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CHARACTERIZATION OF CADMIUM-RESISTANT BACTERIA ISOLATED FROM POLLUTED SOILS IN ALGERIA, AND EVALUATION OF CADMIUM REMOVAL, USING LIVING FREE AND IMMOBILIZED CELLS

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RÉSUMÉ.— *Caractérisation des bactéries cadmium-résistantes isolées de sols pollués en Algérie et évaluation de l'élimination du cadmium en utilisant des cellules libres ou immobilisées*.— La pollution des sols par les métaux lourds est un problème particulièrement préoccupant du fait de leur toxicité et de leur non biodégradabilité. Dans ces environnements, les bactéries développent divers mécanismes de résistance qui leurs confèrent la capacité à accumuler ces métaux. Dans cette étude, vingt-trois bactéries cadmium-résistantes ont été isolées de trois sols algériens et ont été caractérisées. Deux isolats (YL-SS8, YL-SS3), hautement résistants au cadmium, ont été sélectionnés et identifiés par séquençage du gène de l'ARNr 16S, puis testés pour leur capacité à capturer les ions cadmium. Les résultats ont révélé que les bactéries caractérisées appartenaient à neuf familles et dix genres, tandis que les deux souches les plus résistantes sélectionnées, ont été identifiées comme *Bacillus infantis* et *Pseudomonas fluorescens*. Les concentrations minimales inhibitrices ou CMI oscillaient entre 500 µg.mL^{-1} et 1100 µg.mL^{-1}. Les souches YL-SS8 etYL-SS3 ont montré les CMI les plus importantes, de l'ordre de 1100 µg.mL^{-1} et 900 µg.mL^{-1} respectivement. Les cellules libres vivantes de *B. infantis* ont prélevé environ 90 µg.mL^{-1} de cadmium, alors que celles de *P. fluorescens* ont capturé 81 µg.mL^{-1} après 24 heures de contact. Dans le même temps, les cellules immobilisées ont accumulé des concentrations en cadmium légèrement plus importantes avec des valeurs respectives de 93 µg.mL^{-1} et 85 µg.mL^{-1}. En raison de leur forte résistance et de leur importante capacité d'accumulation du cadmium, les deux isolats bactériens pourraient être exploités pour l'assainissement biotechnologique du cadmium dans les sols contaminés par les métaux lourds.

SUMMARY.— Soil pollution by heavy metals is one of the most important problems around the world. Microorganisms in these environments develop various mechanisms of resistance and become able to accumulate these metals. In this study, twenty-three cadmium-resistant bacteria were isolated from three soils and characterized. Two of them (YL-SS8, YL-SS3), highly cadmium-resistant, were selected and identified by the sequencing of the 16S rRNA gene, then tested for their ability to remove cadmium ions. The results revealed that the characterized bacteria belonged to nine families and ten genera, while the most resistant are authentically identified as *Bacillus infantis* and *Pseudomonas fluorescens*. The MIC of bacteria ranged from 500 µg.mL^{-1} to 1100 µg.mL^{-1}, *Bacillus infantis* and *Pseudomonas fluorescens* showed MIC of the order of 1100 µg.mL^{-1} and 900 µg.mL^{-1}respectively. The free living cells of *B. infantis* accumulated about 90 µg.mL^{-1} of cadmium, whereas those of *P. fluorescens* 81 µg.mL^{-1} after 24 hours of contact. During the same time, the immobilized cells accumulated quantities slightly better with respective values of 93 µg.mL^{-1}and 85 µg.mL^{-1}. Due to their strong resistance and high cadmium removal capacity, the two bacterial isolates could be exploited for biotechnological remediation of cadmium and other heavy metals from contaminated soils.

Soil pollution by heavy metals is a serious environmental and social problem, on account of the dangers that can cause these elements, not only for human health but also for biodiversity and structure of soil organisms and microbial populations (Tran & Popova, 2013). Some of heavy metals are essential and required by the organisms as micro-nutrients, while others have no biological role and are detrimental even at very low concentration (Bruins *et al*., 2000). One of the most dangerous heavy metals encountered in soil is cadmium. These metal ions enter agricultural soils with others from pesticides, industrial effluents, phosphate fertilizers and atmospheric deposition, which finally lead to transport to the food chain (Jain *et al*., 2007). Cadmium has a

higher tendency to accumulate in the tissues of plants and affects their growth **(**Lux *et al*., 2010). This metal affects many physiological processes such as membrane functions by changing the fatty acid composition of the lipids, nitrogen metabolism, oxidative stress through increased proteolysis and lipid peroxidation (Chaffei *et al*., 2003; Djebali *et al*., 2005). In human, it affects cell proliferation and differentiation, chromosomal aberrations, modification of transcription factors, skin cancer, prostatic proliferative lesions, pulmonary adenocarcinomas, peripheral neuropathy and peripheral arterial disease (Gunaseelan & Ruban, 2011; Chakravarthi *et al*., 2012). Because of its higher solubility in water, and highest toxicity, the pollutant gains more significance (Katoch & Singh, 2014).

The pollution of the ecosystem by heavy metals is quite special because although these trace elements are mobilized by various organisms, they cannot be degraded into less toxic products and persist indefinitely in the environment, posing a major problem in ecology but also for public health. Their toxicity and persistence in the environment require the development of different methods to reduce the number of contaminated sites. If physico-chemical methods such as excavation and storage or stabilization and containment are used in soil decontamination, most of them tend to be very expensive and many countries encounter difficulties in its implementation (Guzman *et al*., 2016).

In recent years, an important attention has been paid to the problems of soil contamination by heavy metals (Changli *et al.*, 2010; Salam, 2013; Zhou & Guo, 2015; Liu *et al*., 2015; Singh & Prasad, 2015) with interest in a remediation strategy depending on microorganisms and plants: it is bioremediation. Bioremediation is a branch of biotechnology which uses natural biological mechanisms to address environmental problems; it is being explored as an effective and technological solution to the problem of heavy metal pollution (Basha & Rajaganesh, 2014). Bioremediation has been regarded as an environment-friendly, inexpensive and efficient means of environmental restoration (Hrynkiewicz & Baum, 2014) and, in some cases, has been successfully applied in remediating contaminated sites in the developed world (Owolabi & Hekeu, 2015).

A vast array of biological materials, especially bacteria, algae, yeasts and fungi have received increasing attention for heavy metal removal (Wang & Chen, 2008). Heavy metal-resistant bacteria have been demonstrated to exhibit high metal ion removal capacity. Biosorption, bioaccumulation, biotransformation, and biomineralization are the techniques employed by microorganisms for their continued existence in metal polluted environment. These strategies have been exploited for remediation procedures **(**Gadd, 2000; Lin & Lin, 2005). Heavy metal removal can be carried out by living organisms or dead biological materials. Large scale feasibility applications of biosorptive processes have shown that dead biomass is more applicable than the bioaccumulation approach, which involves the use of living organisms and thus requires nutrient supply and a complicated bioreactor system. Also, the toxicity of pollutants, as well as other unfavourable environmental conditions, can contribute to the inability to maintain a healthy microbial population. However, many characteristic attributes of living microorganisms have not been exploited in large scale applications **(**Park *et al.*, 2010).

The choice organism must develop resistance towards metal ions as it comes into contact with the heavy metal pollutant to achieve the goal of remediation. The organism of choice may be native to the polluted environment or isolated from another environment and brought to the contaminated site (Sharma *et al*., 2000). Micro-organisms can also act indirectly as they support the growth of phytoaccumulator plants thus they help in the remediation of heavy metals (Yan-De *et al*., 2007; Zhuang *et al*., 2007; Heshmatpure & Rad, 2012).

However, studies demonstrate that living systems may be inconsistent in heavy metal removal if used as free suspended biomass. Free suspended biomass can promote higher contact with the contaminants during the removal process; often, however, it is not practical as a clean-up method. To obtain a more reliable and reproducible system, bacteria should be immobilized on a solid matrix (Vijayaraghavan & Yun, 2008; Wang & Chen, 2009), some materials employed for this

were clay (Quintelas *et al*., 2009) or synthetic polymers such as alginates and pectates (Pires *et al*., 2011).

The principal aim of the present work was to isolate and characterize cadmium-resistant bacteria from some polluted soils in Algeria, to determine the minimum inhibitory concentration of cadmium and eventually to study the ability of two highly resistant isolates to remove cadmium ions, free or immobilized.

MATERIALS AND METHODS

STUDY AREAS AND COLLECTION OF SOIL SAMPLES

Soil samples were taken from three areas located in the east of Algiers (Algeria). The first is a cultivated soil (Dellys), the second from an industrial zone (Setif) and the third from deposit metal tools next to the railway (Bab-Ezzouar). The samples were collected in sterilized and non-sterilized flasks during the month of February 2014 to a depth of four centimetres from the surface. The samples for microbiological analyses were placed in a cooler (4°C), while those for physic-chemical analyses were transported at ambient temperature.

PHYSICO-CHEMICAL PROPERTIES OF SOIL SAMPLES

The soil samples were dispersed to be dried and then passed through a two millimetres sieve before measuring the various parameters. The pH of each soil was measured according to the AFNOR standard NFX31-10 using a pHmeter electrode in a solution of soil diluted to 1/5 in water. Available nitrogen (N) content in soils was measured by an alkali N-proliferation method, whereas organic material was determined by the $K_2CrO_7 2H_2SO_4$ oxidation method of "Anne" described in the standard NF X31-109.

The determination of heavy metal concentration tested (cadmium, lead, zinc, chrome, copper and nickel) was carried out using Atomic Absorption Spectrometry (AAS) with flame and graphite furnace AA 800 Perkin Elmer. It is based on the detection of very low levels of concentration (of the order of ppb), of numerous mineral elements by using different radiation sources for each mineral element to be measured, compared to solutions the concentrations of which are known, from the element to be assayed. This step comes after mineralization of soil samples undergoes acid digestion using a pure HCl mixture and Supra Pure-concentrated HNO_3 (3:1 v/v; Sigma-Aldrich) with the addition of 3 drops of H_2O_2. Then heating is carried out on a heating plate at 300°C until complete digestion (USEPA, 1996). Soil samples after mineralization, are transferred to volumetric flasks and diluted to desired concentrations. A range of calibration solutions of the sought-after metal makes it possible to plot a calibration curve while respecting its linearity domain. The concentration of metals in the soil samples can be calculated.

Soils texture was determined by the hygrometry method, whereas the electrical conductance was by conductivity meter (HI 2316, HANNA Instruments) (Roane & Kellogg, 1995).

ISOLATION AND IDENTIFICATION OF CADMIUM-RESISTANT BACTERIA

From each sample, 5 g of soil were suspended in 45 mL of nutrient broth [Grams per litre: 5 g peptic digest of animal tissue; 5 g sodium chloride; 1.5 g beef extract; 1.5 g yeast extract (HiMedia laboratories)], then flasks were incubated at 30°C for 48 hours with shaking at 90 rpm. Thereafter, ten-fold serial dilutions of the cultures were prepared. An aliquot (1 mL) of the diluted samples was spread in mass of sterile nutrient agar plates amended with 50 $\mu g.mL^{-1}$of cadmium chloride ($CdCl_2,2H_2O$; Biochem, Chemopharma). After an incubation period (48 h at 30°C), isolated and distinct colonies were sub-cultured on the same media for purification. The purified isolates were identified on the basis of cells morphology, Gram-stain, catalase, oxidase, nitrate reductase enzymes and some biochemical characteristics following Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Commercial biochemical identification systems Api 20E, Api 20NE and Api 50CH used, are provided by Biomerieux.

The most resistant isolates were identified by sequencing of the 16S rRNA gene by GATC laboratories (GATC – biotech laboratories, Germany).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MIC of the cadmium-resistant isolates was determined by gradually increasing the concentration of cadmium 100 $\mu g.mL^{-1}$ each time on nutrient-agar plate until the strains failed to grow on plates even after five days of incubation at 30°C. Cadmium solution used was prepared by dissolving cadmium chloride ($CdCl_2$, $2H_2O$) in ultrapure water (MILLI-Q plus, USA), then sterilized. The MIC was designated as the lowest concentration that inhibited growth of colonies on the medium (Nies, 1999). The range of concentrations tested was from 100 to 1300 $\mu g.mL^{-1}$

CADMIUM REMOVAL BY LIVING BACTERIAL FREE CELLS

Two isolates (YL-SS3 strain and YL-SS8 strain), highly cadmium-resistant were selected and used in this study. Foremost, the evolution of growth was measured in the presence of cadmium ions at pH 7.2. Thereafter, the cadmium removal capacity of living free cells was evaluated by measuring concentrations of cadmium in the medium (residual cadmium) as a function of contact time (incubation time). So, the isolates were cultivated in 100 ml of nutrient broth with 100 $\mu g.mL^{-1}$ of metal at final concentration. The inoculated flasks were incubated in shaking conditions (100 rpm) at 30°C for 72 h **(**Sujitha & Jayanthi, 2014; Benmalek & Fardeau, 2016). Two controls were prepared simultaneously with the experiment cultures for each isolate. The first one was prepared without cadmium to measure bacterial growth and the second without bacterial biomass to determine the artefacts that might arise due to the metal sorption on the glass surface of the culture container. The growth was determined by measuring the optical density at 600 nm. However, the residual concentration of cadmium was evaluated after centrifugation of 10 mL of each bacterial culture at 4000 rpm for 30 minutes and analysis of the supernatant from each sample using AAS. The values so obtained by AAS analysis represent the residual concentration of cadmium in the medium.

The cadmium concentration in the supernatant was determined with a Perkin-Elmer 2380 atomic absorption spectrometer at 228.8 nm with a cadmium lamp.

CADMIUM REMOVAL BY IMMOBILIZED BACTERIAL CELLS

The bacterial cells of the isolates YL-SS3 and YL-SS8 were immobilized as beads of alginate according to the procedure of Leung *et al.* (2000). So, 2 % sodium alginate solution (Biochem, Chemopharma) is prepared in distilled water and sterilized (Tao *et al*., 2009). Thereafter, 100 mL of the solution prepared were cooled to room temperature, then 10 mL of the bacterial cultures were added (10 % v/v). The sodium alginate with cell culture was stirred by shaking to get homogenized mixtures. In a separate beaker, 100 ml of 0.1 M sterilized calcium chloride solution (Riedel-de-Haen) was taken. The sodium alginate containing cell culture suspension was extruded dropwise through a syringe and allowed to fall in the beaker containing sterilized calcium chloride solution. The beads of sodium alginate gel formed were left in the flasks overnight for hardening. Afterwards, beads were washed with sterile distilled water and introduced into flasks containing 100 mL of nutrient broth with 100 $\mu g.mL^{-1}$ of cadmium and incubated with shaking (100 rpm) at 30°C for 72 hours. Controls containing only nutrient broth and beads were prepared in the same experimental conditions. Then, 5 mL of samples were taken at different times, centrifuged at 4000 rpm for 30 minutes and withdrawn for cadmium analysis in the supernatant using AAS.

RESULTS

As shown in Table I, the amount of heavy metals varies according to the soil studied. Thus, the concentration of zinc was important in all samples with values ranging from 55.26 $mg.kg^{-1}$ to 124 $mg.kg^{-1}$. However, the content of cadmium was low relative to AFNOR norms values (2 $mg.kg^{-1}$). The highest amount of zinc was obtained in the industrial zone (124 $mg.kg^{-1}$), while that of nickel (49.89 $mg.kg^{-1}$) was in soil taken from Bab-Ezzouar. Furthermore, the soil pH was slightly higher with values of the order of 7.83 (cultivated soil), 7.49 (industrial zone) and 7.74 (deposit metal tools). The organic matter content of samples ranged between 5.12 % and 5.38 %. Though, the amount of nitrogen fluctuates between 0.01 and 0.11 $mg.kg^{-1}$, while the conductivity ranged from 0.12 to 0.35 $dS.m^{-1}$. Results of the soil structure showed that the clay and coarse sand were the dominant elements.

TABLE I

The basic physicochemical properties and heavy metal concentrations in the soil samples

Parameters studied	Cultivated Soil (Dellys)	Industrial zone (Setif)	Deposit metal tools (Bab-Ezzouar)
Cadmium ($mg.kg^{-1}$)	1.024	1.612	0.854
Chrome ($mg.kg^{-1}$)	60.61	58.49	52,44
Zinc ($mg.kg^{-1}$)	70.01	124.00	55.26
Copper ($mg.kg^{-1}$)	26.99	24.83	27.65
Lead ($mg.kg^{-1}$)	24.13	21.98	12.85
Nickel ($mg.kg^{-1}$)	30.14	20.46	49.89
pH	7.83	7.49	7.74
Organic matter (%)	5.31	5.12	5.38
Conductivity ($dS.m^{-1}$)	0.12	0.35	0.20
Nitrogen (N) ($mg.kg^{-1}$)	0.11	0.056	0.01
Clay (Cl%)	23	10	20
Silts (S%)	14	12	10
Coarse silt (CS%)	8	4	6
Coarse sand (Cs%)	37	21	28

IDENTIFICATION AND MIC DETERMINATION OF THE CADMIUM-RESISTANT BACTERIA

A total of twenty-three cadmium-resistant bacteria were isolated with ten strains from cultivated soil, nine others from the deposit metal tools and only four from industrial zone. According to the results of the biochemical identification, these bacteria belonged to nine families and ten genera: *Chromobacterium*, *Burkholderia*, *Pseudomonas*, *Photobacterium*, *Aeromonas*, *Sphingomonas*, *Staphylococcus*, *Delftia*, *Serratia* and *Bacillus* as shown in Table II. The most resistant bacteria YL-SS3 strain and YL-SS8 strain were selected, 16S rRNA gene sequencing and phylogeny analysis revealed that the strain YL-SS3 was authentically identified as *Pseudomonas fluorescens* with maximum sequence similarity of 99 % and the strain YL-SS8 was identified as *Bacillus infantis* with 98% maximum sequence similarity. Length of sequenced gene was 1492 bases pairs for either YL-SS3 or YL-SS8 strain.

TABLE II

Cadmium-resistant bacteria identified using API system and MIC values

Cadmium-resistant bacteria	Origin of isolates	Similarity rate (%)	MIC (μgmL^{-1})
Burkholderia cepacia	Dellys	99	800
Pseudomonas fluorescens	Dellys	95	700
Pseudomonas fluorescens (YL-SS3	Dellys	99	900
Staphylococcus sp	Dellys	100	500
Delftia acidovoran	Dellys	98	600
Pseudomonas fluorescens	Dellys	90	700
Pseudomonas aeruginosa	Dellys	100	800
Burkholderia cepacia	Dellys	98	800
Bacillus infantis (YL-SS8)	Dellys	99	1100
Pseudomonas fluorescens	Dellys	90	800
Pseudomonas fluorescens	Bab-Ezzouar	90	800
Photobacterium damselae	Bab-Ezzouar	90	800
Aeromonas hydrophila G1	Bab-Ezzouar	95	500
Burkholderia cepacia	Bab-Ezzouar	98	800
Serratia macerans	Bab-Ezzouar	98	800
Burkholderia cepacia	Bab-Ezzouar	90	700
Sphingomona paucimobilis	Bab-Ezzouar	95	700
Pseudomonas aeruginosa	Bab-Ezzouar	96	700
Aeromonas hydrophila	Bab-Ezzouar	90	800
Chromobacterium violacerum	Setif	95	600
Aeromonas hydrophila G1	Setif	90	700
Sphingomonas paucimobilis	Setif	98	600
Aeromonas hydrophila G2	Setif	92	600

Neighbour-joining tree was constructed using both the sequences YL-SS3 (Fig. 1) and YL-SS8 (Fig. 2), and representative sequences from databases.

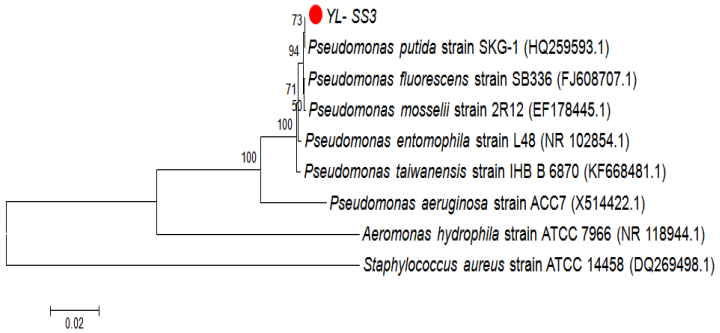

Figure 1.— Neighbour-joining phylogenetic tree based on 1492 bp of 16S rRNA gene sequences of isolate YL-SS3 depicting the phylogenetic relationships of YL-SS3 and its closest relatives in the genera *Pseudomonas. Staphylococcus aureus* strain ACC 14458 was taken as the out-group organism and the scale bar corresponds to the expected number of changes per nucleotide position.

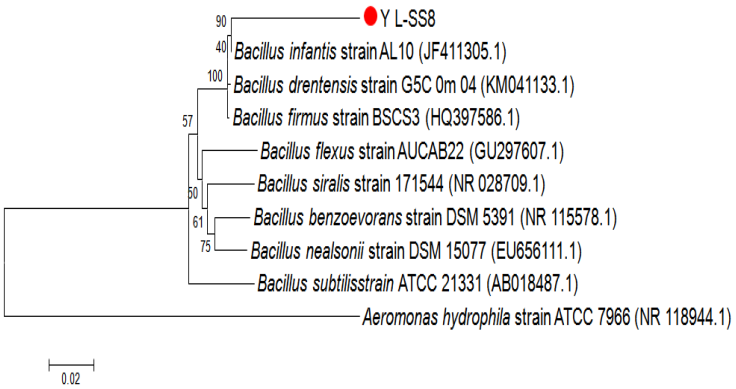

Figure 2.— Neighbour-joining phylogenetic tree based on 1492 bp of 16S rRNA gene sequences of isolate YL-SS8 depicting the phylogenetic relationships of YL-SS8 and its closest relatives in the genera *Bacillus*. *Aeromonas hydrophila* strain ACC 7966 was taken as the out-group organism and the scale bar corresponds to the expected number of changes per nucleotide position.

Cadmium resistance studies showed that the MIC were in interval of 500-1100 µg.mL^{-1} for the isolates of Dellys, 600-700 µg.mL^{-1} for the isolates of Setif and 500-800 µg.mL^{-1} for the isolates of Bab-Ezzouar. Overall, the majority of isolates reached a MIC of 800 µg.mL^{-1} (8 isolates) and 700 µg mL^{-1} (7 isolates). The maximum resistance to Cd was observed in YL-SS8 strain with MIC 1100µg.mL^{-1} and next to it, the resistant bacteria YL-SS3 was reported as the most resistant with MIC 900µg.mL^{-1}.

EFFECT OF CADMIUM ON BACTERIAL GROWTH OF THE STRAINS YL-SS8 AND YL-SS3

To study the effect of cadmium on growth of YL-SS3 strain and YL-SS8 strain, the cells were cultivated in the metal stress. For both strains, the concentration of 100 µgm.L^{-1} inhibited cell proliferation compared to untreated controls, as shown in Fig. 3.

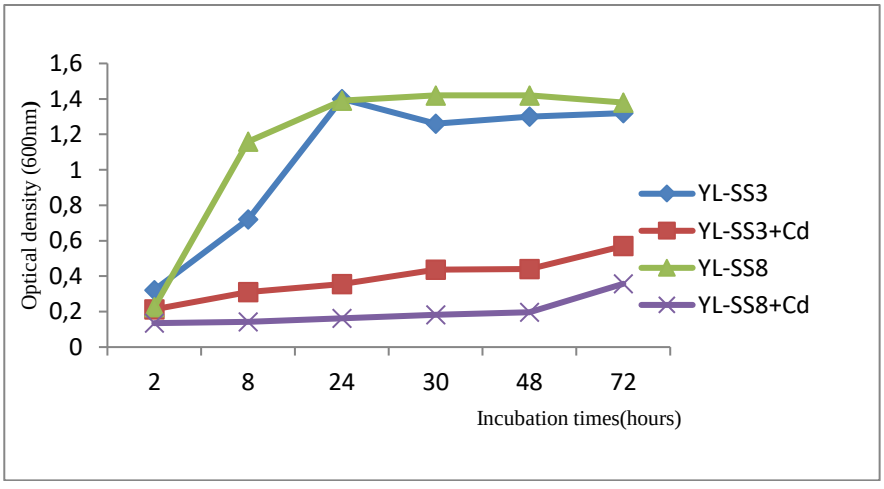

Figure 3.— Growth curves of free living cells of YL-SS3 or YL-SS8 strains in the presence and absence of cadmium. In treated samples cadmium was added at the beginning of experimentation with final concentration of 100 µgmL^{-1}.

The growth of the strain YL-SS8 (*Bacillus infantis*) was low, between the beginning of experimentation until 48 hours of incubation. But after this period, strain YL-SS8 cells were able to adapt and resume growth, translated by an increase in optical density.

However, growth of YL-SS3 strain (*P. fluorescens*) stressed evolved positively as a function of incubation time, but at lower level than the control (Fig. 3).

CADMIUM REMOVAL BY LIVING FREE CELLS

As shown in Figure 4, YL-SS8 strain (*B. infantis*) captured considerable concentrations of cadmium than the strain YL-SS3 (*P. fluorescens*). The cadmium removal level was 90 $\mu g.mL^{-1}$ after 24 hours of incubation, and then decreased to 83 $\mu g.mL^{-1}$ after 48 hours. During this same period, YL-SS3 strain (*P.fluorescens*) removed quantities of the order of 81 $\mu g.mL^{-1}$ then 56 $\mu g.mL^{-1}$.

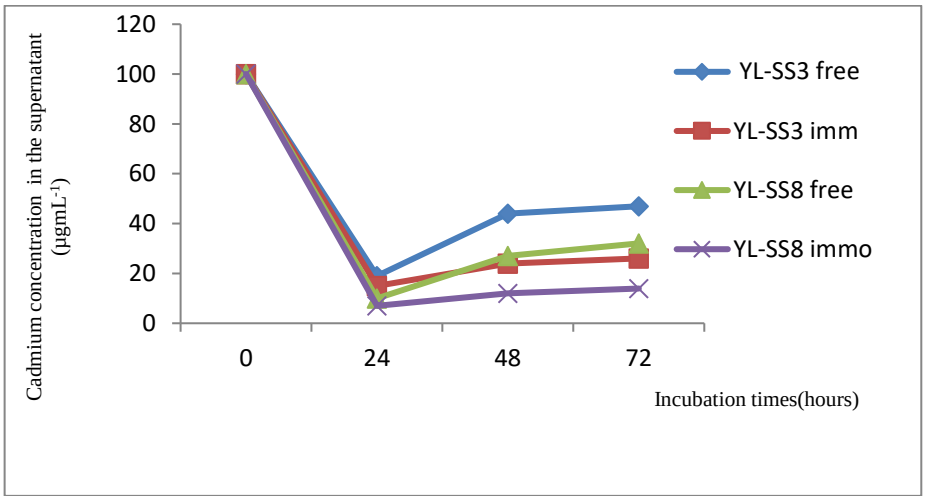

Figure 4.— Cadmium removal by living cells of YL-SS3 and YL-SS8 strains free, or immobilized in alginate beads. Cadmium was added at the beginning of experimentation at final concentration of 100 μgmL^{-1}.

CADMIUM REMOVAL BY LIVING IMMOBILIZED CELLS

The immobilized cells showed better uptake capacity than free cells (Fig. 4). On the first and second day, the removed amounts were 93 $\mu g.mL^{-1}$, 88 $\mu g.mL^{-1}$ for YL-SS8 strain (*B. infantis*) and 85 $\mu g.mL^{-1}$,76 $\mu g.mL^{-1}$ for YL-SS3 strain *(P. fluorescens*). According to these results, *B. infantis* immobilized cells captured higher amounts of cadmium ions.

DISCUSSION

In this study we adopted the AFNOR regulatory standards as a reference for assessing the pollution of soil samples. There is a lack in Africa in terms of regulating the maximum allowable levels of potentially toxic metals in soils.

It was observed that the determined quantities of heavy metals were lower than standards compared to AFNOR norms, except for nickel that displays an important value in the soil from Bab-Ezzouar. According to Baize (2000), most clayey and iron-rich soils approach or exceed the value of 50 mg Ni per kg. However, the zinc content of the three soils could be related to that the

soils samples are the support of many industrial (Setif soil), agricultural (Dellys soil) and urban activities (Bab-Ezzouar soil). The development of these activities leads to a marked increase in the contents of metallic trace elements (MTE) since the high use of zinc in industry and organic fertilizers (Allam, 2012). The cadmium concentrations determined in the three soils are all lower than the AFNOR standards (2 $mg.kg^{-1}$). However, these soils are not free from contamination because these levels greatly exceed the natural concentrations of cadmium in the upper soil horizons which are between 0.2 and 0.4 $mg.kg^{-1}$ (Bourrelier & Berthelin, 1998; Alloway, 1990), showing the share of anthropological contamination resulting from human activities.

However, bacteria isolated from these soils were highly resistant to Cd. Previous results of several laboratory and field experiments concerning the long term heavy metal effects, showed an increased bacterial community tolerance to metals present in soil, with the tolerance increase being correlated with the pollution level (Diaz-Ravina *et al*, 1994; Diaz-Ravina & Baath, 1996a; Pennanen *et al*, 1996; Baath *et al*, 1998).The soil isolates identified belonged to the genera *Bacillus*, *Pseudomonas*, *Burkholderia*, *Staphylococcus*, *Chromobacterium*, *Photobacterium*, *Aeromonas*, *Sphingomonas*, *Delftia* and *Serratia*. These bacterial genera are often encountered in contaminated areas (Ajaz *et al*., 2010; Elsilk *et al*., 2014). 91 % of isolates are gram-negative. It is widely accepted in the scientific literature that gram-negative bacteria are very common at contaminated sites (Khafilzadeh *et al.*, 2013**)**, indicating that gram-negative bacteria have an outer membrane and a negative surface charge of lipopolysaccharides (LPS). However, resistance to Cd has been reported for both gram-positive and gram-negative bacteria (Foster, 1983). In agreement with our results gram-positive bacteria like firmicutes were found to tolerate high concentration of heavy metals (Gupta, 2012). All the isolates have a MIC above 500 $\mu g.mL^{-1}$. The MIC high value translates the great adaptability of these bacterial genera to hostile environments, which is related to their varied energy metabolisms and a wide range of biochemical and molecular processes. Certain bacteria like *Pseudomonas* are able to produce siderophores which are commonly used for Fe transportation and have the capability to chelate heavy metals such as Cd^{2+}, and may be responsible for maintaining metal homeostasis (Złoch *et al*., 2016).

Besides, metallothionein (MT), a thiol-containing, cysteine-rich protein, induced by heavy metals and used to transport and reduce toxic metals, is of great help in the sequestration of metal ions. Thiol groups of cysteine residues in metallothionein can bind Cd^{2+} to form metal-thiolate complexes, thus rendering Cd unavailable to exert toxicity (Khan *et al*., 2015).

Microbes still have other detoxification methods such as glutathione (GSH) system (Wang & Wang, 2010; Barmo *et al*., 2011; Won *et al*., 2011; Cirillo *et al*., 2012).

When cells of strain YL-SS3 and strain YL-SS8 were cultivated in presence of 100 $\mu g.mL^{-1}$ of cadmium, results showed that the metal has an inhibitory effect on growth of both strains YL-SS3 and strain YL-SS8. Furthermore, growth was not completely inhibited at this concentration. It should be noted that a concentration of 10 mM of cadmium was lethal for *E. coli* (Ferianc, 1998), which shows the large capacity of these isolates to adapt to hostile conditions of growth, such as metallic stress and develop various resistance mechanisms towards heavy metals. It is very important to mention that cadmium causes oxidative damage to microorganisms, leading to a decrease of cell activity, a reduction of growth rate and cell density, an inhibition of cell proliferation and therefore a decrease of the bacterial number due to the death of some bacteria (Sihamim and Rehman, 2012).

Although the Cd^{2+} concentration used in this work was much higher than those generally observed in contaminated environments (Wagner, 1993), the inhibition of cell proliferation in presence of cadmium could be linked to an inhibited DNA replication (Mitra *et al.*, 1975; Nystrom & Kjelleberg, 1987) which makes the DNA more susceptible to nucleolytic attack, resulting in single-strand DNA breaks. Therefore, cadmium causes serious damage during the growth of bacteria present in polluted environments (Fahmy, 2013). Strain YL-SS8, growth was low at the beginning of incubation, but after 48 hours of contact with the metal ions, bacterial cells became

able to adapt and resume growth. This period appears to involve repair of cadmium-mediated cellular damage and adjustment of the cell physiology to limit the distribution of toxic ions in the cell. Wiatrovska (2015) reported that Cd was more toxic to *Bacillus sp* than Pb.

YL-SS3 strain growth, was not stopped by Cd; the cells were able to maintain their growth at a low rate and then restart. Gram-negative bacteria like (*Pseudomonas*) are well reported to play a significant role in detoxification of various heavy metals like cadmium (Halder & Basu, 2016); this genus has widely been studied for its well-adapted metal resistance properties (Deb et *al*., 2013).

However, the negative effect of cadmium on bacterial growth did not prevent the capture of cadmium ions, since YL-SS3 strain and YL-SS8 strain were capable of taking up the metal ions. Large amounts of cadmium ions were removed by living free cells of the two species. The results showed that the maximum of cadmium uptake occurred during the first period of incubation (24 h). This period corresponds to low cell biomass level, resulting from the toxic effect of cadmium on the cells of the two strains; cells would probably involve passive mechanisms, as bacteria during this period need to save energy to ensure their adaptation and then their proliferation. Interactions between bacterial cells and metals are governed by passive or active mechanisms (Chang, 1997; Haferburg & Kothe, 2007). The passive mechanisms of uptake are independent of the metabolism and therefore the physiological state of the cells (living or dead), they are fast and reversible. They take place at the cell / solution interface and involve mechanisms such as ion exchange, surface complexation onto the cell wall and other outer layers (Fomina & Gadd, 2014) or precipitation. These processes are grouped in the term of Biosorption.

An analysis of the cell wall components, which vary among the different microorganisms, helps in assessing metal uptake by different microorganisms. The peptidoglycan layer in gram-positive bacteria, which contains alanine, glutamic acid, meso-di-aminopimelic acid, polymer of glycerol and teichoic acid, and that of the gram-negative bacteria, which contains enzymes, glycoproteins, lipopolysaccharides, lipoproteins, and phospholipids, are the active sites involved in metal binding processes (Lesmana *et al*., 2009; Gupta *et al*., 2015). Changli *et al.* (2010) reported that *Pseudomonas fluorescens* biomass includes different functional groups and these functional groups are able to react with metal ions in aqueous solution.

When bacterial growth increased (48-72 h), cadmium remediation could be attributed to active mechanisms. Active mechanisms depend on the metabolism of the cells and are therefore specific to each bacterial strain. They are slower and generally inducible such as efflux pumps. Removal cadmium capacity decreased when incubation time increased, showing that the contact time between bacterial cells and cadmium ions and also the age of the cells could be considered as an important factor affecting metal uptake.

The lack of remediation could be also explained by metal exclusion or by detoxification mechanisms similar to those described for antibiotic resistance, which represent the main defence of bacteria in the presence of external toxicants (Saier, 2003; Pana, 2012).

Two well-studied genetic mechanisms of metal resistance in bacteria include heavy metal efflux systems (Nies & Silver, 1995) and the presence of metal binding proteins (Robinson *et al*., 1990). Many operons of efflux system are known. In the gram-positive bacteria, the plasmid-encoded Cd efflux system, called the *CadA* resistance system, utilizes the *CadA* protein, which is a P-type ATPase (Tsai & Linet, 1993). An analysis of the cadmium tolerance genes of *B. cereus S5*, identified ATPase genes that were associated with cadmium tolerance and involved in the ATP pumping mechanism (Huiqing *et al*, 2016). However, Cd resistance in gram-negative organisms is due to a multi-protein chemiosmotic antiport system (Silver, 1996**)**. Our results showed that during this period (48-72 h), growth of the two strains improved, proving that the medium became less toxic to bacterial cells than at the beginning of incubation. A decrease in the toxicity of copper at a longer feast famine period was attributed to the presence of higher amounts of extracellular polymeric substances (EPS) (Song, 2016). EPS play a defensive action that prevents microbial cell

from toxic heavy metal ions. Its net anionic makeup allows the biopolymer to effectively sequester positively charged heavy metal ions (Gupta & Batul, 2017). Enhanced production of EPS was induced by the so-called stressful culture conditions (Arudhanti & Paul, 2008). *Pseudomonas stutzeri* produced large quantities of EPS under cadmium stress (Debarati & Basu, 2016).

Gram-negative and gram-positive bacteria are able to accumulate the metal ions inside the cell, in the cytoplasm and/or the periplasm, and outside on the outer membrane (Voloudakis *et al.*, 1993).

The immobilized cells of strain YL-SS3 and YL-SS8 strain showed high cadmium uptake capacity. Several researches demonstrated a better remediation of heavy metals by the immobilized organisms as compared to free cells. Numerous authors (Tsekova & Ilieva, 2001; Wuana & Okieimen, 2010; Sujitha & Jayanthi, 2014; Wasi *et al*., 2011) have reported better efficiency bioremediation potential of immobilized *P. fluorescens* SM1 strain compared to free cells. This could be directly linked to the advantage of this process, based on the immobilized biomass which leads to the improvement of the stability of microbial cells, thus allowing a continuous operation of the process and avoiding the need to separate the biomass from the medium. Cellular immobilization leads to the confinement or localization of the viable microbial cells to a certain defined region of the space, so as to limit their free migration and increase their contact surface with the metal and consequently their capacity for biosorption. Immobilized microorganism technology offers a multitude of advantages, such as high biomass, high metabolic activity and strong resistance to toxic chemicals (Cai *et al*., 2011; Liu *et al*., 2012). In other hands, numerous reports verify that attachment of bacterial cells to solid surfaces stimulates the exopolysaccharides production without altering the specific growth rate (Vandevivere & Kirchman, 1993).

A combination of bacterial EPS immobilized in calcium alginate resulted in maximum cadmium and cobalt ion sequestration from aqueous solutions (Ozdemir *et al.*, 2005). Microbial cells in communities display a variety of metabolic differences as compared to their free-living counterparts. The majority of changes observed in immobilized cells result from protection provided by the supports (Zur *et al*., 2016).

CONCLUSION

The present study showed that contaminated areas offer good ecological niches for cadmium resistant bacteria. In terms of bioremediation, immobilized cells removed more cadmium than free cells in both YL-SS3 and YL-SS8. Better remediation of cadmium requires both bacterial strains cadmium-resistant but also a technique that better values their performance. In our study, immobilization in alginate beads proved better than the cells in suspension. However, knowledge about the main physiological responses occurring in immobilized cells may contribute to improving the efficiency of immobilization techniques.

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