Advanced Journal of Microbiology Research Volume 2013
Available online at http://advancedresearchjournals.org/AJMR

Full Length Research paper

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

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Accepted 18 November 2012

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 nutriational 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.

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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 $FeSO_4.7H_20$ and 0.084 mM $NaHPO_4$ dissolved in NINE-SALTS-SOLUTION (0.3 M NaCl; 0.1 M Na_2SO_4; 0.95 mM $NaHCO_3$; 3.37 mM KCl; 0.34 mM KBr; 9.25 mM $MgCl_2.6H_20$; 2.8 mM $CaCl_2.2H_20$; 0.037 mM $SrCl_2.6H_2O$; 0.16 mM H_3 BO_3, pH 7,] (Hermansson et al., 1987) and incubated at 37ºC. 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).

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 95ºC 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 95ºC for 30 s, an annealing step at 52ºC for 45 s and elongation step at 72ºC for 90 s, followed by a final extension, for 10 min at 72ºC.

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 fluores -cens, 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'-ACYTACTGGTAYCACAGCCATTC 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 60ºC. 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

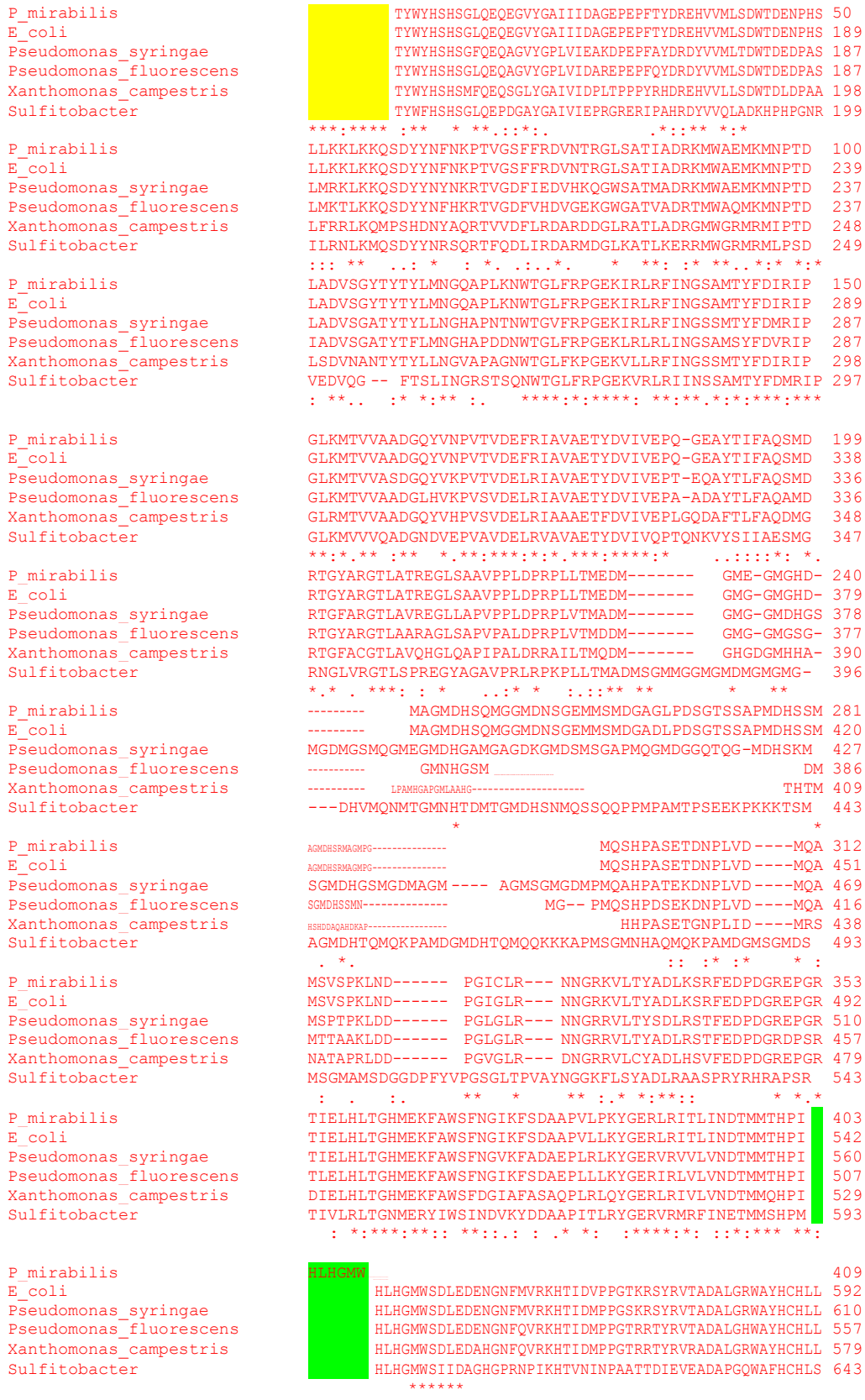

Figure 1a. Multiple alignments of amino acid sequences from various Gram-negative bacterial copper resistance proteins for primer design. The amplified and translated fragment from *P. mirabilis* is included in the alignment.

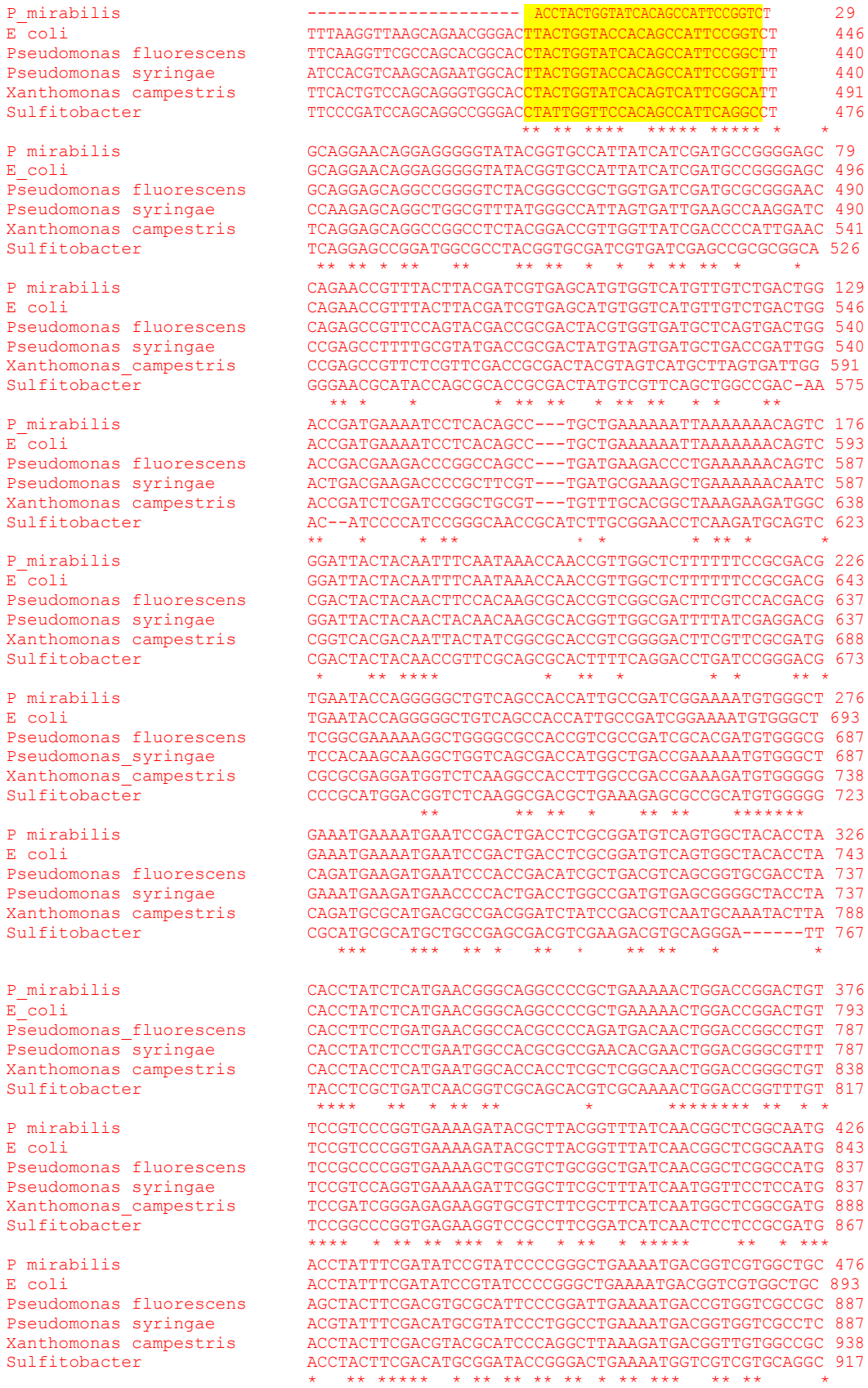
P_mirabilis ---------- ACCTACTGGTATCACAGCCATTCCGGTCT 29
E coli TTTAAGGTTAAGCAGAACGGGACTTACTGGTACCACAGCCATTCCGGTCT 446
Pseudomonas fluorescens TTCAAGGTTCGCCAGCACGGCACCTACTGGTATCACAGCCATTCCGGCTT 440
Pseudomonas syringae ATCCACGTCAAGCAGAATGGCACTTACTGGTACCACAGCCATTCCGGTTT 440
Xanthomonas campestris TTCACTGTCCAGCAGGGTGGCACCTACTGGTATCACAGTCATTCGGCATT 491
Sulfitobacter TTCCCGATCCAGCAGGCCGGGACCTATTGGTTCCACAGCCATTCAGGCCT 476
P mirabilis GCAGGAACAGGAGGGGGTATACGGTGCCATTATCATCGATGCCGGGGAGC 79
E_coli GCAGGAACAGGAGGGGGTATACGGTGCCATTATCATCGATGCCGGGGAGC 496
Pseudomonas fluorescens GCAGGAGCAGGCCGGGGTCTACGGGCCGCTGGTGATCGATGCGCGGGAAC 490
Pseudomonas syringae CCAAGAGCAGGCTGGCGTTTATGGGCCATTAGTGATTGAAGCCAAGGATC 490
Xanthomonas campestris TCAGGAGCAGGCCGGCCTCTACGGACCGTTGGTTATCGACCCCATTGAAC 541
Sulfitobacter TCAGGAGCCGGATGGCGCCTACGGTGCGATCGTGATCGAGCCGCGCGGCA 526
P mirabilis CAGAACCGTTTACTTACGATCGTGAGCATGTGGTCATGTTGTCTGACTGG 129
E coli CAGAACCGTTTACTTACGATCGTGAGCATGTGGTCATGTTGTCTGACTGG 546
Pseudomonas fluorescens CAGAGCCGTTCCAGTACGACCGCGACTACGTGGTGATGCTCAGTGACTGG 540
Pseudomonas syringae CCGAGCCTTTTGCGTATGACCGCGACTATGTAGTGATGCTGACCGATTGG 540
Xanthomonas_campestris CCGAGCCGTTCTCGTTCGACCGCGACTACGTAGTCATGCTTAGTGATTGG 591
Sulfitobacter GGGAACGCATACCAGCGCACCGCGACTATGTCGTTCAGCTGGCCGAC-AA 575
P_mirabilis ACCGATGAAAATCCTCACAGCC---TGCTGAAAAAATTAAAAAAACAGTC 176
E coli ACCGATGAAAATCCTCACAGCC---TGCTGAAAAAATTAAAAAAACAGTC 593
Pseudomonas fluorescens ACCGACGAAGACCCGGCCAGCC---TGATGAAGACCCTGAAAAAACAGTC 587
Pseudomonas syringae ACTGACGAAGACCCCGCTTCGT---TGATGCGAAAGCTGAAAAAACAATC 587
Xanthomonas campestris ACCGATCTCGATCCGGCTGCGT---TGTTTGCACGGCTAAAGAAGATGGC 638
Sulfitobacter AC--ATCCCCATCCGGGCAACCGCATCTTGCGGAACCTCAAGATGCAGTC 623
P_mirabilis GGATTACTACAATTTCAATAAACCAACCGTTGGCTCTTTTTTCCGCGACG 226
E coli GGATTACTACAATTTCAATAAACCAACCGTTGGCTCTTTTTTCCGCGACG 643
Pseudomonas fluorescens CGACTACTACAACTTCCACAAGCGCACCGTCGGCGACTTCGTCCACGACG 637
Pseudomonas syringae GGATTACTACAACTACAACAAGCGCACGGTTGGCGATTTTATCGAGGACG 637
Xanthomonas campestris CGGTCACGACAATTACTATCGGCGCACCGTCGGGGACTTCGTTCGCGATG 688
Sulfitobacter CGACTACTACAACCGTTCGCAGCGCACTTTTCAGGACCTGATCCGGGACG 673
P mirabilis TGAATACCAGGGGGCTGTCAGCCACCATTGCCGATCGGAAAATGTGGGCT 276
E coli TGAATACCAGGGGGCTGTCAGCCACCATTGCCGATCGGAAAATGTGGGCT 693
Pseudomonas fluorescens TCGGCGAAAAAGGCTGGGGCGCCACCGTCGCCGATCGCACGATGTGGGCG 687
Pseudomonas_syringae TCCACAAGCAAGGCTGGTCAGCGACCATGGCTGACCGAAAAATGTGGGCT 687
Xanthomonas_campestris CGCGCGAGGATGGTCTCAAGGCCACCTTGGCCGACCGAAAGATGTGGGGG 738
Sulfitobacter CCCGCATGGACGGTCTCAAGGCGACGCTGAAAGAGCGCCGCATGTGGGGG 723
P mirabilis GAAATGAAAATGAATCCGACTGACCTCGCGGATGTCAGTGGCTACACCTA 326
E coli GAAATGAAAATGAATCCGACTGACCTCGCGGATGTCAGTGGCTACACCTA 743
Pseudomonas fluorescens CAGATGAAGATGAATCCCACCGACATCGCTGACGTCAGCGGTGCGACCTA 737
Pseudomonas syringae GAAATGAAGATGAACCCCACTGACCTGGCCGATGTGAGCGGGGCTACCTA 737
Xanthomonas campestris CAGATGCGCATGACGCCGACGGATCTATCCGACGTCAATGCAAATACTTA 788
Sulfitobacter CGCATGCGCATGCTGCCGAGCGACGTCGAAGACGTGCAGGGA------TT 767
P_mirabilis CACCTATCTCATGAACGGGCAGGCCCCGCTGAAAAACTGGACCGGACTGT 376
E_coli CACCTATCTCATGAACGGGCAGGCCCCGCTGAAAAACTGGACCGGACTGT 793
Pseudomonas_fluorescens CACCTTCCTGATGAACGGCCACGCCCCAGATGACAACTGGACCGGCCTGT 787
Pseudomonas syringae CACCTATCTCCTGAATGGCCACGCGCCGAACACGAACTGGACGGGCGTTT 787
Xanthomonas campestris CACCTACCTCATGAATGGCACCACCTCGCTCGGCAACTGGACCGGGCTGT 838
Sulfitobacter TACCTCGCTGATCAACGGTCGCAGCACGTCGCAAAACTGGACCGGTTTGT 817
P mirabilis TCCGTCCCGGTGAAAAGATACGCTTACGGTTTATCAACGGCTCGGCAATG 426
E coli TCCGTCCCGGTGAAAAGATACGCTTACGGTTTATCAACGGCTCGGCAATG 843
Pseudomonas fluorescens TCCGCCCCGGTGAAAAGCTGCGTCTGCGGCTGATCAACGGCTCGGCCATG 837
Pseudomonas syringae TCCGTCCAGGTGAAAAGATTCGGCTTCGCTTTATCAATGGTTCCTCCATG 837
Xanthomonas_campestris TCCGATCGGGAGAGAAGGTGCGTCTTCGCTTCATCAATGGCTCGGCGATG 888
Sulfitobacter TCCGGCCCGGTGAGAAGGTCCGCCTTCGGATCATCAACTCCTCCGCGATG 867
P mirabilis ACCTATTTCGATATCCGTATCCCCGGGCTGAAAATGACGGTCGTGGCTGC 476
E coli ACCTATTTCGATATCCGTATCCCCGGGCTGAAAATGACGGTCGTGGCTGC 893
Pseudomonas fluorescens AGCTACTTCGACGTGCGCATTCCCGGATTGAAAATGACCGTGGTCGCCGC 887
Pseudomonas syringae ACGTATTTCGACATGCGTATCCCTGGCCTGAAAATGACGGTGGTCGCCTC 887
Xanthomonas campestris ACCTACTTCGACGTACGCATCCCAGGCTTAAAGATGACGGTTGTGGCCGC 938
Sulfitobacter ACCTACTTCGACATGCGGATACCGGGACTGAAAATGGTCGTCGTGCAGGC 917

Figure 1b.

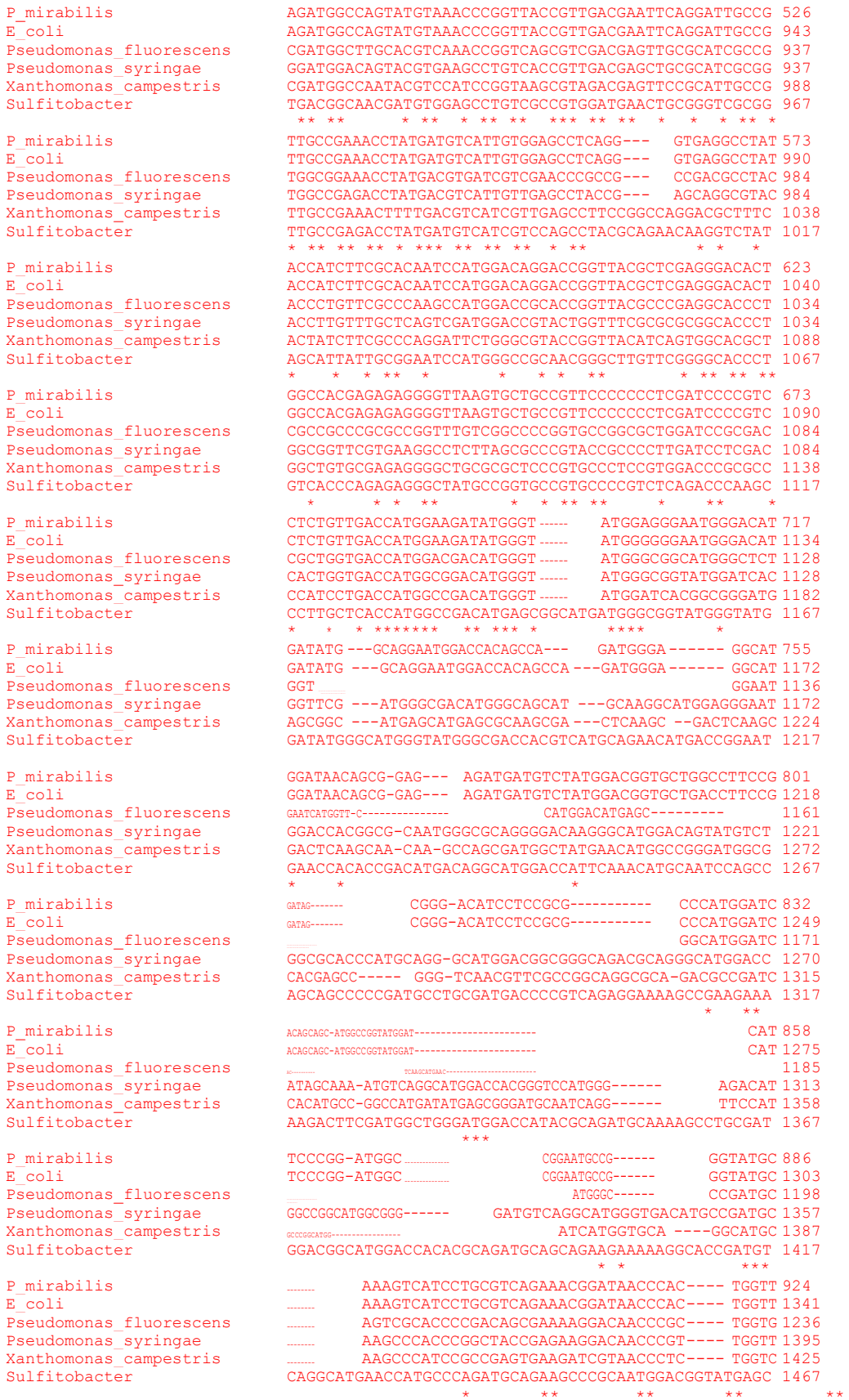

Figure 1b. Cont.

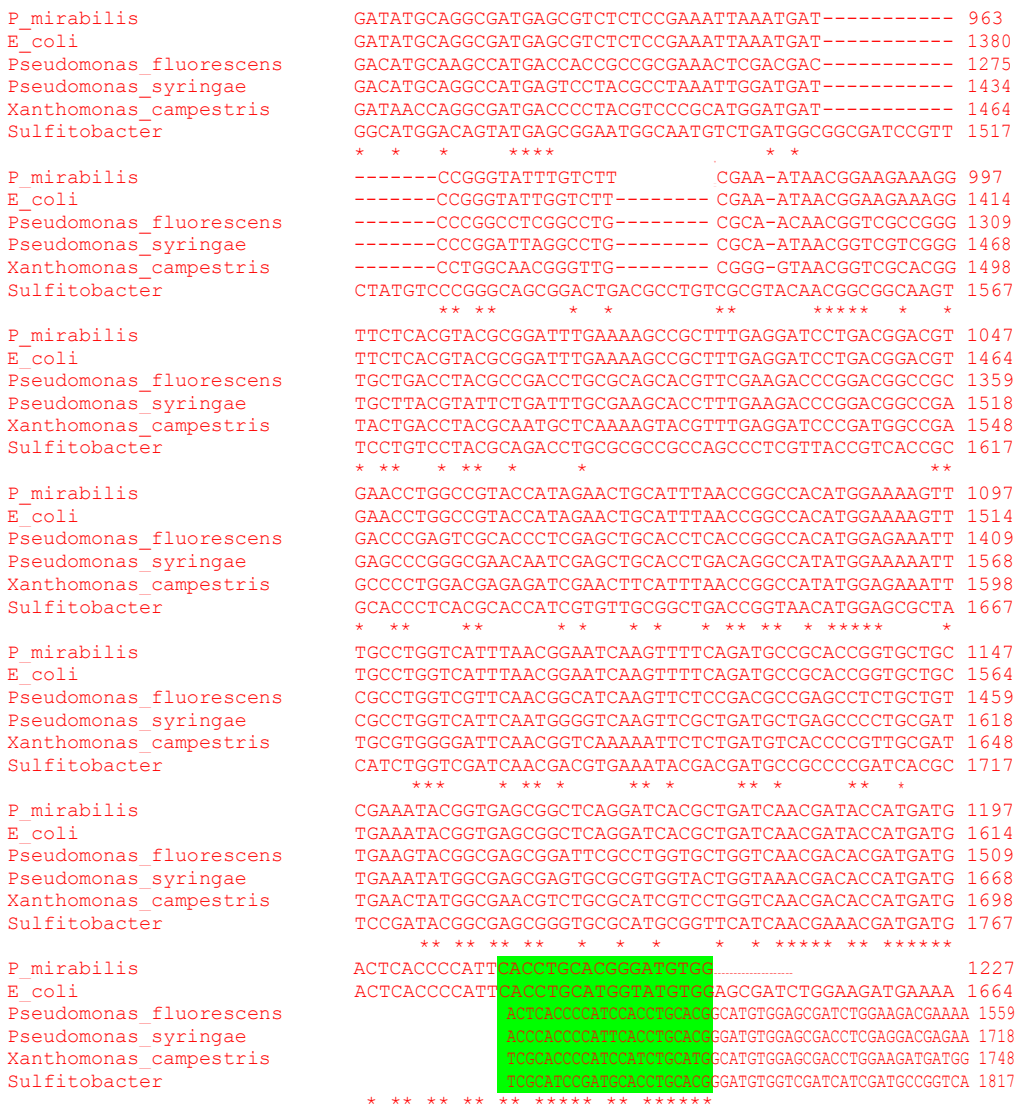

Figure 1b. Multiple alignments of nucleotide sequences from various Gram-negative bacterial copper resistance proteins for primer design. The amplified fragment from *P. mirabilis* is included in the alignment.

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 miminum inhibitory concentration of copper at 2.5 times more than *E. coli* (1 mM MIC) as demonstrated by Nies (1999); 3 times higher than *Vibro* 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

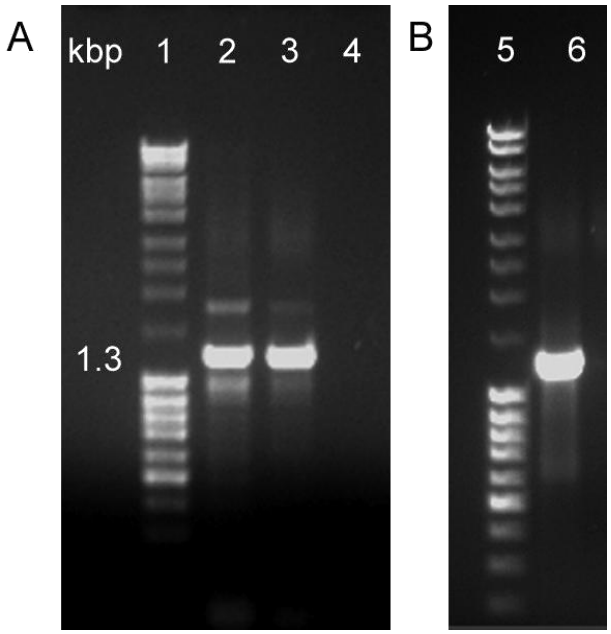

Figure 2. PCR amplification of a *pcoA* fragment in (A) *P. mirabilis* at annealing temperatures of 56.9ºC (Lane 2) and 59ºC (Lane 3) as well as *Bacillus licheniformis* (Lane 4) as negative control, and (B) *E. coli* (Lane 6) as positive control. Lanes 1 and 5 represent the MassRuler^{TM} DNA ladder (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 *pco*A 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 sequests excess copper in the periplasm.

ACKNOWLEDGEMENTS

The authors would like to thank the NRF, South Africa for financial support of this project.

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