these soil organisms are likely to bear systems to survive with toxic metals in their habitats (Cánovas et al. 2003).

Previous results obtained from growing experiments of alfalfa inoculated with *Sinorhizobium meliloti* 2011, showed that the addition of cadmium or a polycyclic aromatic hydrocarbon fluoranthene to the mineral medium, significantly reduced the numbers of root nodules. However, the presence of these bacteria enhanced the expression of transcript DDMs1 2.5- to 3-fold in inoculated alfalfa when compared with control plants. Sequence analysis of DDMs1 revealed a significant overall homology (50% identity) to a hypothetical protein from *Arabidopsis thaliana* with high similarity to a copper transporting ATPase (Neumann and Werner 2000).

It was also found that members of the *Caulobacter*, *Sphingomonas* and *Rhizobium* families isolated from contaminated mine tailings, may be responsible for the reduction and mobilization of arsenic (Macur et al. 2001). These mechanisms of metal resistance are frequently related with transport-related membrane proteins that mediate bacterium's direct metabolic interactions with the complex soil and aquatic environments that inhabits. Cells produce a variety of ABC transporter proteins that shuttle numerous molecules across the plasma membrane to maintain the integrity of the intracellular milieu. Therefore, the analysis of *Sinorhizobium meliloti, Mesorhizobium loti* and *Rhizobium leguminosarum* transport proteins is a key step to unravel the strategies evolved by this microorganism to adapt to complex environmental conditions.

Results from this study with rhizobial strains, namely *Sinorhizobium meliloti, Mesorhizobium loti* and *Rhizobium leguminosarum* not only will facilitate the completion of the puzzle of heavy-metal resistance genetic system, but will contribute, in a near future, to the identification of other process for soil bioremediation.

MATERIALS AND METHODS

Culture Media, Growth Conditions and Maintenance of Rhizobial Strains

Rhizobium sp. strains were isolated from soils polluted with waste waters from pesticides industry, highly contaminated with heavy-metals (Estarreja, Portugal). This study involved 25 strains isolated from nodules of the leguminous plants *Lotus corniculatus* and *Trifolium* sp. Nodules from plants were aseptically removed, kept on ice until frozen in liquid nitrogen, and stored at -70°C with 25% glycerol, as previously described (Sá Pereira et al. 2007). These strains were characterized phenotypically by classical microbiological methods, and were identified as *Mesorhizobium loti*, *Sinorhizobium meliloti* and *Rhizobium leguminosarum* bv . *trifolli* (Table 1). They were selected due to their ability to nodulate their hosts in heavy-metal contaminated soils. The strains were grown in mannitol medium yeast extract (YM) at 28°C, for 1 week and preserved in the same medium containing agar (1.8%, v v^{-1}, YMA) at 4°C.

TABLE 1
Rhizobia strains used in the study

Isolates	Strains	Local
1, 13-C, 15-C 28-C, 16-C, 30-C	*Mesorhizobium loti*	Estarreja eucaliptal
2, 5, 6, 8, 10, 9, 13, 14, 15, 17, 18, 20, 21, 29	*Mesorhizobium loti*	Estarreja Vala de Breja
32 TsIA(C^{+})	*Rhizobium leguminosarum bv trifolli*	Estarreja Póvoa de Baixo
35, 36, 37, 38	*Sinorhizobium meliloti*	

PCR Amplification

For extraction of total DNA, the bacterial biomass grow 5 days in a 6 cm Petri dish, was collected with a pipette tip and introduced in a 1 ml microtube. Bacterial DNA was extracted using the kit Nucleospin Tissue (Macherey-Nagel) according to the supplier's instructions. The oligonucleotide primers, named ChrA, used in this study for PCR amplification, were obtained from literature (Nies et al. 1990; Trajanovska et al. 1997) and degenerated primers, named ChrA1, were designed after alignment of the protein sequences, using the BLOCK MAKER sofware (http://blocks.fhcrc.org/; Henikoff et al. 1995). Consensus-degenerate PCR primers were designed using the Consensus-Degenerate Hybrid Oligonucleotide tool (CODEHOP) (http://blocks.fhcrc.org/blocks/codehop.html; Rose et al. 2003; Lourenço et al. 2004). The ChrA1 primers were obtained using conserved motifs from the multi-alignment of sequences similar to *chrA* (Table 2) using CODEHOP. The primers' sequences and PCR annealing conditions are listed on Table 3.

PCR amplification was carried out in a 25 μl reaction volume containing 1 μl of DNA template (~ 40 ng μl^{-1}); 1 μl of 25 mM $MgCl_2$(Fermentas); 2.5 μl of $(NH_4)_2SO_4$ (10X, Fermentas);

TABLE 2
Sequences chrA or similar for CODEHOP primer design

Microorganism	Accession number
Sinorhizobium meliloti	NP_384859.1
Mesorhizobium loti	NP_106077.1
Mesorhizobium sp.	ZP_00193416.2

TABLE 3
Primers sequences, predicted fragment size and PCR annealing conditions

	Degeneracy	T°C (*)	Primer sequence	Size (pb)
ChrA1F	64	64,9	5′-CGAACAGCGCTTCCTGcaygcnytnaa-3′	960
ChrA1R	48	60,7	5′-CGACGACGGCGgcngtdatngc-3′	

* T°C – Annealing temperature.
F – Forward.
R –Reverse.

2.5 μl of 2 mM dNTPs (Fermentas); 1U of Taq DNA polymerase (Fermentas); 0.4 pmol μl^{-1} of each primer and water MilliQ was added up to final volume. DNA was amplified in a BIORAD *i*Cycler thermocycler with the following temperature profile: an initial denaturation step 95°C for 5 min, in one cycle; 35 cycles of 3 steps - denaturation, 1 min at 95°C; annealing, 1 min at 50°C for ChrA, and 1 min at 60°C, for ChrA1; extension, 45s at 72°C - a final extension was applied for 5 min at 72°C. Amplified DNA fragments were separated using gel electrophoresis: 1 to 2% agarose (w v^{-1}, Bioline) in TAE (1X, Qbiogene), with EtBr at 2% (v v^{-1}) (stock solution, 10 mg ml^{-1}, Macherey-Nagel). After running the gel at 100 V, constant current, for 1 h, the fragments migration was analysed and registered by photography.

Cloning Procedures

The PCR products were cloned into pTZ57R/T vector using InsT/Aclone™ PCR Product Cloning Kit according to supplier's instructions (Fermentas).

DNA Sequencing

Sequencing reactions were carried out according to standard protocols (ABI Sequencing Reaction Kit V3.1, Applied Biosystems), using either the original PCR primers (ChrA e ChrA1), or the InsT/Aclone™ cloning kit sequencing primers. Nucleotide sequencing was performed with an ABI Prism 310 automated sequencer (Applied Biosystems).

Data Analysis

Sequences were assembled with the BioEdit sequence alignment editor (Hall 1999) with local BLAST (Altschul et al. 1990) and CLUSTALW algorithms (Thompson et al. 1994). The BLAST server at http://www.ch.embnet.org/software/bBLAST.html was also used. All sequences were obtained from the EMBL database at http://srs6.ebi.ac.uk/.

Chromosome Walking

To complete the ABC transporter sequence amplified with ChrA1 primers, genomics libraries were constructed using the Genome Walker™ kit (Clontech) with the following modification: Genomic DNA of *Sinorhizobium meliloti* was digested with *Sma*I, *Pvu*II, *Eco*RV, *Stu*I e *Aha*II, to generate blunt-ended fragments. Adapters AP1 and AP2 (from the genome walker kit, from Clontech) were ligated onto the 5′-and 3′-terminals of digested DNA, respectively. PCR amplification was then performed with gene-specific primers (Table 4) and with the adapter primers AP1 and AP2. For primary PCR amplification, CWE-R and the primer AP1were used to walk downstream, and CWE-F and AP1 primers to walk upstream, of the sequence. For secondary PCR amplification, CWI-R/AP2 and CWI-F/AP2 were used to walk downstream and upstream, respectively.

RESULTS

Using the ChrA primer sequences suggested by Nies et al. (1990) several PCR were performed in order to amplify the fragments of interest. Gel electrophoresis revealed the amplification of multiple bands with sizes that ranged from 2000 bp to 400 bp (Fig. 1). However, an amplicon with 750 bp, of strong intensity was obtained for two samples. Figure 2 shows a partial nucleotide sequence of the 750 bp containing the supposed amino acid translation products of the predicted ORF's.

The size of putative translation products of the *chr*A gene, for each sample, was consistent with the predicted size for homologue sequences of a hybrid sensor histidine kinase (GenBank BAB50556) and a two-component sensor histidine kinase (GenBank AAY51205), respectively, sample 36 and sample 37.

To confirm this information, CODEHOP ChrA1 primers were designed, using the multi-alignment of protein sequences from *Rhizobiaceae* family functionally related with ChrA, a chromate efflux pump (Table 2).

TABLE 4
Gene-specific primers for chromosome walking primary and secondary PCR

Primers	Sequences
CWE-Forward	5′TTCTGCAGAGCCTCGTCACCAACACC GGTTC/3′
CWI-Forward	5′TCTTCGGCTCGGAACTGACGGTTGC CATAATC3′
CWE- Reverse	5′TACTGGCTGTGCTACGGCCGGTCG CGCTT3′
CWI -Reverse	5′CCTCTCTAGCTAGACCGGGCTATA GTGTGT3′

E-external; I-internal.

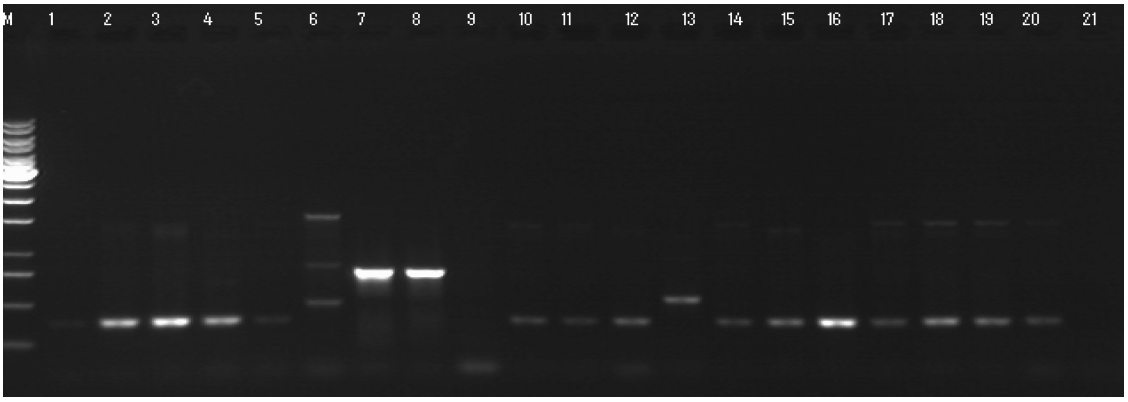

FIG. 1. Electrophoresis in a 1% (w v^{-1}) agarose gel of the products amplified in PCR using the ChrA primers. M- 1Kb Marker 1Kb, 1- 1-C (Isolate 1-C), 2- 8, 3- 10, 4- 9, 5- 20, 6- 35, 7- 37, 8- 36, 9- 38, 10- 2, 11- 21, 12- 28, 13- C^+, 14- 13, 15- 15, 16- 14, 17- 17, 18- 18, 19- 5, 20- 6, 21- Negative control.

The primers were selected based on specific criteria such as: high annealing temperature, higher fragment size and lower degeneracy. The primers forward and reverse ChrA1 match to the sequences EQRFLHALN and AITAAVVG (Figure 3).

The PCR results with the primers ChrA1, amplified a fragment of $\approx$ 960 bp, with the expected size, that *in silico* search, to have strong homology with an anion ABC transporter (GenBank ZP_002212691) and a peptide ABC transporter (GenBank NP_766950), for the isolates 9 (GenBank DQ398941) and 37 (Figure 4). Analyzing the translated genes, the ORF -1 of both amplicons showed to have homology with a sulphate transporter and with an ABC transporter ATP-binding region (GenBank NP_52017).

Surprisingly, the amplified transporter does not show homology with the chromate efflux protein. However, this fragment may correspond to part of the gene that codifies for a heavy metals ABC transporter. Janulczyk et al., (1999) described in *Streptococcus,* the operon *mt*ABC that codifies for a ABC transporter with multi-specificity for metallic cations (zinc and iron), what support the obtained result for the isolates *S. meliloti* and *M. loti*. On the other hand, Tam and Saier (1993), verified that the ABC transporters proteins that translocate Ni^{2+}, Mn^{2+} and Fe^{2+}, are closely related to transporters that mobilize peptides and sugars, what reinforces the hypothesis that this amplified protein can correspond to a heavy metal ABC transporter, with homology to a peptide ABC transporter (GenBank NP_766950).

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         10        20        30        40        50        60        70        80        90       100
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
CGCCAGCTCTAATTTTNCTNACTATANCGGAAAGCTTGCATGCAGGCCTCTGCAGTCGACGGGCCCGGGATCCGATTGTAATGGCATTCAGTCGCTTGCC

        110       120       130       140       150       160       170       180       190       200
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
ACCCGACAAGGCGGTGATGGAAAACAGCATCGTCTTGCCGTCGGCATGGCGGAACTGGCGTGCCGAAACGCTGCCGGCACGGATCTCATCCAGCCGGCTG

        210       220       230       240       250       260       270       280       290       300
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
GCGAGATAGCGCTCCCAATCCGGCTGGGCGATGTCGCCATAAATACCATTGCGCCGATTGATCTCCATCAACAGGCTGAAGGGTGCGCCGACGGCCACAT

        310       320       330       340       350       360       370       380       390       400
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
CGCCTTCCGACATTTTGGAGATGTCACGATAGGCCCTGTTGATGATCAGCGTGTCGAGCTTCGCGTCGAGAAGCACGATCCCCATGCGCATGGCGTCCAT

        410       420       430       440       450       460       470       480       490       500
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
CGTCCGCTCGAGATCGGCAAGGTGCCCGGCGGTGTCGCCGCCGACCGGCGGTTCCTGCCTCGGCTCGATGTCTTCGAACGCGATGTCTTCNAAGCGCTGG

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CN
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FIG. 2. Histidine kinase partial nucleotide sequence amplified with ChrA primers.

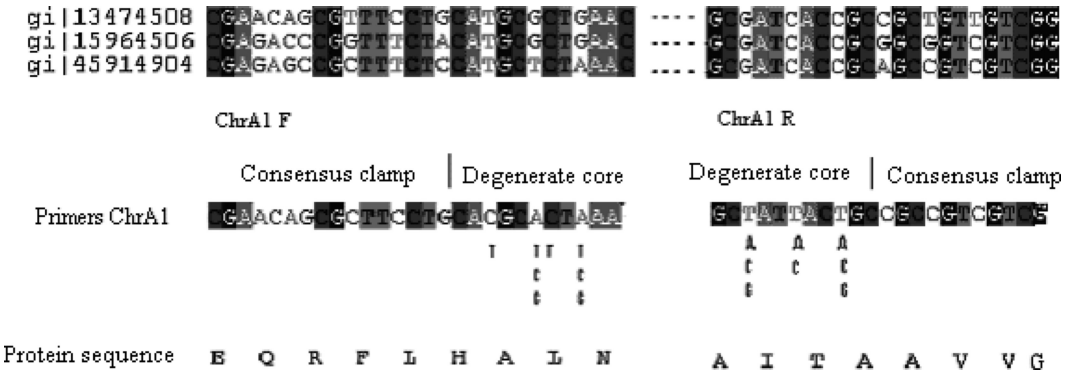

FIG. 3. Alignment of the hybrid CODEHOP primers with the NCBI sequences. The primer ChrA1 R is the reverse of the complement.

Confirming the results, the DNA fragments amplified in both isolates, have homology with those that are functionally equivalent to heavy metal resistant organisms, such as *Ralstonia solanacearum* (NP_ 52017) (Mergeay et al. 2003), *Chromobacterium violaceum* (GenBank AAQ60686) (Hungria et al. 2004) and *Pseudomonas syringae pv. Tomato* (GenBank ZP_00205487) (Nies et al. 1998a).

Another fragment with 750 bp was amplified in *Rhizobium leguminosarum* and *Sinorhizobium meliloti*. BLASTX alignment revealed that the fragment amplified on *R. leguminosarum*

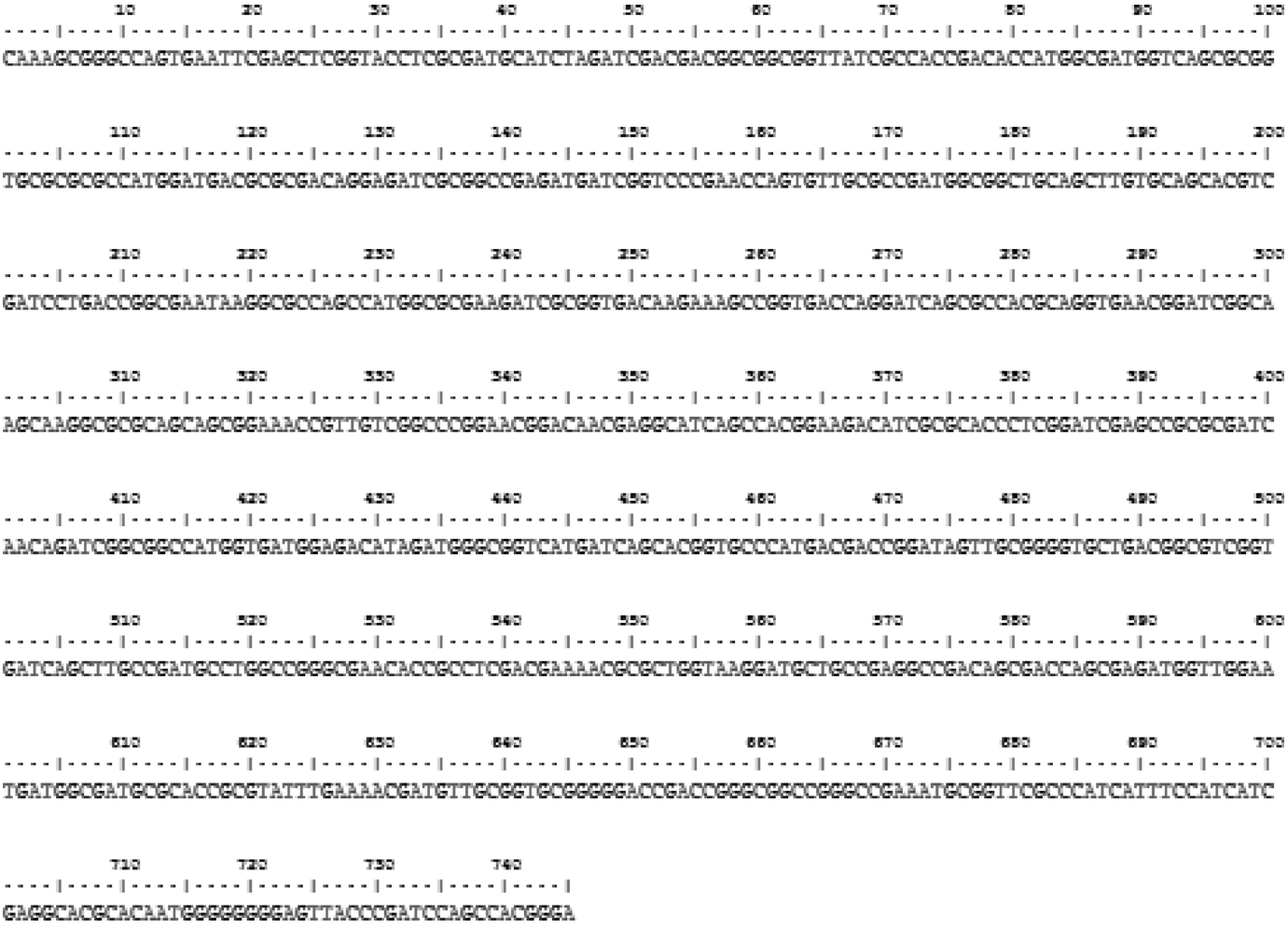

FIG. 4. Peptide ABC transporter partial nucleotide sequence amplified in *Sinorhizobium meliloti* (isolate 37) with ChrA1 primers.

is a putative protein homologue to a cation/multidrug efflux pump component (GenBank DQ398937) that has homology with cation/multidrug efflux pumps. Alignment of the fragment obtained from *S. meliloti* (Fig. 5), has shown homology with three ABC transporters, a ribose (GenBank NP_107469), a dipeptide (GenBank AAV96075), oligopeptide (GenBank NP_615274), and a nickel ABC transporter (GenBank YP_223216).

To identify the entire ABC transporter sequence amplified from *Sinorhizobium meliloti,* using Chromosome Walking, gene-specific primers were synthesized using the partial ABC transporter gene sequence amplified with ChrA1 primers (Fig. 5). This method allowed the amplification of downstream and upstream regions of the unknown sequence through the design of internal and external primers. For primary PCR amplification, CWE-R and the primer AP1 were used, to walk to the left side and CWE-F and AP1 primers to walk to the right. For secondary PCR amplification, the sets of primers used were CWI-R and AP2 and CWI-F and AP2.

Analyzing the PCR results, only the genomic libraries with *Pvu*II enzyme and *Aha*II produced single fragments that were cloned and sequenced. The analysis of the sequenced fragments correspondent to the *Pvu*II and *Aha*II restriction libraries obtained in the secondary PCRs, did not increase any bp to the previous sequence. However, using reverse primers in *Pvu*II restriction library, the secondary PCR revealed a single amplicon that was cloned and sequenced (Figure 6, Line 7R). This new fragment increased more 302 bp to the ABC transporter previous sequence. The BLASTX alignment of these 302 bp revealed that have homology with an ATPase from PP superfamily (GenBank ZP_00197146.1) (Figure 7).

DISCUSSION

At a molecular level, the *chr* operon mediates resistance against the toxic oxyanion chromate (Nies et al. 1989, 1990). Chromate enters the cell by the sulphate uptake system and is effluxed by the ChrAB proteins, which form a sulphate-chromate antiporter (Nies et al. 1998b). Several additional *chr* genes were detected downstream of *chr*AB. In *Ralstonia metallidurans* resistance system, these genes encode a superoxide dismutase (ChrC) and a sigma factor of the heat-shock family, ChrH. The genes surrounding the core *chr*BA operon increase uptake of chromate, reduction of chromate, and probably, the export of

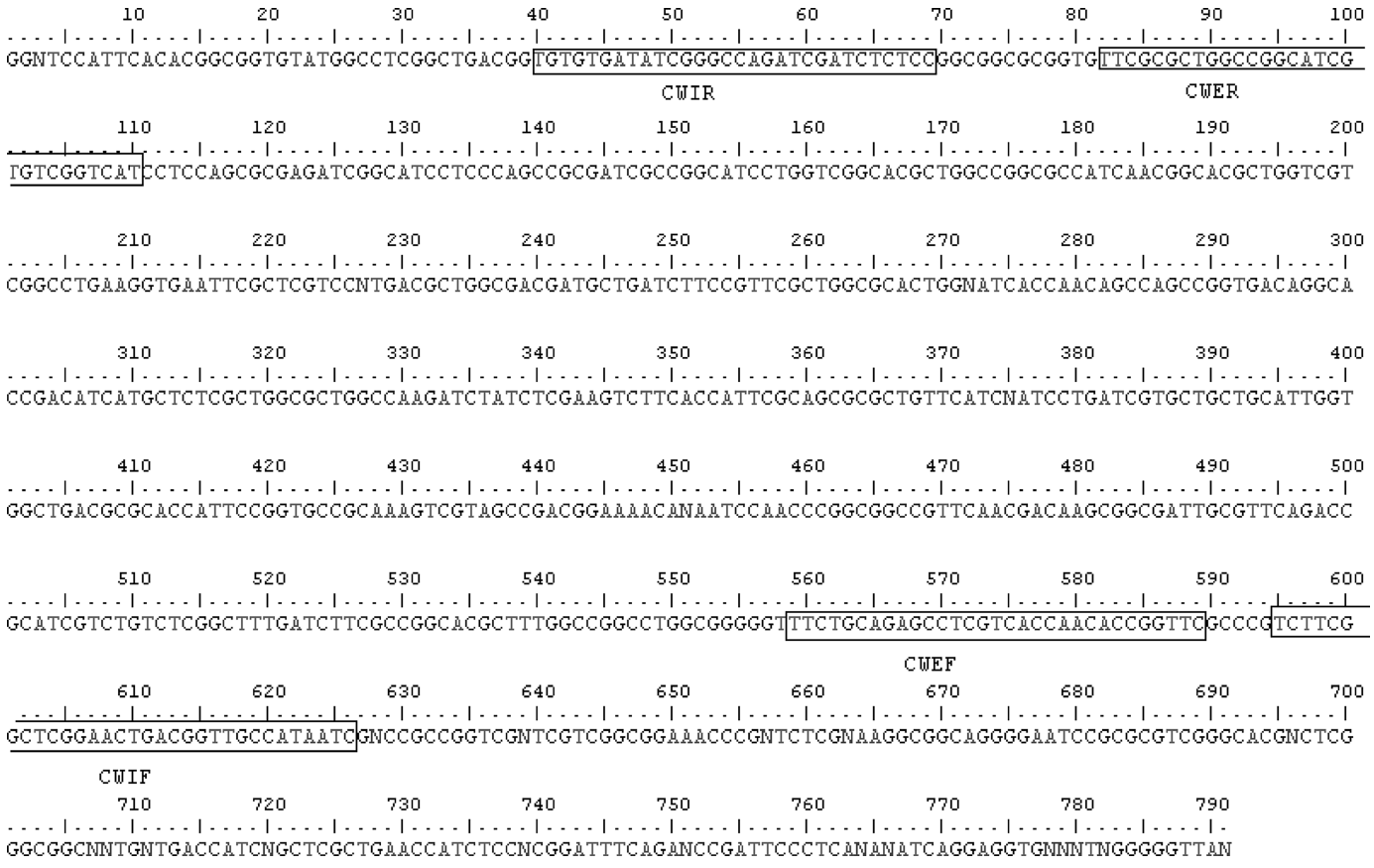

FIG. 5. ABC transporter partial nucleotide sequence amplified with ChrA1 primers.

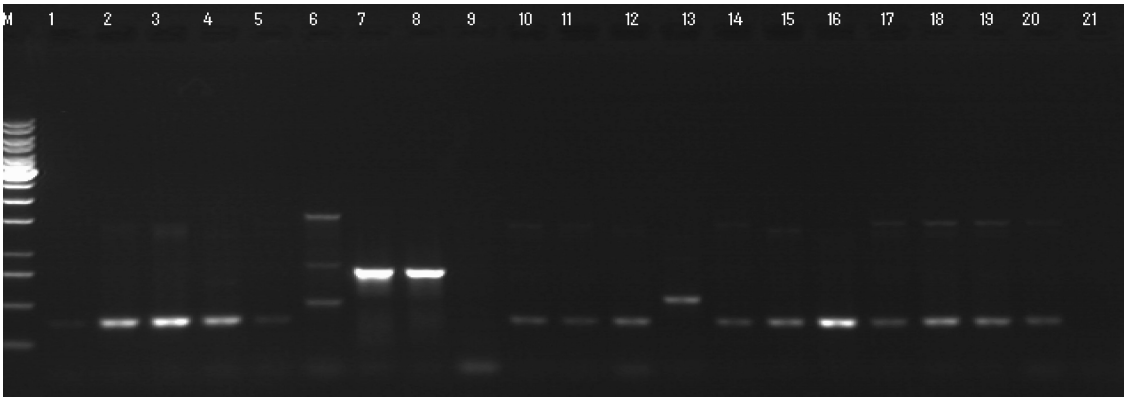

FIG. 6. Electrophoresis in 1% (w v^{-1}) agarose gel of primary and secondary PCRs - Forward and Reverse primers. M – 100 pb marker, 1F - Restriction library-*Pvu*II. *Pvu*II; 2F -. *Sma*I, 3F - *Aha*II, 4F - *Stu*I, 5F - *Eco*R(V), 6F –Negative control, 7F - *Pvu*II, 8F - *Sma*I, 9F - *Aha*II, 10F - *Stu*I, 11F –. *Eco*R(V), 12F –Negative control; 1R – *Pvu*II; 2R - *Sma*I, 3R - *Aha*II, 4R- D. *Stu*I, 5R -. *EcoR*(V), 6R –Negative control, 7R - D. *Pvu*II, 8R - D. *Sma*I, 9R - D. *Aha*II, 10R-D. *Stu*I, 11R - D. *Eco*R(V), 12R – Negative control. F -Forward, R-Reverse; 1-6F and 1-6R - products amplified in primary PCR, 7-12F and of the 7-12R - products amplified in secondary PCR.

the product, Cr^{3+}. However, this process is far from being completely understood.

The amplified transporters belong to the same family, the multidrug transporters, which are split in two major classes on the basis of bioenergetics and structural criteria. One class consists of secondary multidrug transporters, use transmembranar electrochemical potential of a proton or sodium ion; the other consists in ATP-binding cassette (ABC) transporters which utilize the free energy of ATP hydrolysis to transport the solute from the cells (Lee et al. 2003).

Signal-responsive components of transmembrane signal-transducing regulatory systems in heavy-metal transport are complex and difficult to ascertain. However, the identification of a two-component hybrid sensor kinase and a cation/multridug efflux pump in *S. meliloti* and *Rhizobium leguminosarum*, suggests that it was identified a newly different structure related to chromium resistance, and possibly, to other heavy-metals.

For the hybrid sensor kinase amplified with ChrA primers, similar components responsible for the regulation of homeostasis of some heavy-metals were found in the system *pcoRS* (that regulates the transcription of the copper resistance genes) and in the system *czcRS* (that regulates cadmium, zinc and cobalt) (Kim et al. 2001). This newly identified sensor, maybe a regulator of chromium uptake/efflux pump. The results obtained with CODEHOP primers are in agreement with those revealed by the Rose et al. (1998) that suggests that the CODEHOP hybrid primers can be used to isolate several members of a multi-gene family, simultaneously. With the primers ChrA1, it was amplified an ABC transporter that corresponds to a cation/multridug efflux pump, that belongs to the same family of the ABC proteins. Chromosome walking procedure, revealed an ATPase confirming the previous result.

Similar to other microorganisms resistance genetic systems (Tam and Saier 1993; Janulczyk et al. 1999), the function of ABC transporters in these rhizobial strains, possibly involves translocation of chromium through a pore formed by two integral membrane protein domains. Efflux systems pump the toxic ions out of the cell, avoiding their accumulation to

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          10         20         30         40         50         60         70         80         90        100
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
AAAAACAACAAGCCATGGGAATTCGAGCTCGGTACCTCGCGAATGCATCTAGATTCTACATgTCCACGGACAGTGGCCGACCGAATAGGTGTCCTGGTAC

         110        120        130        140        150        160        170        180        190        200
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
TCGATGCGGTGCGCAATGCCATTGGCGTTGAGGTAGTCCGGCAGGATGTGCTTGGGAAAGTTCGGCTGGCCCTGGTCGAGATTGCAGGCCAcGCAATCGA

         210        220        230        240        250        260        270        280        290        300
----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
CCGGCAGCATCCCGCGCCATTTGAGATCGAGCAAGATAGCGAGCAGGCCGTAGGAATCCTTGCCGCCCGACAGCGCGACCAGCCAGCGCTGGGCCGGCTC

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CA
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FIG. 7. ATPase nucleotide sequence amplified with Chromosome Walking technique.

levels that possibly will inhibit growth, or cause cell death, are the most common mechanism of heavy-metal resistance (Silver 1998).

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