

Full Length Research paper

Identification and initial characterization of a copper resistant South African mine isolate

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South African mine isolates were screened for resistance to copper, and an isolate showing the highest resistance was identified as *Proteus mirabilis*, a Gram-negative bacterium, by 16S rDNA gene analysis and Biolog test. A higher minimal inhibitory copper concentration at 150 µg/ml was obtained using Vätäänen-Nine-Salts-Solution as a less complex medium. Studies pertaining to the copper resistance mechanism of *P. mirabilis* showed the organism to possess a *pcoA*-like gene which encodes PcoA, a putative multicopper oxidase known to protect organisms from copper-mediated toxicity at high concentrations.

Key words: *Proteus mirabilis*, copper resistance gene, multicopper oxidase.

INTRODUCTION

Copper ions pose a dual challenge to both eukaryotic and prokaryotic cells as these serve as a micronutrient for both kingdoms, but do however become toxic above optimum levels (Munson et al., 2000). In this regard, copper is an essential ion that is involved in some metabolic processes such as being a component (co-factor) of many metalloenzymes where it plays a role in the active sites of these enzymes (Cervantes and Guitierrez-Corona, 1994; Harris, 2000).

The ability however of this metal to generate free radicals has contributed to its potential toxicity. As such, copper ions are capable of catalyzing harmful redox reactions which result in the oxidation of lipid membranes and damage to nucleic acids (Hoshino et al., 1999). Some bacteria have, in turn, developed detoxification systems to protect themselves from toxic concentrations of copper ions while still ensuring that these ions meet their nutritional requirements (Hoshino et al., 1999). These mechanisms vary from active efflux to sequestration, cell wall modification and bioprecipitation (Choudhury and Srivastava, 2001). Such heavy metal resistant microorganisms are very useful in biotechnology for the remediation of metal contaminated environments and can also be used in the construction of biomarkers for the detection of the presence of metals.

The present work reports the first identification of *Proteus mirabilis* as a copper-resistant environmental isolate and explores its resistance mechanism through investigation of the organism's genetic determinants for copper resistance.

MATERIALS AND METHODS

Enrichment and isolation of a copper resistant bacterium

Soil and water samples were collected from mining and refining sites of Consolidated Murchinson Mine near Gravelott in the Limpopo Province of South Africa since these sites have high concentrations of heavy metals. The samples were collected aseptically and placed on ice at the sites. Aerobic enrichments were performed in TYG medium containing 5 g/l tryptone (Biolab), 3 g/l yeast extract (Biolab) and 1 g/l glucose (Holpro) at pH 7 (Botes et al., 2007).

Cultures obtained from the enrichment medium were streaked onto fresh TYG plates to obtain pure cultures, and these subsequently streaked onto copper-supplemented TYG and VNSS plates [1.0 g/l peptone; 0.5 g/l yeast extract; 2.78 mM glucose; 0.036 mM FeSO₄·7H₂O and 0.084 mM NaHPO₄ dissolved in NINE-SALTS-SOLUTION (0.3 M NaCl; 0.1 M Na₂SO₄; 0.95 mM NaHCO₃; 3.37 mM KCl; 0.34 mM KBr; 9.25 mM MgCl₂·6H₂O; 2.8 mM CaCl₂·2H₂O; 0.037 mM SrCl₂·6H₂O; 0.16 mM H₃BO₃, pH 7,] (Hermansson et al., 1987) and incubated at 37°C. The isolates capable of growing in the presence of copper were used for further experiments.

The MIC of copper for the isolates, as well as *E. coli* were determined using both TYG and VNSS agar containing various concentrations (25-1000 µg/ml) of copper sulfate (Miranda and Rojas, 2006).

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Identification of the copper resistant bacterium

The isolate showing the highest MIC was identified by sequence analysis of the 16S rDNA fragment, amplified from genomic DNA extracted with the celite method (Boom et al., 1990), using universal bacterial primers (10 µM) 27F and 1492R (Weisburg et al., 1991). PCR cycling started with a 5 min at 95°C hot start step of the genomic DNA-containing master mix (0.2 mM dNTP's final concentration, Fermentas; 10X buffer and 25 mM magnesium chloride) after which cycling was paused, 0.5 µl (5 units/µl) Super -Therm (Southern Cross Biotechnology) *Taq* polymerase added and cycling resumed. Amplifications were run for 30 cycles in a thermal cycler PxE 0.2 (Thermo electron corporation) following a denaturation step at 95°C for 30 s, an annealing step at 52°C for 45 s and elongation step at 72°C for 90 s, followed by a final extension, for 10 min at 72°C.

The PCR product was purified with the GFX PCR DNA purification kit (Amersham Biosciences) and ligated into a pGEM[®]-T Easy vector according to the manufacturer's instructions (Promega). The ligation reaction was transformed into *Escherichia coli* Top10 competent cells, followed by small scale plasmid isolation using the Gene JET[™] plasmid Miniprep Kit manual (Fermentas) according to the manufacturer's instructions.

Sequencing was performed with the ABI 377 Genetic Analyser (Applied Biosystems) using the SP6 and T7 sequencing primers and the results interpreted with BLASTn searches. The results were further confirmed with a Biolog assay using the GN2 Microplate[™] (Biolog, Inc.), following inoculation onto the microplate according to manufacturer's instructions and identification using the Biolog's Microlog[™] 1 software.

Gene-specific PCR amplification of a copper resistance gene

Sequences of copper resistance genes from Gram-negative bacteria (*E. coli*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Xanthomonas campestris*, and *Sulfitobacter* sp. NAS-14.1) were obtained from GenBank and their amino acid sequences used to identify conserved regions. Based on these, the corresponding nucleotide sequences were aligned and primers 5'-ACYACTGGTAYCACAGCCATTC and 5'-CCACATVCCRTGCAGGTG designed for amplification of a similar copper resistance (*pcoA*) gene in *P. mirabilis*.

Genomic DNA was extracted as previously described, and the putative *pcoA* fragment amplified with the designed primer set using the previously described PCR cycling conditions, with the exception of the annealing temperature that was set over a gradient of 54 to 60°C. The PCR product was again purified, ligated, transformed and small scale plasmid isolation performed followed by sequencing as described.

RESULTS AND DISCUSSION

Isolation of a copper resistant bacterium

After 48 h, the growth on both TYG and VNSS agar plates with different copper sulfate concentrations were evaluated, including the negative controls. Many mine isolates showed resistance towards copper when compared to the MIC displayed by *E. coli*: 200 µg/ml copper sulfate in TYG agar and 50 µg/ml in VNSS copper sulfate-containing agar (data not shown). One isolate in particular showed marked ability to interact with copper and was able to grow on 100 µg/ml VNSS copper sulfate-containing agar with maximum MIC at 150 µg/ml and

TYG copper sulfate- containing agar at 400 µg/ml MIC (data not shown). In this regard, TYG is a complex medium and it is known that such media complex copper from solution thereby reducing the concentration of copper (Ramamoorthy and Kushner, 1974; Zevenhuizen et al., 1979). These results signified the importance of using a less complex medium and hence VNSS was selected as such for further studies.

Identification of the copper resistant isolate

The extracted genomic DNA was used for amplification of the 16S rDNA fragment using the universal bacterial primers, and visualization by UV illumination confirmed amplification of the 1.5 kb fragment (data not shown). The near full length 16S rDNA sequence was deposited in the NCBI database under accession number EU287466. BLASTn analysis revealed 100% identity with *P. mirabilis* 16S ribosomal RNA gene, partial sequence (EF 626945.1). This identification was further confirmed by Biolog Microlog[™] analysis (data not shown) with 100% probability.

Amplification of a copper resistance gene

A fragment of a *pcoA* gene was amplified from *P. mirabilis* by PCR using the designed gene-specific primers. Based on the conserved regions obtained from the Gram-negative bacteria copper resistance amino acid sequences. Figure 1a, primers were designed with the corresponding nucleotide sequences (Figure 1b) and PCR performed for the amplification of a 1250 bp fragment (Figure 2). Amplification of a *pcoA* gene in *E. coli*, a Gram-negative bacterium was also performed to serve as a positive control. No amplification using *Bacillus licheniformis*, a Gram-positive bacterium, as a negative control was obtained.

Selected clones were sequenced, the files retrieved and compared to sequences deposited in the NCBI (National Center for Biotechnology Information) database. The BLASTn analysis yielded sequences similar to *pcoA*, a plasmid-bound gene that encodes a periplasmic protein PcoA which can functionally substitute CueO, a chromosomally encoded multicopper oxidase gene (Grass and Resing, 2001) and a potential multicopper oxidase gene which was 97 - 98% identical to the *E. coli pcoA* gene. *E. coli* PcoA, CueO and *P. syringae* CopA belong to the multicopper oxidase family but each of these proteins have different functions in resisting deleterious effects of high copper concentrations. PcoA functions in reduced cellular accumulation of copper while *P. syringae* CopA mediates the increase in copper uptake and sequestration (Brown et al., 1995). Also, in *E. coli* CueO is a periplasmic protein responsible for safe-guarding from copper toxicity by preventing oxidative damage in the periplasm and possibly prevent uptake of Cu(I) into the cytoplasm by oxidizing this ion to Cu(II) (Grass and

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P_mirabilis TYWYHSHSGLQEQEGVYGAIIDAGEPEPFYDREHVVMSDWTDENPHS 50
E_coli TYWYHSHSGLQEQEGVYGAIIDAGEPEPFYDREHVVMSDWTDENPHS 189
Pseudomonas_syringae TYWYHSHSGFQEQAGVYGPLVIEAKDPEPFAYDRDYVVMLTDWTDDEDPAS 187
Pseudomonas_fluorescens TYWYHSHSGLQEQAGVYGPLVIDAREPEPFQYDRDYVVMLSDWTDEDPAS 187
Xanthomonas_campestris TYWYHSHSMFQEQSGLYGAIVIDPLTPPYRHDREHVVLSDWTDLDPAA 198
Sulfitobacter TYWFHSHSGLQEPDGYGAVIEPRGRERI PAHRDYVVQLADKHPHPGNR 199
***:*** :** * **.:*:. .:*** **

LLKLLKQSDYYNFKPTVGSFFRDVNTRGLSATIADRKMWAEMKMNPTD 100
E_coli LLKLLKQSDYYNFKPTVGSFFRDVNTRGLSATIADRKMWAEMKMNPTD 239
Pseudomonas_syringae LMRLLKQSDYYNFKPTVGSFFRDVNTRGLSATIADRKMWAEMKMNPTD 237
Pseudomonas_fluorescens LMKTLKQSDYYNFKPTVGSFFRDVNTRGLSATIADRKMWAEMKMNPTD 237
Xanthomonas_campestris LFRRLKQMPSHDNYAQRVTVDFLRDARDGLRATLADRGMWGRMRIPTD 248
Sulfitobacter ILRNLLKQSDYYNRSQRTFQDLIRDARMDGLKATLKERRMWRMRMLPSD 249
::: ** .: * : * .:..* . * ** : * **..* * *

LADVSGYTYTYLMLNGQAPLKNWTGLFRPGEKIRLRFINGSAMTYFDIRIP 150
E_coli LADVSGYTYTYLMLNGQAPLKNWTGLFRPGEKIRLRFINGSAMTYFDIRIP 289
Pseudomonas_syringae LADVSGATYTYLLNGHAPNTNWTGVFRPGEKIRLRFINGSSMTYFDMRIP 287
Pseudomonas_fluorescens IADVSGATYTYFLMNGHAPDDNWTGLFRPGEKLRLLRINGSAMSFYDVRIP 287
Xanthomonas_campestris LSDVNANTYTYLLNGVAPAGNWTGLFKPGEKVLLRFINGSSMTYFDIRIP 298
Sulfitobacter VEDVQG -- FTSLINGRSTSQNWTGLFRPGEKVLRIINSSAMTYFDMRIP 297
: *.. : * ** * . : * ** : * ** : * ** : * ** : * **

P_mirabilis GLKMTVVAADGQYVNPVTVDEFRIAVAETYDVIVEPQ-GEAYTIFAQSM 199
E_coli GLKMTVVAADGQYVNPVTVDEFRIAVAETYDVIVEPQ-GEAYTIFAQSM 338
Pseudomonas_syringae GLKMTVVASDGQYVKPVTVDELRIAVAETYDVIVEPT-EQAYTLFAQSM 336
Pseudomonas_fluorescens GLKMTVVAADGLHVKPVSVDELRIAVAETYDVIVEPA-ADAYTLFAQSM 336
Xanthomonas_campestris GLRMTVVAADGQYVHPVSVDELRIAAAETFVIVEPLGQDAFTLFAQDMG 348
Sulfitobacter GLKMVVQADGNDVPEVADELRVVAETYDVIVQPTQNKVYSIIAESMG 347
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RTGYARGTLATREGLSAAVPLDPRPLLTMEDM----- GME-GMGHD- 240
E_coli RTGYARGTLATREGLSAAVPLDPRPLLTMEDM----- GMG-GMGHD- 379
Pseudomonas_syringae RTGFARGTLAVREGLLAPVPLDPRPLVTMADM----- GMG-GMDHGS 378
Pseudomonas_fluorescens RTGYARGTLAARAGLSAPVPLDPRPLVTMDDM----- GMG-GMGSG- 377
Xanthomonas_campestris RTGFACGTLAVQHLQAPIALDRRAILTMQDM----- GHGDGMHHA- 390
Sulfitobacter RNGLVVRGTLSPREYAGAVPRLRPKPLLTADMDSGMMGMGMGMGMGMG- 396
* . * . ** : * * . . : * * * . : : * * * * *

----- MAGMDHSQMGMDNSGEMMSMDGAGLPDSGTSSAPMDHSSM 281
E_coli ----- MAGMDHSQMGMDNSGEMMSMDGADLPDSGTSSAPMDHSSM 420
Pseudomonas_syringae MGDMDGSMQMGEMDHDGAMGAGDKGMDSMSGAPMQGMDGGQTQG-MDHSKM 427
Pseudomonas_fluorescens ----- GMNHGSM ----- DM 386
Xanthomonas_campestris ----- LPAMHGAQGLAAHG----- THTM 409
Sulfitobacter ---DHVMQNMNTGMNHTDMTGMDSNMQSSQQPPMPAMPSEKPKKKTSM 443
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AGMDHSRMAQMG----- MQSHPASETDNPLVD----MQA 312
E_coli AGMDHSRMAQMG----- MQSHPASETDNPLVD----MQA 451
Pseudomonas_syringae SGMDHSGMDMAGM---- AGMSGMGDMPMQAHATEKDNPLVD----MQA 469
Pseudomonas_fluorescens SGMDHSSMN----- MG-- PMQSHPDSEKDNPLVD----MQA 416
Xanthomonas_campestris HSHDQAQAKAP----- HHPASETGNPLID----MRS 438
Sulfitobacter AGMDHTQMOKPAMDGMHTQMQQKKKAPMSGMNHAAQMQKPAWDGMSGMDS 493
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MSVSPKLN----- PGICLR--- NNGRVLTYADLKSREFDPDGREPGR 353
E_coli MSVSPKLN----- PGIGLR--- NNGRVLTYADLKSREFDPDGREPGR 492
Pseudomonas_syringae MSPTPKLDD----- PGLGLR--- NNGRVLTYADLKSREFDPDGREPGR 510
Pseudomonas_fluorescens MTTAAKLD----- PGLGLR--- NNGRVLTYADLKSREFDPDGRDPSR 457
Xanthomonas_campestris NATAPRLD----- PGVGLR--- DNGRVLTYADLKSREFDPDGREPGR 479
Sulfitobacter MSGMAMSDGGDPFVYVPGSGLTPVAYNGGKFLSYADLRAASPRYRHRAPS 543
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TIELHLTGHEKFAWSFNGIKFSDAAPVLKYGRLRITLINDTMMTHPI 403
E_coli TIELHLTGHEKFAWSFNGIKFSDAAPVLLKYGRLRITLINDTMMTHPI 542
Pseudomonas_syringae TIELHLTGHEKFAWSFNGVFKADAEPLRLKYGERVRLVNDTMMTHPI 560
Pseudomonas_fluorescens TLELHLTGHEKFAWSFNGIKFSDAEPLLLKYGRLRILVNDTMMTHPI 507
Xanthomonas_campestris DIELHLTGHEKFAWSFDGIAFASAQPLRLQYGERLRIVLVNDTMMTHPI 529
Sulfitobacter TIVLRLTGNMERYIWSINDVKYDAAPIITLRYGERVRRMFINETMMSHPM 593
: * ** : * ** : * ** : * ** : * ** : * **

P_mirabilis HLHGMWSDLEENGFVVRKHTIDVPPGTRKRSYRVTTADALGRWAYHCHLL 409
E_coli HLHGMWSDLEENGFVVRKHTIDVPPGTRKRSYRVTTADALGRWAYHCHLL 592
Pseudomonas_syringae HLHGMWSDLEENGFVVRKHTIDVPPGTRKRSYRVTTADALGRWAYHCHLL 610
Pseudomonas_fluorescens HLHGMWSDLEENGFVVRKHTIDVPPGTRRTRVTTADALGRWAYHCHLL 557
Xanthomonas_campestris HLHGMWSDLEAHGNFQVRKHTIDVPPGTRRTRVTTADALGRWAYHCHLL 579
Sulfitobacter HLHGMWSIIDAGHGPRNPIKHTVNIINPAATDIEVEADAPGQWAFHCHLS 643
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Figure 1a. Multiple alignments of amino acid sequences from various Gram-negative bacterial copper resistance proteins for primer design. The amplified and translated fragment from *P. mirabilis* is included in the alignment.


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P_mirabilis      AGATGGCCAGTATGTAACCCGGTTACCGTTGACGAATTCAGGATTGCCG 526
E_coli           AGATGGCCAGTATGTAACCCGGTTACCGTTGACGAATTCAGGATTGCCG 943
Pseudomonas_florescens CGATGGCTTGCACGTCAAACCCGGTCAGCGTCGACGAGTTGGCCATCGCCG 937
Pseudomonas_syringae  GGATGGACAGTACGTGAAGCCTGTCACCGTTGACGAGCTGCGCATCGCCG 937
Xanthomonas_campestris CGATGGCCAAACCTACGTCCATCCGGTAAGCGTAGACGAGTTCCCGATTGCCG 988
Sulfitobacter    TGACGGCAACGATGTGGAGCCTGTCGCCGTGGATGAACTGCGGGTCCGGC 967
  * * * * *
P_mirabilis      TTGCCGAAACCTATGATGTCATTGTGGAGCCTCAGG--- GTGAGGCCAT 573
E_coli           TTGCCGAAACCTATGATGTCATTGTGGAGCCTCAGG--- GTGAGGCCAT 990
Pseudomonas_florescens TGGCGAAACCTATGACGTGATCGTCGAACCCGCCG--- CCGACGCCTAC 984
Pseudomonas_syringae  TGGCCGAGACCTATGACGTGATGTTGAGCCTACCG--- AGCAGCGCTAC 984
Xanthomonas_campestris TTGCCGAAACCTTTGACGTGATCGTTGAGCCTTCCGGCCAGGACGCTTTC 1038
Sulfitobacter    TTGCCGAGACCTATGATGTCATCGTCCAGCCTACGCAGAACAAAGTCTAT 1017
  * * * * *
P_mirabilis      ACCATCTTCGCACAATCCATGGACAGGACCGGTTACGCTCGAGGGACACT 623
E_coli           ACCATCTTCGCACAATCCATGGACAGGACCGGTTACGCTCGAGGGACACT 1040
Pseudomonas_florescens ACCCTGTTCCGCCAAGCCATGGACCGCACCGGTTACGCCCGGACCCCT 1034
Pseudomonas_syringae  ACCTTGTTGCTCAGTCGATGGACCGTACTGGTTTCGCGCGCGGCACCT 1034
Xanthomonas_campestris ACTATCTTCGCCAGGATCTGGGCGTACCGGTTACATCAGTGGCAGCT 1088
Sulfitobacter    AGCATTATTGCGGAATCCATGGGCGCAACGGGCTTGTTGCGGGCACCT 1067
  * * * * *
P_mirabilis      GGCCACGAGAGAGGGGTTAAGTGCTGCCGTTCCCCCCTCGATCCCCGTC 673
E_coli           GGCCACGAGAGAGGGGTTAAGTGCTGCCGTTCCCCCCTCGATCCCCGTC 1090
Pseudomonas_florescens CGCCGCCCGCGCGGTTTGTCCGCCCGGTCGCCGCGCTGGATCCGCGAC 1084
Pseudomonas_syringae  GGCGGTTGCTGAAGGCTCTAGCGCCCGTACCGCCCTTGATCCTCGAC 1084
Xanthomonas_campestris GGCTGTGCGAGAGGGCTGCGCGCTCCCGTCCGTCGCTCCGTGGACCCGCGC 1138
Sulfitobacter    GTCACCCAGAGAGGGCTATGCCGTTGCCGTTCCCGCTCTCAGACCCAAAGC 1117
  * * * * *
P_mirabilis      CTCTGTTGACCATGGAAGATATGGGT----- ATGGAGGGAATGGGACAT 717
E_coli           CTCTGTTGACCATGGAAGATATGGGT----- ATGGGGGGAATGGGACAT 1134
Pseudomonas_florescens CGCTGTTGACCATGGAAGATATGGGT----- ATGGGCGGATGGGCTCT 1128
Pseudomonas_syringae  CACTGTTGACCATGGCGGACATGGGT----- ATGGGCGGATGGATCAC 1128
Xanthomonas_campestris CCATCCTGACCATGGCCGACATGGGT----- ATGGATCAGCGCGGGATG 1182
Sulfitobacter    CCTTGCTCACCATGGCCGACATGAGCGGCATGATGGGCGGATGGGTATG 1167
  * * * * *
P_mirabilis      GATATG---GCAGGAATGGACCACAGCCA--- GATGGGA----- GGCAT 755
E_coli           GATATG---GCAGGAATGGACCACAGCCA---GATGGGA----- GGCAT 1172
Pseudomonas_florescens GGT----- GGAAT 1136
Pseudomonas_syringae  GGTTCG---ATGGCGCATGGGCAGCAT---GCAAGGCATGGAGGAAT 1172
Xanthomonas_campestris AGCGCG---ATGAGCATGAGCGCAAGCGA---CTCAAGC--GACTCAAGC 1224
Sulfitobacter    GATATGGGCATGGGTATGGGCGACCACGTCATGCAGAACATGACCGGAAT 1217
  * * * * *
P_mirabilis      GGATAACAGCG-GAG--- AGATGATGTCTATGGACGGTGTGGCCTTCCG 801
E_coli           GGATAACAGCG-GAG--- AGATGATGTCTATGGACGGTGTGACCTTCCG 1218
Pseudomonas_florescens GAATCATGGTT-C----- CATGGACATGAGC----- 1161
Pseudomonas_syringae  GGACCACGGCG-CAATGGGCGCAGGGGACAAGGGCATGGACAGTATGTCT 1221
Xanthomonas_campestris GACTCAAGCAA-CAA-GCCAGCGATGGCTATGAACATGGCCGGATGGCG 1272
Sulfitobacter    GAACCACACCGACATGACAGGCATGGACCATTCAAACATGCAATCCAGCC 1267
  * * * * *
P_mirabilis      GATG----- CGGG-ACATCCTCCGCG----- CCCATGGATC 832
E_coli           GATG----- CGGG-ACATCCTCCGCG----- CCCATGGATC 1249
Pseudomonas_florescens ----- GGCATGGATC 1171
Pseudomonas_syringae  GGCGCACCCATGCAGG-GCATGGACGGCGGGCAGACGCAGGGCATGGACC 1270
Xanthomonas_campestris CACGAGCC----- GGG-TCAACGTCGCGCGCAGGGCA-GACGCGCAT 1315
Sulfitobacter    AGCAGCCCCGATGCCTGCGATGACCCCGTCAGAGGAAAAGCCGAGAAA 1317
  * * * * *
P_mirabilis      ACAGCAGC-ATGGCCGGTATGGAT----- CAT 858
E_coli           ACAGCAGC-ATGGCCGGTATGGAT----- CAT 1275
Pseudomonas_florescens ----- CAT 1185
Pseudomonas_syringae  ATAGCAA-ATGTCAGGCATGGACCACGGTCCATGG----- AGACAT 1313
Xanthomonas_campestris CACATGCC-GGCCATGATATGAGCGGGATGCAATCAGG----- TTCCAT 1358
Sulfitobacter    AAGACTTCGATGGTGGATGGACCATACGCAGATGCAAAAGCCTGCGAT 1367
  * * * * *
P_mirabilis      TCCCGG-ATGGC----- CGGAATGCCG----- GGTATGC 886
E_coli           TCCCGG-ATGGC----- CGGAATGCCG----- GGTATGC 1303
Pseudomonas_florescens ----- ATGGC----- CCGATGC 1198
Pseudomonas_syringae  GGCCGGCATGGCGG----- GATGTCAGGCATGGGTGACATGCCGATGC 1357
Xanthomonas_campestris ----- ATCATGGTGCA ---GGCATGC 1387
Sulfitobacter    GGACGGCATGGACCACACGCAGATGCAGCAGAAGAAAAGGCACCCGATGT 1417
  * * * * *
P_mirabilis      ----- AAAGTCATCCTGCGTCAGAAACGGATAACCCAC---- TGTT 924
E_coli           ----- AAAGTCATCCTGCGTCAGAAACGGATAACCCAC---- TGTT 1341
Pseudomonas_florescens ----- AGTCGCACCCCGACAGCGAAAAGGACAACCCGCG---- TGTT 1236
Pseudomonas_syringae  ----- AAGCCACCCCGCTACCGAGAAGGACAACCCGCT---- TGTT 1395
Xanthomonas_campestris ----- AAGCCATCCGCGGAGTGAAGATCGTAACCCCTC---- TGTT 1425
Sulfitobacter    CAGGCATGAACCATGCCAGATGCAGAAAGCCGCAATGGACGATGAGC 1467
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Figure 1b. Cont.

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P_mirabilis      GATATGCAGGCGATGAGCGTCTCTCCGAAATTAATGAT----- 963
E_coli           GATATGCAGGCGATGAGCGTCTCTCCGAAATTAATGAT----- 1380
Pseudomonas_fluorescens GACATGCAAGCCATGACCACCGCCGCGAAACTCGACGAC----- 1275
Pseudomonas_syringae  GACATGCAAGCCATGAGTCTTACGCTAAATTTGGATGAT----- 1434
Xanthomonas_campestris GATAACCAGGCGATGACCCCTACGTCCCGCATGGATGAT----- 1464
Sulfitobacter    GGCATGGACAGTATGAGCGGAATGGCAATGTCTGATGGCGCGATCCGTT 1517
* * * * *
P_mirabilis      -----CCGGGTATTTGTCTT          CGAA-ATAACGGAAGAAAG 997
E_coli           -----CCGGGTATTTGTCTT----- CGAA-ATAACGGAAGAAAG 1414
Pseudomonas_fluorescens -----CCCGGCTCGGCCTG----- CGCA-ACAACGGTCCGCCGG 1309
Pseudomonas_syringae  -----CCCGGATTAGGCCTG----- CGCA-ATAACGGTCCGCCGG 1468
Xanthomonas_campestris -----CCTGGCAACGGGTTG----- CGGG-GTAACGGTCCGCACGG 1498
Sulfitobacter    CTATGTCCCGGGCAGCGGACTGACGCTGTCCGGTACAACGGCGCAAGT 1567
* * * * *
P_mirabilis      TTCTCACGTACGCGGATTTGAAAAGCCGCTTTGAGGATCCTGACGGACGT 1047
E_coli           TTCTCACGTACGCGGATTTGAAAAGCCGCTTTGAGGATCCTGACGGACGT 1464
Pseudomonas_fluorescens TGCTGACCTACGCCGACTGCGCAGCACGTTGGAAGACCCGGACGGCCGC 1359
Pseudomonas_syringae  TGCTTACGTATTCTGATTTGCGAAGCACCTTTGAAGACCCGGACGGCGA 1518
Xanthomonas_campestris TACTGACCTACGCAATGCTCAAAGTACGTTTGAGGATCCCGATGGCCGA 1548
Sulfitobacter    TCCTGTCTACGCAACCTGCGCGCCGCCAGCCCTCGTTACCGTCACCCG 1617
* * * * *
P_mirabilis      GAACCTGGCCGTACCATAGAAGTGCATTTAACC GGCCACATGGAAGAAAGTT 1097
E_coli           GAACCTGGCCGTACCATAGAAGTGCATTTAACC GGCCACATGGAAGAAAGTT 1514
Pseudomonas_fluorescens GACCCGAGTCGCACCCCTCGAGCTGCACCTCACC GGCCACATGGAGAAAT 1409
Pseudomonas_syringae  GAGCCCGGGCGAACAATCGAGCTGCACCTGACAGG CCATATGGAAAAAT 1568
Xanthomonas_campestris GCCCCTGGACGAGAGATCGAACTTCATTTAACC GGCCATATGGAGAAAT 1598
Sulfitobacter    GCACCTCAGCCACCATCGTGTTCGGGCTGACCGGTAACATGGAGCGGTA 1667
* * * * *
P_mirabilis      TGCCTGGTCATTTAACGGAATCAAGTTTTTCAGATGCCGCACCGGTGCTGC 1147
E_coli           TGCCTGGTCATTTAACGGAATCAAGTTTTTCAGATGCCGCACCGGTGCTGC 1564
Pseudomonas_fluorescens CGCCTGGTCGTTCAACGGCATCAAGTCTCCGACGCCGAGCCTCTGCTGT 1459
Pseudomonas_syringae  CGCCTGGTCATTCAATGGGGTCAAGTTCGCTGATGCTGAGCCCTCGCAT 1618
Xanthomonas_campestris TGCGTGGGATTCAACGGTCAAAAATCTCTGATGTCACCCGTTGCGAT 1648
Sulfitobacter    CATCTGGTCGATCAACGACGTGAAATACGACGATGCCGCCCGATCACCG 1717
* * * * *
P_mirabilis      CGAAATACGGTGAAGCGGCTCAGGATCACGCTGATCAACGATACCATGATG 1197
E_coli           TGAAATACGGTGAAGCGGCTCAGGATCACGCTGATCAACGATACCATGATG 1614
Pseudomonas_fluorescens TGAAGTACGGCGAGCGGATTCGCCTGGTGGTCAACGACACCGATGATG 1509
Pseudomonas_syringae  TGAAATATGGCGAGCGAGTGCAGCTGGTACTGGTAAACGACACCATGATG 1668
Xanthomonas_campestris TGAAGTATGGCGAAGCTGTGCGCATCGTCTGGTCAACGACACCATGATG 1698
Sulfitobacter    TCCGATACGGCGAGCGGTTGCGCATGCGGTTTCATCAACGAAACGATGATG 1767
* * * * *
P_mirabilis      ACTCACCATTACCTGCATGGGATG----- 1227
E_coli           ACTCACCATTACCTGCATGGGATGAGCGATCTGGAAGATGAAAA 1664
Pseudomonas_fluorescens ACTCACCATTACCTGCATGGGATGAGCGATCTGGAAGATGAAAA 1559
Pseudomonas_syringae  ACTCACCATTACCTGCATGGGATGAGCGATCTGGAAGATGAAAA 1718
Xanthomonas_campestris TGCATGGGATGAGCGATCTGGAAGATGAAAA 1748
Sulfitobacter    TGCATGGGATGAGCGATCTGGAAGATGAAAA 1817
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Figure 1b. Multiple alignments of nucleotide sequences from various Gram-negative bacterial copper resistance proteins for primer design. The amplified fragment from *P. mirabilis* is included in the alignment.

Resing, 2001).

Conclusions

The use of copper as an anti-microbial agent for surface cleaning of hospitals in the rural areas has been proposed since this is considered to be cheap and easily accessible in comparison to antibiotics (Copper Development Association Africa, 2006). However, some bacteria are able to protect themselves from toxicity of copper as a result of the detoxification systems they possess. This is either located within their chromosomes or on plasmid-borne genes, and their resistance mechanisms vary from active efflux to sequestration, cell wall modification and bioprecipitation (Choudhury and Srivastava, 2001).

In this study, we report on the first isolation of *P. mirabilis* from heavy metal contaminated mining environments, confirmed by Biolog and analysis of its 16S rDNA gene. Here, *P. mirabilis* exhibits a high minimum inhibitory concentration of copper at 2.5 times more than *E. coli* (1 mM MIC) as demonstrated by Nies (1999); 3 times higher than *Vibrio* sp. (0.787 mM) described by Miranda and Rojas (2006).

A possible copper resistance mechanism of *P. mirabilis* was also investigated through the assessment of a copper resistance gene. The amplified gene fragment showed that *P. mirabilis* possesses *pcoA*, a potential multicopper oxidase gene, which constitutes a group of periplasmic protective enzymes (Lee et al., 2002). To date, *P. mirabilis* resistance has only been studied with

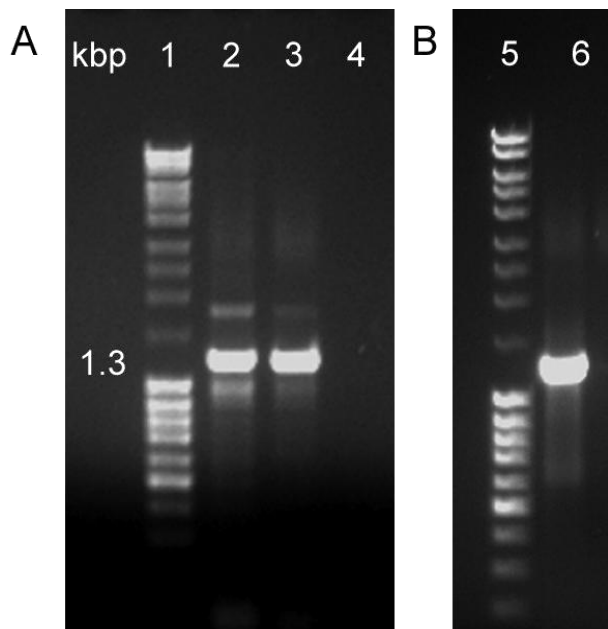


Figure 2. PCR amplification of a *pcoA* fragment in (A) *P. mirabilis* at annealing temperatures of 56.9°C (Lane 2) and 59°C (Lane 3) as well as *Bacillus licheniformis* (Lane 4) as negative control, and (B) *E. coli* (Lane 6) as positive control. Lanes 1 and 5 represent the MassRuler™ DNA ladder (Fermentas).

regard to antibiotics (Wachino et al., 2006), and a swarming-defective mutant of *P. mirabilis* lacking a putative cation-transporting membrane P-type ATPase (Lai et al., 1998). This study is therefore the first pertaining to this organism and its copper-resistance characteristic. Also, given that *P. mirabilis* is second only to *E. coli* as a pathogenic organism causing urinary tract infections in individuals with structural abnormalities or indwelling catheters (Mobley and Belas, 1995); this study emphasizes the understanding of copper resistance in light of the application of copper as an anti-microbial agent. Furthermore, *P. mirabilis* could be a good candidate for biotechnological applications, such as a heavy metal biosensor. Having *pcoA* in *P. mirabilis* similar to *E. coli* as revealed by BLASTn analysis, with further investigations regarding the mechanism of copper resistance in *P. mirabilis*, this organism is a prospective organism for soil bioremediation of copper-contaminated environments since PcoA in *E. coli* is similar to CopA in *Pseudomonas syringae* that sequesters excess copper in the periplasm.

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