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Presence of Cell Wall Lytic Enzyme in Stable Staphylococcal L-Form

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ABSTRACT

The stable L-form cells derived from *Staphylococcus aureus* 209P were examined for the presence of cell wall lytic enzymes. The enzyme preparations from cells and culture fluid of the parent strain lysed both *Micrococcus lysodeikticus* cells and *S. aureus* cells, whereas the enzyme preparations from the L-form lysed *M. lysodeikticus* cells but not *S. aureus* cells. Lipoteichoic acid, which has been reported to be a regulator of the lytic enzyme, inhibited both enzyme preparations from the parent strain and the L-form. However, the susceptibility of the enzyme preparation from the L-form to lipoteichoic acid was lower than that from the parent strain.

Stable L-form bacteria can grow without a rigid cell wall. The L-form strain must differ greatly from its parent strain. Although the L-form strain lacks a cell wall, it possesses some enzymes of cell wall biosynthesis[8]. We examined whether the L-form produces cell wall lytic enzymes or not. Cell wall lytic enzymes are thought to be necessary for bacterial elongation, division, separation and cell wall turn over[3,5-7]. In *Staphylococcus aureus*, the major cell wall lytic enzymes in the cell wall are *N*-acetylmuramyl-l-alanine amidase and β-*N*-acetylglucosaminidase[6].

The paper describes the presence of cell wall lytic enzyme in the staphylococcal stable L-form and the difference between the enzymes of the L-form and the parent strain.

MATERIALS AND METHODS

Organisms and growth conditions

The organisms used were *Staphylococcus aureus* 209P and its stable L-form 209PL which was kindly provided by Dr. Kanemasa (Okayama University School of Medicine, Okayama, Japan).

The L-form was grown in 3 liters of brain heart infusion broth (Difco Laboratories, Detroit, Mich, USA) containing 5% NaCl. A 5% inoculum was cultured at 37°C for 24 hr with gentle stirring. The parent strain was grown in 3 liters of brain heart infusion broth to the late log phase with rotary shaking.

Preparation of cell wall lytic enzyme

The *S. aureus* 209P parent strain and L-form cultures were centrifuged at 8,900 × *g* for 20 min at 4°C. The Extracellular enzyme preparation (EEP) was prepared by precipitating the supernatant with 70% saturated ammonium sulfate, and dissolving and dialyzing the precipitate against 0.01 M phosphate buffer pH 7.0. The cellular enzyme preparation (CEP) was prepared by washing the bacterial cells in 5% NaCl for the L-form and in normal saline for the parent strain for three times and extracted in 120 ml of 2% Triton X-100 at 4°C for 3.5 hr with stirring. The cells were removed by centrifugation and the supernatant was precipitated with

70% saturated ammonium sulfate. The precipitate was dissolved and dialyzed against 0.01 M phosphate buffer pH 7.0.

DEAE-Sephadex column chromatography

Crude EEPs from the parent strain and the L-form strain were applied on DEAE-Sephadex A-25 column (16 × 50 mm, Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.02 M phosphate buffer pH 7.3, washed with the same buffer, and then eluted with 0.2 M ammonium sulfate. Ammonium sulfate eluted factions were concentrated with polyvinlylpyrrolidone and dialyzed against 0.01 M phosphate buffer at pH 7.0.

Assay for cell wall lytic activity

Micrococcus lysodeikticus NCTC 2665 and *S. aureus* 209P heat-killed and lyophilized cells were used for the substrate. Half ml of the 1 mg/ml substrate, 0.5 ml of 0.2 M phosphate buffer at pH 7.0, 0.5 ml of enzyme preparation and 0.5 ml of distilled-water or inhibitor were incubated at 37°C with reciprocal shaking. At zero time and every 1 hr interval, the turbidity at 660 nm was measured and the percent of initial turbidity was calculated.

For the enzyme inhibition assay, lipoteichoic acid (LTA) extracted from *S. aureus* 209P cells or cardiolipin was added to the assay system instead of distilled-water.

RESULTS

Fig. 1. shows the lytic activity of EEP and CEP from the *S. aureus* parent and L-form strain. EEP and CEP from the parent strain markedly lysed *M. lysodeikticus* cells (Fig. 1A). The CEP from the parent strain also lysed the *S. aureus* cells, whereas EEP hardly lysed the *S. aureus* cells (Fig. 1B). *M. lysodeikticus* cells were lysed by both EEP and CEP from the L-form (Fig. 1C). However, EEP and CEP from the L-form could not lyse the *S. aureus* cells (Fig. 1D).

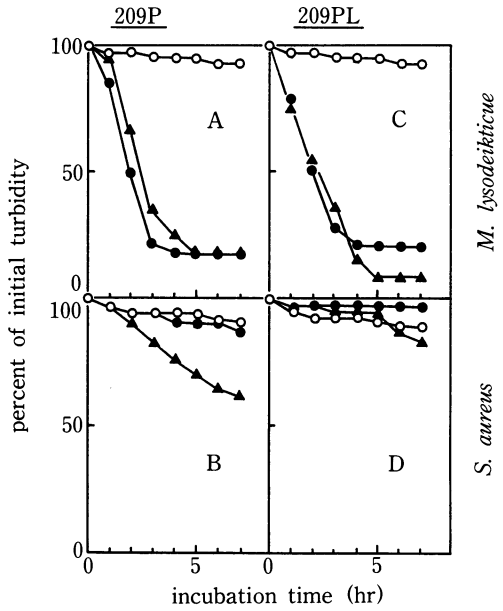

Fig. 1. Lytic activities of EEP and CEP from the parent strain 209P and L-form 209PL. Half ml of substrate (1 mg/ml), *M. lysodeikticus* or *S. aureus* cells, 0.5 ml of buffer, 0.5 ml of enzyme preparation and 0.5 ml distilled water were incubated at 37°C and turbidity at 660 nm was measured.
○ , control; ● , EEP; ▲ , CEP.

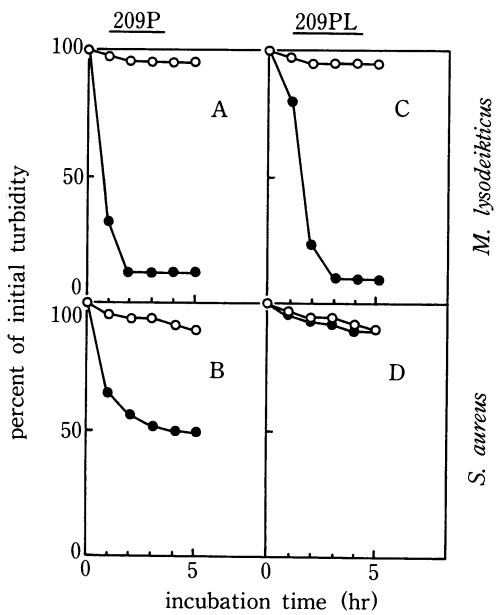

Fig. 2. Lytic activities of DEAE-Sephadex fraction of EEP from parent strain 209P and L-form 209PL. EEPs from parent strain and L-form were applied on DEAE-Sephadex and eluted with 0.2 M ammonium sulfate. Lytic activities against *M. lysodeikticus* and *S. aureus* cells were assayed. For assay system, see legend to Fig. 1.
○ , control; ● , DEAE-Sephadex eluate.

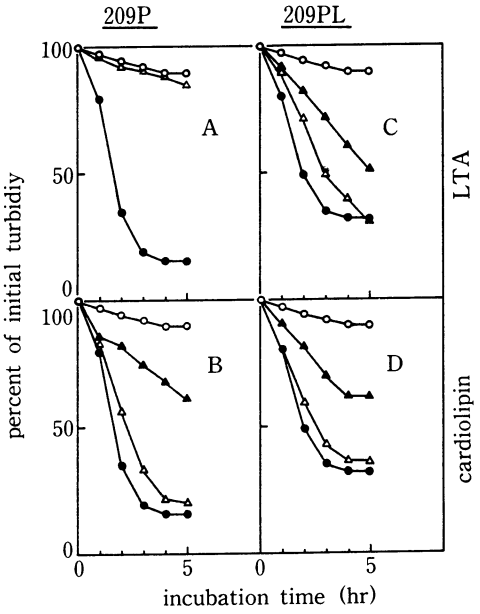

Fig. 3. Effects of LTA and cardiolipin on *M. lysodeikticus* lytic activity of EEP from parent strain 209P and L-form 209PL. LTA or cardiolipin was added to the assay system. For assay system, see legend to Fig. 1.
○ , control; ● , no additional; △ , 400 nmole LTA, 70 nmole cardiolipin; ▲ , 800 nmole LTA, 140 nmole cardiolipin.

Fig. 2 shows the lytic activity of the DEAE-Sephadex eluate of the EEPs from the parent strain and L-form. The DEAE-Sephadex eluate of EEP from the parent strain lysed the *S. aureus* cells (Fig. 2B), but the EEP not passed through the DEAE-Sephadex column could not (Fig. 1B). The EEP from the L-form did not lyse the *S. aureus* cells even after DEAE-Sephadex column chromatography (Fig. 2D), whereas it lysed the *M. lysodeikticus* cells (Fig. 2C).

Fig. 3 shows the effects of LTA and cardiolipin on the *M. lysodeikticus* lytic activities of EEP from the parent strain and L-form. The addition of 400 nmole of LTA completely inhibited the EEP from the parent strain, but even 800 nmole of LTA failed to inhibit the EEP from the L-form (Fig. 3A, C). Cardiolipin inhibited the EEPs from both the parent strain and L-form similarly (Fig. 3B, D).

DISCUSSION

A cell wall lytic enzyme was present in the staphylococcal stable L-form cells and its culture fluid. Both lysed *M. lysodeikticus* cells, but the L-form could not lyse the *S. aureus* cells. The EEP and CEP from the parent strain lysed both *M. lysodeikticus* and *S. aureus* cells. These findings indicate that one of the three enzymes that exist in the parent strain[6] was lost by the conversion to the L-form. Although the enzyme was not characterized, the findings that the enzyme from the L-form lysed the *M. lysodeikticus* cells but not the *S. aureus* cells, suggests that the L-form lost glucosaminidase but still possesses amidase.

Lytic enzymes are necessary for bacterial elongation, division, separation and cell wall turnover[3,5-7]. Since the L-form produced a lytic enzyme that seemed to be unnecessary, and since the L-form possesses a penicillin binding protein, an enzyme of cell wall synthesis[8], not all the components related to the cell wall were lost by the conversion to the L-form. The presence of *N*-acetylmuramyl-l-alanine amidase in the L-form of *Bacillus licheniformis*[2] also supports this assumption.

The effects of LTA and cardiolipin, inhibitors and regulators of cell wall lytic enzymes[1,4,9], were also examined. Both LTA and cardiolipin inhibited the lytic activity of the EEP from the L-form, but the susceptibility of the EEP from the L-form to LTA was lower than that of the EEP from the parent strain. This is considered to be due to a difference in the lytic enzyme composition or the presence of a substance inhibiting the action of LTA.

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