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Effects of Silver Ion Exchanged Water Treatment Agent upon *E. Coli* RB 797 and *Bacillus* sp.

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Abstract

The effects of the silver ion-exchanged water treatment agent (Ag-Os) upon *E. Coli* RB 797 and *Bacillus* sp. have been discussed in this study. Silver ion causes a number of toxic effects with no known biological function. Silver ion-exchanged water treatment agent (Ag-Os) using oyster shell here showed antimicrobial activities. The soluble form of silver ion in water is more toxic to the growth of *Bacillus* sp. than that of *E. Coli* RB 797. The minium amount of Ag-Os needed for growth inhibition is 0.2 mg/ml for *E. Coli* RB 797 and 0.02 mg/ml for *Bacillus* sp., which is consistant with the data of the survival cell fractions. Binding studies suggested that binding of silver to the cell surface was a rapid, metabolic-independent process and different from active transport. *Bacillus* sp. showed more binding than *E. coli* RB 797. Reducing substances of the cell cultures in the presence of Ag-Os was detected using Methylen blue as an indicator. From these results, we suggest that Ag-Os is effective as an antimicrobial agent on *E. Coli* RB 797 and *Bacillus* sp. and silver binds to the cells through rapid, metabolic-indepedent process and might complex to sulfur group in the cells for its toxicity.

Key words : *silver, antimicrobial, bacteria, water treatment agent*

Introduction

Silver ion has been used for centries as one of antimicrobial agents in medical and other applied fields[1,2,3]. However, little is known about molecular aspects of silver toxicity. Biologically nonessential metal, silver shows its toxic effect in prokaryotic and eukaryotic cells. Silver ion decreases the activities of lactate dehydrogenase and glutathione peroxidase, and the peroxidation of membrane lipids. Silver ions might complex to sulfhydryl groups of sulfur-rich proteins[4]. It is likely that silver ions are bound to a certain cellular component and transported into the cell to exert its toxic effects. In fact, silver was reported to bind to basal membrane and cellular components[4], and be transported into the cell by a copper transport mechanism, p-type ATPase, CopB ATPase in membrane vesicle of *Enterococcus hirae*[5]. The interaction between azurin in *Pseudomonas aeruginosa* and silver ions was also investigated[6]. Silver ion has a high affinity for reduced azurin and completely displaces the copper ion from the native binding site[6].

Silver-resistant bacteria have been isolated and studied[7,8,9,10,11], but were not examined directly for the silver resistancy, and toxicity mechanism. It is also shown that

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silver resistance can be plasmid-encoded[12]. However, the mechanism of silver toxicity is not understood and there is little information on silver uptake and silver binding to specific components in cells.

In this study, silver ions are coupled to the water treatment agent using oyster shell and the effects of this silver ion exchanged water *treatment agent on E. Coli* RB 797 and *Bacillus* sp. have been discussed.

Materials and Methods

Production of antimicrobial water treatment agent

Fine-powder form of crushed oyster shell (average particle size of 100~200 μm) was used to make 10~20% slurry. After pH of the water treatment agent was controlled, antimicrobial metal, silver, was added to make 1~2% final concentration by ion exchange. This slurry was dried and crushed more. The final product was fine milky colored powder. Whole procedures were illustrated in Fig. 1. The antimicrobial water treatment agent (Ag-Os) has been used for this study.

Bacterial strains and growth conditions

To investigate silver toxicity, *E. Coli* RB 797 as a Gram-negative microorganism and Bacillus sp. as a Gram-positive microorganism were used. Fresh overnight cultures (about 4×10^7 cells were inoculated) were grown in 5 ml LB broth for 12 h at 37℃ for *E. Coli* RB 797 and 25℃ for *Bacillus* sp. with shaking at 120 rpm with or without Ag-Os. When Ag-Os was used in broth or agar, a stock solution was added separately to give the desired concentrations. The concentrations of Ag-Os used were 0.02 mg /ml, 0.2 mg/ml and 2 mg/ml in final.

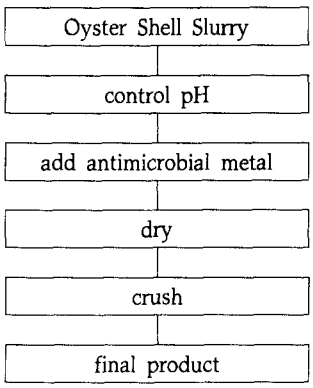

Fig. 1. Flow Diagram for the Production of Antimicrobial Water Tratment Agent Using Oyster Shell Powder

After culture inoculation, cell culture turbidity was measured spectrophotometrically at 600 nm in every 3 h. Tubes of LB broth were inoculated with mid-logarithmic phase cells in the absence of Ag-Os with same condition. Double beam spectrophotometer was used.

Counting survived cells in the presence of Ag-Os

To count survived cells, fresh overnight cell cultures (about 4×10^2, 4×10^3, 4×10^4 cells) were pre-incubated with Ag-Os (0.02 mg/ml, 0.2 mg/ml, 2 mg/ml in final) for 20 minutes and inoculated on agar plates. These plates were incubated overnight. Survived cells or resistant cells were counted.

Production of reducing substances

LB agar plates containing Ag-Os and methylene blue 0.002% (w/v) as a redox indicator were used to determine if reducing compounds were produced during the growth of cells. A positive test for reducing compounds is a clearing (reduction of methylene blue to colorless) around bacterial colonies[13]. All plates were incubated at the desired temperature for 1 week in the dark. Assay for volatile reducing compounds was by the inverted plate technique of Belly and Kydd[13]. An uninoculated agar plate containing methylene blue was inverted over an inoculated plate of LB agar with Ag-Os and the plates were sealed and incubated at desired temperature.

Binding study

Since accurate amout of the soluble form of Ag^+ from

Ag-Os was not easy to be checked, log-phase cultures in LB broth containing 20 mM glucose with 0.1 mM or 1.0 mM $AgNO_3$ (instead Ag-Os), or without $AgNO_3$, were centrifuged at 10000g for 10 min at 20℃. The cells were resuspended in 5 mM MES buffer (2[N-morpholino]ethanesulphonic acid, pH 6.5) centrifuged again and resuspended in the same buffer. In Ag^+ binding studies, cell suspensions were starved for 2 h at 37℃ to metabolise intracellular carbon sources before adding glucose, as recommended by Packer[14]. Ag^+ concentrations in the resting cell suspensions were measured with an Orion 407A ion meter and siver specific electrode (Orion Research Incorporated, Cambridge, MA, USA). MES buffer was used because it has negligible metal binding properties. Ag^+ binding and accumulation was measured at 4℃ and 37℃.

Results and Discussion

Effect of silver ion on cell growth

Fresh overnight culture cells (approximately 1×10^7 *E. Coli* RB 797, 2×10^7 *Bacillus* sp.) were incubated with Ag-Os of 0.02 mg/ml, 0.2 mg/ml, and 2 mg/ml in final, and cell density turbidity was measured photospectrometrically at 600 nm (Fig. 2&3). The effect of silver ion on the growth of *E. Coli* RB 797, a Gram-negative microorganism, shows in Fig. 2. *E. Coli* RB 797 was capable of growth in the presence of 0.02 mg/ml, but not at 2 mg/ml. When the incubation period was extended to 21 h, *E. Coli* RB 797 in the presence of 0.2 mg/ml Ag-Os recovered nomal growth. This recovery of cell growth after 9 h inhibition might indicate that cell, somehow, obtain resistancy to silver, which is remained for the furture study. The minimum amount of Ag-Os as an antimicrobial agent is at least 0.2 mg/ml for *E. Coli* RB 797. Same experiment was done with *Bacillus* sp., a Gram-positive microorganism (Fig. 3). The final concentration of Ag-Os at 0.02 mg/ml shows growth inhibition in Bacillus sp. Bacillus sp. is more sensitive to Ag-Os than that of *E. Coli* RB 797, which could suggest that Bacillus sp. might have more transport systems. However, further more experiments are needed to prove this.

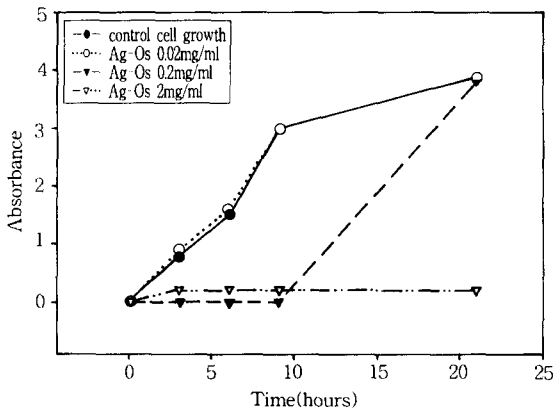

Fig. 2. The effects of Silver lons on Growth of *E. coli* RB797

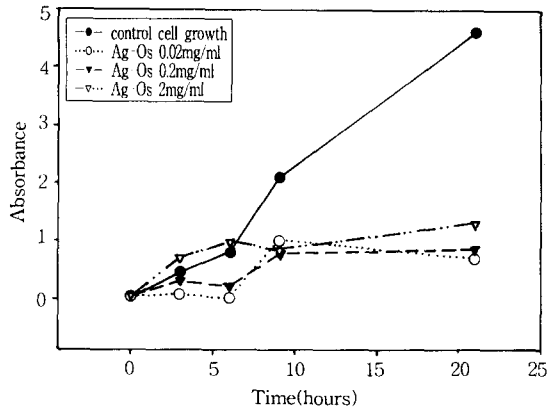

Fig. 3. The effects of Silver lons on Growth of *Bacillus* sp.

Survived fractions of microorganism in the presence of Ag-Os

Plate counts of *E. Coli* RB 797 and *Bacillus* sp. strains on LB agar with Ag-Os of 0.02 mg/ml, 0.2 mg/ml, 2 mg/ml in final concentrations were done to check the

survived fractions of microorganisms in the presence of Ag-Os. The results show that the survived fractions of *E. Coli* RB 797 is more than that of *Bacillus* sp., which is consistant to the data of growth inhibiton in Fig. 2&3 (Table 1). Survived colonies is currently being investigated for genetic studies of silver resistancy.

Table 1. Survived Cell Fractions

Inoculated cell strains	Ag-Os 0.02 mg/ml	0.2 mg/ml	2 mg/ml
E. Coli RB 797 4×10^2	390	3	0
Bacillus sp.	2	11	0
E. Coli RB 797 4×10^3	1680	11	0
Bacillus sp.	3	0	0
E. Coli RB 797 4×10^4	ND	9	0
Bacillus sp.	45	3	0
E. Coli RB 797 4×10^5	ND	148	0
Bacillus sp.	ND	15	0

ND : not determined

Production of reducing substances

In order to study whether silver interacts with sulfhydryl groups, possible reducing substances were checked in the cell culture plate in the presence of Ag-Os. Experiments were done as described in Materials and Methods. Fresh overnight culture cells were inoculated into the plates with indicated amount of Ag-Os and incubated for 1 week at desired temperature in dark. An uninoculated agar plate containing methylene blue was inverted over an inoculated plate of LB agar with Ag-Os and the plates were sealed and incubated at desired temperature. Reducing compounds were detected by the fading of the methylene blue indicator when *E. Coli* RB 797 and *Bacillus* sp. was streaked on LB agar with Ag-Os. This is a qualitative test for the presence of reducing compounds. In the absence of silver, no fading of the methylene blue was observed. Control plates did not show any change in the indicator after 1 week. However, since this experiment is an estimate of reducing activity, more sensitive and quantitative experiments, ie. lead acetate paper method[15], are required for the accurate observation of reduction.

Because reducing substances, probably, H_2S is toxic to most bacteria, it would not be beneficial to overproduce H_2S unless the sulphide complexed with metals like silver to form insoluble precipitates[16].

Silver Binding and accumulation study

Binding and Accumulation of metals into bacterial cells usually requires specific transport systems[17]. Accumulation may occur via a two stage process : a rapid, metabolic-independent surface binding to cells followed by a metabolic-dependent intracellular accumulation of the metal. Active transport is usually inhibited at 4℃, and is also decreased in the absence of an energy source.

After nonspecific binding of silver ions was eliminated by washing with 5 mM MES buffer, silver ions bound to the cells were measured by a specific silver electrode as described in Materials and Methods. Binding of Ag^+ occured in < 1 min in cell suspensions of *E. Coli* RB 797 and *Bacillus* sp. (Table 2). Ag^+ binding was not decreased by incubation at 4℃, nor incresed in the presence of glucose at the desired temperature in cells grown in the presence of $AgNO_3$. Preliminary data shows that cells grown without $AgNO_3$ did not initially bind as much Ag^+ as cells grown in the presence of $AgNO_3$ (data not shown). Incubation at 4℃, or in the absence of glucose, did not significantly decrease the binding of Ag^+ to cells. About 50% of the Ag^+ was

Table 2. Ag^+ Binding by Cell Suspensions

strains	Ag^+ binding (μmol/min) Temp	1	4	12
E. Coli RB797	37℃	4.70	7.80	8.95
	4℃	4.60	7.40	8.34
Bacillus sp.	25℃	5.35	8.40	11.20
	4℃	5.90	9.40	13.50

removed from the assay solutions during the first minute ; this rapid removal was probably binding, followed by a gradual accumulation of silver. However, other direct experiment like TEM or energy dispersive x-ray analysis could prove this accumulation of silver inside cells.

Ag^+ is not an essential metal and it is unlikely that there is a specific energy dependent transport system for it, but Ag^+ could enter cells via a transport systems for an essential metal.

Conclusions

In this study, an antimicrobial water treatment agent using oyster shell was made and its effects as an antimicrobial water treatment agent were tested on *E. Coli* RB 797 and *Bacillus* sp. Although silver ions have been used for centries as an antimicrobial metal, mechanisms of silver toxicity in bacteria are not well understood[18].

Bacillus sp. is more sensitive than *E. Coli* RB 797 in growth inhibition study (Fig. 2&3), suggesting that at least the antimicrobial water treatment agent shows different effective amount on different microorganism. The minimun effective amount as antimicrobial water treatment agent was 0.2 mg/ml for *E. Coli* RB 797, and 0.02 mg/ml for *Bacillus* sp. This is consistant to the survived fraction of the cells (Table 1).

Since Ag^+ is not an essential metal to bacteria, it is unlikely that there is a specific transport system for it. Howerer, binding and accumulation of metals into bacterial cells usually requires specific transport systems[17]. Binding of Ag^+ occured in $<$ 1 min in cell suspensions of *E. Coli* RB 797 and *Bacillus* sp. (Table 2). Ag^+ binding was not decreased by incubation at 4℃, nor incresed in the presence of glucose at 37℃ in cells grown in the presence of $AgNO_3$, suggesting that Ag^+ could enter cells via a transport systems for an essential metal.

It has been known that silver ions complex strongly to sulfhydryl groups of sulfur-rich proteins inside cells[4]. To check this, possible reducing substances could be assayed in the cell culture plate in the presence of Ag-Os. Reducing compounds were detected by the fading of the methylene blue indicator when *E. Coli* RB 797 and *Bacillus* sp. was streaked on LB agar with Ag-Os. Although this is a qualitative test for the presence of reducing compounds, this data suggest that silver might enter inside of the cells and coupled to sulfhydryl groups of cellular components.

For the conclusions, this study suggests followings ;

1) Silver ion exchanged water treatment agent here is effective on *E. Coli* RB 797 and *Bacillus* sp.
2) The minimum effective amount is 0.2 mg/ml for *E. Coli* RB 797 and 0.02 mg/ml for *Bacillus* sp.
3) The survived fractions of the cell strains show consist results.
4) Binding of silver is a rapid, metabolic-independent mechanism.
5) Bacillus sp. showed more binding than E. Coli RB 797.
6) Silver might complex to sulfur group of the cells.

Acknowledgements

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초록 : 수처리제 은이온이 *E. Coli* RB 797과 *Bacillus* sp.에 미치는 영향

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본 연구에서는 은이온이 이온 교환되어 있는 수처리제(Ag-Os)의 영향을 *Bacillus* sp.와 *E. Coli* RB 797을 사용하여 연구하였다. *Bacillus* sp.의 성장이 *E. Coli* RB 797의 성장에서보다 더 은이온에 민감하게 억제됨을 보였다. 성장억제에 필요되어지는 Ag-Os양은 0.2 mg/ml 이상에서 *E. Coli* RB 797를 0.02 mg/ml 이상에서 *Bacillus* sp.의 성장을 저해하며 Ag-Os 수처리제의 존재하에서 생존 할 수 있는 세포 수도 *E. Coli* RB 797이 더 많음을 보여 윗 결과와 일치함을 보였다. 세포에 bind되는 것은 몇 분안에 일어 나는 과정이며 starved cells에서도 일어나는 에너지를 필요치 않는 과정임을 Binding연구는 나타내고 있다. Binding은 4℃에서도 아무런 영향을 미치지 않음으로 active transport와는 다름을 알 수 있다. 또한 *Bacillus* sp.의 은이온 binding이 더 많이 일어남을 보여준다. 수처리제의 존재하에서 reducing substances가 생성됨을 methylene blue를 indicator로 사용하여 관찰하였다. 이상의 결과로 이 수처리제는 *E. Coli* RB 797과 *Bacillus* sp.에 대해 효과적이며 은이온은 빠르고 에너지를 필요로 하지 않는 과정에 의해 세포에 bind한 후 세포내로 들어가 sulfur group과 반응할 것으로 사료된다.