ROLE OF EXOPOLYMER AND ACID-TOLERANCE IN THE GROWTH OF BACTERIA IN SOLUTIONS WITH HIGH COPPER ION CONCENTRATION

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(Received April 3, 1991)

When copper-resistant *Escherichia coli* HB101 cells were transferred from regular to copper-amended, 1/3 strength Tryptic Soy Broth (TSB), there was an increase in the amount of exopolymer produced. The copper binding abilities of exopolymer for *Pseudomonas aeruginosa* 7, *P. pseudomallei* 13-1 (both copper-resistant) and from *P. putida* 3 (copper-sensitive), were also determined. If treated with phenol, about 50 to 75% of the copper binding ability of these exopolymers was removed. Although the amount of copper ion bound by the exopolymer isolated from *P. putida* 3 was about the same as from the resistant organisms, it did not grow in medium containing 0.5 ppm copper ion. When free copper ion concentration in 1/3 strength TSB increased to 0.5 ppm, the pH value of the broth dropped from 6.8 to 4.9. We believe this was what prevented the growth of the *P. putida* 3 in the solution containing the high concentration of copper ion.

Many metals (e.g., copper) when in low concentrations are essential for microbial growth but they become toxic in high concentrations. Some microorganisms, exposed to high levels of heavy metals, have evolved resistance mechanism(s), such as active exclusion (14), forming cysteine-rich proteins (8), increasing ω -cyclohexyl fatty acids level in the membrane (12), detoxification by redox conversion (3), and producing hydrogen sulfide (6) and extracellular polysaccharide (1). This enables them to maintain their functions in the presence of high concentrations of heavy metals.

Low pH or high concentrations of metal ions inhibit various microbial activities. Also the solubility of metal increases as the pH of the solution decreases. Wastewater released from the plating industry has low acidity and high concentrations of metal ions. Yet, some bacteria do proliferate in them. Hence, we were curious to know what the major factor(s) are that allow them to do so.

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MATERIALS AND METHODS

Bacterial strains. Heavy metal-resistant *Pseudomonas* spp. were isolated from the sediment of a ditch which had been receiving effluent from a metal processing plant for more than a decade. Samples were diluted in saline (0.85% NaCl; w/v) and plated on 1/3 strength Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI, USA) plates containing different amounts of copper sulfate. The plates were then incubated in the dark at 30°C for 72 h. Colonies were isolated and purified on the 1/3 strength TSA plate amended with the same amount of copper sulfate. Bacteria which have high copper-resistant ability were selected for further study. Isolates 7 and 13-1 were identified as *Pseudomonas aeruginosa* and *P. pseudomallei*, respectively, by using API 20NE (38390 Montalieu-Vercieu, France). Spontaneous copper-resistant *Escherichia coli* HB101 was obtained by plating a cell suspension on copper-amended 1/3 strength TSA plates. *Pseudomonas putida* 3 (copper-sensitive) was obtained from the culture collection of the Department of Microbiology, Soochow University, Taipei, Taiwan, Republic of China.

Growth of the selected bacteria in the presence of copper ion. The concentration of copper ion in 1/3 strength TSB medium was determined using a copper ion electrode (HNU Systems Inc., Newton Highlands, MA, USA), and adjusted to 0.001, 0.01, 0.5, 1, 2, 3, and 4 μg per ml by increasing the amount of copper sulfate added. The media were then filter-sterilized and distributed aseptically into pre-sterilized glass test tubes. After inoculation with the selected bacteria, the tubes were then incubated at 30°C in a rotating tube shaker (120 rpm) for 72 h. The turbidity increase was determined by measuring the absorbance at 600 nm with a Spectrophotometer (Spectronic 20; Bausch & Lomb, Analytical Systems Division, Rochester, NY, USA).

Copper-resistant *Escherichia coli* HB101 was first cultivated in the TSB medium overnight. Cells were then collected by centrifugation and washed with 0.85% NaCl solution. The cell suspension was added to flasks containing the TSB medium with or without copper amendment, and their optical densities (at 600 nm) were adjusted to 0.05. These flasks were then incubated on a rotary shaker (200 rpm) at 37°C. The change in turbidity was measured as above.

Isolation of exopolymer. *Pseudomonas* spp., after different periods of incubation in the 1/3 strength TSB medium at 30°C on a rotary shaker (120 rpm), were harvested by centrifugation at $8,000 \times g$ for 10 min and washed three times with saline (0.85% NaCl). The cell pellet was suspended in a solution consisting of 20 mM EDTA and 0.1 M Tris-HCl (pH 7.0) then mixed vigorously for 2 min. The supernatant fluid was separated from cells by centrifugation at $8,000 \times g$ for 10 min. Isopropyl alcohol was added to the above supernatant to a final concentration of 45% (v/v) and placed at 4°C for 36 h. The precipitate was collected by centrifugation at $10,000 \times g$ for 15 min. Alcohol was removed by heating this precipitate at 85°C overnight. This precipitate was then dissolved in distilled water and

dialyzed against deionized water at 4°C for 24 h. The insoluble material was removed by centrifuging at $20,000\times g$ for 60 min. The water in the above supernatant was removed by lyophilization. For copper-resistant *E. coli*, exopolymers were isolated from cultures that had reached an early stationary phase. Exopolysaccharides of *Pseudomonas* spp. were prepared by using phenol extraction to remove protein from the exopolymers that had been isolated.

Copper ion binding by exopolymer. Known amounts of exopolymer were first dissolved in 5 ml of deionized water prepared by using the Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). This solution was then added to 95 ml of solution with known copper ion concentration. In the control experiment, 5 ml of deionized water only was added to 95 ml of the above copper ion solution. The copper ion concentration was measured with an ion electrode.

Growth of test bacteria in broth with different pH values. The pH value of 1/3 strength TSB medium was adjusted to 5.5, 5.0, 4.5, and 4.0 with 0.1 N HCl. These media were filter-sterilized and portions (5.0 ml) of them were distributed into each of the pre-sterilized glass test tubes. The growth of the test bacteria in them was measured by the method mentioned above.

Relationship between copper ion concentration and the pH of the growth medium. Various amounts of copper sulfate were added to the 1/3 strength TSB medium. The ionic concentration of copper in the medium and its pH were determined with an ion electrode and a pH meter, respectively.

RESULTS

Growth of bacteria in TSB medium amended with copper ion

When the actual copper ion concentration in the TSB medium varied from 0.001 μg per ml to 4 μg per ml, growth of *P. putida* 3 occurred only in media containing copper ions up to 0.01 μg per ml. But *P. aeruginosa* 7 and *P. pseudomallei* 13-1 still grew in the presence of 3 and 4 μg per ml of copper ion, respectively (Table 1).

Table 1. Growth of *Pseudomonas* spp. in Tryptic Soy Broth amended with different concentrations of copper ion.

Copper ion concentration ($\mu\text{g/ml}$)	<i>Pseudomonas</i> species		
	<i>Pseudomonas</i> <i>putida</i> 3	<i>Pseudomonas</i> <i>aeruginosa</i> 7	<i>Pseudomonas</i> <i>pseudomallei</i> 13-1
0.001	+	+	+
0.01	+	+	+
0.5	—	+	+
1	—	+	+
2	—	+	+
3	—	+	+
4	—	—	+

Fig. 1. Growth of copper-resistant *E. coli* in TSB medium. Without copper amendment (●), with copper amendment (▲). Insert figure describes the growth of copper-sensitive *E. coli* in TSB medium with (▲) or without (●) copper amendment. Arrow indicates copper sulfate was added.

When copper-resistant *E. coli* HB101 was inoculated into medium containing 100 ppm of copper sulfate, there was a significant decrease in growth rate compared to the growth rate in the normal TSB broth (Fig. 1).

Binding of copper ion by exopolymers

As shown in Table 2, the amount of exopolymer produced by *P. putida* 3, *P. aeruginosa* 7, and *P. pseudomallei* 13-1 after incubation for 5 h was 580 μ g, 422 μ g, and 70 μ g per mg of oven-dried cells, respectively. If the period of incubation was extended up to 24 h, the amount of exopolymer produced by the above pseudo-

Table 2. Binding of copper ion by exopolymers produced^a by *Pseudomonas* spp.

Organism	Incubation period (h)	Exopolymer produced ($\mu\text{g}/\text{mg}$ dried cell)	Copper ion bound ($\mu\text{g}/\text{g}$ exopolymer)
<i>P. putida</i> 3	5	580	127 (66) ^b
	24	94	106 (40)
<i>P. aeruginosa</i> 7	5	422	125 (67)
	24	118	84 (60)
<i>P. pseudomallei</i> 13-1	5	70	107 (39)
	24	61	98 (ND) ^c

^a Average of three samples.^b Binding of copper ion by phenol-treated exopolymers.^c ND=Not done.

Fig. 2. Relationship between copper ion concentration (●) and the pH (▲) of the growth medium.

monads was 94 μg , 118 μg , and 60 μg per mg of oven-dried cells, respectively. The copper ion binding ability of the exopolymer produced by each of the *Pseudomonas* spp. after different periods of incubation were about the same. The only exception was that after incubation for 24 h the copper ion binding ability of the exopolymer produced by *P. aeruginosa* 7 was lower than the others (Table 2). If treated with phenol, about 50% of the copper ion binding ability of exopolymers isolated from *P. putida* 3 and *P. aeruginosa* 7 was lost and 70% was lost from the exopolymer isolated from *P. pseudomallei* 13-1.

When copper-resistant *E. coli* HB101 were inoculated into media with or

Table 3. Growth of *Pseudomonas* spp. in Tryptic Soy Broth with different pH values.

pH	<i>Pseudomonas</i> species		
	<i>Pseudomonas putida</i> 3	<i>Pseudomonas aeruginosa</i> 7	<i>Pseudomonas pseudomallei</i> 13-1
5.5	+	+	+
5.0	+	+	+
4.5	—	+	+
4.0	—	—	+

without copper supplement and incubated for 5.5 h, the amounts of exopolymer produced were 533 ng and 206 ng per mg of cell, respectively.

Relationship between copper ion concentration and pH of the growth medium

The copper ion concentrations in the growth medium measured by the ion electrode was significantly different from the actual amount of copper sulfate added. When the amount of added copper sulfate increased from 10 μ g per ml to 100 μ g per ml, the pH of the growth medium decreased from 6.8 to 5.0. As the amount of copper sulfate added increased from 100 μ g per ml to 450 μ g per ml, the subsequent pH of the growth medium only decreased from 5.0 to 4.0 (Fig. 2).

Growth of the test bacteria in media with different pHs

When *P. pseudomallei* 13-1, *P. aeruginosa* 7, and *P. putida* 3 were inoculated into TSB media of different pHs, the lowest pH that they could proliferate at were 4.0, 4.5, and 5.0, respectively (Table 3).

DISCUSSION

It is well known that microorganisms are responsible for many important processes, including nutrient cycling, in the environment. When they are under stress, these functions might be hindered and cause some unfavorable effect on the balance of the ecosystem (2, 7, 11). For bacteria to grow in the presence of heavy metal, one of the methods adapted was to prevent the metals from entering into their cytoplasm, as in forming metal-binding protein or exopolysaccharide. Mittelman and Geesey (10) reported that exopolysaccharide from a bacterial strain isolated from the metal-laden sediment of a freshwater river, bound up to 253 nM of copper ion per mg of carbohydrate. In this study, there was a significant increase in the amount of exopolymer produced by the copper-resistant *E. coli* when this bacterium was grown in copper-amended medium as compared to the normal medium. But when comparing the copper binding abilities of the exopolymers isolated from either copper-resistant or copper-sensitive pseudomonads, the differences are too small to account for the former's copper-resistant ability. When exopolymer was treated with phenol to remove protein, about 50 to 75% of the

copper-binding capacity of the exopolymer was also removed. However, the copper binding abilities of the remaining exopolysaccharide still did not account for the differences in copper resistance among pseudomonads tested.

From the results reported by various researchers, it is clear that the total amount of heavy metals in the environment does not always coincide with the extent of their toxicities (4, 5, 7, 13). It was shown that the toxicity of copper to the estuarine microbial community was a quantitative function of free copper ion activity (9). Acidity (pH) is one of the factors which affects the solubility of heavy metals. Consequently, in a solution, the concentration of heavy metals increased as the pH decreased. We have observed a close relationship between the abilities of the test organisms to grow in medium with low pH and their ability to tolerate high levels of copper ion. Clearly, the growth of the *Pseudomonas* spp. used in this study, in the presence of copper ion were not related to either the amount of exopolymer produced or to the copper ion binding ability of these exopolymers, but to their abilities to tolerate the acidity of the surrounding environment. Therefore, for microorganisms to proliferate properly in environments containing high concentrations of copper ion, they must first overcome the limitation of acidity before various protective mechanisms (e.g., producing copper ion binding exopolymer) are turned on.

This work was supported by the National Science Council, Republic of China, grant NSC77-0414-B031-01Z, NSC78-0421-B031-01Z.

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