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ORIGINAL ARTICLE

Utilization of phenol in the presence of heavy metals by metal-tolerant nonfermentative gram-negative bacteria isolated from wastewater

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ABSTRACT. Strains of Cr-tolerant or Hg-tolerant Gram-negative bacteria were isolated from Chemistry School's sewage water and these were studied in relation to their ability to use phenol as sole carbon source in the presence of $K_2Cr_2O_7$ and $HgCl_2$. These metals showed inhibitory effect in the assimilation of this aromatic compound. However, one Cr-tolerant strain (*Burkholderia cepacia* - JT50) got phenol metabolized in the presence of high concentration of $K_2Cr_2O_7$ (until concentration of 200 µg/ml). Additional investigation of this strain in minimum medium with phenol and chromate indicated that the tolerance mechanism did not involve chemical reduction from Cr^{6+} to Cr^{3+}, neither any changes in the total-chromium levels in that medium.

Key words: Phenol-degradation, heavy metal tolerance, mercury, chromate.

RESUMEN. Se estudiaron linajes de bacterias Gram negativas tolerantes a $K_2Cr_2O_7$ o a $HgCl_2$ aisladas de aguas residuales de una escuela de química en lo que respecta a la capacidad de utilización de fenol como única fuente de carbono en presencia de esos metales. Ambos metales mostraron un significativo efecto de inhibición al utilizar el compuesto aromático, pero un linaje Cr-tolerante (*Burkholderia cepacia* - JT50) logró metabolizar fenol en presencia de hasta 200 µg/ml de $K_2Cr_2O_7$. Algunos estudios adicionales con ese linaje indicaron, que en las condiciones de los ensayos utilizados, su mecanismo de tolerancia no involucró procesos de reducción química de Cr^{6+} a Cr^{3+}, ni tampoco variación en los niveles del cromo total en el medio de cultivo.

Palabras clave: Degradación de fenol, tolerancia a metales pesados, mercurio, cromato.

INTRODUCTION

Heavy metals represent a serious environmental problem due to their stability in nature and accumulation in the food chain. Two important metal pollutants are chromium and mercury. The chromates are considered carcinogenic and mutagenic,[1-3] and it is presented as environmental pollutant emitted by metal finishing industry, petroleum refining, leather tanning, paints and pigments, steel production, textile manufacturing and pulp production. This metal may exist at various oxidation levels, however the most stable and common forms are the hexavalent (Cr^{6+}) and trivalent (Cr^{3+}). The hexavalent form, considered the most toxic form of Cr, is highly soluble in water and is usually associated with oxygen as chromate (CrO_4^{2-}) or dichromate (CrO_4^{7-}) ions. Cr^{3+} is much less toxic and tends to form insoluble hydroxides.[4] Some bacteria have been shown to reduce chromate to the trivalent form.[5-7]

The mercury is highly toxic even at very low levels.[8,9] Antropogenic environmental source of Hg as pollutant includes burning of fossil fuels, smelting metal ores, mercury mining, fungicides, and waste incinerators and crematories. The solubility of inorganic and organic mercury compounds in lipids as well as their binding to sulfhydryl groups of proteins in membranes and enzymes account for their cytotoxicity.[10]

Among the organic environmental pollutants aromatics compounds, as phenol, and phenolic compounds are detached. They are commons constituents of waste water originating from many industries including pharmaceutical, polymeric resin production, petroleum and coal refining. The toxicity of these compounds to microorganisms seems include changes in their membranes, even at low concentrations.[11,12]

Many types of bacteria may degrade these compounds in aerobiosis and anaerobiosis conditions, despite of their toxicity.[13,14] So, many isolated strains have been studied with the objective to apply to bioremediation process.[15-17]

Often the environments contaminated by aromatic compounds also receive discharges from toxic metal pollutants. In this case, in addition of affecting the viability of the microbiota, the metal activity may compromise the

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biodegradable processes of the aromatic compounds. Therefore, some studies related to the association of the bacterial tolerance properties to metals and degradation of phenolic compounds may be relevant to applications in bioremediation processes.

In the present study we evaluate the capacity of Gram negative Hg and Cr tolerant bacteria isolated from wastewater to use phenol in the presence of the respective metals. Additionally, the behavior of a strain of *Burkholderia cepacia* phenol degrader and tolerant to high concentrations of chromium isolated in this work was investigated.

MATERIALS AND METHODS

Samples: eleven samples of sewage water taken from a chemistry school (CS) located in the northern region of the city of Rio de Janeiro were analyzed. The school's sewage was chosen to study because aromatic compounds and heavy metals are used everyday in their laboratories. Amounts of 100 ml of sewage were collected from the meeting point of the CS's sanitary sewage with the sewage from its laboratories using sterile bottles.

Isolation of Cr and Hg tolerant-bacteria: After clarifying filtration using filter paper, aliquots of 0.1 ml of wastewater saline dilutions were spread onto Petri dishes containing Nutrient Agar (Merck), with 100 μg/ml of cycloheximide and $HgCl_2$ (60, 80, 100, 120 μg/ml) or $K_2Cr_2O_7$ (110, 160, 210, 260, 310, 360 μg/ml) in addition to Nutrient Agar without metal. Previous experiments with sewage samples spread onto Nutrient agar supplemented with different concentrations of Cr or Hg allowed the choice of these concentrations. Three replicates of each dilution were plated and incubated at 35ºC for 24 hours. The metallic salts employed were of analytical grade and its solutions sterilized by Millipore membrane filtration with 0.22 μm pores. Standard strains of Gram negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 25619 and *Escherichia coli* ATCC 25922 with previously determined levels of sensitivity to metals at tested concentrations were inoculated for control of the metal activity in the medium. Colony forming units (cfu) were corrected to c.f.u./ml of sewage. Cr-tolerant and Hg-tolerant c.f.u./ml percentages were calculated by comparison with the results obtained in the medium without metal.

Characterization of isolates: The Gram-positive species and the fermentative Gram-negative species isolated in this study were only stocked to further research. The samples were processed by means standard procedures. The Gram-negative strains, after testing to confirm their growth capacity in the Hg or Cr concentrations noted in the primary isolation, were submitted to glucose fermentation and oxidation tests. The glucose-nonfermentative strains were identified by conventional tests, such as colony morphology, growth capacity in MacConkey Agar and Cetrimide Agar (Merck), nitrate reduction with or without producing gas, oxidase reaction, pigment production, fluorescence in F Agar, pyocyanine research in P Agar, growth at 42ºC , motility using the hanging drop, arginine dihydrolase, urease, gelatin hydrolysis,[18,19] and the API 20 NE identification system (bioMérieux , Marcy l'Etoile, France).

The determination of the capacity of using phenol by metal-tolerant strains: Initially, the strains were seeded in a minimal medium with glucose (g/L: Na_2HPO_4.12 H_2O: 15.1; KH_2PO_4: 3.0; NaCl: 0.5; NH_4Cl: 1.0; distilled water to 1L; autoclaving for 15 min at 121ºC; sterile solution of 1 M $MgSO_4$: 1ml; sterile solution of 0.01 M $CaCl_2$: 10 μl; sterile 20 % solution of glucose: 10 ml; pH 7.0). After having identified the strains with capacity of growing in this medium, subsequently aliquots of 10 μl of cultures with approximately 10^8 ufc/ml were inoculated in tubes containing 2 ml of minimal medium (pH 7.0) containing phenol as the sole carbon source (100 μg/ml), in addition to minimal medium (pH 7.0) with and without glucose (control). After incubation at 35ºC/48h a visual reading was taken, by way of a turbidity test. The property of strains to use phenol as their unique source of carbon in the presence of Hg or Cr was analyzed by inoculation in minimal medium (pH 7.0) with phenol and $HgCl_2$ (3, 5, 10, 20 µg/ml) or $K_2Cr_2O_7$ (40, 60, 80, 100, 200 µg/ml) added. The inoculation, incubation and reading conditions were the same as previously described.

Additional tests with B. cepacia Cr-tolerant strain: Among the microorganisms isolated during this study a strain identified as *B. cepacia* (strain JT50) was highlighted. Aliquots of 100 µl of the strain JT50 cultivated for 24h in minimal medium with phenol (150 µg/ml) and $K_2Cr_2O_7$ (80 µg/ml) were inoculated, respectively, in Erlenmeyer flasks containing 400 ml of minimal medium with 150 µg/ml of phenol, and minimal medium with the addition of 150 µg/ml of phenol and 80 µg/ml of $K_2Cr_2O_7$. Minimal medium with phenol and $K_2Cr_2O_7$ without bacterial inoculum was used as control. The flasks were incubated for 48 h at 35ºC by stirring at 140 rpm, and at time intervals aliquots were removed and filtered using a 0.45-µm-pore-size filter for total chromium and Cr^{6+} concentration analysis. The filtrates for chromium analysis were acidified with hydrochloric acid (100 µl/5 ml of sample). Aliquots from the medium with phenol and phenol plus chromium were removed at time intervals to follow the bacteria growth, through optical density readings in UV-visible spectrophotometer.

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The total content of chromium was determined by atomic absorption spectrophotometry (Perkin Elmer, model 3100), using an air-acetylene flame with reduction characteristics (excess acetylene). The wavelength of the absorbance measurements was 357.9 nm, with a slit opening of 0.7 nm. The calibration curve was constructed using Cr^{6+} solutions in a concentration range of 0.25 to 4.0 ppm (mg/l). Prior to performing the measurements, the samples were diluted 25 times.

For the determination of Cr^{6+}, the classic spectrophotometric procedure was employed, based on the reaction of the $Cr_2O_7^{2-}$ ion with diphenylcarbazyde in acid medium, producing a colored compound that absorbs radiation in the visible range. The calibration curve was constructed using Cr^{6+} solutions in a concentration range of 0.2 to 2.0 ppm (mg/l). The absorbance measurements were performed on a Micronal spectrophotometer, model B 342 II, with a wavelength of 546 nm. Both methodological determinations (chromium and Cr^{6+}) were described by Marczenko.[20]

The confirmation of the phenol degradation was performed spectrophotometrically based on the reaction of the phenols with the 4-aminoantipyrin reagent at a pH of 7.9, and the presence of ferricyanide.[21] Such reaction produces a colored compound that absorbs radiation in the visible range. The calibration curve of phenol in a concentration range of 0.2 to 1.0 ppm (mg/l) was constructed using standard solutions. The measurements were performed on a Micronal spectrophotometer device, (model B 342 II), with a wavelength of 506 nm using a cuvette with a 1 cm optical path.

The tests with the strain *B. cepacia* JT50 were done in triplicate and they showed similar results. The mean of these 3 tests was considered as final result.

RESULTS AND DISCUSSION

Plate count analysis (u.f.c./ml) for Hg-tolerant bacteria ranged from 2% in 60 μg/ml concentration to less of 0.1% at concentration of 120 μg/ml of $HgCl_2$. For the $K_2Cr_2O_7$, the range were approximately from 1% in 60 μg/ml to less of 0.1% at 310 µg/ml. Therefore, the concentrations of metals used in this study allow the isolation of a number of metal-tolerant bacteria very small in relation to the whole number. It is well known that there are no currently acceptable concentrations of metal ions which can be used to distinguish metal-resistant from metal-sensitive bacteria. As consequence, the aim of this study was to work with very high concentrations of these metals. In this way, it was possible to isolate strains with high degree of tolerance to Cr or Hg.

Among the Gram negative bacteria isolated in the presence of Hg or Cr, an absolute predominance of nonfermentative species over the fermentative species was observed. In the highest concentrations of the two metals (≥ 210 µg/ml to $K_2Cr_2O_7$ and ≥ 120 µg/ml to $HgCl_2$), the occurrence of nonfermentative was 100%.

Seventy one metal-tolerant nonfermentative Gram-negative strains were studied. As presented at Table 1, more than 70% of Hg-tolerant strains tested showed the property of using phenol as sole carbon source. The numeric distribution (not included in Table 1) of these strains was: *P. fluorescens* (8), *P. putida* (3), *P. aeruginosa* (3), *Pseudomonas sp* (3), *B. cepacia* (2), *Alcaligenes* sp (3). The capacity of growth of these microorganisms in minimal medium with phenol was very affected by the Hg^+ ions. In spite of high

Table 1. Utilization of phenol (100 μg/ml) in the presence of $HgCl_2$ by non-fermentative Gram negative Hg-tolerant strains

Add compounds to mineral medium [µg/ml]	Number of strains tested	Degrading strains Number/(%)
Phenol	31	22 (70.9)
Phenol + $HgCl_2$ [3]	22	5 (22.7) *P. fluorescens* (2); *P. putida* *P. aeruginosa*; *Alcaligenes* sp.
Phenol + $HgCl_2$ [5]	22	2 (9.0) *P. putida*; *Alcaligenes* sp
Phenol + $HgCl_2$ [10]	22	0
Phenol + $HgCl_2$ [20]	22	0

Table 2. Utilization of phenol (100 μg/ml) in the presence of $K_2Cr_2O_7$ by non-fermentative Gram negative Cr-tolerant strains

Add compounds to mineral medium [µg/ml]	Number of strains tested	Degrading strains Number/(%)
Phenol	40	18 (45.0)
Phenol + $K_2Cr_2O_7$ [40]	18	3 (16.6) *P. fluorescens* -2 *B. cepacia*
Phenol + $K_2Cr_2O_7$ [60]	18	3 (16.6) *P. fluorescens* -2 *B. cepacia*
Phenol + $K_2Cr_2O_7$ [80]	18	1 (5.5) *B. cepacia*
Phenol + $K_2Cr_2O_7$ [100]	18	1 (5.5) *B. cepacia*
Phenol + $K_2Cr_2O_7$ [200]	18	1 (5.5) *B. cepacia*

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tolerance to metal identified in the previous described experiments, only 2 strains presented turbidity during the period of incubation (48h) in presence of 5 μg/ml of $HgCl_2$.The property of using phenol was less pronounced (45 %) in the Cr-tolerant non-fermenting strains. The numeric distribution (not included in Table 2) of these strains was: *P. putida* (7), *P. fluorescens* (5), *B. cepacia* (2), *P. aeruginosa* (1), unidentified (3). Predominant inhibitory effect was also observed to chromium onto the bacterial property to phenol utilization. However, it is possible to detach the notable behavior of one of Cr-tolerant strains, identified as *Burkholderia cepacia* (JT50). This strain showed phenol assimilation in the presence of high concentration of $K_2Cr_2O_7$ (until 200 μg/ml) (Table 2).

Some studies relating to the influence of heavy metals in the degradation of benzenic compounds have pointed to the occurrence of an inhibitory effect on the process, even in very low concentrations of metallic elements. Researching the action of sub-inhibitory concentrations of Cd^{2+}, Cu^{2+}, Cr^{6+} e Hg^{2+} on the biotransformation and biodegradation of aromatic compounds by an anaerobic bacteria consortium, Kuo and Sharak Genthner[22] observed that the phenol degradation was suppressed by 1 ppm of Hg^{2+}, while for the Cr^{6+} this effect was reached with 5 ppm. Fijalkowska et al.[23] also showed a complete inhibition in the degradation of anthracene by *Rhodococcus* in the presence of lead acetate, in spite of the elevated resistance to metal demonstrated by the studied strain. Nevertheless, Pahan et al.[24] demonstrated that one strain of Hg-resistant *Bacillus pasteuri* showed, at the same time, capacity of using aromatic compounds and capacity of removing mercury from its growth medium.

The strain identified as *Burkholderia cepacia* (JT50) was chosen for additional study because its extraordinary capacity of growing in minimal medium with phenol in the presence of high $K_2Cr_2O_7$ concentration. The aim of this study was to evaluate the interaction of that strain with $K_2Cr_2O_7$ in that medium.

The lag period of samples in minimum medium with phenol and samples in minimum medium with phenol and chromium was similar, indicating that the metal did not cause a retardation in the growth. However, after 48h the optical density of control sample was 1.4 times bigger (Figure 1). Despite of this, the analysis of phenol in the filtrates, after 48 h, revealed that the aromatic compound was completely degraded.

The experiment to evaluate the capability of this strain to remove dichromate from the surrounding medium, or convert it into trivalent chromium, allowed to conclude that neither of these processes occurred, because as it was showed in the Figure 2, the levels of total chromium in the filtrates of the culture remained practically unchanged during whole experiment. It excludes the possibility of bioaccumulation/biosorption processes as survival mechanism in the presence of toxic metal. In fact, some studies about the chromate resistance mechanism have shown that it seems more related to the reduction of accumulation of the metallic salt by the resistant cells.[25-27] Since the chromate can enter the bacterial cell using the sulfate transportation system,[26,28] it is possible the JT50 strain possesses an efflux mechanism to ensure the elimination of the metal to the extracellular medium, as was detected by Alvarez et al.[29]

Based on Figure 2 it can be also be observed that there is a difference in the zero-time between the total chromium

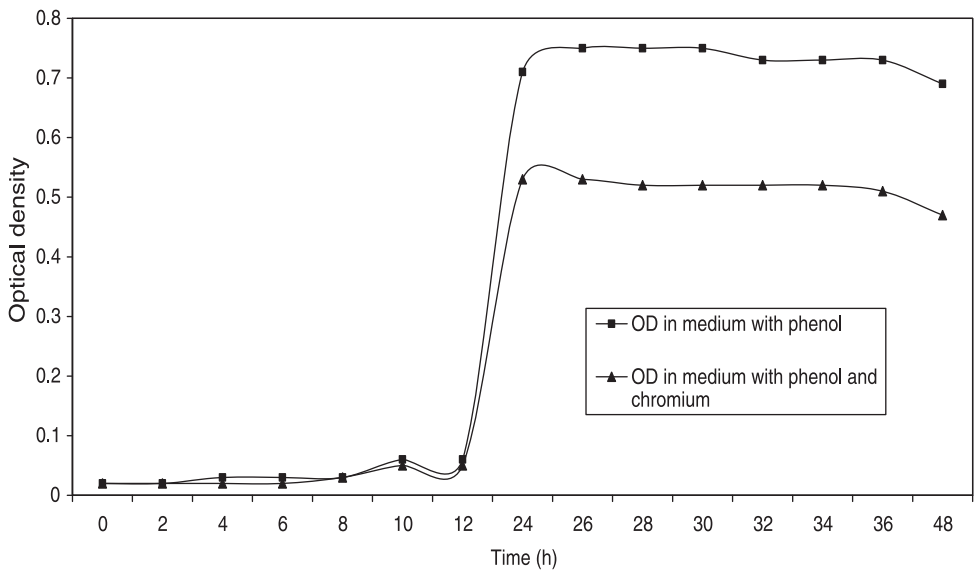

Figure 1. *Growth curve of strain B. cepacia JT50.*

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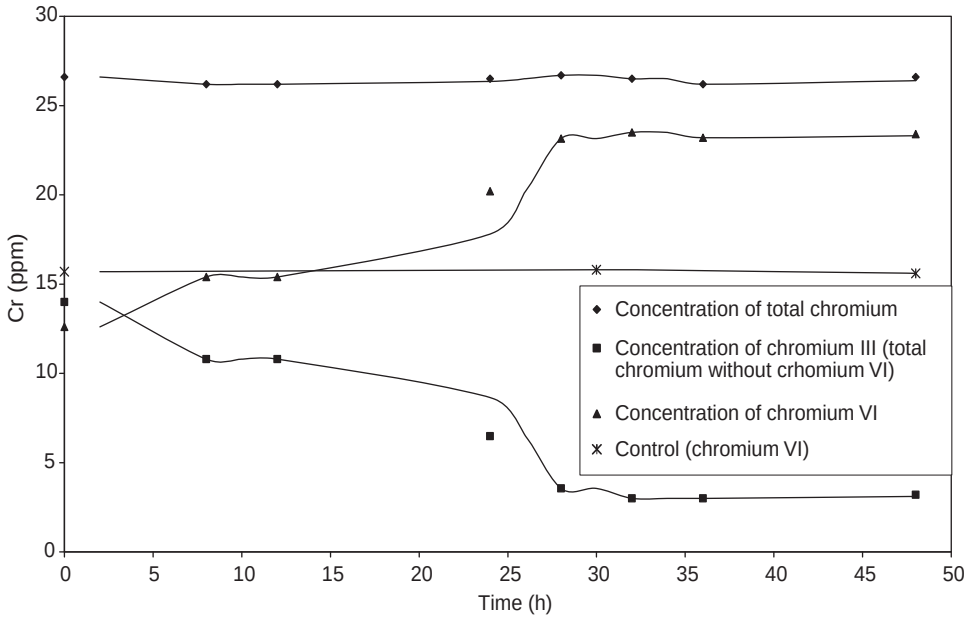

Figure 2. *Levels of total chromium, chromium VI and chromium III.*

and Cr^{6+} concentrations (total chromium: 26.6 ppm; Cr^{6+}: 12.6 ppm), which suggest the occurrence of a chemical reduction on the part of Cr^{6+} when interacting with medium components. This is also evidenced by the fact that the control medium, that is, without bacteria, showed similar decline in the value of Cr^{6+}, when compared to the value of total chromium. However, during the incubation of the strain JT50, it was observed that the Cr^{6+} concentration increased from 12.6 to 23.4 ppm, showing, therefore, the corresponding decline of Cr^{3+} (from 14 to 3.2 ppm). That is, in face of this result, it is admitted that, in some way, this microorganism promoted the oxidation to Cr^{6+} of almost every Cr^{3+} previously formed from the chemical reduction of Cr^{6+} by the components of the medium.

This result is interesting since the existing descriptions refer generally to microbial processes of chromium reduction and not the oxidation of Cr^{3+} to Cr^{6+}.[5,6] Otherwise, it's important to emphasize that evidence that phenol can be utilized as electron donors for microbial reduction of Cr^{6+} was not shown. The simultaneous chromium reduction and phenol degradation seems to be possible occur only in systems of co-culture, in which a bacterial strain degrades the phenol, while the other promotes the chemical reduction of Cr^{6+}.[30]

Therefore, the results of this study point out that the use of phenol as sole carbon source is very common in metal-tolerant strains isolated from environment exposed to contamination by these chemicals. The metals tested show pronounced effect on the capacity that strains to degrade phenol in minimal medium, despite of high degree of metal-tolerance of strains tested. However, the JT50 strain showed capacity to biodegrade this aromatic compound in presence of high chromate concentration. This process was developed without change of concentration of total chromium in the medium, and without promoting the reduction from Cr^{6+} to Cr^{3+}.

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