Bacteriolyses of Bacterial Cell Walls by Cu(II) and Zn(II) Ions Based on Antibacterial Results of Dilution Medium Method and Halo Antibacterial Test

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Abstract

Bacteriolyses of bacterial cell walls by copper (II) ions and zinc (II) ions based antibacterial results of broth dilution medium method and halo antibacterial test were investigated. From dilution medium method, MIC=625mg/L, MBC=1250mg/L for Cu²⁺ solution as bactericide action were obtained against *Staphylococcus aureus*, and also from halo antibacterial test, the high antibacterial effects for Cu²⁺, Zn²⁺ ions were obtained against *Staphylococcus epidermidis*. Bacteriolysis of *S. aureus* peptidoglycan (PGN) cell wall by Cu²⁺ ions is ascribed to the inhibition of PGN elongation due to the damages of PGN biosyntheses TG, TP and the activations of PGN autolysins. The other, bacteriolysis of *E. coli* outer membrane cell wall by Cu²⁺ ions is attributed to the destruction of outer membrane structure and to the inhibition of PGN elongation due to the damage of PGN biosynthesis TP and the activations of PGN autolysins. Furthermore, bacteriolysis of *S. aureus* PGN cell wall by Zn²⁺ ion is due to the inhibition of PGN elongation owing to the activations of PGN autolysins of amidases. The other, bacteriolysis of *E. coli* cell wall by Zn²⁺ ions is attributed to the destruction of outer membrane structure due to degradative enzymes of lipoproteins at N-, and C-terminals, whereas is dependent on the activities of PGN hydrolases and autolysins of amidase and carboxy peptidase-transpeptidase. Cu²⁺ and Zn²⁺ ions induced ROS such as O₂[•], H₂O₂[•], OH[•] producing in bacterial cell wall occur oxidative stress.

Keywords: MIC, MBC, CFU measurements and Halo antibacterial test, Cu²⁺ and Zn²⁺ ions, PGN cell wall, Outer membrane lipoproteins, Biosynthesis and autolysin, Reactive oxygen species(ROS).

Introduction

Silver, copper, and zinc of transition metals have highly antibacterial activities and are utilized as chemotherapy agents. Recently, antibacterial activities of copper, zinc and these complexes call attention to potential treatments such as prevention of serious diseases [1], Exploitation during bacterial pathogenesis [2], and cancer and tumor cell [3]. Cu²⁺ ions could kill cancer cell by Cu(II)-Cu(I) redox-cycle, the other, Zn²⁺ ions may kill tumor cell by bivalent state of Zn(II), unfortunately, the killing mechanism by Cu²⁺, Zn²⁺ ions for cancer cell remains unclear. Cancer arises from a fault in a cell. This single faulty

cell then multiplies to form a cluster of cells, namely, a tumor, that these tumor cells then spread to the whole body and these metastases can eventually kill the person [4]. Hence, the zinc homeostasis with apoptosis and necrosis for cancer cell should be eventually established. It has become apparent that zinc ions inhibit mitochondria [5], lysosomes [6], DNA [7], and nucleus [8] of cancer cell, and that regulate cell proliferation and growth [9,10], and metastasis[11] of tumor cell. However, no confirmed common relationships of zinc ions with cancer development and progression have been identified, in which killing mechanisms of cancer cell and tumor cell by Cu²⁺, Zn²⁺ ions are not yet definitely elucidated.

In this study, the broth dilution medium method test against *S. aureus* and *E. coli*, and the halo antibacterial susceptibility test against *Staphylococcus epidermidis* were carried out, where in it was turned out that antibacterial effects of Cu²⁺ and Zn²⁺ ions were examined. On the basis of the high antibacterial activities for these copper and zinc ions, the processes of bacteriolyses and destructions of bacterial cell walls by copper and zinc ions had been considered against *S. aureus* peptidoglycan (PGN) and *E. coli* outer membrane cell walls. Furthermore, the bacteriolytic mechanisms by copper (II) ion and zinc(II) ion solutions have been also revealed against both Gram-positive and Gram-negative bacteria.

Method

Two-fold broth dilution medium method tests for Cu²⁺ ion solutions

This method is quantitatively obtained for the antibacterial activity on the bactericidal assay. Bacteria intended for two-fold broth dilution medium method were treated as *Staphylococcus aureus* (NBRC12732) and *Escherichia coli* (ATCC25922). The other, the antibacterial copper ion of commercial copper (II) ion agent (Japan ion production Ltd., original Cu²⁺ solution; 500 mg/L) are used as bacteriostasis, and copper nitrate

(Cu(NO₃)₂·3H₂O, Wako Pure Reagents) of special class reagent was used as bactericide action. Firstly, the sample test tube of Cu²⁺ ion concentration of 10,000 mg/L have been prepared in heart infusion agar medium (Nissui). Next, the diluted solutions of 10-stages by two-fold dilution solution method was adjusted in tenth sample tubes for Cu²⁺ ion solution concentration of 9.8~5,000 mg/L. Afterwards, the adjustment solution within final solution of 5×10⁵ cfu/mL was prepared, and then with a sterile micropipette, fungous liquid 1 mL of bacterial suspension was respectively transferred from tube No 1 to other tubes that were inoculated into the respective tubes. Finally, the tubes were incubated at 35°C for 24 hours, in which the incubated solutions were afforded to minimum inhibitory concentration (MIC), minimum bactericide concentration (MBC), colony forming unit (CFU) measurements.

Halo antibacterial susceptibility test procedure

This method is characteristics of finding of inhibitory halo-zone measurements as less qualitative antibacterial activity assay. Halo antibacterial tests have been carried out for the nitrate and sulfate aqueous solutions against *Staphylococcus epidermidis*. The other, the antibacterial reagents were prepared metallic ions 100 mM/L aqueous solutions from metallic salt reagents. The preparation method is shown in Table 1, wherein the crystalline powders of metallic salts of 0.01 mol are dissolved in distilled water of 100 cc, preparing metallic ion concentration of 100 mM/L as antibacterial reagents (crystalline powders of 0.005 mol for silver sulfate and aluminum sulfate were used).

Firstly, *Staphylococcus epidermidis* that were collected from

the inside of the arms, in which were incubated in physiological saline aqueous water solution of salt at a thermostat of constant temperature of 35°C through a week. And then, after the bacteria are incubated in the standard planar agar-medium, the generated colonies are incubated in gradient medium above a week. Secondly, the incubated cells were suspended in physiological saline solution in which they were painted and swabbed at 100 μl share to newly prepared planar medium. Finally, the paper discs that the metallic ion solutions are stained and placed on the center of planar medium at 35°C for a week, in which the antimicrobial liquid is swabbed and spread. Afterwards, the presence or absence of an inhibitory area (zone of inhibition, W) around the disc identifies the bacterial sensitivity to the metallic ions. The diameter of growth inhibition halo is examined and measured by ruler, and then, reports are provided as susceptible, resistant, or intermediate. Measurement of the inhibition halo must be done always with ruler. Inhibitory zone width W is represented as $W = (X - 8 \text{ mm}) / 2$ (in mm) from measured inhibitory diameter X and paper-disc diameter of 8 mm, in which W is calculated from measured X.

Search and Analysis

The surface envelop cell structures of *S. aureus* as representative of Gram-positive bacterium and *E. coli* as representative of Gram-negative bacterium, molecular structures of these cell walls, molecular structure of peptidoglycan (PGN), and PGN biosyntheses and autolysins were searched in detail. Further, the reaction and the behavior of metallic ions and bacterial cell, molecular bonding manner, and zinc ion characteristics were also searched.

Table 1: Preparing metallic ions 100 mM/L aqueous solutions from metallic salt reagents

Metallic salt reagents Nitrates and Sulfates	Molecular weight	Mass[g] of metallic salt reagents dissolved in the distilled water of 100cc. Metallic ion concentration ; 100 m mol/L		
		Dissolved. in water	Metallic salts	Metallic ion conc.
Nitrates				
Silver nitrate ; AgNO ₃	169.87	1.6987g	10.8g	10,800mg/L
Copper nitrate; Cu(NO ₃) ₂ ·3H ₂ O	241.60	2.4160	6.35	6,350
Zinc nitrate; Zn(NO ₃) ₂ ·6H ₂ O	297.47	2.9747	6.54	6,540
Aluminum nitrate; Al(NO ₃) ₃ ·9H ₂ O	375.13	3.7513	0.27	2,698
Lead nitrate ; Pb(NO ₃) ₂	331.21	3.3121	20.7	20,720
Sulfates				
Silver sulfate ; Ag ₂ SO ₄	311.8	1.5590	21.6g	10,800mg/L
Copper sulfate ; CuSO ₄ ·5H ₂ O	249.68	2.4968	6.35	6,350
Zinc sulfate ; ZnSO ₄ ·7H ₂ O	287.56	2.8756	6.54	6,540
Aluminum sulfate ; Al ₂ (SO ₄) ₃ ·14~18H ₂ O	324.14	1.6207	0.54	2.698

Results

Bacteriostatic and bactericide actions of Cu^{2+} ion solution by the broth dilution medium method

Table 2 shows the bacterio stasis as disinfection agent inhibiting the bacteria growth and multiplying organism of Cu^{2+} ion, in which minimum inhibitory concentration, MIC=50mg/L

above was obtained for Cu^{2+} ion concentration range of 0.10~50 mg/L[12]. The other, table 3 indicates the results as bactericide action, in which MIC=625 mg/L and minimum bactericide concentration, MBC=1250 mg/L were obtained for Cu^{2+} ion concentration range of 9.8~5000 mg/L [13]. The killing curve of Cu^{2+} ions is shown in Figure 1(measurement's error= $\pm 6\%$), in which killing effects for the copper (II) ions appear sufficiently.

Table 2: MIC measurement of commercial Cu ²⁺ solution agents as a bacteriostatic action against <i>E.coli</i> by broth dilution medium method											
Cu ²⁺ solution agent Original conc 500 mg/L	Cu ²⁺ solution concentration(mg/L)										MIC 50 mg/L above
	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	
	+	+	+	+	+	+	+	+	+	+	
(+) ; Visible bacterial growth (-) ; No visible bacterial growth											

Table 3: MIC, MBC, and CFU of Cu^{2+} in $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ Solution as a bactericidal action against *S.aureus* by 10- fold diluted solution medium method

	Cu ²⁺ concentration (mg/L)									
Antibacterial agent $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$	5000	2500	1250	625	313	156	78	39	20	9.8
MIC	-	-	-	-	+	+	+	+	+	+
MBC	-	-	-	+	+	+	+	+	+	+
CFU(cfu/ml)	<10	<10	<10	1.1×10^2	3.1×10^8	4.0×10^8	4.5×10^8	5.1×10^8	5.5×10^8	5.3×10^8
(+) ; Bacterial growth(Visible turbidity), (-) No Visible Bacterial growth

Figure 1: Relationship between increasing Cu^{2+} concentration(mg/L) and viable counts(CFU/mL) against *S.aureus*

Halo antibacterial susceptibility tests

Figure 2 indicates the bar-graphs of the relationships between various metallic ions and halo inhibitory zones width. Figure 3 shows the sample surface appearances of inhibitory zone after halo antibacterial tests for nitrate and sulfate solutions against *Staphylococcus epidermidis*. For the nitrate solutions, it is found

that the antibacterial effect has nothing for alkalimetals, alkali earth metals, but, various ions of Al^{3+} , Zn^{2+} , Pb^{2+} , Cu^{2+} , Ag^+ indicate the antibacterial effects. The order of the antibacterial effect is as following; $\text{Cu}^{2+} > \text{Zn}^{2+} > \text{Ag}^+ > \text{Pb}^{2+} > \text{Al}^{3+}$. The other, in sulfate solutions, Al^{3+} , Zn^{2+} , Cu^{2+} , Ag^+ have higher antibacterial activities. From these observations, the antibacterial order is $\text{Zn}^{2+} > \text{Cu}^{2+} > \text{Ag}^+ > \text{Al}^{3+}$, in which Zn^{2+} ions indicate the highest antibacterial effect.

Figure 2: Relationship of halo inhibitory zone (in mm) and some metallic ions of aluminum, zinc, lead, copper and silver nitrates and sulfates against *Staphylococcus epidermidis*

Figure 3: Halo test sample appearances forming the inhibitory zone of bacterial growth for the nitrates (A) $\text{Cu}(\text{NO}_3)_2$, (B) $\text{Zn}(\text{NO}_3)_2$, (C) AgNO_3 , (D) $\text{Pb}(\text{NO}_3)_2$, (E) $\text{Al}(\text{NO}_3)_3$, and for the sulfates (F) ZnSO_4 , (G) CuSO_4 , (H) Ag_2SO_4 , (I) $\text{Al}_2(\text{SO}_4)_3$

Results of Search and Analysis

(1) *S. aureus* and *E. coli* Cell walls, Action Sites of PGN biosyntheses of transglycosylase TG and transpeptidase TP and PGN autolysins

S. aureus surface cell envelop consists of teichoic acids, lipoteichoic acids, and thick peptidoglycan (below PGN) cell wall [14], where as *E. coli* cell wall comprised of lipid A, lipopolysaccharide, porin proteins, outer membrane of lipoprotein, and thinner 2-7 nm PGN layer in 30-70 nm periplasmic space[14]. Figure4 shows the molecular structure of *S. aureus* PGN cell wall, including the action sites of PGN biosynthesis enzymes of TG/TP, and PGN for the autolysins and Lysostaphin enzyme. Furthermore, figure 5 represents the molecular structure of *E. coli* cell wall and periplasmic peptidoglycan, containing the action sites of the hydrolases of lipoproteins, the peptidoglycan biosynthetic enzymes TG/TP, and the autolysins. Further, interactions of PGN molecular structure, PGN syntheses and autolysins influence

essentially in any event the bacteriolysis of bacterial cell walls.

(2) Characteristics of Zinc Sulfate Solution

Zinc is redox-inert and has only one valence state of Zn (II). In proteins, the coordination is limited by His, Cys, Glu, and sulfur donors from the side chains of a few amino acids. In zinc sulfate solution, ZnSO_4 is dissociated into aqua zinc ion $[\text{Zn}(\text{H}_2\text{O})_6]^{2+}$ and sulfuric ion $(\text{SO}_4)^{2-}$. Aqua zinc ions are liable to be bound to ligand L having negative charge. The sulfuric ion has bactericidal inactivity [15].

By the reaction of Zn^{2+} ions with *S. aureus* surface, zinc-proteins are formed, on the ground that is due to formation of S-atom containing Zn-cysteine complex in bacteria [16].

Figure.4: The molecular structure of *S. aureus* PGN cell wall, and the action sites of PGN biosynthesis enzymes of TG/TP, PGN for the autolysins, and Lysostaphin enzyme

Figure.5: Molecular structure of *E.coli* cell wall and periplasmic PGN, and the action sites of the hydrolases of LPT, the PGN synthetic enzymes TG/TP, and the autolysins

Discussions

Bacteriolysis of *S. aureus* PGN cell wall by Cu²⁺ ions

(1) Bacteriolysis by balance deletion between biosynthesis enzyme and decomposition enzyme (autolysin) in PGN cell wall

For the sake of growth of *S. aureus* PGN cell wall, there is necessarily required for the adequate balance between PGN biosynthesis and PGN autolysin. When the balance is broken by Cu²⁺ penetration, Cu²⁺ ions are self-catalytically treated as coenzyme, that this is indicated that activation of autolysin is proceeded, in which bacteriolysis and killing may result. Hence, bacteriolysis of *S. aureus* PGN cell wall by Cu²⁺ ions is due to inhibition of PGN elongation owing to the damages of PGN synthetic TG/TP and the activations of PGN autolysins.

(2) Inhibition of polymerization of glycan chains bonding and cross-linking of side peptide

Cu²⁺ ions inhibit polymerization of glycan chains, forming copper complex in which is partial action sites of glycan saccharide chains. L is coordinated molecular.

Copper-complexes on saccharide chains may be

The other, Cu²⁺ ions inhibit cross-linked reaction by peptide copper complex formation bonding to side-peptide chains.

Peptide copper complex may be 3N-Cu-O, Cu (Gly-L-Ala)₂H₂O.

Specially, Cu^{2+} ions react with cross-molecular penta glycine(Gly)₅, copper-glycine complex may be formed.

Amino acid: $\text{Cu}^{2+} + \text{Gly}^- \rightarrow \text{Cu}(\text{Gly})^+$, $\text{Cu}(\text{Gly})^+ + \text{Gly}^- \rightarrow \text{Cu}(\text{Gly})_2$

Peptido: $\text{Cu}^{2+} + \text{GlyGly} \rightarrow \text{Cu}(\text{GlyGly})$, $\text{Cu}(\text{GlyGly}) + \text{Gly}^- \rightarrow \text{Cu}(\text{GlyGlyGly})^-$

Bacteriolysis and destruction of *E. coli* outer membrane cell wall by Cu^{2+} ions

(1) Inhibition of outer membrane cell wall

Cu^{2+} ions inactivate catalyst enzyme with forming Cu^+ ions.

By the penetration of Cu^{2+} ions, as shown in figure 5, the activations of amidase enzyme of N-terminal and endopeptidase enzyme of C-terminal are enhanced[17,18]. Accordingly, the activations of decomposition at N-, C-terminals of lipoproteins may occur with the destruction of outer membrane structure.

(2) Inhibition of biosynthesis and activation of autolysin, or regulation and deletion of autolysin.

Inhibition of *E. coli* PGN by Cu^{2+} ions is reported [19], however, the site of concrete action is not described. In *E. coli*, it is unlikely thought that Cu^{2+} ions inhibit both TG and TP [20]. The other, it is unclear that Cu^{2+} inhibit the polymerization of NAM and NAG chains. It is perhaps simpler to think that TP enzyme of cross-linked reaction is inhibited by Cu^{2+} ions and the activation of PGN autolysin occurs. By the accumulation of Cu^{2+} ions in periplasmic space, it might be possible that bacteriolysis of cell wall occur by the activation of PGN autolysin within periplasmic space. Many autolysins of *E. coli* are regulated by metals ion such as Hg^{2+} , Cu^{2+} [21]. This regulation or deletion of decomposition enzyme inhibits PGN elongation, in which the bacteriolysis of the cell wall is induced. These facts are consistent with that the destruction by bacteriolysis of cell wall had been observed against *E. coli*.

Hence, bacteriolysis of *E. coli* cell wall by Cu^{2+} ions occurs by destruction of outer membrane structure due to degradation of lipoprotein at N-, C-terminals, damage of TP enzyme and activations of PGN autolysins. Furthermore, deletion of PGN autolysin also becomes bacteriolytic factor.

(3) Antibacterial activities of cell membrane and cytoplasm

Reactive oxygen species (ROS) O_2^- and H_2O_2 generated in cell wall, permeate into cell membrane and cytoplasm, in which in cell membrane high reactive $\cdot\text{OH}$ and OH^- are formed by Haber-Weiss and Fenton reactions.

Haber-Weiss reaction[22]; $\text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2$

Fenton reaction [23]; $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Cu}^{2+}$

Furthermore, new ROS productions occur by Fenton-like type. L=Ligand

$\text{LCu(II)} + \text{H}_2\text{O}_2 \rightarrow \text{LCu(I)} + \cdot\text{OOH} + \text{H}^+$

$\text{LCu(I)} + \text{H}_2\text{O}_2 \rightarrow \text{LCu(II)} + \cdot\text{OH} + \text{OH}^-$

As above-mentioned, the bactericidal processes of bacteriolysis of the *S. aureus* and *E. coli* cell walls by Cu^{2+} ions, and also the antibacterial activities of cell membrane and cytoplasm are shown in Table 4.

Bacteriolysis of *S. aureus* PGN Cell Wall by Zn^{2+} Ions

(1) PGN biosynthesis enzymes of transglycosylaseTG and transpeptidase TP

Wall teichoic acids are spatial regulators of PGN cross-linking biosynthesis TP[24], however, it is not explicit whether zinc ions could inhibit both TG and TP enzymes of the PGN, wherein is due to uncertain relation between wall teichoic acids biosynthesis and PGN biosynthesis.

(2) Inhibition of PGN elongation due to the activations of autolysins

Zn^{2+} binding Rv3717 showed no activity on polymerized PGN and but, it is induced to a potential role of N-Acetylmuramyl-L-alanine Amidase [25], PGN murein hydrolase activity and generalized autolysis; Amidase MurA [26], Lytic Amidase LytA [27], enzymatically active domain of autolysin LytM [28], Zinc-dependent metalloenzyme AmiE [29] as prevention of the pathogen growth, and Lysostaphin-like PGN hydrolase and glycylglycine endopeptidase LytM [30]. It is thought that the activations of these PGN autolysins could be enhanced the inhibitions of PGN elongation simultaneously, with bacteriolysis of *S. aureus* PGN cell wall.

(3) Production of reactive oxygen species (ROS) against *S. aureus*

O_2^- and H_2O_2 permeate into membrane and cytoplasm, that DNA molecular is damaged by oxidative stress [31]. For the penetration of zinc ions to PGN cell wall, the ROS production such as superoxide anion radical O_2^- , hydroxyl radical $\cdot\text{OH}$, hydrogen peroxide H_2O_2 occurred from superoxide radical O_2^- molecular[32]. O_2^- and H_2O_2 permeate into membrane and cytoplasm, and then, DNA molecular is damaged by oxidative stress [31].

Bacteriolysis and destruction of *E. coli* Cell Wall by Zn^{2+} Ions

(1) Permeability of Zinc Ions into *E. coli* Cell Wall

E. coli cell wall is constituted of lipo polysaccharide (LPS), lipoproteins (LPT), and PGN, thinner layer within periplasmic space. The first permeability barrier of zinc ions in the *E. coli* cell wall is highly anionic LPS with hydrophobic lipid A, core

Table 4 : Bactericidal action processes of Cu^{2+} solutions within the cell wall/the cell membrane/the cytoplasm against *S.aureus* and *E.coli*

Cu ²⁺ ion solution	Cell wall			Cell membrane	Cytoplasm
Cu^{2+}	<i>S.aureus</i> cell wall				
	PGN layer cell wall			• Cu-ammine complex and Cu-protein formations	• Cu-complex and Cu-3N-O complex formations
	Cu^{2+} , Cu^+			Cu^+ , Cu^{2+}	Cu^{2+}
	<ul style="list-style-type: none"> • Bacteriolysis of PGN cell wall by TG,TP synthesis inhibitions and activations of <i>S. aureus</i> PGN autolysins • Cu complex of glycan saccharide chain and Cu peptide complex formations • Reactive oxygen species O_2^-, H_2O_2 			O_2^-	• OH
Cu^{2+}	<i>E.coli</i> cell wall			H_2O_2	OH^-
	Lipopolysaccharide (LPS)	Outer membrane(OM) Lipoprotein(LPT)	Periplasmic space(PS)	• OH	(O_2^-)
	Corepolysaccharide	OmpF,A,C,	PGN layer	OH^-	H_2O_2
	Phosphorus lipid,	Porin, Protein	Miscible protein	• OOH	
	Cu^{2+} , Cu^+	Cu^{2+} , Cu^+	Cu^{2+} , Cu^+	• OH • formed by Haber-Weiss/Fenton reactions	• DNA/RNA synthesis inhibition
	• Variation of charge properties	• Cu ion binding proteins	• Cu ²⁺ ions accumulation	• Accumulation of O_2^- and H_2O_2	Substitution of Cu^{2+} ions into DNA hydrogen bond base pairs
	• Cu ion induced increase in permeability	• Damages of outer membrane structure due to the degradable enzymes of lipoprotein at N-, C-terminals and the activation of PGN hydrolases	• Inhibitions of PGN elongation by the deletions of PGN-TP enzyme and PGN autolysins		
	• O_2^- , Cu^+	• Cu^+ , H_2O_2	• Cu^+ , $\text{HO}^\bullet$, H_2O_2		

polysaccharide, O-polysaccharide, in which zinc ions may be possible for the inhibition of LPS biosynthesis, owing to that promotes formation of metal-rich precipitates in a cell surface [33]. In zinc ion uptake across the outer membrane, the lipoproteins of Omp A, Omp C, Omp F porins have a role for at least some of these proteins in Zn^{2+} uptake, in which the lipoproteins have metallic cation selective and hydrophilic membrane crossing pore, to be effective for zinc transfer [34]. Zinc (II) ions react with -SH base, and then H_2 generates. Zinc bivalent is unchangeable as $-SZn-S-$ bond 4-coordinated.

(2) Destruction of outer membrane structure of *E. coli* cell wall by hydrolases of lipoproteins at C-, N-terminals

ZnPT (zinc pyrithione) and Tol (Tol proteins)-Pal (Protein-associated lipoprotein) complex are antimicrobial agents widely used, however, it has recently been demonstrated to be essential for bacterial survival and pathogenesis that outer membrane structure may be destroyed [35,36].

(3) Inhibition of PGN elongation due to the damage of PGN synthesis enzyme of zinc-protein amidase in periplasmic space, and the activities of PGN autolysins

The zinc-induced decrease of protein biosynthesis led to a partial disappearance of connexin-43 of protein synthesis in neurons [37], but it is unknown whether PGN biosynthesis is inhibited. Further, it is also unclear whether the both TG/TP should be inhibited by the zinc ions [38,39,40]. The other, zinc ions were accumulated in *E. coli* periplasmic space, in which the zinc ions are spent to the activation of bacteriolysis of the cell wall. Zinc depending PGN autolysin, amidase PGRPs [41], zinc

metallo enzymes AmiD [42], zinc-containing amidase; AmpD [43], zinc-present PGLYRPs [44] serve to be effective for the PGN autolysins. It is particularly worth noting that enhancement of the activities of autolysins is characterized on PGN carboxypeptidase-transpeptidase IIW [45] requiring divalent cations. Accordingly, the inhibition of PGN elongation had occurred by zinc ion induced activities of PGN hydrolases and autolysins.

(4) ROS production and oxidative stress against *E. coli*

Zinc ions reacted with -SH, and H^+ generates. In *E. coli*, free radicals O_2^- , OH^- , $\cdot OH$ and H_2O_2 are formed as follows [46]:

In the cell wall, reacting with polyunsaturated fatty acids:

Zinc-containing Peptidoglycan Recognition Proteins (PGRPs) induce ROS production of H_2O_2 , O_2^- , $HO\cdot$, the ROS occur the oxidative stress, and killing by stress damage [47].

Thus, from above-mentioned results, the processes of the bacteriolysis of *S. aureus* PGN and *E. coli* outer membrane cell walls by the permeability and the antibacterial activities of Zn^{2+} ions are summarized in Table 5.

Table 5 Bacteriolytic processes of *S.aureus* PGN and *E.coli* outer membrane cell walls by the permeability and the antibacterial activities of Zn^{2+} ions

Zn^{2+} ions solution	<i>S.aureus</i> Cell Wall		
	Teichoic acid, Lipoteichoic acid, Peptidoglycan layer, Proteins		
Zn^{2+}	$\xrightarrow{\quad} Zn^{2+} \quad O_2^-, H_2O_2, \cdot OH, \cdot NO, ONOO^-$ <ul style="list-style-type: none"> • Teichoic acids are spatial regulators of biosynthesis of PGN cross-linking TP enzyme • Zn^{2+} binding proteins • It is unknown whether zinc ions inhibit the PGN biosynthesis TG/TP enzymes • Activations of PGN autolysins of amidases • Bacteriolysis and destruction of PGN cell wall due to inhibition of PGN elongation • ROS productions and the oxidative stress 		
Zn^{2+} ion solution	<i>E.coli</i> cell wall		
	Lipopolysaccharide(LPS) Lipid A, Core polysaccharide	Outer Membrane Lipoprotein, Porins OmpF,A,C	Periplasmic Space PGN layer
Zn^{2+}	$\xrightarrow{\quad} Zn^{2+}, H^+$ <ul style="list-style-type: none"> • Negative charge • Hydrophobic Lipid A • Inhibition of LPS biosynthesis • $Zn^{2+} + 2(-SH) \rightarrow -SZn-S- + H^+$ 	$\xrightarrow{\quad} Zn^{2+}, O_2^-, H_2O_2,$ <ul style="list-style-type: none"> • Porin proteins of hydrophilic channels • Zn binding proteins • Destruction of outer membrane structure due to degradative hydrolases of lipoprotein at C- and N-terminals • $LOO\cdot, L\cdot$(Fatty acid) 	$\xrightarrow{\quad} Zn^{2+}, O_2^-, H_2O_2, OH^-, \cdot OH$ <ul style="list-style-type: none"> • Zn accumulation in periplasmic space • Inhibition of PGN elongation due to activations of PGN autolysins of amidase and carboxypeptidase-transpeptidase

Conclusions

(1) From the result of antibacterial activities of Cu^{2+} ion solution by the two-fold broth dilution medium method, for bacteriostasis $\text{MIC}=50$ mg/L above was obtained in Cu^{2+} concentration range of $0.10\sim 50$ mg/L against *E. coli*. The other, for bactericide action $\text{MIC}=625$ mg/L and $\text{MBC}=1250$ mg/L were obtained in Cu^{2+} concentration range of $9.8\sim 5,000$ mg/L against *S. aureus*.

(2) From halo-antibacterial susceptibility tests of metallic ion concentration of 100 mM/L against *Staphylococcus epidermidis*, the order of bacterial effect for nitrate solutions is as follows: $\text{Cu}^{2+}>\text{Zn}^{2+}>\text{Ag}^{+}>\text{Pb}^{2+}>\text{Al}^{3+}$. The other, in the sulfate solutions, the order is $\text{Zn}^{2+}>\text{Cu}^{2+}>\text{Ag}^{+}>\text{Al}^{3+}$. The appearance of the highest antibacterial activity is found to be the zinc sulfate solution.

(3) Bacteriolysis of *S. aureus* PGN cell wall by Cu^{2+} ions is caused for the inhibition of PGN elongation due to damages of PGN synthetic TG/TP and activation of PGN autolysins. The other, bacteriolysis of *E. coli* outer membrane cell wall by Cu^{2+} ions is attributed to the destruction of outer membrane structure and to the inhibition of PGN elongation due to the damage of PGN biosynthesis TP and the activation of PGN autolysins.

(4) Bacteriolysis and destruction of *S. aureus* PGN cell wall by Zn^{2+} ions are due to the inhibition of PGN elongation by the activities of PGN autolysins of amidases. The other, bacteriolysis of *E. coli* cell wall by Zn^{2+} ions are due to destruction of outer membrane structure by degrading of lipoprotein at C-, N-terminals, owing to PGN formation inhibition by activities of PGN autolysins of amidase and carboxypeptidase-transpeptidase.

(5) By the penetration of copper, or zinc ions into bacterial cell wall, productions of O_2^- , H^+ , H_2O_2 , ONOO^- occurs. The other, in *E. coli* cell wall, the productions of O_2^- , H^+ in outer membrane, and H_2O_2 , OH^- , $\cdot\text{OH}$ in periplasmic space occur. These ROS and H_2O_2 damage the cell membrane and the DNA molecules by oxidase stress.

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