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The Cell and Genetic Characteristics of Slime Forming Bacteria on Antibiotic Resistance in the Paper-making Process*1

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ABSTRACT

The seven strains, *Pseudomonas paucimobilis*, *Pseudomonas cepacia*, *Staphylococcus auricularis*, *Staphylococcus saprophyticus*, *Acidovorax* spp., *Acinetobacter calcoaceticus*, and *Actinobacillus capsulatus* were tested with three slimicides. Most of the tested bacteria were inhibited with slimicide K (an isothiazolin based compound), even at its low concentration, except for *Actinobacillus capsulatus* and *Staphylococcus auricularis*. Both slimicides B (an organic bromine based compound) and S (aldehydes) also couldn't prevent these two strains even at their highest concentration. Five different sizes of plasmid DNA were isolated from *Actinobacillus capsulatus*. *Staphylococcus auricularis*, a gram-positive bacteria, showed the slimy substances around its cell distinctively. The results suggest that two strains, *Actinobacillus capsulatus*, *Staphylococcus auricularis*, have presumably developed a resistance to the slimicide, by plasmid DNA or slimy substance.

Our findings also suggest that not only gram-negative bacteria, but also gram-positive bacteria should not be neglected.

Keywords: slime, slime-forming bacteria, gram-negative bacteria, gram-positive bacteria, bacterial growth, slimicide, scanning electron micrograph, protein, plasmid, electrophoresis

1. INTRODUCTION

Paper manufacturing entails the processing of large amounts of natural materials under conditions favorable for the growth of microorganisms. Unfortunately, with the recent trend towards more closure of the white water system because of environmental constraints, the scale up and the use of higher secondary fibers, the control of microorganisms becomes more challenging. One of the noticeable troubles caused by microorganisms is the forming of slime, a deposit which results from the growth of microorganisms. The term "slime" is a broad

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one covering a wide range of viscous, or mucous materials and mixtures found in aqueous environments. Bacteria itself secretes the mucous materials called slime, or cell-surface polysaccharides, which forms a capsule around the cell. They serve a structural role, benefiting the bacterium by enabling attachment to the surface, providing protection from environmental stresses (Torres-Cabassa *et al.*, 1987). These slime secreting bacteria are already known for forming slime or biofilm in paper and board machines (Delaporte *et al.*, 1960). The first step in the process of slime formation is that organic materials are adsorbed to the surface of machinery, which leads bacteria to be attached to the surface. The second step is bacteria, which is capable of forming slime, accumulates with organic and inorganic components (Au C *et al.*, 1995), and causes paper breaks, holes, spots, discoloration and unpleasant odors in product as well as induce corrosion in machinery. Then, of course, this leads to significant losses in profit (Bandala *et al.*, 1987). Historically, slime formation has been treated by the addition of antimicrobial agents to industrial water, often referred to as biocides or slimicides. They have been critical for controlling microorganism growth in the nutrient-rich environment of pulp and paper manufacturing. But, biocides that mills used to control slime tend to lose effectiveness over time, and microorganisms can develop a resistance to a given biocide (Araki *et al.*, 1990). To prevent the slime formation, it would be unrealistic to isolate and identify all types of microorganisms that are present. However, knowing that slime secreting bacteria cells initiate the process of adhesion by binding to the surface and give the resistance of biocides and slimicides (Araki *et al.*, 1990), we can not overlook presently prevailing bacteria and their characteristics in the paper making process.

In this study, the slimicides currently used in paper mills are tested on the bacteira isolated from the paper-making process. Moreover, their genetic and cellular properties are characterized by isolating plasmid, and scanning electron microscopy observation. The relationship between the bacteria's characteristics and their resistance to the slimicides are also determined.

2. MATERIALS and METHODS

2.1 Bacterial strains

Bacteria used in this work are as follows; *Pseudomonas paucimobilis* (*Ps. paucimobilis*), *Pseudomonas cepacia* (*Ps. cepacia*), *Staphylococcus auricularis* (*St. auricularis*), *Staphylococcus saprophyticus* (*St. saprophyticus*), *Acidovorax* spp. *Acinetobacter calcoaceticus* (*Ac. calcoaceticus*), *Actinobacillus capsulatus* (*Ac. capsulatus*). They were incubated from frozen stocks which were maintained at -70°C in TYE broth (10 g bacto tripton, 5 g yeast, 5 g NaCl/1 L d.d. water) containing 25% glycerol.

2.2 Isolation of plasmid DNA from bacteria

Overnight cultures were pelleted, and resuspended in 350 μL STET buffer (0.1 M NaCl, 10 mM Tris HCl, 1 mM EDTA, 5% Triton X-100), 25 μL lysozyme (10 mg/mL, 10 mM Tris HCl pH 8.0) and vortexed for 3 minutes. The suspension was incubated in boiling water for 40 seconds and centrifuged for 10 minutes at 25°C. After the pellet was removed, 2.5 M NaOAcetate (pH 5.2) was added to the supernatant, and incubated at 25°C for 5 minutes. The suspension were centrifuged for 5 minutes at 4°C and vacuum removed. 1 mL 70% ethanol was added and centrifuged for 5 minutes at 4°C. After centrifugation, ethanol were vacuum

removed. The pellet was resuspended in 50 μL TE buffer (50 mM Tris, 5 mM EDTA, pH 7.5). This DNA was loaded in 1% agarose gel and run at 100 V for 20 minutes. The gel were illuminated on a short wave transilluminator and photographed with polaroid UV 55 mm.

2.3 Antimicrobial activity of slimicide

2.3.1 Slimicide concentration and inoculum

Slimicide used in this work are as follows: an organic bromine based compound as slimicide B, an isothiazolin based compound as slimicide K, aldehydes as slimicide S. This slimicides were used at concentration of 5, 10, 15 and 20 ppm in antimicrobial assay. For the inoculum, nutrient broth(beef extract 3 g, peptone 5 g, agar 15~20 g, distilled water 1 L, Difco) was dissolved in 1 L distilled water and adjusted to pH 7 with 0.1 N HCl and then agar 15 g was added and autoclaved at 121°C for 15 minutes. 0.1 mL of each strains from overnight liquid culture was spread out on the plate and incubated for more than 24 hours at 37°C. Colonies were collected with a autoclaved cotton rod and diluted in autoclaved 0.8% saline solution to an absorbance 0.85~0.89 at wave length 660 nm. This adjusted bacteria suspension was used as an inoculum.

2.3.2 Culture condition and measurement of activity

Nutrient broth was adjusted to pH 7 with HCl and distributed 9 mL to a test tube, then autoclaved at 121°C for 15 minutes. After slimicides and inoculum 0.1 mL were added, these cultures were incubated, shaking in 60 rpm at 37°C. After shaking incubation for 24 hours, test tubes were voltexed, and microbial growth was measured by reading the observance at wave length 660 nm by UV spectrophotometer (Shimadzu UV-120-02, Japan).

2.4 Scanning electron microscopy

The culture, incubated at 37°C for 24 hours, was filtered with 0.2 μm millipore filter. The collected cells were fixed in 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.1) at 4°C for 2 hours. Twice washed in 0.1 M cacodylate (pH 7.1) for 15 minutes, then the cells were refixed in 1% OsO_4 for 1.5 hours at 4°C and washed in the same buffer two times for 15 minutes. The samples were dehydrated in an ethanol series (60, 70, 90, 95%) and dehydrated in absolute ethanol three times for 15 minutes each. Finally, the specimens were twice treated with *t*-butyl alcohol for 15 minutes, by a freezing dryer and observed under a scanning electron microscope.

2.5 Protein pattern of *Staphylococcus auricularis* treated by the selected concentration of slimicide

1 mL precultures was inoculated into 200 mL TYE and incubated, shaking at 37°C till reaching 100 KU. 0.3 mM IPTG (isopropyl-D-thiogalacto-pyranoside) was added and incubated, shaking at 30°C for two hours. After the shaking incubation, cells were pelleted at 12,000 rpm for 10 minutes, and resuspended in 5 mL cell lysis buffer (20 mM Tris base, 500 mM NaCl, 0.1% EDTA, 0.1× TritonX-100). They were sonified 1 minute action / 1 minutes break / 5 times at 20% duty cycle and output 6.5. This clarified cell was loaded in glycine gel (resolving gel: d.d. water 2.3 mL, 38% acrylamide mix 1.3 mL, 1.5 M Tris (pH 8.0) 1.3 mL, 10% SDS 0.05 mL, 10% ammonium persulfate 0.05 mL, TEMED 0.003 mL, stacking gel: d.d. water 1.4 mL, 38% acrylamide mix 0.25 mL, 1.5 M Tris (pH 6.8) 0.25 mL, 10%

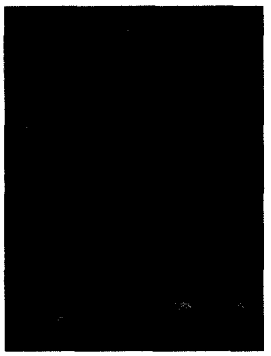
SDS 0.02 mL, 10% ammonium persulfate 0.02 mL, TEMED 0.002 mL), and ran at 30 V for 1.5 hours and 70 V till the end.

3. RESULTS and DISCUSSION

3.1 Isolation of plasmid DNA from bacteria

Bacteria have several factors which enable it to resist antibiotics and other toxic agents. One of them is R plasmid (resistance plasmid). To determine the resistance of bacteria to slimicides in pulp and paper-making process, plasmids were isolated from the bacteria. The result showed, as in Photo 1, that only two strains among the seven bacteria had the plasmids.

There were five different plasmids for *Ac. capsulatus* and only one for *Ac. calcoaceticus*. The sizes of the isolated plasmids from the two strains were 14, 12, 7.4, 6.0, and 4.0 kb for *Ac. capsulatus* and 2.1 kb for *Ac. calcoaceticus*. The growth of *Ac. capsulatus* with 5 plasmids in the culture with slimicides was rather good in comparison to other bacteria tested with slimicides, which made the result very unique. Considering the results, one or more of the plasmids isolated in *Ac. capsulatus* are presumed to be the genes that give the host cell resistance to antibiotics and other toxic agents. Some plasmids isolated from the bacteria are known as being a resistance plasmid, and they can spread rapidly by conjugative transfer through the bacterial population. Knowing that *Ac. capsulatus* existed in the white water tank (Oh *et al.*, 1997), which is considered the origin of the slime problem, and is believed to be a slime secreting bacteria that is able to grow in a wide pH range (Freis *et al.*, 1984), the result implies that *Ac. capsulatus* presumably plays a role in causing the slime problem in the tested paper-making process.



M 1 2 3 4 5 6 7

Photo 1. Plasmids patterns isolated from the bacteria.
M. marker
1. *Pseudomonas paucimobilis*
2. *Staphylococcus saprophyticus*
3. *Pseudomonas cepacia*
4. *Actinobacillus capsulatus*
5. *Staphylococcus auricularis*
6. *Acidovorax* sp.
7. *Acinetobacter calcoaceticus*

3.2 Slimicide susceptibility of bacteria

Three different slimicides were tested against seven bacteria from the paper making process at concentrations of 5, 10, 15 and 20 ppm. The result of the bactericidal effect are illustrated in Figs. 1~4.*

The growth of *Ps. paucimobilis* was effectively inhibited by all the applied slimicides at the concentration of above 5 ppm. The growth of *St. saprophyticus* was prevented 6~60% by B and S slimicides at the concentration of 5 and 10 ppm, but was inhibited 100% by slimicide B and S at the concentration of 15 and 20 ppm, and was inhibited 100% by slimicide K at a low concentration. Also, slimicide K effectively

* 1. *Ps. paucimobilis*; 2. *St. saprophyticus*; 3. *Ps. cepacia*; 4. *Ac. calcoaceticus*; 5. *St. auricularis*; 6. *Acidovorax* sp.; 7. *Ac. capsulatus*

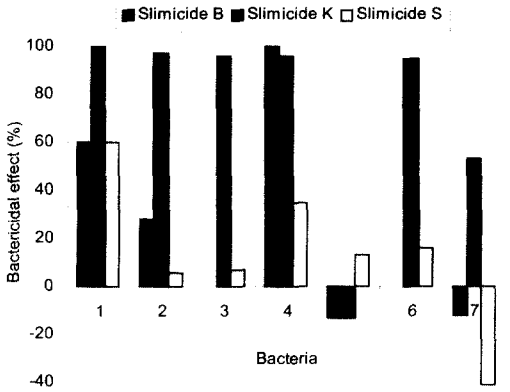


Fig. 1. Bactericidal effect at 5 ppm of slimicides.

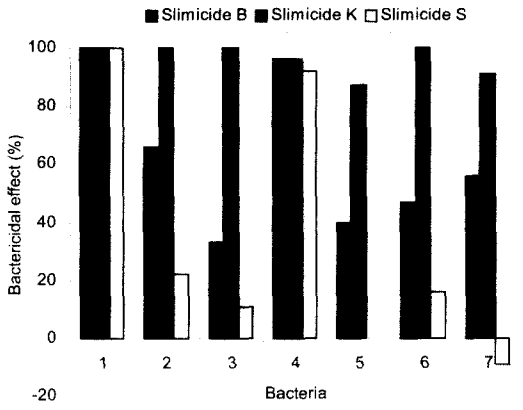


Fig. 2. Bactericidal effect of 10 ppm of slimicides.

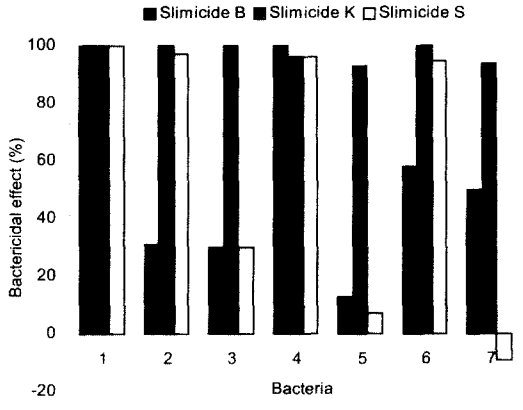


Fig. 3. Bactericidal effect of 15 ppm of slimicides.

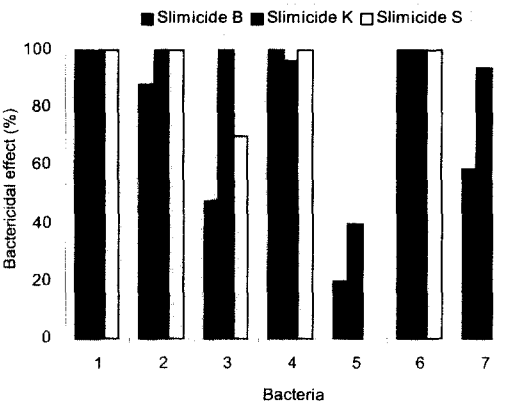


Fig. 4. Bactericidal effect of 20 ppm of slimicides.

inhibited *Ps. cepacia* at all concentrations applied. Slimicide B and S exhibited little effectiveness on *Ps. cepacia* in the concentration of below 15 ppm. *Ac. calcoaceticus and Acidovorax* sp. were also inhibited 100% by all of the applied slimicides at the concentration of 20 ppm, but *Acidovorax* sp. was less affected by the slimicide B and S at the concentration of below 15 ppm. *Ac. calcoaceticus* was barely survived only at the concentration of 5 ppm of slimicide S. Slimicides did not prevent the growth of *St. auricularis*, showing 0~30% efficacy. Also, Slimicide B had less effect on *St. auricularis*, showing less than 40% at the most concentrations. *St. auricularis* has shown the most resistance with the consistent viability among the tested bacteria against all applied slimicides. Even slimicide K, which was a very powerful inhibiting agent on every bacteria tested, showed less effectiveness on *St. auricularis*. The growth of *Ac. capsulatus* was not affected by slimicide S at all, and still had 60% viability at the concentration of 20 ppm of slimicide B. But, as resulted with the other bacteria, *Ac. capsulatus* was also inhibited by slimicide K, except at the concentration of 5 ppm.

3.3 Cell observation by SEM and protein patterns of *Staphylococcus auricularis* tested with selected concentrations of slimicides

To determine the nature of the bacteria morphology differences, the bacteria treated with slimicides were observed by scanning electron microscopy. Selected results are shown in Photos 2~5.

SEM observation showed that most of the bacteria, whether it is gram negative (Photo 2) or gram-positive (Photo 3), underwent the bacteriolysis of cells. The most noticeable finding was that *St. auricularis*, a gram-positive bacteria with a distinctive coccus type (Oh *et al.*, 1997) excretes a slimy, gluelike substance on the surface of the cell, and this substance was stretched out directly to the neighboring cells, which were also secreting the same substance. As the result of the slimicide treatment that showed *St. auricularis* to be one of the most resistance strains among the tested bacteria, this suggests that this slimy substances might promote cell aggregation, and this aggregation may shield individual cells from being inhibited by slimicides. Futhermore, the cells treated with 5 ppm slimicide K and with 5 ppm slimicide B

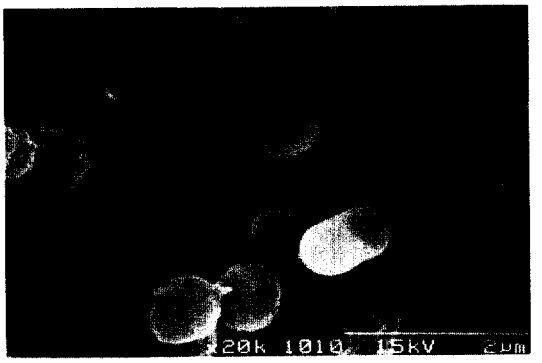


Photo 2. Scanning electron micrograph of *Ac. capsulatus* treated with S slimicide 5 ppm.

Photo 3. Scanning electron micrograph of *St. auricularis* treated with B slimicide 5 ppm.

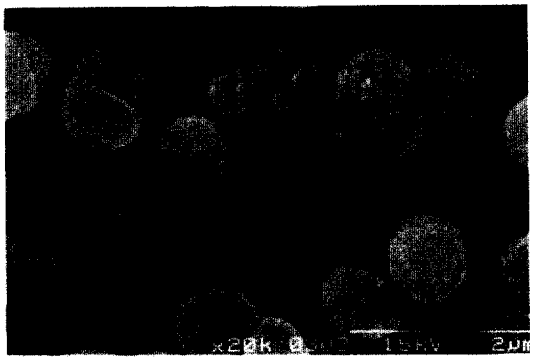


Photo 4. Scanning electron micrograph of *St. auricularis* treated with K slimicide 5 ppm.

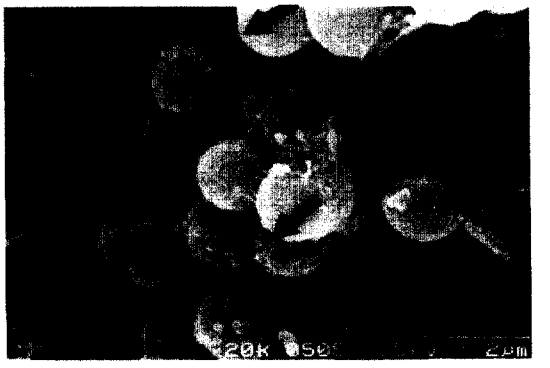


Photo 5. Scanning elecrton micrograph of *St. auricularis* treated with S slimicide 10 ppm.

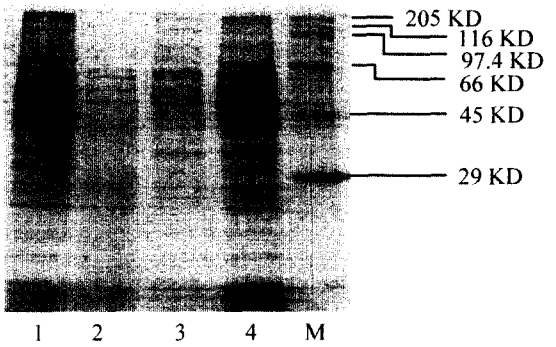


Photo 6. protein patterns of *St. auricularis* tested with selected concentrations of slimicides.
1. Cells treated with K slimicide 5 ppm
2. Cells treated with S slimicide 15 ppm
3. Cells treated with B slimicide 10 ppm
4. *St. auricularis*
5. Marker

didn't show much of the substances. But the cells treated with 10 ppm slimicide S showed the slimy substance very distinctively. Even though the amount of the substance was not measurable, this still implies that the slimy substance is responding differently to the treated conditions. This result can be confirmed in protein patterns (Photo 6), which were prepared from each cell selected by slimicide efficacy on *St. auricularis*, showing clear differences on its component.

Many of the resistance determinants identified in *St. auricularis* are said to be plasmid encoded (Lyon *et al.*, 1987), even though our experiment didn't show them. Whether it is because of plasmid or slimy substance that give resistance to this bacteria, it should be noted that *St. auricularis* could be the main concern in the paper-making process.

4. CONCLUSIONS

The major motivation for establishing this study was to determine the response of the bacteria from the paper making process on three different slimicides. The results showed that *Ps. paucimobilis*, which has been reported by several overseas paper mills (Cornelia *et al.*, 1993) and is commonly known as one the slime forming bacteria with *Acinetobacter* sp. (Araki *et al.*, 1990; Hernandez-Mena *et al.*, 1993; Martin *et al.*, 1988) is not a big problem, even though this bacterium was prevalent through the paper-making process (Oh *et al.*, 1997). They were well inhibited by all of the applied slimicides in this study. Slimicide K had a good effect on most of the tested bacteria except for two strains, *Ac. capsulatus* and *St. auricularis*. As a result of isolating plasmid, *Ac. capsulatus* had five different sizes of plasmid DNA. Even though *Ac. capsulatus* is known to be a slime secreting bacteria, a slimy substance was not observed. Among the bacteria tested, a slimy substance on the surfaces of the cells was observed only in *St. auricularis*, which is a gram-positive bacteria. Considering the results of the slimicide treatment, which showed that these strains were the most resistant among the tested bacteria, two strains have presumably developed a resistance to the slimicide, whether by plasmid DNA or by slimy substance. Bacteriolysis of the cells was observed in all tested bacteria. Until recently, gram-negative bacteria was considered to be the main factor that caused slime formation among bacteria, but our findings suggest that not only gram-negative, but also gram-positive bacteria should not be neglected. Every paper mill is unique as far as the presence of different microorganisms is concerned. Thus, it becomes imperative to understand and respond to the problem of each particular mill differently. So, it is expected that this study will improve the state of understanding of the nature of slime formation bacteria and will facilitate applied effort which involves controling slime formation in the paper making industry.

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