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Effect of antibiotics inhibiting cell wall synthesis on growth of staphylococcal L-phase variant

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Penicillin, a typical inhibitor of cell wall synthesis, inhibited the colony formation of L-phase variant of *Staphylococcus aureus*, which has no cell wall.

The inhibition was observed only when the L-colony was formed in a culture medium with a low concentration of NaCl (0.35 M). Lowering concentration of yeast extract and incubation temperature suppressed inhibition. L-colony formation in a culture medium without Mg⁺⁺ was dependent on the presence of penicillin.

The same effects, inhibition and dependence, were observed in the treatment with D-cycloserine and bacitracin. However, bacitracin inhibited L-colony formation even in the medium with 0.9 M NaCl. Vancomycin inhibited formation of L-colony but did not support L-colony formation without Mg⁺⁺. Phosphomycin neither inhibited nor supported L-colony formation.

The cell wall structure was not detected by electron microscopic observation in the L-phase variant cell grown under the condition in which L-colony formation was inhibited by penicillin.

In a previous study (MAKINO, 1983), the induction of staphylococcal L-phase variant was investigated. Protoplast, prepared by the treatment of parental cells with lysostaphin, was readily transformed to L-phase variant on the surface of an agar medium which contained an osmotic stabilizer (NaCl), Mg⁺⁺, serum and penicillin in addition to nutrient components. Penicillin was added to prevent colony formation of the remaining parental type cells. Although L-phase variant was not susceptible to penicillin because it does not synthesize cell wall, inhibition by and dependence on penicillin were observed under a certain restricted condition. Penicillin is a typical inhibitor of cell wall synthesis. It combines with penicillin binding protein (GEORGO-PAPADAKOU *et al.*, 1976), which is found in cell membrane and inhibits the action of transpeptidase. Therefore, any effect of penicillin on cell wall-less bacteria is inconsistent with the current view.

The purpose of this communication was to describe a condition which affects the action of penicillin on L-phase variant and to present some possible explanations of its mechanism.

Materials and Methods

Bacterial strain

Staphylococcus aureus 209P was used throughout this experiment.

Culture

Tryptic soy broth (Difco, Detroit) supplemented with 0.5% yeast extract (Difco, Detroit) was used for the growth of parental cells. The basal medium for L-phase variant cells consisted of 2% casamino acid (Difco, Detroit), 0.5% yeast extract, 1% Na-lactate (Nakarai, Kyoto), 5.2% NaCl and 0.8% Nobel agar (Difco, Detroit), and pH was adjusted to 7.0 with Tris. Casein (0.005%) and Mg-acetate (20 mM) were autoclaved separately and added to the culture medium before preparation of agar plate (CLYS-agar medium).

Chemicals

Lysostaphin and antibiotics except penicillin were purchased from Sigma (St. Louis). Penicillin was purchased from Banyū (Tokyo).

Induction of L-phase variant

Cells harvested from the middle of a logarithmic culture (cell density was about $10^9/ml$) were washed once with physiological saline and resuspended in the original volume of 1.4 M NaCl solution with 0.01 M Tris-HCl buffer, pH 7.4. After treatment with 20 $\mu g/ml$ lysostaphin at 37°C for 3 hours, almost all the parental cells were transformed to protoplast. The protoplast was spread on the surface of CLYS-agar medium after appropriate dilution. A typical fried egg shaped L-form colony was formed after incubation at 37°C 3 days.

Electron microscopi microscopic observation

The sample of electron microscopic observation was prepared by the rapid-freezing and substitution fixation method (AMAKO *et al.*, 1986). L-phase variant cells grown on CLYS-agar medium at 37°C were mounted directly on a Teflon specimen support.

This part of the research was carried out by KAZUNOBU AMAKO and AKIKO UMEDA at School of Medicine in Kyūshū University.

Results

Inhibition of L-colony formation by penicillin

Under the ordinary condition in which the culture medium contained 0.9 M NaCl, penicillin did not inhibit L-colony formation. However, when the concentration of NaCl was decreased, L-colony formation was inhibited by penicillin (Fig. 1). Minimum inhibitory concentration for L-phase variant was the same as that for parental cells (0.2 unit/ml). Fig. 1 also shows that

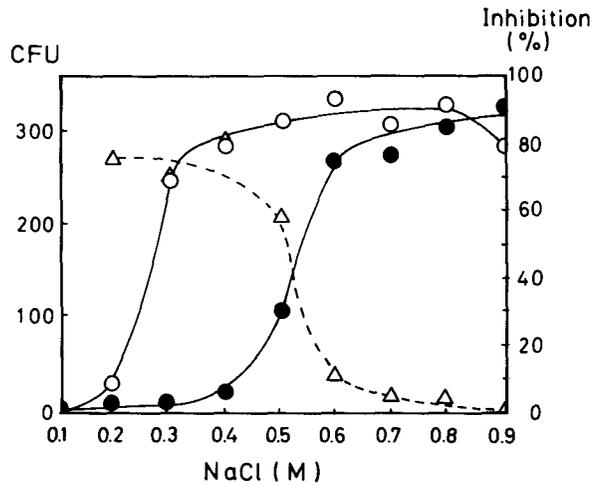


Fig. 1. Effect of NaCl on inhibition of L-colony formation by penicillin. ○: control, ●: with penicillin (1 unit/ml), △: inhibition rate.

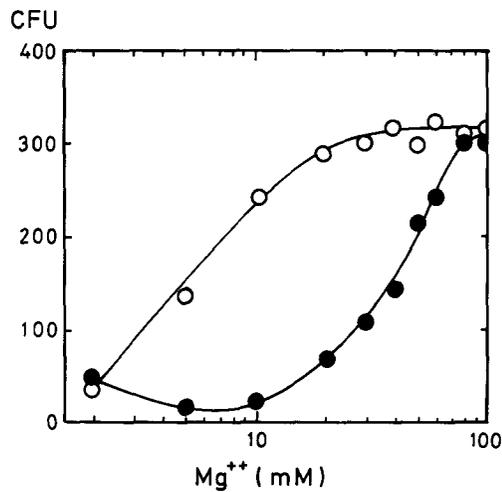


Fig. 2. Effect of Mg⁺⁺ on inhibition of L-colony formation by penicillin. L-colony was formed on CLYS-agar medium of which NaCl concentration was reduced to 0.35 mM. ○: control, ●: with penicillin (1 unit/ml).

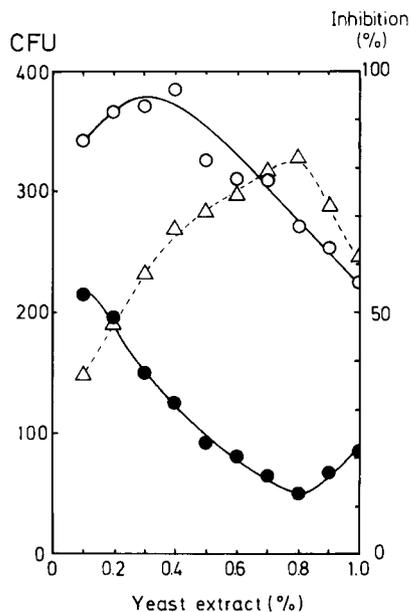


Fig. 3. Effect of yeast extract on inhibition of L-colony formation by penicillin. L-colony was formed on CLYS-agar medium of which NaCl concentration was reduced to 0.35 M. Symbols are as defined in the legend to Figure 1.

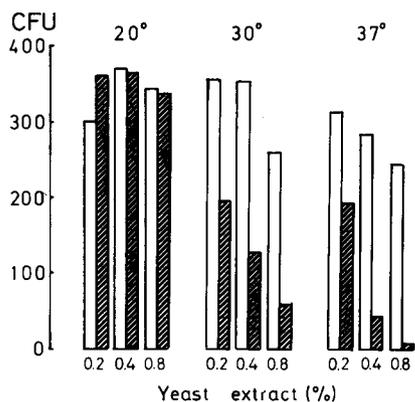


Fig. 4. Effect of temperature on inhibition of L-colony formation by penicillin.

L-colony was formed on CLYS-agar medium of which concentration was reduced to 0.35 M.

□ : control, ■ : with penicillin (1 unit/ml)

0.3 M of NaCl was the minimum concentration required to protect the L-phase variant cell from low osmolarity.

Fig. 2 shows the effect of Mg^{++} on L-colony formation and its inhibition by penicillin. Mg^{++} was essential for the L-colony formation. Higher concentrations of Mg^{++} suppressed the penicillin inhibition.

Yeast extract was also effective factor of penicillin inhibition. As yeast extract was an essential nutrient for L-phase variant, less than 0.1% of yeast extract could not support the growth of L-phase variant but higher concentrations of yeast extract gave a higher inhibition rate. The highest inhibition rate was demonstrated at a concentration of 0.8% of yeast extract (Fig. 3).

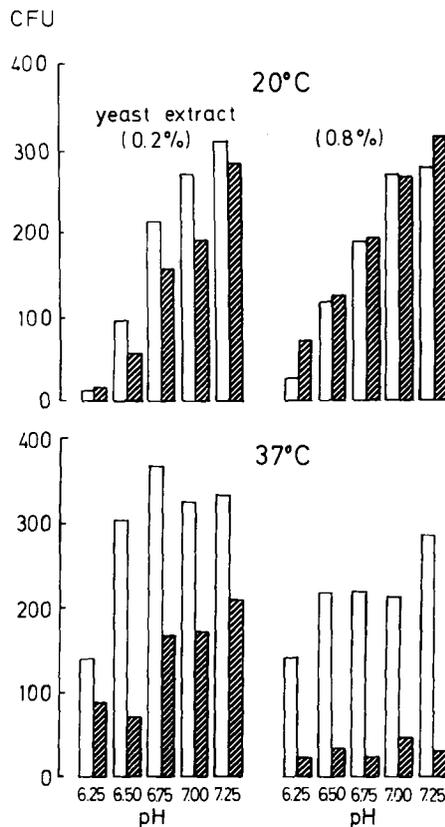


Fig. 5. Effect of pH on inhibition of L-colony formation by penicillin. L-colony was formed on CLYS-agar medium of which NaCl concentration was reduced to 0.35 M. Symbols are as defined in the legend to Figure 4.

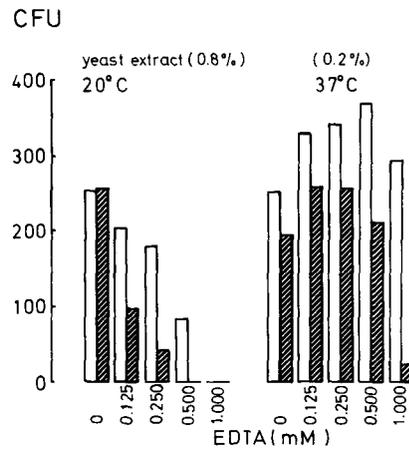


Fig. 6. Effect of EDTA on inhibition of L-colony formation by penicillin. L-colony was formed on CLYS-agar medium of which concentration of NaCl was reduced to 0.35 M. Symbols are as defined in the legende to Figure 4.

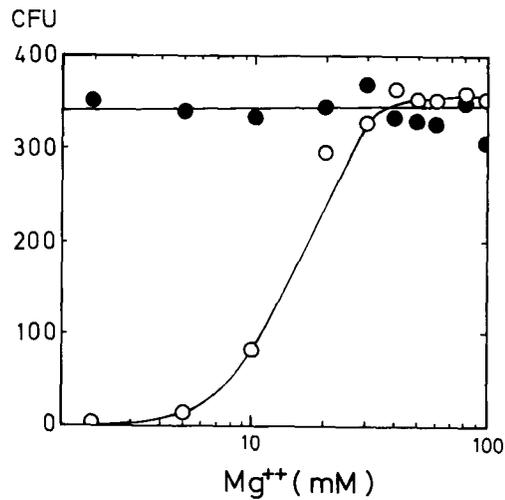


Fig. 7. Dependence of L-colony formation on penicillin. ○ : control, ● : with penicillin (1 unit/ml)

Incubation temperature was another factor which affected the penicillin inhibition of L-phase variant (Fig. 4). At 20°C, penicillin did not show any significant effect on L-colony formation. At 30°C, inhibition was demonstrated but its rate was less than that at 37°C. Fig. 4 also shows the effect of yeast extract on penicillin inhibition at various temperatures.

Fig. 5 shows the effect of pH, yeast extract and incubation temperature on penicillin inhibition. Yeast extract and temperature gave almost the same results as those described above. There was no significant effect of pH on the susceptibility of L-phase variant to penicillin in the range of pH tested (6.25–7.25).

Fig. 6 shows the effect of EDTA on L-colony formation and its inhibition by penicillin. The cells growing at 20°C and with 0.8% yeast extract had higher sensitivity to EDTA. At a concentration of 0.5 mM, the colony size became small and it was hard to count individual colonies. No colony was detected at 1.0 mM of EDTA. The cells growing at 37°C and with 0.2% of yeast extract were comparatively resistant to EDTA. Inhibition by penicillin was observed when concentration of EDTA was 1.0 mM.

Dependence of L-colony formation on penicillin

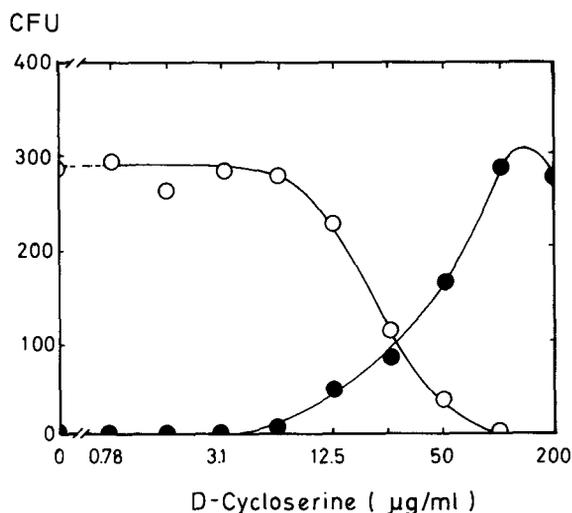


Fig. 8. Effect of D-cycloserine on L-colony formation.
 ● : L-colony was formed on standard CLYS-agar medium omitted Mg⁺⁺,
 ○ : L-colony was formed on CLYS-agar medium of which NaCl concentration was reduced to 0.35 M.

L-phase variant required Mg^{++} for its colony formation ; however, as shown in Fig. 7, if penicillin was present, it could grow without Mg^{++} . In this case the amount of penicillin required was 0.02 unit/ml, which was much less than MIC.

Effect of other antibiotics which inhibit cell wall synthesis

Antibiotics other than penicillin which are known as inhibitors of cell wall synthesis were examined for their ability to affect L-colony formation. D-Cycloserine inhibited and supported L-colony formation by the same way as penicillin (Fig. 8). Bacitracin also inhibited L-colony formation in the medium with 0.35 M NaCl and supported it without Mg^{++} . However, it inhibited L-colony formation even in the medium with 0.9 M NaCl (Fig. 9).

Vancomycin did not support L-colony formation without Mg^{++} but inhibited it under both conditions of 0.35 M and 0.9 M NaCl (Fig. 10). Phosphomycin neither inhibited nor supported L-colony formation.

Electron microscopic observation of the L-phase variants grown with 0.35 M NaCl

Since there must be a possibility that the L-phase variant grown with 0.35 M NaCl can synthesize cell wall, the cell structures were examined by an electron microscope. Fig. 11 shows that no structures resembling cell wall were recognized, even in the cells grown in the medium with 0.35 M NaCl and 0.8% yeast extract.

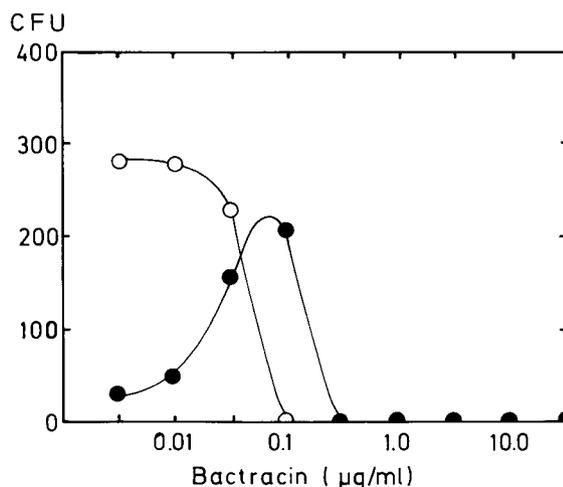


Fig. 9. Effect of bacitracin on L-colony formation. Symbols are as defined in the legend to Figure 8.

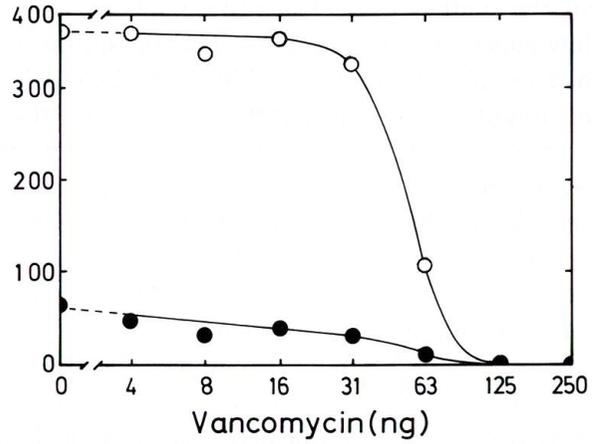


Fig. 10. Effect of vancomycin on L-colony formation. Symbols are as defined in the legend to Figure 8.

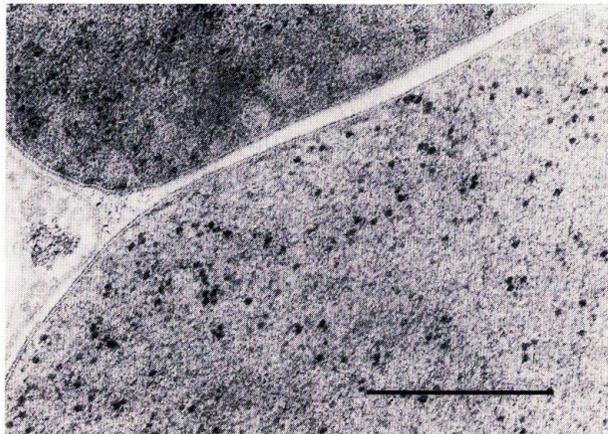


Fig. 11. Thin sectioned L-phase variant cell grown on CLYS-agar medium of which NaCl concentration was reduced to 0.35 M. Bar = 1 μ m

Discussion

One of the possible explanations of the inhibition of L-colony formation by penicillin was the synthesis of cell wall by L-phase variant grown in the medium with low concentration of NaCl. This possibility was ruled out by the electron microscopic observation. Although chemical analysis of cell wall components might be necessary, at the present stage the number of cells obtainable was insufficient because L-phase variant cells can grow only on the surface of agar medium and most part of the colony is formed in the agar layer. However, the L-phase cells grown under the restricted condition were also susceptible to low osmolarity (data not shown). This meant that they did not retain an enough amount of cell wall structure to prevent the cells from plasmolysis due to low osmolarity.

All the antibiotics which inhibit L-colony formation had a common characteristic: the cells inhibited by these antibiotics accumulated uridine nucleotide peptide (PARK, 1952; PARK and STROMINGER, 1957; PARK, 1958; 1961; REYNOLDS, 1964). On the contrary, the antibiotic that had no effect, phosphomycin, is known to inhibit NAC-pyruvate transpeptidase (MATA *et al.*, 1969). Some relationship may exist between the accumulation of uridine nucleotide peptide and the inhibition by and dependence on penicillin of L-colony formation.

There was an interesting similarity between the penicillin inhibition of L-phase variant and methicillin resistant *Staphylococcus aureus* (MRSA). As is well known MRSA isolates frequently have a characteristic referred to as heterogeneity (BARBAER, 1964; DYKE, 1969; SABATH and WALLACE, 1971; SABATH *et al.*, 1972; SABATH, 1977). This term refers to the fact that within a given strain of MRSA, only a small proportion of CFU are able to express resistance to β -lactam antibiotics. Modification of growth conditions, incubation temperature, an increase in osmolarity, and the addition of EDTA, can increase the proportion of CFU that expresses resistance to various degrees. In the case of penicillin inhibition of L-phase variant, osmolarity, temperature and EDTA also gave a similar effect. In MRSA, penicillin binding protein 2 (PBP 2) was replaced with PBP2a which had low affinity to penicillin and its relation with the phenotypic expression of MRSA was suggested (HARTMAN and THOMASZ, 1984; 1986). While the presence of PBP in L-phase cell was reported (MARTIN *et al.*, 1980), its detail remained to be elucidated. Effect of culture conditions on PBP formation in L-phase cells now under investigation.

One of the characteristic aspects of L-phase variant was pleuromor-

phism (DIENS, 1970; HIJIMANS *et al.*, 1969). The cell division of L-phase variant was irregular, and sometimes even the budding of daughter cell was observed. This phenomenon seemed to be the result of the loss of cell wall. In other words, the loss of cell wall induced irregular cell division. It was conjectured that the cell wall synthesizing system may have the capability to control cell division. It is well known and established evidence that the bacterial chromosome is attached to the cell surface, providing a means of regulating both initiation of DNA replication and chromosome segregation. SANDER and KEYMAN (1988) described an association of DNA, including newly synthesized regions, with a specific region of the cytoplasmic membrane, and the attachment was prevented by inhibitor of cell wall synthesis, vancomycin and bacitracin.

A clinical isolate of *Staphylococcus aureus* which was tolerant to a number of β -lactam antibiotics showed initially stimulated and then severely inhibited RNA synthesis by the treatment with oxacillin, an inhibitor of cell wall synthesis. Protein synthesis of this strain was not inhibited initially but the rate of protein synthesis declined after 50 min of antibiotic treatment (JABLONSKI and MYCHAJLONKA, 1988).

The phenomena above mentioned might be suggestive to explain the mechanism of growth inhibition of L-phase variant with cell wall inhibiting antibiotics.

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