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Metal-loaded zeolite remediation of soils contaminated with pandrug-resistant _Acinetobacter baumannii_

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Due to the development of resistance to antimicrobial agents, bacterium _Acinetobacter baumannii_ is nowadays a leading cause of nosocomial outbreaks. Clinically relevant _A. baumannii_ outside hospital settings including natural soils affected by human waste represents a public-health risk for humans and animals. The aim of this study was to investigate the potential of metal-loaded zeolites to eliminate viable _A. baumannii_ from artificially contaminated natural soils. _A. baumannii_ isolate was subjected to the activity of natural zeolitised tuff (NZ) and Cu-modified (CuNZ) or Ag-modified zeolite (AgNZ) in wet, slightly acidic _terra rossa_ and slightly alkaline red palaeosol. _A. baumannii_ survived in _terra rossa_ and _red palaeosol_ supplemented with 1 wt% of NZ for seven days and four months, respectively. The addition of 1 wt% of CuNZ to _terra rossa_ and red palaeosol shortened the survival of _A. baumannii_ to three and 14 days, respectively. The addition of 0.1 wt% of AgNZ to both soils resulted in complete removal of viable _A. baumannii_ within 1 h of contact, while the total native heterotrophic bacterial counts remained high. Since AgNZ is prepared with a simple modification of cost-effective and environmentally friendly natural zeolite, it is a promising material for the remediation of soils contaminated with pandrug-resistant _A. baumannii_.

KEY WORDS: copper; environment; natural zeolite; pathogens; public health; silver

_Acinetobacter baumannii_ is a neutrophilic, aerobic, Gram-negative, non-sporulating bacterium (1) that has no minimal infectious dose and is capable of developing pandrug resistance (resistance to all antimicrobial agents). This is why _A. baumannii_ is an emergent opportunistic pathogen causing nosocomial infections worldwide (2–4). Infections have also been evidenced outside hospital environments (5), but the source for these community-acquired infections has not yet been determined beyond doubt.

However, soil has scarcely been investigated as a possible source of _A. baumannii_. One _A. baumannii_ strain similar to a clinical isolate was reported in _terra rossa_ contaminated with illegally disposed human solid waste (6). Three drug-resistant isolates of _A. baumannii_ were recovered from _terra rossa_ developed at one dumpsite (7). However, judging by a study of _A. baumannii_ in pristine soils (8), this pathogen is not native to uncontaminated soils, which clearly points to contamination (teaching) from human solid waste.

_A. baumannii_ can survive in a wide moisture, temperature, and pH range (9–11), which suggests long-term persistence in soil. In a water suspension of strongly acidic palaeosol, _A. baumannii_ survived for one day (6), while in a pH-neutral technosol it survived for 38 days (7).

As drug-resistant _A. baumannii_ in soil poses a public-health risk for humans and can also affect animals (12), we need to look for ways to remove this pathogen from contaminated soils. One of the ways to do that could be to use transition metal-containing natural zeolites due to their antibacterial properties. They have an advantage over free metal cations with antibacterial activity, since the aluminosilicate lattice of zeolites slowly releases metal cations into surroundings (13, 14). However, experiments with zeolites were carried out in physiological solution at 36 °C and are not applicable to the conditions in soil. To address that shortcoming, we investigated the antibacterial potential of metal-loaded zeolites directly in contaminated natural soils.

MATERIALS AND METHODS

A. baumannii isolate properties

The _A. baumannii_ isolate (named EF7) chosen for the experiments was recovered from the effluent of a secondary wastewater treatment plant and deposited at the University of Zagreb Faculty of Science, Department of Biology, Zagreb, Croatia.

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of Zagreb Faculty of Science. This isolate is highly related to clinical isolates and is resistant to all tested antibiotics, which classifies it as pandrug-resistant (15, 16).

**Soil sampling and characterisation**

Based on results of a previous investigation (17), we used two soil samples representative of slightly acidic and alkaline soil from Istria, Croatia – *terra rossa* and red palaeosol from Cretaceous limestone – dug from the upper 30 cm of 50 cm deep pits. Samples were aseptically collected in sterile plastic bags and analysed in the laboratory within 24 h of collection.

Soil pH value was measured with a WTWSenTix81 electrode (WTW, Weilheim, Germany) after triplicate suspension in distilled water (1:2.5 wt/v). The procedures for chemical (ICP-ES/MS following a lithium borate fusion and dilute nitric digestion) and mineralogical analyses (X-ray powder diffraction of <2 mm and <2 μm fractions) were described in detail in Durn et al. (18).

**Preparation of metal-loaded zeolites**

Natural zeolitised tuff (NZ) was obtained from a Zlatokop sedimentary deposit in Serbia and consisted of 73 % clinoptilolite, 14 % plagioclase, and 13 % quartz (wt). To load NZ with metals, we used the ion-exchange procedure described in our earlier study (14). Briefly, it consisted of the following steps: 1) conversion of NZ into Na-enriched form (NaNZ) by treating NZ with 2 mol/L of NaCl solution at 25 °C for 48 h; 2) treatment of NaNZ with 6 mmol/L of Cu(NO$_3$)$_2$ or AgNO$_3$ solution in a water bath at 25 °C for 24 h; and 3) recovering of the metal-containing products (CuNZ and AgNZ) by filtration.

**Leaching test**

The leaching of cations from zeolites to soil samples is technically hard to track. Moreover, our previous investigation (13) showed negligible leaching of Cu$^{2+}$ from CuNZ into the water medium. This is why we investigated only Ag$^+$ leaching in this study. One gram of red palaeosol was supplemented with 1 mg (0.1 wt%) of AgNZ and added to 100 mL of autoclaved commercially available spring water and left in the dark at 25 °C for 1 h. The solid was separated from water by filtration through 0.45 µm syringe filters and the concentration of the leached Ag determined in water solution by atomic absorption spectrophotometer (AAS Varian, Spectra AA 55b, Agilent Technologies, Santa Clara, CA, USA).

**Experimental setup and bacterial counting**

The *A. baumannii* isolate was grown on CHROMagar Acinetobacter supplemented with CR102 (CHROMagar, Paris, France) and 15 mg/L of cefsulodin (Sigma-Aldrich, St. Louis, MO, USA) at 36 °C for 24 h. The biomass was suspended in 300 mL of autoclaved commercial spring water. Based on preliminary experiments, this suspension was used to adjust soil moisture to their maximum water-holding capacity of 30 wt%.

Both soils were distributed in 100 g quadruplicate samples to laboratory glasses. *A. baumannii* suspension was added gradually by mixing it in with a sterile spatula. The content of eight glasses was mixed with 1 g of NZ, CuNZ, or AgNZ or 0.1 g of AgNZ. These experimental glasses were covered with parafilm and incubated in the dark at 25 °C until all viable *A. baumannii* disappeared. Soil moisture was kept constant (30 wt%) in all systems by adding autoclaved spring water as needed, which was determined by weekly gravimetrical measurements of soil moisture (by drying soil at 105 °C to constant weight) in a system supplemented with NZ.

At the beginning of the experiment and at specified time points (Figures 1 and 2), the soil samples were mixed well and subsamples taken for bacteriological analysis in triplicate. One gram of wet soil was suspended in sterile...
oxides and trace elements. The only difference was the higher content of MgO and CaO in red palaeosol, which corresponds to its mineral composition (presence of calcite and dolomite) and slight alkalinity.

The samples of modified zeolites contained similar amounts of metal cations per gram of dry sample: 0.37 mmol Cu\(^{2+}\) (CuNZ) or 0.50 mmol Ag\(^{+}\) (AgNZ).

Remediation of terra rossa contaminated with A. baumannii

In terra rossa supplemented with 1 wt% of NZ (Figure 1) A. baumannii survived for seven days, when its count dropped from the baseline of 6.7 log CFU/g to below the detection limit (1 CFU/g). No statistically significant drop was observed for the native heterotrophic bacteria over these seven days. As NZ does not exhibit antimicrobial activity (14), this drop in A. baumannii count can be attributed to soil acidity, to which A. baumannii as a neutrophilic species is not adapted.

Statistical analysis

Statistical significance (p<0.05) of the difference between the baseline and end bacterial counts was established with Student’s t-test for independent variables using Statistica 13.3 (TIBCO Software, Inc., Palo Alto, CA, USA).

RESULTS AND DISCUSSION

Characteristics of soils and modified zeolites

Terra rossa was slightly acidic (pH 5.40±0.21), and red palaeosol slightly alkaline (pH 8.43±0.14). Both soils were dominated by quartz and clay minerals (illitic material and mica, kaolinite and mixed-layer clay minerals), followed by plagioclase, K feldspar, haematite, and goethite. Unlike terra rossa, red palaeosol also contained chlorite, 14 Å clay minerals, dolomite and calcite. Terra rossa and red palaeosol had similar chemical composition of both major oxides and trace elements. The only difference was the higher content of MgO and CaO in red palaeosol, which corresponds to its mineral composition (presence of calcite and dolomite) and slight alkalinity.

The samples of modified zeolites contained similar amounts of metal cations per gram of dry sample: 0.37 mmol Cu\(^{2+}\) (CuNZ) or 0.50 mmol Ag\(^{+}\) (AgNZ).

In terra rossa supplemented with 1 wt% of NZ (Figure 2) A. baumannii was removed by the end of the experiments, as indicated by the bacterial counts. The detection limit was 1 CFU/g.
The addition of 1 wt% CuNZ (Figure 1) significantly accelerated the removal of A. baumannii to only three days of contact, while the native heterotrophic bacteria were not affected. This suggests a selective bactericidal effect of CuNZ on A. baumannii.

The addition of 1 wt% of AgNZ (Figure 1) resulted in complete removal within 1 h of contact. The same result was achieved with 0.1 wt% of AgNZ. Although this effect was accompanied by a statistically significant reduction of heterotrophic bacteria, their count was still high (5.2±0.2 log CFU/g) after 24 h of contact.

Remediation of red palaeosol contaminated with A. baumannii

In red palaeosol supplemented with 1 wt% of NZ (Figure 2) A. baumannii count dropped significantly compared to baseline, but viable cells were detected throughout the four months of monitoring (2.2 log CFU/g). The bacteria did not multiply, probably due to low soil organic carbon content (0.215 wt%). An earlier study (11) showed that A. baumannii survived for a long time (150 days) in spring water with a pH of 8.1, which is very close to the pH of our red palaeosol samples (8.43±0.14). As moisture was kept to the maximum water holding capacity, desiccation can be eliminated as the cause of A. baumannii count drop. Anaerobic conditions can also be excluded, as the soil layer in glasses was not thicker than 3 cm and was regularly stirred. Rises and falls in A. baumannii counts observed from day 68 on could be explained by the “bust and boom” survival strategy, where weak cells die to provide sustenance for the remaining cells (9). However, the difference between the 32 % survival in red palaeosol in this study and >90 % survival in spring water in our earlier study (11) suggests that nutrient deprivation is not the only cause of lower A. baumannii survival in red palaeosol. Perhaps it was the presence of Fe in wet red palaeosol that contributed to the drop in A. baumannii count via oxidative stress (20).

The difference in A. baumannii survival between red palaeosol (four months) and terra rossa (seven days), both treated with NZ, is clearly owed to their difference in soil pH (8.43 vs 5.40, respectively), which suggests that priority in soil treatment should be given to slightly alkaline soils such as red palaeosol.

The addition of 1 wt% of CuNZ (Figure 2) completely removed A. baumannii after 14 days without affecting native heterotrophic bacteria by the end of the experiment.

Considering the long survival of A. baumannii in red palaeosol (more than four months), we feel that the promising activity of AgNZ in slightly alkaline red palaeosol deserves more attention than in acidic terra rossa. AgNZ particles are bactericidal in direct contact with bacteria in soil and through leached Ag cations (14, 21). However, this antibacterial activity will depend on soil chemistry and the distribution of AgNZ particles and their contact with bacteria in soil. In our leaching test with red palaeosol 0.038 mg/L of Ag leached from 0.1 wt% (1 mg/g) of AgNZ added to the soil. This concentration of leached Ag was enough to eliminate 6.8 log CFU/g of A. baumannii.

Table 1 Native soil heterotrophic bacterial isolates determined with MALDI-TOF MS in fresh red palaeosol before and at the end of treatment with 1 wt% of NZ or CuNZ or AgNZ.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Fresh soil</th>
<th>NZ</th>
<th>CuNZ</th>
<th>AgNZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthrobacter sp.</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arthrobacter oxydans</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Arthrobacter scleromae</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthrobacter polychromogenes</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Bacillus sp.</td>
<td>2</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Bacillus cereus</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Bacillus megaterium</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Bacillus thuringiensis</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Brevibacillus sp.</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkholderia sp.</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkholderia caledonica</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Paenibacillus sp.</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Streptomyces sp.</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Streptomyces chartreus</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Variovorax sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* spore-formers
The addition of AgNZ to soil resulted in removal of 6.8 log CFU/g of viable A. baumannii within 1 h of contact. AgNZ particles in the mass fraction of 0.1 wt% (1 g per 1 kg of soil) could easily be dispersed onto soil contaminated with A. baumannii. Since AgNZ is prepared with a simple modification of cost-effective and environmentally friendly natural zeolite, it is a promising material for the remediation of soils contaminated with pandrug-resistant A. baumannii.

Acknowledgements

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Conflicting interests

None to declare.

REFERENCES


Zeoliti modificirani metalima u remedijaciji tala zagađenih bakterijom *Acinetobacter baumannii* otpornom na antibiotike

Bakterija *Acinetobacter baumannii* u današnje vrijeme je osjećala izazove u medicinskom položaju, pojavljujući se u većini svake bolničke zalihe. Neki medicinski radovi štete i putem i primjene antibiotika, što rezultira rastom otpornosti bakterija na antibiotike. U ovom radu su ispitivani efekti na bakterije *Acinetobacter baumannii* članice *Acinetobacter baumannii* iz umjetno zagađenih prirodnih tala. Isolat *Acinetobacter baumannii* iz umjetno zagađenih prirodnih tala je bila podvrgнутa djelovanju prirodnog zeolita (NZ), te NZ-a modificiranog bakrom (CuNZ) ili srebro (AgNZ).

U slučaju dodatka 1 wt% NZ-a, *Acinetobacter baumannii* preživio u *terra rossa* sedam dana, a u crvenom paleotlu čak četiri mjeseca. Dodatak 1 wt% CuNZ-a u tla skratio je preživljavanje *Acinetobacter baumannii* na tri dana u *terra rossa* i 14 dana u crvenom paleotlu. Dodatak samo 0,1 wt% AgNZ-a u tla rezultirao je kompletnim uklanjanjem *Acinetobacter baumannii* tijekom jednog sata kontakta, pri čemu je brojnost ukupnih heterotrofnih bakterija, prirodno prisutnih u tlu, ostala visoka. Čestice AgNZ-a pripremaju se jednostavnom modifikacijom jeftinoga i okolišno prihvatljivoga prirodnoga zeolita. Mala koncentracija (1 g AgNZ-a po 1 kg tla) mogla bi se jednostavno raspršiti po tlu koje je zagađeno bakterijom *A. baumannii*. Stoga je AgNZ obećavajući materijal za remedijaciju tala zagađenih tom bakterijom koja je otporna na sve dostupne antibiotike.

KLJUČNE RIJEČI: bakar; okoliš; patogeni; prirodni zeolit; srebro; zdravstvena zaštita