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# Heavy Metal Tolerance and Chelating Activity of Bacteria Associated with Mediterranean Polychaetes

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## Introduction

Several species of benthic invertebrates are known as potential bioindicators of polluting impact in marine environments, thanks to their considerable filtering activity on the surrounding water and their important ecological role as hotspot of marine diversity. The large filtration rate makes them accumulation matrices for a lot of pollutants and recalcitrant compounds, such as plastics, hydrocarbons, and heavy metals (HMs). Particles suspended in the water column could be retained inside the body of these aquatic organisms and then transferred to higher trophic levels [1,2]. HM pollution is one of the most worrying forms of contamination affecting the marine environment [3,4], mainly correlated to the rapid industrialization and to the spillage of wastewater with high levels of these inorganic pollutants directly into the water bodies. Differently from organic pollutants, HMs are persistent and not biodegradable into innocuous carbon dioxide and water [5], so that their removal from environmental matrices is a very complex issue. During last decades, research has been oriented mainly to the restoration of environments contaminated by metals by using traditional techniques, such as chemical surfactants or metal chelating agents [6], which are not always eco-friendly and often cause further damages. HMs tends to accumulate in sediments, because of their chelating properties, prior to be transferred into the food chain through benthic organisms. Microbial surface-active metabolites, called biosurfactants (BSs), have been reported as metal-complexing agents, effective in the remediation of HM-contaminated environments [5,7]. They are promising alternative agents for remediation purposes because they are less toxic, with better environmental compatibility and biodegradability [8]. Most BSs reported till today are obtained from microorganisms of terrestrial origin, and HMs removal has been reported mostly for sediments and, even if at a lesser extent, polluted waters. Hence, the exploitation of biological matrix potential for the isolation of BSs of marine origin with HMs chelating properties becomes an intriguing research topic. In this study, bacterial strains previously isolated from marine filter feeders (Polychaeta, Annelida) and also able to produce BSs and degrade hydrocarbons were used [9,10]. Among them, *Joostella* sp. A8 was reported as optimal and competitive BS-producer, and was further tested for HM tolerance and BS-production in liquid culture with the addition of different metal solutions [11]. The interesting obtained results have strengthened the interest in improving the chelating abilities of the BSs produced by Polychaete-associated bacteria. For this reason, a selection of bacterial isolates were first tested for HM tolerance, and then two of them were monitored in Zn-amended liquid cultures for growth, emulsification activity and surface tension reduction.

## Materials and Methods

Bacterial strains (30) tested for HM tolerance is listed in Table 1. All *Joostella* strains derived from *Megalomma claparedei* (Gravier, 1906) enrichment cultures [9], while the other strains derived from specimens of *B. luctuosum* (Bl), *M. claparedei* (Mc) and *Sabella spallanzanii* (Ss) [10]. HM tolerance was tested on agar plates against cadmium (Cd), copper (Cu), and zinc (Zn), as reported by Rizzo et al. [11].

Two strains (i.e. *Joostella* sp. A8, J, and *Alcanivorax* sp. A53, A), selected on the base of their interesting properties as BS-producing strains [12], were used for shake flask biodegradation experiments, carried out in 150 ml Bushnell Haas Broth (BH, Difco) supplemented with NaCl (3%, w/v), tetradecane (2%, w/v), as a carbon source, and zinc chloride (100ppm, w/v). Sterilized culture medium was inoculated with 10% (v/v) of overnight bacterial preculture. The culture flasks were incubated for 480 h under shaking and used to perform BS-production screening tests. Cultures were monitored at regular intervals of 48 h by measuring optical density spectrophotometrically,

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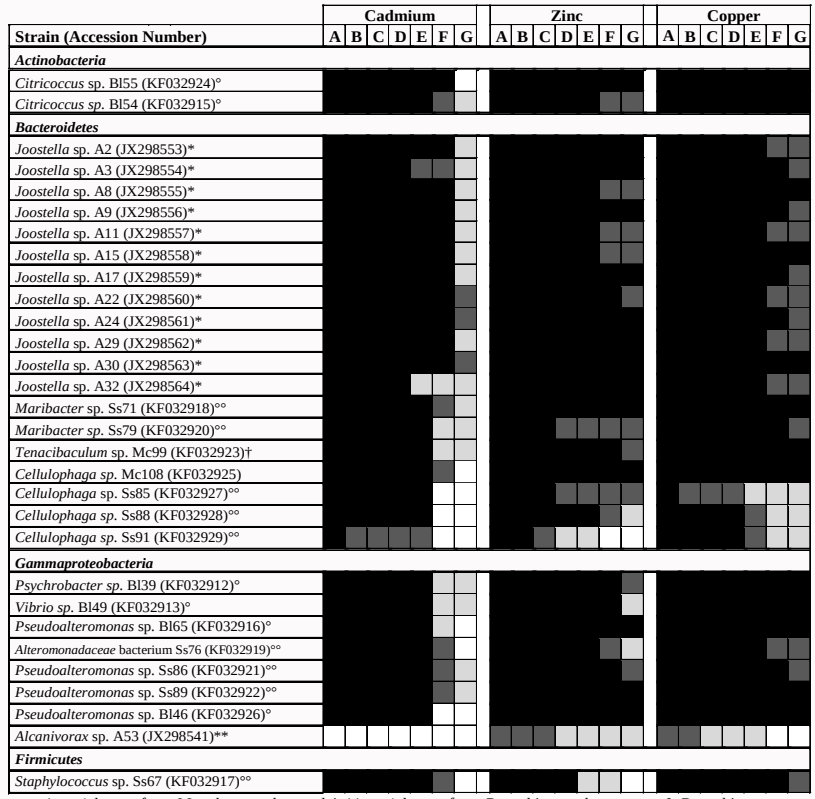


*, enrichment from *Megalomma claparadei*; **, enrichment from *Branchiomma luctuosum*; °, *Branchiomma luctuosum*; °°, *Sabella spallanzanii*; †, *Megalomma claparadei*.

| *Legend* |
|---|
| Complete growth (100%) |
| High growth (>50%) |
| Low growth (<50%) |
| Absent growth (0%) |

A: 10 ppm; B: 50 ppm; C: 100 ppm; D: 500 ppm; E: 1000 ppm; F: 5000 ppm; G: 10000 ppm.

**Table 1:** Heavy metal tolerance of bacterial strains from different origin.

emulsifying activity [13], stable emulsion production [14], and surface tension reduction [15]. BS production screening test were performed on supernatants obtained after centrifugation at 4700rpm for 20min at 4°C, and removal of tetradecane with hexane (1:1, v/v) [16].

## Results

Bacterial tolerance patterns are shown in Table 1 (results for *Joostella* sp. A8 were taken from Rizzo et al. [11], and here added for major completeness). For all strains HM tolerance was in the order of toxicity Cd>Zn>Cu. *Joostella* spp. resulted particularly tolerant also to Cd up to 10000ppm, even if with different growth levels. The most sensible strains resulted those affiliated to the genus *Cellulophaga*, which seldom showed low tolerance also toward Zn. HM tolerance of *Alcanivorax* sp. A53 was tested in liquid culture, because of its obligate growth in the presence of hydrocarbons. It showed a medium growth rate in presence of Zn and Cu, but not in presence of Cd.

*Joostella* sp. A8 and *Alcanivorax* sp. A53 were chosen for further analyses in force of their strong potentialities in terms of bioremediation, and in order to understand if the presence of multiple pollutants could affect their BS-producing activity. In terms of bacterial growth, *Joostella* sp. A8 showed higher optical density values, despite both strains presented the same pattern and achieved the exponential phase after about 144 and 192h of incubation (Figure 1). Emulsification abilities were in the range 9-24% and 7.5-21% for *Joostella* sp. A8 and *Alcanivorax* sp. A53, respectively. In terms of $E_{24}$ index, *Joostella* sp. A8 started to produce stable emulsion after 48 h of growth and achieved maximum percentages during the exponential phase with value of 52.5%. *Alcanivorax* sp. A53 started to produce stable emulsion during the exponential phase, with its maximum values of 42.5% after 240h of incubation (Figure 2). No remarkable reduction of surface tension was recorded for both strains. The formation of flocculate corpuscles was observed after 48h of incubation.

## Discussion

The search for new products of natural origin able to chelate toxic substances like HMs may find crucial applications in the treatment of polluted waste water [4]. Many filter-feeding organisms have been just reported as both potential sites of contaminant accumulation [17] and optimal source for specialized microbial communities, with ability in the uptake and removal of recalcitrant compounds [9,10]. This is particularly true in contaminated environments. The removal of contaminants by bacteria is often mediated by surface-active molecules, such as BSs. It has been shown that BSs could remove HMs from contaminated matrices by binding and mobilizing those [18]. The complexes with metals are formed thanks to attraction between anionic surfactants and non-ionic metal forms, or by mobilization through micelles [19,20]. Several invertebrates, such as bivalve molluscs, sponges and polychaetes, were reported as pollutant bio accumulators [21,22] and some of them were proposed as source of

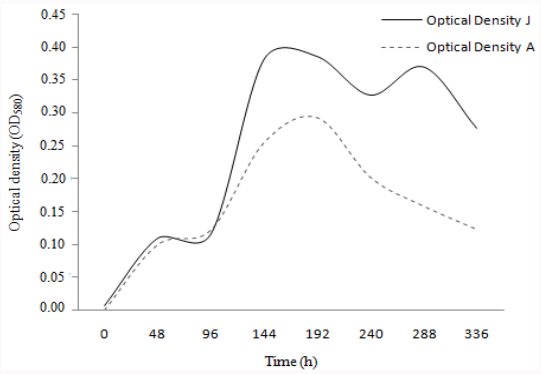


**Figure 1:** Optical density values for *Joostella* sp. A8 (J) and *Alcanivorax* sp. A53 (A) during incubation in presence of tetradecane and zinc chloride.

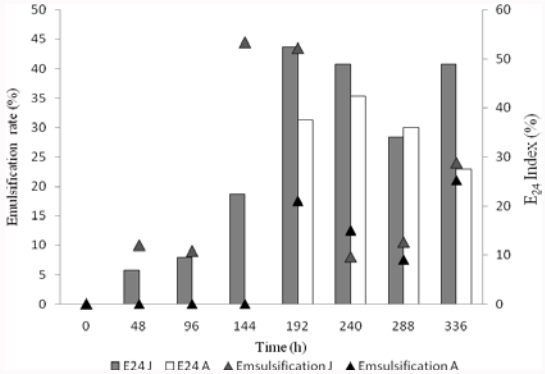


**Figure 2:** Emulsification rate and stable emulsion production ($E_{24}$) for *Joostella* sp. A8 (J) and *Alcanivorax* sp. A53 (A) during incubation in presence of tetradecane and zinc chloride.

specialized bacteria [23,24]. Recently, the potential of sabellids as a novel source of BS-producing bacteria has been reported [9,10].

In this study all tested strains showed an optimal tolerance towards HMs, even if those deriving from enrichment cultures exhibited a higher resistance in terms of multiresistance and tolerance of highest HM concentration. Despite this, the preliminary screening was a good starting point to investigate deeply the filter-feeder associated bacteria also in the field of metals remediation. *Joostella* sp. A8 was reported as optimal candidate for BS production and also for its tolerant capacities toward HMs [11]. Here we reported a microcosm experiment set up with *Joostella* sp. A8 and *Alcanivorax* sp. A53, and hydrocarbon-degrading strain isolated from *B. luctuosum* and reported as BS producer together with *Joostella* sp. A8. *Joostella* sp. A8 is here confirmed as a competitive strain in the field of bioremediation, and showed superior performance than *Alcanivorax* sp. A53, thus suggesting its possible use in the concomitant recovery of pollutants of different nature. In our previous report, *Alcanivorax* sp. A53 achieved emulsification rate of about 50% and a stable emulsion production of 66%, in addition to a surface tension reduction of about 20mN/m, higher than that exhibited by *Joostella* sp. A8 [12]. The observed lower emulsification and $E_{24}$ values, in addition to the absence of surface tension reduction, led us to suppose that the presence of HMs probably strongly affect its ability to produce BSs and degrade hydrocarbons.

To the best of our knowledge, *Alcanivorax* spp. strains have been scarcely reported for potential HM removal from contaminated environments. The removal of HMs from aqueous matrices is also poorly reported. Further insights will be needed to improve the knowledge about these promising strains and their potentiality in HM removal. It could be interesting to study the effect of crude BSs of both strains on the removal of metals, from both solid and liquid matrices. Alternatively, also the assessment of a bacterial consortium of the two strains can be considered to deepen their potential in presence of multiple environmental stressors. In conclusion, if previously the use of benthic matrices has been proposed and confirmed as optimal sources of BS-producing strains with potentialities in hydrocarbon degradation, here their validity also in the field of HM pollution was confirmed.

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