Williopsis saturnus yeast killer toxin does not kill *Streptococcus pneumoniae*

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Abstract
Streptococcus pneumoniae represents an important human bacterial pathogen, and the increase in antibiotic resistance demands the development of new antimicrobial compounds. Several reports have suggested that yeast killer toxins show activity against bacteria and we therefore investigated the activity of K9 killer toxin from the yeast *Williopsis saturnus* var. *mrakii* NCYC 500 against *S. pneumoniae*. However, no inhibition of bacterial growth was observed with concentrated K9 preparations in agar diffusion assays and in liquid culture. Although cell morphology was slightly affected by K9 treatment, no effect on cellular viability was detectable, and K9 had no stimulatory effect on cell lysis induced by beta-lactams or TritonX100. This indicated that K9 did not contribute to cell wall damage. Moreover, flow cytometry was used as a sensitive assessment of integrity of cells exposed to killer toxin. No significant damage of *S. pneumoniae* cells was evident, although minor changes in fluorescence suggested that K9 killer toxin may interact with bacterial surface components.

Introduction

The increase of drug-resistant bacterial strains represents a major global medical challenge. Antibiotic therapy is becoming ineffective against a significant number of nosocomial infections and there is therefore a pressing need to identify and characterize new antibacterial agents. Although the antimicrobial effects of antibiotics, bacteriophages and bacteriocins have been known for a long time, the antimicrobial activity of yeast-derived metabolites has been demonstrated only relatively recently (Bevan and Makover 1963).

Killer yeasts secrete proteinaceous toxins that have potential in medicine and biotechnology (Schmitt and Breinig 2002). They have potential in the biocontrol of contaminant microbes in fermentation processes (Palpacelli et al. 1991), and for intraspecific characterization of industrially and clinically interesting yeast cultures (Buzzini and Martini 2000). The use of

selected toxins as antimycotic agents against pathogenic yeasts that cause systemic mycoses has also been suggested (Hodgson et al. 1995; Walker et al. 1995). Very little is known about the antibacterial mode of action of killer yeasts. Most killer toxins are small secreted peptides below 20 kDa, but some *Williopsis* strains secrete active compounds of much larger size (85 kDa) (Guyard et al. 2002). Previous research has shown that killer toxins of *Hansenula anomala, Hansenula (Williopsis) mrakii, Kluyveromyces drosophylarum, Kluyveromyces lactis* and *Candida tropicalis* have potential growth inhibitory activity against Gram-positive pathogenic and nonpathogenic bacteria (Polonelli and Morace 1986; Izgü and Altinbay 1997). However, in these studies only growth inhibition zones of bacteria on agar plates surrounding the growth of yeast strains was recorded, and the toxic activity of yeast metabolic compounds rather than killer toxin could not be excluded. In the present paper, we reassessed the activity of yeast killer toxin on a Gram-positive bacterium using flow cytometry. This technique is a useful method for determining antimicrobial effects, and has been used for studies in bacteria for many years (Boye et al. 1983). It has also been used to assess influence of antifungal agents against yeast using the membrane integrity fluorescent dye, propidium iodide (Green et al. 1994). Our attention was drawn to the killer toxin of *Hansenula (Williopsis) mrakii* K9, because it is stable at high temperatures (100°C, for 10 min) and retains activity over a wide range of pH values (pH 2 to 11) (Yamamoto et al. 1988; Buzzini et al. 2004). We chose *Streptococcus pneumoniae* as test organism since it is a major human pathogen and the recent emergence of drug resistance in this species worldwide exemplifies the need for new antibacterial compounds (Brussow and Desiere 2001; Henriques-Normark 2007).

Materials and methods

Microbial strains and media

The K9 killer toxin producer yeast strains *Williopsis saturnus var. mrakii* NCYC 2251, NCYC 500, and killer toxin sensitive *Saccharomyces cerevisiae* NCYC 1006 were obtained from the yeast collection of Abertay University of Dundee. Yeast cells were grown in Yeast Medium (YM) (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose) broth or on YM agar plates (YM broth with 1% agar). For preparation of crude K9 killer toxin, *Williopsis saturnus var. mrakii* was grown in Edinburgh Minimal Medium 3 (EMM3) (Creanor and Toyne 1993). Cells used for K9 activity assays were grown in YM or EMM3 medium for 72 h at 28°C.

E. coli NCIMB 10000 (previously named ML30) has been described Cohn and Horibata (1959), was grown in Brain Heart Infusion (BHI) broth aerobically, or on BHI agar (1 % agar) plates. *Streptococcus pneumoniae* R6 - an un-encapsulated derivate of the Rockefeller University strain R36A (Avery et al. 1944) and the highly virulent strains *S. pneumoniae* TIGR4 previously named JNR 7/87 contains a type 4 capsule (Bricker and Camilli 1999) were grown in Brain-Heart-Infusion (BHI) broth or in the semi-synthetic C-medium supplemented with 0.1% yeast extract (Difco) at 37°C without aeration (Lacks and Hotchkiss 1960). As solid medium, D-agar (Alloing et al. 1996) supplemented with 3 % defibrinated sheep blood (Oxoid, Wesel, Germany) were used. Other Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) were grown in Luria Bertani (LB) medium. Growth of *S. pneumoniae* was followed with Nephelometry (Nephelometer Units = N) or by determination of OD_{660} (Ultrospec 110pro, Amersham Biosciences).

Preparation of crude K9 killer toxin

Williopsis saturnus var. mrakii NCYC 500 was grown in 10 l EMM3 broth (pH 4.5) for 48 h at room temperature (20°C). Cells were harvested by continuous centrifugation (Heraeus, Japan) at 1000 x g and the supernatant containing the crude killer toxin was filtered and concentrated 25fold using a 10 kDa cut-off membrane filter system (Millipore, Japan) in order to remove acid and other metabolic products with potential antimicrobial activity. This preparation was used for flow cytometric analyses. Using an Amicon Ultracel_10 regenerated cellulose 10 kDa cut of membrane filter, an approximately 250fold concentrated K9 toxin preparation was obtained which was used for testing antibacterial activity during bacterial growth in liquid medium. Protein concentration of crude toxin preparations was determined using a Coomassie (Bradford) protein assay kit (Pierce, Rockford. IL).

Agar diffusion assay for killer yeast activity

Bacteria were diluted in 20 ml agar medium favorable for bacterial growth (as described above) at ca. 1 x 10^6 CFU before distribution into Petri dishes; alternatively cells were plated on the agar surface. No differences concerning K9 activity were observed between these two procedures. After solidification of the agar, the surface was spotted with live cells of killer-yeast, sensitive yeast, crude K9 killer toxin and yeast growth medium (20 µl each). The plates were incubated at 37°C for 24 h. As positive control for K9 activity, the sensitive yeast strain *Saccharomyces cerevisiae* NCYC 1006 was used grown in buffered YM agar plates (pH 4.5) or non buffered YM agar (pH7.0) and the plates incubated at 28°C for 24 h.

Effect of K9 killer toxin on S. pneumoniae cells grown in liquid media

In addition to the agar diffusion assays described above, the activity of crude K9 preparations against *S. pneumoniae* in liquid media was also tested, as follows.

S. pneumoniae R6 and TIGR4 were grown in C-Medium or BHI broth and allowed to grow at 35°C until reaching a cell density of N = 15 (30) and divided into several aliquots. Samples of concentrated K9 toxin (final concentration1 µg/ml) and/or benzylpenicillin (1 µg/ml final concentration) or EMM medium (as negative control) were added, and growth followed at 30 min intervals by nephelometry (N). Untreated growing cultures of *S. pneumoniae* were used as positive controls.

Flow cytometric assay of yeast killer toxin activity

Bacterial growth in liquid media was monitored at regular intervals using a Novospec II (Pharmacia) photometer. Culture samples were taken at three different time points during growth, and stained with propidium iodide (PI; Invitrogen, USA). At the same time, cells were examined microscopically. After washing with saline (0.9 % NaCl) (3x), cells were diluted in saline to a final OD_{600} = 0.1 prior to addition of K9 toxin or antibiotic. Fluorescence measurements were made with a FloMax flow cytometer (Partec, Germany) using 532nm excitation and emission at 570 to 610nm.

Results and Discussion

Activity of K9 killer toxin on cells grown on agar plates

Williopsis saturnus var. mrakii NCYC 500 was grown in minimal medium for 24 hours, and a crude concentrated preparation of the growth medium was used for testing the activity of the K9 killer toxin. It has previously been suggested (Polonelli and Morace 1986) that activity of yeast toxins against Gram-negative as well as Gram-positive bacteria may be due to acid or other metabolic products in the preparations obtained from citrate-buffered medium which are generally

inhibitory to bacteria (Izgü and Altinbay 1997). Therefore, YM (which does not contain citrate) was used for growth of yeast cells in all experiments conducted.

In order to assess antimicrobial activity of the K9 toxin, live cells of *Saccharomyces cerevisiae* NCYC 1006 served as a sensitive control culture. Toxin-sensitive cells were grown on agar plates, and K9 preparations were spotted onto the agar surface. The K9 preparation as well as both live cells of *W. saturnus var. mrakii* strains showed clear activity against the sensitive yeast strain S *cerevisiae* NCYC1006 (Fig. 1A). No activity of yeast cells or concentrated K9 was observed with both *S. pneumoniae* indicator strains, the virulent serotype 4 strain TIGR4, and the un-encapsulated laboratory strain R6 (Fig. 1B). Occasionally, faint inhibitory zones were visible, but these effects may not be attributable to yeast killer toxin as other media-derived components may inhibit bacterial cells. The same negative results were obtained using other Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* (not shown).

Effect of K9 killer toxin on cells grown in liquid culture

The activity of crude K9 preparations against *S. pneumoniae* was tested in two liquid media as a more subtle assay of bacterial growth inhibition compared with agar diffusion. These media do not contain blood, which could affect killer toxin activity, and all undefined ingredients are similar to those in yeast medium which have no effect on killer toxin activity. Nevertheless, no effect on generation time and stationary phase cell lysis was observed with K9 in both, *S. pneumoniae* R6 and TIGR4 cultures, independent of the choice of growth medium (see Fig. 2A and B for examples), and viability was also unaffected when compared to the untreated control cultures (not shown). However, morphology of the *S. pneumoniae* cells was slightly affected after 2 hours of K9 treatment with the appearance of aberrant cells (Fig. 3A and B).

Effect of K9 toxin on cellular lysis induced by beta-lactams and Triton X100

As there was some indication of minor cellular damage to bacteria due to K9 treatment, we assumed that lysis induced by other cell envelope damaging agents may augmented synergistically with yeast killer toxin. *S. pneumoniae* is highly susceptible to lysis induced by penicillins and detergents, thus benzylpenicillin (1 µg/ml) and Triton X100 (0.001 %) was used with and without additional K9 treatment. No significant difference in the rate of lysis was observed, either if K9 and benzylpenicillin were added simultaneously, or if cells were pretreated with K9 for 20 min before the addition of the lytic compound. Fig. 4 shows the results obtained with benzylpenicillin; similar results were obtained with Triton X100 (not shown).

Flow cytometry analysis

Flow cytometric analyses were performed in order to detect subtle changes induced by K9 treatment to *S. pneumoniae* cells. *S. pneumoniae* R6 cultures were grown in liquid medium and treated with K9 killer toxin. In addition, R6 cells were treated with a lytic beta-lactam antibiotic for comparison. Growth was measured and flow cytometric analysis performed immediately before treatment, 4h after treatment and from cultures exposed to killer toxin (Fig. 5B). *E. coli* NCIMB10000 (Fig. 5 C) and *Saccharomyces cerevisiae* NCYC1006 (Fig. 5A) were used as a negative and positive control, respectively.

Sensitive yeast cells clearly showed intense fluorescence of the entire population (Fig. 5A), whereas no effect was seen with *E. coli* (Fig. 5C). *S. pneumoniae* when treated with crude K9 killer toxin showed a slightly enhanced PI staining, indicating some changes as a result of K9 activity. The K9 effect, however, was significantly less when compared to penicillin treatment (Fig. 5B). Changes were more pronounced after overnight treatment. In contrast, treatment with the beta-

lactam resulted in increased PI staining of the bacterial cells documenting massive cell damage (Fig. 5B).

Conclusions

We could not detect anti-Streptococcal activity of K9 toxin produced by *Williopsis saturnus var. mrakii* NCYC 500, that has been observed with other yeast strains where only growth inhibition on agar plates has been recorded (Polonelli and Morace 1986; Izgü and Altinbay 1997). Growth of *S. pneumoniae*, either on agar plates or in liquid media, was virtually unaffected by exposure to K9. Additionally, no effect of K9 was observed on stationary phase lysis typical for *S. pneumoniae* grown in liquid medium, and K9 did not enhance bacterial cell lysis triggered by beta-lactams or the detergent Triton X100. However, we could not exclude some form of molecular interaction of the yeast toxin with *S. pneumoniae* cells since slight alterations of cell morphology, as well as a slight shift in PI fluorescence staining using flow cytometry of K9 treated pneumococci were observed. Electron microscopy of yeast killer toxin treated bacteria may provide some insight regarding cell wall damage, as has been reported for yeast cells (Vadasz et al. 2000). For bacteria, biochemical analyses of cell wall composition and cell wall associated proteins may additionally provide information on yeast toxin induced cell wall associated changes. In this context it is interesting that monoclonal antibodies representing the internal image of another yeast killer toxin produced by a *Pichia anomala* ATCC96603 have been shown to exert activity against a wide spectrum of Gram-positive bacteria including *S. pneumoniae* by an unknown mechanism, but most likely via interaction with cell surface polysaccharides (Conti et al. 2000; Conti et al. 2002). The binding of yeast killer toxins to bacterial surface components represents an interesting basis which could be exploited for the development of antibacterial compounds. Further biochemical characterization of antibacterial yeast killer toxins are required, together with understanding of their

molecular interaction with specific cell surface binding sites on bacteria in order to evaluate their potential as novel therapeutic agents.

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Figure Legends

Figure 1. Activity of yeast killer toxin on yeast and *S. pneumoniae* cells. (A) The indicator strain *Saccharomyces cerevisiae* NCYC1006 was grown on YM agar plates. 1, *S. cerevisiae* NCYC1006; 2, *Williopsis saturnus var. mrakii* NCYC500; 3, EMM3 Medium. (B) *S. pneumonia* TIGR4 was grown on blood agar plates. Samples: 1, 250fold concentrated medium of *Williopsis saturnus var. mrakii* NCYC500; 2, 250fold concentrated killer K9 toxin; 3, EMM3 Medium (20 µl).

Figure 2. Activity of K9 toxin on *S. pneumoniae* in liquid culture.

S. pneumoniae R6 (A) and TIGR4 (B) were grown in BHI and allowed to grow at 35°C until reaching a cell density of N = 30. At the time indicated, concentrated K9 toxin (■ 1 µg/ml), EMM medium (▲) or benzylpenicillin (1 µg/ml final concentration) was added, and growth followed by nephelometry (N). ●: untreated control.

Figure 3. Effect of K9 toxin on *S. pneumonia* morphology. Exponentially growing *S. pneumoniae* R6 (A, B) and TIGR4 (C, D) in BHI medium were treated with a concentrated K9 toxin preparation at N = 30 for 60 min (B, D). Pictures were taken with a Nikon *Eclipse* E600 microscope in phase contrast.

Figure 4. Effect of K9 toxin on benzylpenicillin induced lysis. An exponentially growing culture of *S. pneumoniae* R6 in BHI was divided into seven aliquots at N = 15 (t = 0), and treated with benzylpenicillin (1 µg/ml) and/or K9 toxin (solid arrow); the open arrow indicates time of addition of benzylpenicillin at 20 min. Growth was monitored throughout the experiment by Nephelometry. ●: untreated control; ▲K9 toxin; □ benzylpenicillin; ○

K9 plus benzylpenicillin; Δ K9 followed by benzylpenicillin at 20 min; ♦ benzylpenicillin at 20 min.

Figure 5. Flow cytometry analysis of K9 killer toxin activity on yeast and bacterial cells. As controls, *S. pneumoniae* R6 was also treated with benzylpenicillin (1 µg/ml), and *E. coli* with ampicillin (1 µg/ml); cells killed by incubation at 100°C for 60 min were also used. Samples were taken before the addition of K9 toxin or the antibiotic, after 4 h and after overnight treatment, and prepared for flow cytometry as described in Materials and Methods. A: susceptible *Saccharomyces cerevisiae* NCYC1006; B: *S. pneumonia* R6 grown in BHI; C: *E. coli* NCIMB 10000 grown in BHI medium. Compounds were added to bacterial cultures at OD_{600} = 0.3.

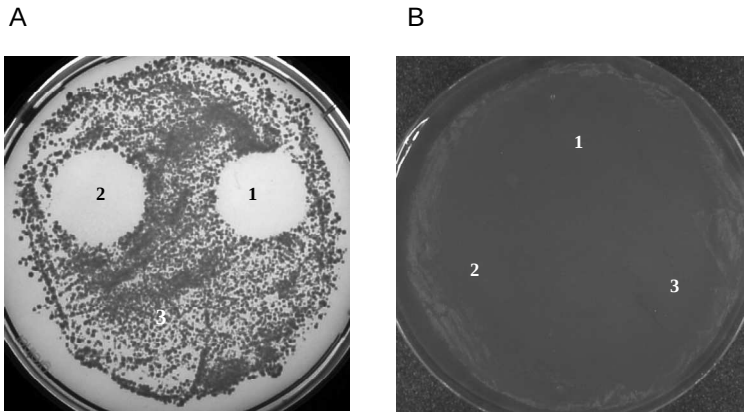
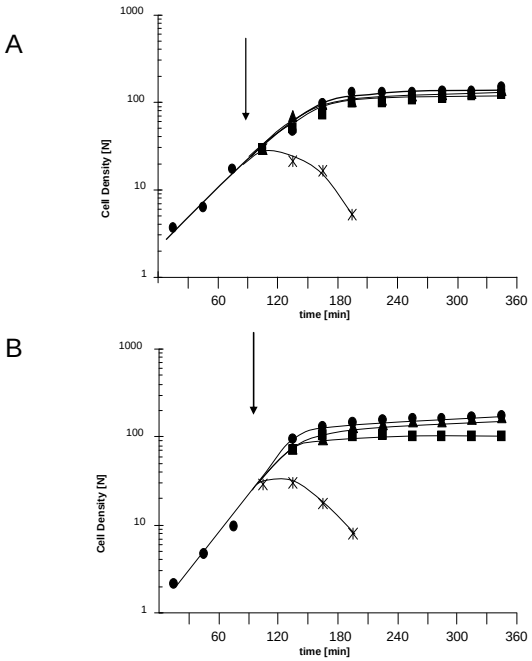


Figure 1 Oshigawa et al.



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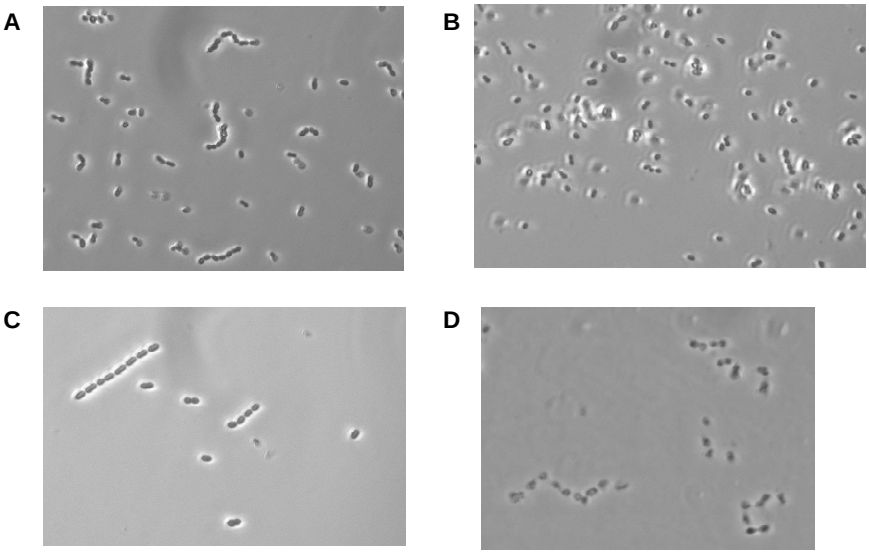

Figure 3 Oshigawa et al.

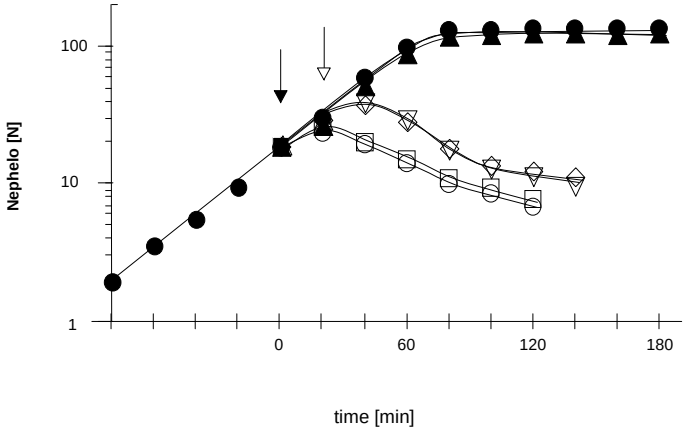

Figure 4 Oshigawa et al.

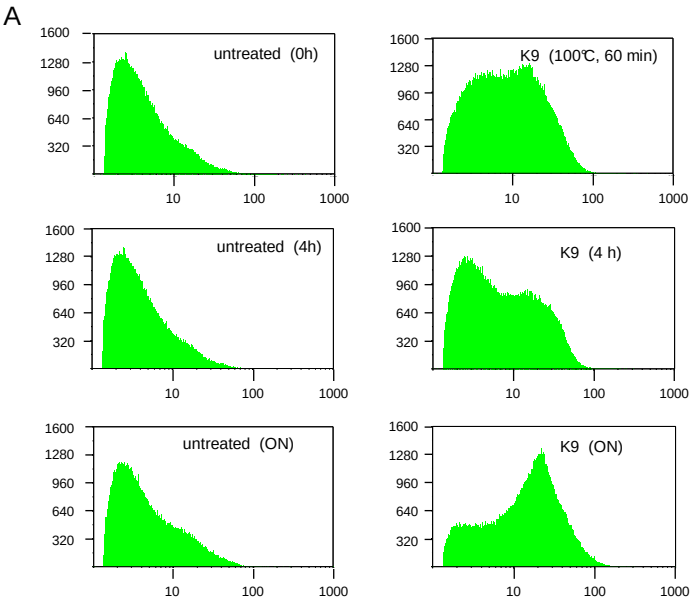

Figure 5A Oshigawa et al.

B *Streptococcus pneumoniae* R6

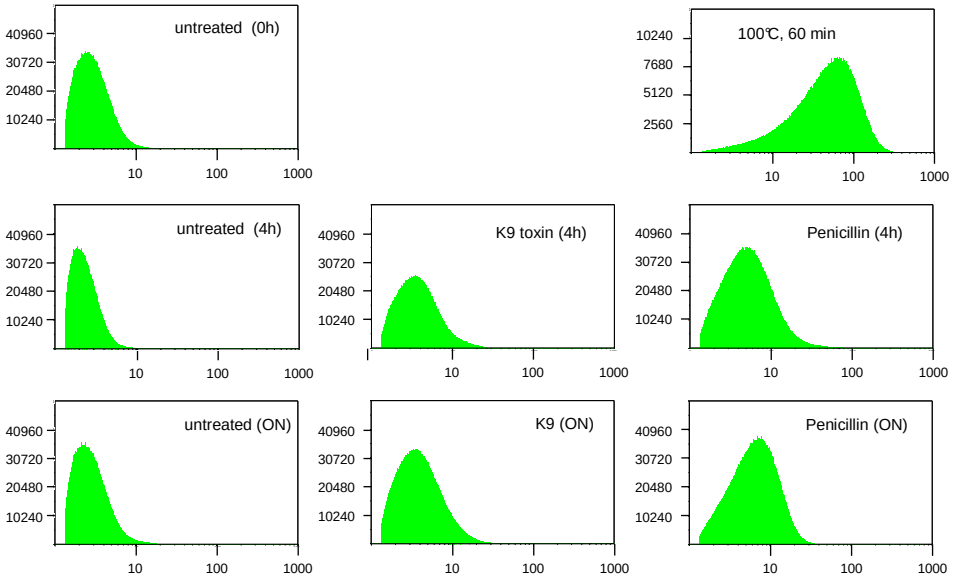

Figure 5B Oshigawa et al.