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Copper retention by a strain of *Bacillus*

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SUMMARY

A survey has been made of the copper accumulation by resting cells of bacteria selected as copper-resistant, isolated from activated sludges. The best selected strain, classified as *Bacillus*, retained copper at up to 3.8% of its cell dry weight. These values were lower in the presence of glucose, unlike a type culture of *Bacillus cereus*, in which the retention of copper was higher when glucose was present. Possible reasons for these changes in uptake of both strains are suggested.

INTRODUCTION

In spite of the fact that copper is an essential heavy metal, since it is present in a good number of enzymes and other proteins [19], its toxicity is relatively high, chiefly to heterotrophic bacteria of aquatic environments [1]. Otherwise, it is an important pollutant presently discharged from various industries, such as petroleum refineries and paper factories, or habitually used as a component of fertilizers and pesticides [8].

Copper recovery from effluents by means of microorganisms would prevent a metal increase in the environment. For a better understanding of the general microbiological methods for the recovery of metals, see the review by Kelly et al. [13]. The literature on copper accumulation by microorganisms has been reviewed by Baldry and Dean [2].

There is a certain relationship between the heavy-metal accumulation by microorganisms and their resistance, since the mechanisms involved, when they are known, are very often the same, such as intracellular uptake of a copper-tolerant green alga [24], surface adsorption by the lipopolysaccharide of *Escherichia coli* [11], formation of extracellular polymers, as in the case of *Zoogloea ramigera* [17], or extracellular precipitation following a metal biotransformation, as in the case of *Sphaerotilus* and other iron bacteria [20].

In this work a strain was selected from copper-resistant bacteria for its accumulation capacity, and its retention of copper at different external concentrations was studied, as was the influence of the presence of a carbon supply. Assays were made with the cells resuspended in a resting state in Pipes

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buffer (piperazine-*N,N'*-bis(2-ethanesulphonic acid; Sigma), as described previously [18], because this buffer has negligible metal-chelating properties, unlike most of the usual components of microbial growth media [21] or other buffers usually employed, such as Tris [6,16].

MATERIALS AND METHODS

Samples. Four different samples were taken from activated sludges of aerobic wastewater treatment plants (one urban and three industrial ones), near Tarragona (Catalonia, Spain), an industrial and populated area.

Homogenization. In order to disperse flocs of sludges, and microorganisms included therein, samples were mixed with a 10% volume of a previously autoclaved homogenization solution [10], containing sodium chloride 90 g, Lubrol PX (Sigma) 1 g and sodium pyrophosphate 1 g in 1 litre of distilled water. After homogenization for 1 min with a Turmix homogenizer, the resultant suspension was centrifuged for 5 min at 500 × *g*.

Isolation of copper-resistant strains. The supernatants obtained were diluted and plated on tryptic soy agar (TSA, Difco) with $CuSO_4$, to give final concentrations of 2–10 mM Cu^{2+}. These plates were incubated for 72 h at 30°C and then a number of morphologically different colonies were isolated. These colonies were subcultured on TSA with 2 mM Cu^{2+} and their culture purity was checked. The levels of copper-tolerance for the isolated strains were tested individually by subculturing them on TSA plates with different concentrations of the metal: 0, 2, 5, 10 and 20 mM Cu^{2+}. After incubation for 48 h at 30°C, their growth was checked. From the strains isolated as resistant, those which grew well at up to 5 mM Cu^{2+} were selected.

Organisms. Strain QT 117 was isolated as copper-resistant as described above. Procedures for its identification were extracted from Abstracts of Microbiological Methods by Skerman [25]. A strain of *Bacillus cereus* (ATCC 14579) was also employed.

Copper accumulation by resting cells. To check the capacity of metal accumulation by selected strains, these were inoculated in 20 ml of tryptic soy broth (TSB, Difco) and incubated at 30°C in a shaking bath (ca. 100 strokes/min) for 20 h. Then, 0:8 ml of these cultures were transferred to 40 ml of fresh sterile TSB and incubated under the same conditions until early exponential phase, which was detected by measuring the absorbance at 540 nm. These data were converted to biomass by previously determined absorbance–dry weight plots.

Cells were harvested by centrifugation, washed twice with 8 ml of sterile saline solution (8.5 g NaCl in 1 litre distilled H_2O), and resuspended in 40 ml 5 mM Pipes buffer adjusted to the desired pH using a solution containing 10% tetramethylammonium hydroxide [18]. Cell suspensions were previously adjusted by dilution with Pipes buffer to approximately 0.2–0.4 mg dry wt. · ml^{-1}. Then, a volume of a solution of $CuSO_4$ in pure deionized water (Milli-Q, Millipore) was added to give the desired final concentration of Cu^{2+}. After that, suspensions were placed at 30°C and 3 ml samples were taken at intervals. All experiments were repeated three times.

Copper content of cells. Samples taken during accumulation assays were filtered through 0.45 μm pore size, 47 mm diameter membrane filters (Millipore), and these were washed immediately with 10 ml Pipes buffer. Filters with cells were digested with 4 ml 6 M HNO_3, low metal content (Merck), for 3 h at 110°C in Teflon-lined pressure digestors, to release cell-associated metal ions. Samples were cooled and made up to 5 ml with pure deionized water (Milli-Q, Millipore) and the copper content was analysed by flame atomic absorption spectrophotometry, using a specific copper lamp with an IL 551 (Instrumentation Laboratory).

RESULTS AND DISCUSSION

Copper-tolerant strains

Solutions proceeding from homogenized samples of activated sludges were plated on TSA with $CuSO_4$ at concentrations of 2–10 mM. After growth, 132 colonies were picked and isolated, and

after testing their resistance levels 40 strains were chosen. All of them grew well on TSA with 2–10 mM Cu^{2+}.

The study of the relationship between heavy metals and microorganisms always presents the problem of heavy metal ion binding by components of microbial growth media, such as peptones and casamino acids [21]. It is highly unlikely that the strains used here, isolated in TSA with Cu^{2+} concentrations of 2 mM or above, would tolerate such high copper levels in minimal media. Nevertheless, if the isolation were done in minimal media, a part of the natural population would not be able to grow on it because of unsatisfied unknown nutritional requirements. However, study of copper accumulation by selected strains has been carried out, following Norris and Kelly [18], in Pipes buffer, which does not bind metals. Therefore, in these assays, the metal retention is only due to the cells.

Retention of copper by the system

Blank accumulation assays without cells were done, filtering Pipes buffer at pH 7.0 with 1 mM Cu^{2+}, and retained Cu^{2+} after filter digestion was analysed. Values obtained were very high, about 26 mg/l, representing 41% of the total copper in solution. The reason for this is the low solubility of copper in Pipes buffer at pH 7.0, as in other non-complexing aqueous systems [3]. This precipitation on filters was confirmed by resuspension of 1 mM Cu^{2+} in Pipes buffer at different pH values. The results obtained for copper retention by the filters are shown in Table 1. On the basis of these data, accumulation assays made with cells were carried out at pH 5.5, where Cu^{2+} precipitation was minimal.

Table 1

Influence of pH on copper retention by the system without cells

pH	Cu^{2+} retained	
	$mg \cdot l^{-1}$	%[a]
11.0	37.14	58.50
7.0	37.02	58.30
6.0	8.88	13.98
5.5	0.30	0.47
4.0	0.42	0.66
2.0	0.60	0.94

[a] % of Cu^{2+} retained with respect to the added 1 mM.

Selection of a copper-accumulating strain

Once the 40 resistant strains had been tested against 1 mM Cu^{2+} in Pipes buffer at pH 5.5, strain QT 117 was selected because it showed the highest retention of copper. Cells of this strain retained 4 mg/l Cu^{2+} after 4 h of assay, which was equivalent to 6% of added copper. Cells of this strain were endospore-forming rods, gram-positive, aerobic and catalase-positive. According to the descriptions in Bergey's Manual [12], it was classified as a strain of *Bacillus*.

Copper retention by QT 117 and B. cereus ATCC 14579

Copper accumulation by QT 117 was tested at different concentrations of Cu^{2+} in the buffer: 0, 0.4, 0.75 and 5 mM Cu^{2+}. The amount of copper retained with respect to cell dry weight is shown in Fig. 1A. Values for *B. cereus* in the presence of 5 mM Cu^{2+} are also shown in Fig. 1C. The average biomass concentration for both strains was 0.2 mg/ml dry weight. Cell concentration remained constant during assays, since these were done in buffer and cells could not grow. On the other hand, pH did not change during assays, remaining about 5.5. This eliminates the possibility of metal retention by surface precipitation due to a pH increase for some extracellular compounds.

As can be seen, Cu^{2+} retention by QT 117 is higher than that shown by *B. cereus* for an external concentration of 5 mM. For QT 117, there is a relation between the amount of Cu^{2+} retained and the concentration in the buffer, and for every concentration there is a slight increase with time. These values of copper retained at 2 h of assay represented 2.6–3.8% of cell dry weight, which is physiologically important, and suggests a higher total retention in the case of a larger quantity of biomass. These values represent 19.7% of Cu^{2+} retained by cells with respect to external copper in the case of

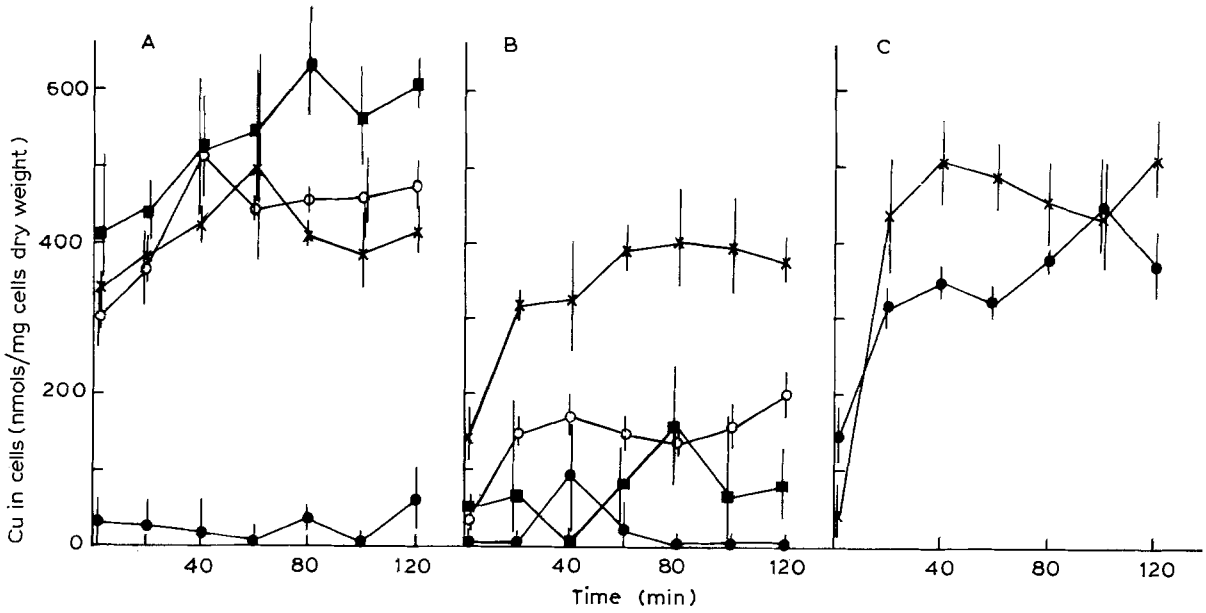

Fig. 1. (A) Copper retention by QT 117 in Pipes buffer, for 0 (●), 0.4 (×), 0.75 (○) and 5 (■) mM Cu^{2+} added. (B) Copper retention by QT 117 in Pipes buffer with 10 mM glucose, for 0 (●), 0.4 (×), 0.75 (○) and 5 (■) mM Cu^{2+} added. (C) Copper retention by *B. cereus* in Pipes buffer without (●) and with (×) 10 mM glucose, for 5 mM Cu^{2+} added. All data are the mean values from three replicates, and vertical bars represent ± S.D.

0.4 mM Cu^{2+} added, 12.1% for 0.75 mM Cu^{2+} added, and 2.9% for 5 mM Cu^{2+}. On repeating the same accumulation experiments for strain QT 117 in the presence of 10 mM of glucose added to Pipes buffer, the results shown in Fig. 1B were obtained. Values for *B. cereus* under the same conditions are shown in Fig. 1C. As can be seen, retention of copper by QT 117 in the presence of glucose was lower when the concentration of Cu^{2+} added was higher than 0.4 mM, unlike *B. cereus*, which even increased its copper content in the presence of glucose.

These results suggest different mechanisms for the two strains tested. In the type culture of *B. cereus*, copper would be retained by a metabolic process related to the uptake of some essential cation, which would be favoured in the presence of energy supplies, similarly to the situations described for yeasts, such as *Candida utilis* [14] and *S. cerevisiae* [22], or for bacteria with other heavy metals, like nickel [26], manganese [23], cadmium [27] or zinc [7]. These energy-dependent systems have also been described in different species of *Bacillus* for the uptake of Ni^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} [28,29].

On the other hand, a possible explanation of copper retention in strain QT 117 would be a non-active uptake mechanism, since the presence of glucose did not increase the cellular content of Cu^{2+}; on the contrary, the presence of glucose caused a decrease in metal concentration. So, retention of copper by this strain in the resting state, in the absence of carbon supplies, could be explained by a non-metabolic, external adsorption of copper, as the one studied for the cell walls of *B. subtilis* [4,5] or that observed for the capsule of *B. megaterium* [9]. In relation to that, the presence of glucose could cause some changes in the composition of external parts of cells, making the passive adsorption of copper more difficult.

It also seems possible that in the absence of glucose some intracellular uptake could occur, following alteration of membrane permeability due to the binding of metal to cell surfaces, rather than utilization of transport systems, as has been suggested by other authors [13]. In that case, the presence of glucose would reinforce external cellular parts, such

as capsules and cell walls, maintaining the correct membrane permeability, and so excluding copper. Another explanation would be the activation by glucose of some regulatory mechanism, which would turn off the possible uptake of copper. Bearing in mind that QT 117 is a sporulated bacterium, and that it has been reported that the Cu^{2+} content of spores is always much higher than that of vegetative cells [15], a situation of resting cells, without glucose, would be more predisposed to initiate sporulation, and to retain copper, than the reverse situation with glucose. A more detailed study of the copper retention by this strain is being carried out by the authors.

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