Calcium (II) - and dipicolinic acid mediated-biostimulation of oil-bioremediation under multiple stresses by heat, oil and heavy metals

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The oil-producing Arabian Gulf states have hot summer seasons of about 7-month in length. Therefore, environmental oil spills should be bioremediated by the activity of indigenous, hydrocarbonoclastic (hydrocarbon-degrading) microorganisms with optimum growth at about 50 °C. Soils in such arid countries harbor thermophilic bacteria, whose oil-consumption potential is enhanced by calcium (II) - and dipicolinic acid (DPA)-supplement. Those organisms are, however, subjected to additional stresses including toxic effects of heavy metals that may be associated with the spilled oil. Our study highlighted the resistance of indigenous, thermophilic isolates to the heavy metals, mercury (II), cadmium (II), arsenic (II) and lead (II) at 50 °C. We also detected the uptake of heavy metals by 15 isolates at 50 °C, and identified the merA genes coding for Hg²⁺-resistance in 4 of the studied Hg²⁺-resistant isolates. Hg²⁺ was the most toxic metal and the metal toxicity was commonly higher in the presence of oil. The addition of Ca²⁺ and DPA enhanced the Hg²⁺-resistance among most of the isolates at 50 °C. Crude oil consumption at 50 °C by 4 selected isolates was inhibited by the tested heavy metals. However, Ca²⁺ and DPA limited this inhibition and enhanced oil-consumption, which exceeded by far the values in the control cultures.

Globally, the Arabian Gulf states are major oil producers. Consequently, oil spills during oil production, transportation and use as an energy source are more frequent in that region than elsewhere. This region is an arid zone with a harsh climate. It is characterized by hot summer seasons, usually extending from mid-April to mid-October. This is also the case in several other oil-producing countries over the globe. Summer temperatures of about 50 °C and higher are usual in the Gulf region, leading to minimized activities of indigenous, mesophilic microorganisms that are responsible for oil bioremediation world-wide1-4. For bioremediation to occur under these hot conditions, thermophilic microorganisms (growing best at about 50 °C) with hydrocarbon degradation potential should be involved5.

Although there is currently a wealth of information on thermophilic microorganisms and their practical applications6-9, thermophiles with hydrocarbonoclastic potential have been only poorly investigated. The first report on a thermophilic bacterium capable of oil degradation appeared in the 196010. Subsequently, it took a number of decades for more reports on this subject to appear11-13. Thermophilic bacteria with hydrocarbonoclastic potential reportedly belonged to the genera Bacillus / Geobacillus, Thermomonas and Thermooleophilum. Our group contributed to such studies at that time by a publication on a local Bacillus stearothermophilus strain capable of crude oil utilization at about 50 °C...
bonoclastic activities of all the tested organisms were enhanced in response to treating the cultures with Ca2+
and DPA-amendment in resisting the toxic effects of heavy metals and DPA-amendment may enhance
microbial tolerance to heat, and may thus stimulate their potential for spilled-oil bioremediation in the thermo-
philic range. As mentioned, we have already provided experimental evidence for the validity of this assumption14.
However, the thermophilic, hydrocarbonoclastic isolates
have to operate under multiple stresses. In addi-
tion to standing stresses due to toxic, aromatic oil constituents17-19 and heat, the microorganisms should with-
stand the toxicity exerted by heavy metals associated with crude oil20-22. Therefore, we now have elaborated on
our earlier study by focusing on the effects of crude oil and heavy metals on the microbial isolates. Furthermore,
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we have investigated the probable roles of Ca2+- and DPA-amendment in resisting the toxic effects of heavy metals
that lead to reduced growth and hydrocarbon consumption activity of those thermophiles at 50 °C. The results are
expected to be useful in designing biotechnologies for bioremediating environmental oil spills in hot countries.

**Results and Discussion**

Nineteen thermophilic bacterial species with hydrocarbonoclastic potential were used; they had been isolated as
pure cultures, and characterized by sequencing their 16 S rDNA regions as described earlier14. Four heavy metals, Hg2+
Cd2+, As2+ and Pb2+, were chosen for this study, based on their frequent use in earlier similar studies on
mesophilic microorganisms23.

**Minimum inhibitory concentrations of heavy metals at 50 °C.** Heavy-metal resistance/tolerance is measured either as “maximum tolerated concentrations” (MTC)24-26 or as “minimum inhibitory concentrations” (MIC)27,28. In this study, we determined the MIC values for four heavy metals for the 19 studied strains at 50 °C in the absence and presence of crude oil vapor. The results are presented in Table 1. As expected, the various bacterial species exhibited different MIC values depending on their identities and on the heavy metal used. In the absence of oil vapor, the MIC values were lowest with Hg2+ (<5–90 ppm) and highest with As2+ (70 – > 14,800 ppm), reflecting highest and lowest toxicities, respectively. Cd2+ with MIC values between 5 and 700 ppm was next to Hg2+ in toxicity to the tested microorganisms. Kocuria rosea (MIC of 5,600 ppm Cd2+) was exceptionally tolerant to this metal. With MIC-values between 50 and 4000 ppm, Pb2+ was relatively less toxic to most of the isolates than As2+. In many cases, the MIC values of the heavy metals were much lower in the presence of crude oil than in its absence, reflecting higher microbial sensitivity. Those differences were more pronounced with Hg2+, Cd2+ and Pb2+ than with As2+. However, exceptions were also recorded. For example, the MIC-values with Bacillus niabensis treated with Cd2+ was higher (>600 ppm) in the presence of oil vapor than in its absence (only 5 ppm). With As2+, the MIC values measured for Amycolatopsis thermoﬂava, Chelativorans multitrrophicus, Isoptericola variabilis and Nocardia farcinica in the absence and presence of oil vapor were quite similar.

| Bacteria | Minimal inhibitory concentrations of heavy metals, MIC (ppm) |
| --- | --- |
| | On nutrient agar | On nutrient agar + oil |
| | Hg | Hg + Ca2+ | Cd | As | Pb | Hg | Hg + Ca2+ | Cd | As | Pb |
| Amycolatopsis thermoﬂava | 90 | 90 | 300 | >14,800 | 4000 | 20 | 20 | 50 | >14,800 | 500 |
| Bacillus carboniphilus | 30 | 30 | 50 | >14,800 | 140 | <5 | <5 | 50 | 70 | <10 |
| Bacillus firmus | <5 | 10 | 700 | >14,800 | 4000 | <5 | <5 | 5 | 2000 | <10 |
| Bacillus foraminis | 50 | 50 | 700 | >14,800 | 500 | <5 | <5 | 70 | 1200 | <10 |
| Bacillus licheniformis | <5 | 90 | 5 | 2000 | 500 | <5 | <5 | 70 | 1000 | <10 |
| Bacillus niabensis | 90 | 90 | 5 | 10000 | 500 | <5 | <5 | 5 | 2000 | <10 |
| Bacillus thermomylolovorans | 30 | 30 | 300 | 10000 | 500 | <5 | <5 | 5 | 2000 | <10 |
| Chelativorans multitrrophicus | 50 | 170 | 700 | >14,800 | 1200 | <5 | 20 | >600 | >14,800 | 150 |
| Isoptericola variabilis | 30 | 50 | 50 | >14,800 | 1200 | <5 | 20 | 500 | >14,800 | 150 |
| Nocardia farcinica | 10 | 20 | 50 | >14,800 | 1200 | <5 | <5 | 50 | >14,800 | >1050 |
| Aeribacillus pallidus | <5 | 50 | 50 | >14,800 | 1200 | <5 | <5 | 5 | 3000 | >1050 |
| Aneurinibacillus danicus | 30 | <210 | 50 | >14,800 | 500 | <5 | <5 | 5 | 2000 | 150 |
| Bevrivibacillus borsteidenis | <5 | 170 | 700 | 70 | 1200 | <5 | <5 | 50 | 70 | 150 |
| Bevrivibacillus thermoruber | <5 | 10 | 700 | 70 | 50 | <5 | <5 | 50 | 70 | <10 |
| Geobacillus kaustophilus | <5 | 20 | 50 | 500 | 140 | <5 | 30 | 50 | 70 | <10 |
| Geobacillus subterraneus | <5 | 10 | 700 | 5000 | 1200 | <5 | 50 | 50 | 2000 | >1050 |
| Kocuria rosea | <5 | 10 | >5600 | 10000 | 1200 | <5 | <5 | 50 | 5000 | <10 |
| Marinobacter lataeensis | <5 | 10 | 50 | 70 | 50 | <5 | <5 | 50 | 70 | <10 |
| Paenibacillus lautus | <5 | 10 | 700 | 70 | 500 | <5 | <5 | 5 | 70 | <10 |

Table 1. Minimum inhibitory concentrations (MIC) [ppm] of heavy metals for 19 hydrocarbonoclastic bacteria at 50 °C on nutrient agar with and without oil vapor. Values are means of triplicates.
There are no relevant reports in the available literature on thermophiles (nor on mesophiles) with which to compare our findings. An explanation for the lower MIC heavy metal values in the presence rather than the absence of oil may be that oil exerts additional stress on the strains. Oil is known to contain polyaromatic hydrocarbons with toxic effects. Organisms whose MIC values were not affected by oil were probably active in biodegradation of the polyaromatic hydrocarbons. The results in Table 1 indicate that the heavy metals may be arranged according to their toxicities to the studied isolates in the following order: \(\text{Hg}^{2+} > \text{Cd}^{2+} > \text{Pb}^{2+} > \text{As}^{3+}\).

This toxicity order is more or less similar to that described by earlier workers on mesophiles. A new finding in our study is that the amendment with \(\text{Ca}^{2+}\) as \(\text{CaSO}_4\) significantly enhanced the resistance of many isolates to \(\text{Hg}^{2+}\) (\(p < 0.05\)). This enhancement was particularly pronounced with \(\text{Bacillus licheniformis}, \text{B. thermoamylolovorans}, \text{Chelatovorans multitrophicus}, \text{Isopericala variabilis}, \text{Aeribacillus pallidus}, \text{Aneurinibacillus danicus}, \text{Brevibacillus borstelensis}, \text{Geobacillus kaustophilus}\) and \(\text{G. subterraneus}\). The two \(\text{Geobacillus}\) species in cultures that had been supplemented with \(\text{CaSO}_4\) surprisingly tolerated \(\text{Hg}^{2+}\) more effectively than in its absence. Again, there are no reports in the available literature on the resistance of microorganisms to heavy metals as affected by crude oil for comparison. This study thus provides some novel and important information in this area of research.

**Figure 1.** Uptake of heavy metal by hydrocarbonoclastic bacteria at 50 °C. Most of the amounts of the heavy metals added decreased in the media and accumulated in the cells by all the studied organisms during incubation. Each value was the mean of three parallel readings, and the deviation values were < 5.8% of the means.

**Uptake of heavy metals at 50 °C.** To reduce the extensive set-up associated with this experiment, we selected the fifteen isolates that revealed (in preliminary experiments) high heavy metal resistance. The results in Fig. 1 show the uptake of \(\text{Hg}^{2+}\) as supplied by \(\text{HgCl}_2\), \(\text{Cd}^{2+}\) as \(\text{CdSO}_4\), and \(\text{Pb}^{2+}\) as \(\text{Pb(NO}_3)_2\) by the studied hydrocarbonoclastic isolates at 50 °C. The four cations were taken up successfully by all the strains. Most of the \(\text{Pb}^{2+}\) was taken up from the medium relatively quickly, i.e. within the first day, and was maintained intracellularly.
through the 8 days of incubation. The uptake of Hg$^{2+}$ and Cd$^{2+}$, on the other hand, continued through the 8 day incubation period, although most of the cations were taken up during the first 3 days (left graphs). In most cases, the cells lost proportions of the bound Hg$^{2+}$ and Cd$^{2+}$ (but not Pb$^{2+}$), at late phases of incubation (right graphs), yet not into the surrounding medium. It is well known that microorganisms have the potential for reduction of some heavy metals into volatile forms (e.g. Hg$^{2+}$ into Hg). Probably, Bacillus thermoamylovorans cells in the closed batch cultures (top-graph at the right, Fig. 1) again took up much of the volatilized Hg leading to increased amounts of cell-bounded Hg$^{2+}$. In field treatment, the summer heat (about 50 °C or more) would enhance the volatilization process in the open atmosphere. This transformation (Hg$^{2+}$ reduction to volatile Hg) is mediated by enzymes encoded by merA genes. Therefore, our Hg$^{2+}$-resistant species (based on preliminary experiments, see also Table 1) were analyzed for those genes.

The results in Fig. 2 and Table 2 offer experimental evidence for the occurrence of merA genes in the genomes of 9 Hg$^{2+}$-resistant bacterial species (see Fig. 2).

Table 2. Information related to sequencing of MerA genes in the genomes of 9 Hg$^{2+}$-resistant bacterial species (see Fig. 2).
primer sets for their common amplification. New primer sets for this purpose may will be constructed in the future.

**Consumption of crude oil at 50 °C.** Due to the extensive work needed for this analysis, we selected, as representative species, the four isolates with the best growth potential (in preliminary experiments) in a mineral medium with oil as a sole source of carbon and energy. The results in Fig. 3 show, after 2 weeks at 50 °C in the absence of heavy metals, that the four tested isolates consumed significant proportions of the crude oil available in the medium. *Isoptericola variabilis* and *Nocardia farcinica* with consumption values of 41 and 37%, respectively, were most effective, whereas *Chelativorans multitrophicus* and *Amycolatopsis thermoflava* with consumption values of 22 and 14%, respectively, were least effective. The oil-consumption values by the tested organisms decreased in the presence of the four tested heavy metals. In all cases, the consumption values were significant, with \( p < 0.05 \). The GLC profiles show that individual oil constituents (individual peaks) were consumed rather evenly by all the tested microorganisms.

An interesting and new finding is that the amendment with Ca\(^{2+}\) alone or in combination with DPA significantly \( (p < 0.05) \) stimulated oil consumption at 50 °C, even in the presence of inhibitory concentrations of heavy metals (see Methods, Uptake of heavy metals). In response to such amendments, the consumption values reached 87% of the amount available at time zero. From a biotechnological viewpoint, this result is quite impressive. However, the biochemical basis for the mechanisms of such biostimulation is still far from clear. In the available literature, there are no related studies with which to compare our data. However, endospores of *Bacillus* spp. are heat- and stress-resistant, like the studied thermophiles, and are known to contain DPA complexed with Ca\(^{2+}\) at about 15% of their dry weights. Calcium-dipicolinate has been reported to stabilize nucleic acids and proteins (enzymes) at high temperature. Should this be commonly the case, this interpretation could also be adopted to the effects of Ca\(^{2+}\) and DPA on the active cells of thermophilic, hydrocarbonoclastic bacteria, most of which are

![Figure 3. GLC-Profiles of residual crude oil showing the effects of Ca\(^{2+}\) and dipicolinic acid (DPA) on oil-consumption by bacteria at 50 °C in the presence of heavy metals. Percent values on the individual profiles are those of residual oil. Each value is the mean of 3 parallel analyses ± standard deviation. All changes in the oil concentrations compared to the 100% controls were significant with \( p < 0.05 \).](image-url)
actually endospore-producers. As endospores, they would remain of course dormant without hydrocarbonoclastic or any other activities.

In conclusion, although exposed to multiple stresses (toxic oil-constituents, namely the polyaromatics, toxic heavy metals, heat), thermophilic, hydrocarbonoclastic bacteria indigenous to hot regions are still capable of consuming spilled oil. Their oil bioremediation potential can be effectively biostimulated by amendment with Ca\(^{2+}\) and DPA. These novel findings should be considered in designing biotechnologies for controlling oil-spills in hot regions. Based on the results of this contribution, the thermophiles, Amycolatopsis thermoflava, Chelativorans multitrophicus, Isoptericola variabilis and Nocardia farcinica may be suggested for bioremediation of oily soils under multiple stresses. These organisms may be biostimulated by amendment with Ca\(^{2+}\) and DPA.

**Methods**

**Thermophilic bacteria.** The nineteen hydrocarbonoclastic, thermophilic bacterial species used in this study are listed in Table 2. They had been isolated from oil-contaminated soil samples from Kuwaiti oil fields on a selective medium at 50 °C. This is a mineral medium with oil vapor as the sole source of carbon and energy\(^35\). It consisted of (g l\(^{-1}\)): 5.0 NaNO\(_3\), 0.6 KH\(_2\)PO\(_4\), 0.9 Na\(_2\)HPO\(_4\), 0.2 K\(_2\)SO\(_4\), 0.4 MgSO\(_4\) 7H\(_2\)O, 0.7 CaCl\(_2\) 2H\(_2\)O, 2.5 ml of trace element mixture (g l\(^{-1}\)): 2.3 ZnSO\(_4\), 1.8 MnSO\(_4\), 0.6 H\(_3\)BO\(_3\), 1.0 CuSO\(_4\), 0.4 Na\(_2\) MoO\(_4\), 0.4 CoCl\(_2\), 0.7 KI, 1.0 EDTA, 0.4 FeSO\(_4\), 0.004 NiCl\(_2\), and 20.0 g agar. Oil vapor was made available by fixing in the dish lids filter papers impregnated with 2 ml crude oil and sealing the dishes. The isolated strains were purified and characterized by comparing the sequences of their 16S rRNA-coding genes with those of strains in the GenBank database. Detailed information of these organisms is available in our earlier publication\(^{14}\).

**Uptake of heavy metals.** A common inoculum was prepared for each tested strain by homogenizing a loopful of 48-h biomass in 10 ml sterile water. One ml of this suspension was inoculated into 200 ml nutrient broth (g l\(^{-1}\): 5.0 peptone, 2.0 yeast extract) provided with either 15 ppm Hg\(^{2+}\) as HgCl\(_2\), 112 ppm Cd\(^{2+}\) as CdCl\(_2\) or 207 ppm Pb\(^{2+}\) as Pb(NO\(_3\))\(_2\). Three parallel replicates were prepared for each analysis. Cultures were incubated at 50 °C on an electrical shaker, 100 rpm, for 8 days. From each culture, 10 ml were taken at time zero and then daily. The aliquots were centrifuged at 6000 \(\times\) g for 10 min at 4 °C, and the pellets were washed 3 times with nutrient broth. The washed pellets, the combined supernatants and 1 ml of the control (cell-free medium containing the heavy metal) were quantitatively analyzed for the heavy metals. In experiments with Hg\(^{2+}\), biomass and supernatant samples were first digested with a mixture of concentrated HNO\(_3\) and HCl, 2:1, v/v, on a hot plate. The digested solutions were filtered, diluted with 1% HNO\(_3\) and the total mercury concentrations were measured by an inductively coupled plasma-atomic emission spectrophotometer (ICP-AES; Thermo Elemental, Franklin Lakes, NJ, USA, or JY 2000 Ultrace, Jobin–Yvon Horiba) using a certified reference soil standard containing mercury solution. For Cd\(^{2+}\) and Pb\(^{2+}\) an inductively coupled plasma mass spectrophotometer (ICP-MS, varian-820-MS, NJ, USA) was used to measure the concentrations directly. The percent values of heavy metals remaining in the medium were calculated.

**The merA gene analysis.** Total genomic DNA was extracted from 24-h bacterial biomass using a PrepMan Ultra Kit (Applied Biosystems, Foster City, CA, USA). The merA region was amplified by PCR using 9 different primer pairs (sequences are listed in Table 3). The reaction mixture contained puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, UK), 1 µl (25 ng) of DNA template and 1 µl each of the primer combinations.

**Table 3.** Primer sets used for merA gene amplification\(^{33}\).
The reaction volume was completed to 25 μl with molecular water. Amplification was carried out in a Veriti Thermal Cycler (Applied Biosystems, USA) and the PCR conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 50–64 °C for 30 sec, 72 °C for 1–3 min and one cycle of final extension at 72 °C for 10 min.

Sequencing of amplicons was performed by a BigDye version Terminator Kit (Applied Biosystems, USA); 20 ng of the DNA template was added to 2 μl of a BigDye v 3.1 terminator and 2 μl of BigDye Terminator v 1.1, v 3.1 5x sequencing buffer; 1 μl of either the forward or reverse primer was added, and the final volume was brought up to 10 μl with molecular water. Labeling was completed in a Veriti Thermal Cycler (Applied Biosystems, USA) using one cycle of 96 °C for 1 min, then 25 cycles of 1 min at 96 °C, 5 s at 50 °C and 4 min at 60 °C. The pure template DNA samples were processed in a 3130xl Genetic Analyser (Applied Biosystems, USA). Sequencing analysis version 5.2 software (Applied Biosystems, USA) was used to analyze the results. Sequences were subjected to basic local alignment search tool analysis with the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) GenBank database.

Heavy metal toxicity. To determine the minimum inhibitory concentrations (MIC) of the heavy metals, 10 μl aliquots of a cell suspension (48-h biomass in 5 ml water) of the tested strain were spot inoculated on nutrient agar containing a concentration gradient range for each heavy metal based on results of preliminary experiments: 0 to 210 ppm Hg²⁺, 0 to 5600 ppm Cd²⁺, 0 to 14800 ppm As⁶⁺ and 0 to 4400 ppm Pb²⁺. To study the effect of crude oil on the MIC values, the experiment was repeated using nutrient agar plates exposed to oil vapor, as described above. The effect of CaSO₄ (2.5 M) and DPA (8% w/v) on the MIC values using both media was also studied. Triplicates were done throughout and the inoculated cultures were incubated at 50 °C for 3 days. They were examined for growth and the lowest heavy metal concentration at which growth ceased (MIC) was recorded.

Crude oil consumption. The oil sample used was Kuwaiti light crude, which according to the Kuwaiti oil company consisted of 60% aliphatics, 29% aromatics, 8% asphalins and 3% resins. To measure the oil-consumption by the tested bacteria, 1 ml-aliquots of cell suspensions (loopful of 48-h biomass in 10 ml water) were inoculated in 250 ml flasks containing 100 ml mineral medium provided with 0.3% (w/v) light crude as the sole source of carbon and energy. Control flasks were prepared similarly but were inoculated with previously autoclaved cell suspensions. The oxygen tension in the media (as measured by Orion Star Meter, Thermo Scientific, USA) was 6.7 ± 0.1 mg l⁻¹. The flasks were sealed (to avoid oil loss by volatilization) and shaken at 100 rpm and 50 °C. To study the effect of Ca²⁺ and DPA amendment on the oil consumption, additional sets of flasks were prepared and treated either with 2.5 M CaSO₄ or 2.5 M CaSO₄ + 8% w/v DPA; these concentrations were selected based on results of preliminary experiments. Three replicate flasks were prepared throughout. Whole flask contents were harvested after 2 weeks. Residual hydrocarbons were extracted with three successive 15-ml aliquots of pentane. The combined extract was completed to 50 ml using pentane and 1 ml was analyzed by gas liquid chromatography (GLC). The GLC analysis was done using a Chrompack CP-9000 instrument equipped with a FID (Chrompack Middelburg, The Netherlands), a WCOT-fused silica CP-SIL-5 CB capillary column (Chrompack), and a temperature program of 45–310 °C with temperature rising 10 °C min⁻¹ using N₂ as a carrier gas. The detector temperature was 300 °C and the injector temperature 270 °C. The percentage of hydrocarbons remaining in the media was calculated as the percentage of total hydrocarbon peak areas based on the areas of gas. The detector temperature was 300 °C and the injector temperature 270 °C. The percentage of hydrocarbons remaining in the media was calculated as the percentage of total hydrocarbon peak areas based on the areas of

Statistical analysis. As already mentioned, all the readings were means of 3 replicates. Mean values of the triplicates ± standard deviation values were calculated using Microsoft Excel 2007. Statistical Package of Social Science, version 12, was used to assess the degree of significance, where the t-test and analysis of variance were used to differentiate between the means of the tested parameters.

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Author Contributions
Suggested the topic and designed experiments: S.S.R. Contributed to experimental design and carried out the microbiological and molecular analyses: D.M.A. Performed experimental work: M.K.K. Wrote the paper: S.S.R. Suggested the topic and designed experiments: S.S.R. Contributed to experimental design and carried out the microbiological and molecular analyses: D.M.A. Performed experimental work: M.K.K. Wrote the paper: S.S.R. All authors revised the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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