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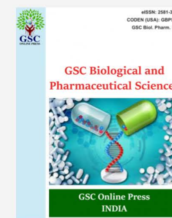

(Research Article)

Effects of LytR on bacterial morphology in *Streptococcus pyognes*

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Abstract

A robust cell envelope is the first line of defense in infectious pathogens when encountering host immune defenses. In Gram-positive bacteria, LytR-CpsA-Psr (LCP) family proteins play a major role in the synthesis and assembly of the cell envelope. The Gram-positive bacterium *S. pyogenes* causes a wide range of diseases from pharyngitis to septicemia, but the involvement of LytR in the synthesis and assembly of the cell wall envelope in this bacterium is not clear. Therefore, we investigated whether LytR of *S. pyogenes*, like LytR of other streptococci, is involved in cell wall formation. The *lytR* gene-deficient strains were used to investigate the bacteria's ability to form a chaining, drug sensitivity to penicillin G, an inhibitor of cell wall synthesis, bacterial biofilm formation, and the morphological structure of bacteria by transmission electron microscopy, and the expression of *pbp2b*, which encodes a penicillin-binding protein, compared with the wild-type strain. Our results showed that LytR-deficient strains had reduced bacterial chaining compared to wild-type strains. Compared to wild-type strains, LytR-deficient strains were also more drug sensitive to the cell wall synthesis inhibitor penicillin G. This genetic study was accompanied by increased expression of the *pbp*2b gene in the LytR-deficient strains. In addition, the LytR-deficient strain showed a reduced ability to form biofilms, and the *lytR* gene-deficient strain showed morphological irregularities with abnormal bacterial septa. These results indicate that the *lytR* gene is involved in cell wall synthesis in *S. pyognes*.

Keywords: *S. pyogenes*; LytR; morphology; Biofilm; PCG susceptibility

1. Introduction

S. pyogenes has demonstrated a high capacity to colonize a variety of tissue sites and cause a wide variety of diseases. They range from mild superficial infections of the skin (e.g., impetigo) and mucous membranes (e.g., pharyngitis) to severe invasive infections (e.g., streptococcal toxic shock-like syndrome and necrotizing fasciitis) and autoimmune sequelae (e.g., rheumatic fever) [1]. The cell walls of Gram-positive bacteria contain a variety of glycopolymers (CWGPs), a significant proportion of which are covalently bound to peptidoglycan (PGN) scaffold structures CWGPs play important roles in bacterial cell function, morphology, and pathogenicity. Despite obvious differences in composition, structure, and underlying biosynthetic pathways, the final ligation step of CWGP to the PGN backbone involves a conserved class of enzymes, LytR-CpsA-Psr(LCP) transferases. Thus, the catalytic mechanism of this biologically important class of enzymes is of interest as a new target for antimicrobial drug discovery to combat the emergence of multidrug-resistant bacteria [2]. LytR investigations are ongoing in the Gram-positive bacteria, especially Streptococcus species, but there are few reports on *S. pyogenes*. Previously, we have shown that *lytR* mutants have higher cysteine protease activity than the wild type, that the amount of the cysteine protease SpeB in the *lytR* mutant is higher than in the wild-type, that the level of *speB* mRNA in the *lytR* mutant is also increased compared to the wild-type, and that mouse infection models have shown that *lytR* mutant-infected mice had lower survival rates than wild-type mice, and

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the size of wound lesions in mice carrying the *lytR* mutant was larger than in wild-type mice [3]. Therefore, in the present study we investigated whether LytR, like other streptococcal LytRs, is involved in cell wall formation.

2. Material and methods

2.1. Bacteria and culture

The M1 serotype *S. pyogenes* 1529 strain used in this study was a clinical isolate from a Japanese hospital patient with invasive *S. pyogenes* infection [4]. Cultures were usually incubated in 20 mL of Todd Hewitt broth (Difco Laboratories) with 0.3% yeast extract (Difco Laboratories, MI, USA) (THY) for 18 hours at 37 °C and 5% CO_2 without stirring. *E. coli* DH5α (Takara Bio, Otsu, Japan) was cultured on Luria-Bertani (LB) agar medium (Difco Laboratories) or LB broth at 37 °C with aeration. Antibiotic concentrations were ampicillin (Sigma Aldrich, MO, USA) 100 μg/mL (*E. coli*); spectinomycin (Sigma Aldrich) 100 μg/mL (*E. coli* and *S. pyogenes*); kanamycin (Sigma Aldrich) 100 μg/mL (*E. coli* and *S. pyogenes*) as appropriate.

2.2. Gene-deficient mutant strains

E. coli DH5α was used for growth of plasmid construction. As previously reported, a gene-deficient strain was used in which the nonpolar inactivation of *lytR* gene (Spy1733) mutant was constructed by double-crossover of the chromosome of *S. pyogenes* 1529 [3].

2.3. Morphological evaluation of bacteria

Bacterial morphology was observed under an optical microscope (Olympus Corporation, Tokyo, Japan) after Gram staining with Gram-Hacker stain solution (Mutoh Chemical Co., Ltd., Tokyo, Japan). The number of bacteria in a chain was measured visually.

2.4. Drug susceptibility testing

Drug susceptibility testing was performed by the disk diffusion method. Kirby Bauer discs "EIKEN" containing penicillin G (10U = 0.6 μg/disc) (Eiken Chemical, Tokyo, Japan) were used. After *S. pyogenes* were cultured overnight in THY, bacteria were applied on Pearl Core-Mueller-Hinton S agar medium (Eiken Chemical) supplemented with sheep defibrinogenic blood (Japan Biological Material Center, Tokyo, Japan) at a ratio of 5%, and Kirby Bauer discs were placed on the agar medium. After 24 hours of incubation at 37 °C under 5% CO_2, the bacterial diameter of the zone inhibition was measured.

2.5. Evaluation of Bacterial Biofilm

Biofilm assays were performed with slight modifications [5]. Each bacterium (1×10^6 CFU) was seeded into 96-well polystyrene plates (Thermo Fisher Scientific, MA, USA) and incubated in THY broth at 37 °C for 48 hours. After removal of the medium, the plates were washed three times with PBS, and adherent bacteria were stained with 0.2% Crystal Violet (Fujifilm Wako Pure Chemicals) at room temperature for 10 minutes, followed by gentle washing three times with PBS. Each biofilm was quantified by measuring absorbance at 570 nm (A570). Wells incubated without bacteria were used as blanks. The absorbance of the blank wells was subtracted from the test values.

2.6. Evaluation of bacterial morphology by transmission electron microscopy

Bacterial morphology was examined by ultra-thin section method using transmission electron microscopy (TEM). Samples were observed with a transmission electron microscope (JEM1011J; JEOL, Tokyo), and digital images were captured with a MegaView slow-scan camera (JEOL).

2.7. Northern blotting assay

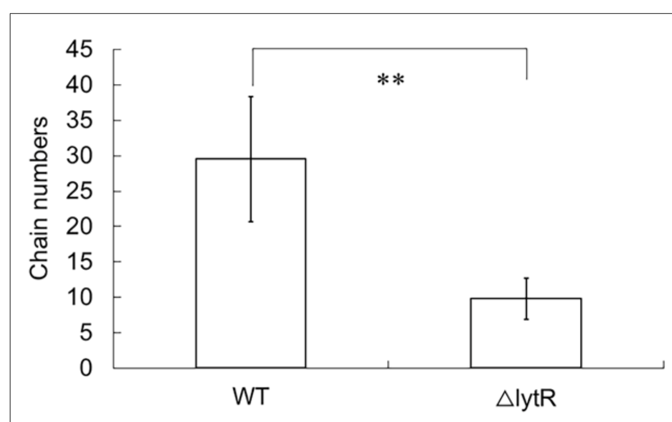
Total RNA was extracted and purified as previously reported [6]. Briefly, bacterial cells were cultured in 5 mL THY; cells were harvested when the optical density at 660 nm was approximately 0.8 (late log phase) Total RNA was extracted and purified using ISOGEN (Fujifilm Wako Pure Chemical Industries, Osaka, Japan). Approximately 2 μg of each total RNA preparation was electrophoresed on a 1% agarose gel containing 1.1 m formaldehyde (Fuji Film Wako Pure Chemical Industries, Ltd.) RNA was transferred to Hybond-N+ membrane (GE Healthcare, Waukesha, WI, USA). The pbp2b DNA for the probe was amplified using oligonucleotide primers pbp2b-F1 (CCTATATCCTCCAAACAGCCT) and pbp2b-R1 (GGTCAATTCCTGTCGCAGTA). The probe was isotopically labeled using the Random Primer DNA Labeling Kit Ver2 (Takara Bio, Otsu, Japan) and [α-32P] dCTP (PerkinElmer, MA, USA). The membranes were then autoradiographed and analyzed at room temperature using a bioimaging analyzer (BAS-1800II; Fujifilm, Tokyo, Japan).

2.8. Statistical Analysis

Study data were expressed as mean ± standard deviation (SD). Statistical analysis of differences between groups was performed using the t-test. All experiments were performed at least five times. The p-value < 0.05 indicates statistical significance.

3. Results

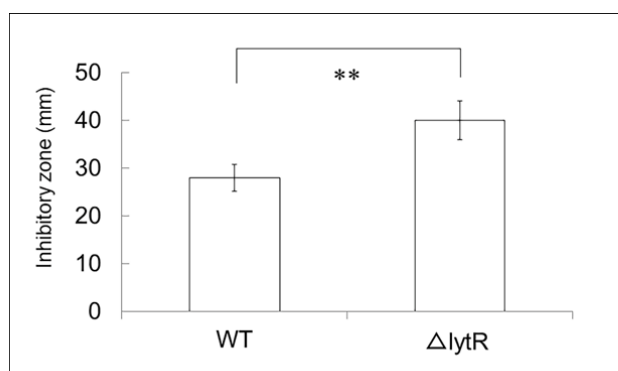
To begin, the morphology of the wild and mutant strains was observed under an optical microscope with Gram staining of the bacteria. The wild-type strain was identified as a long chain of bacteria. However, only short chains of bacteria were identified in the mutant strain. Comparison of the number of bacteria in the streptococcal chain showed that the wild-type strain had an average of 30 bacteria in the chain, while the mutant strain had an average of only 10 bacteria in the chain, indicating a clear and significant difference in the number of bacteria in the chain (Figure 1).



The numbers of bacteria were measured under an optical microscope. WT: 1529 wild-type strain,ΔlytR: 1529 *lytR*-deficient strain. **: $p < 0.01$.

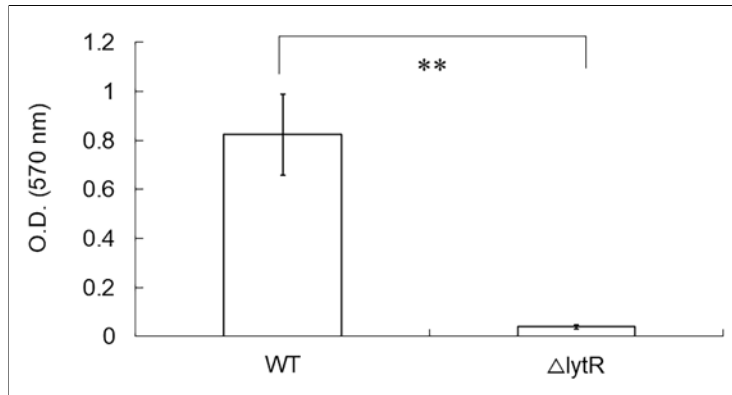
Figure 1 Number of bacterial chaining

Assuming that *lytR* mutant strain had some cell wall alteration, we next measured the drug sensitivity of the streptococci to penicillin G, a cell wall synthesis inhibitor, by the disk diffusion method (Figure 2). In the wild-type strain, the average zone of inhibition was 30 mm, but in the mutant strain, the average zone of inhibition increased to 40 mm, a clear and significant difference. Since bacterial cell walls are also involved in bacterial biofilm formation, bacterial biofilms were stained and measured using the crystal violet staining method (Figure 3). The results showed that the absorbance of the biofilm of the strain was significantly lower than that of the wild strain. In addition, microscopic cell morphology, including cell walls, was observed using transmission electron microscopy (Figure 4). The results showed that in the mutant strain, the cell morphology was irregular, cell division was defective, and the size of each cell was enormous. In addition, the cell walls of the mutant strains tended to be thin.



The inhibitory zone of bacteria by penicillin G was measured. WT: 1529 wild-type strain,ΔlytR: 1529 *lytR*-deficient strain. **: $p < 0.01$.

Figure 2 Drug sensitivity to penicillin G



Bacterial biofilms were stained with crystal violet and absorbance was measured. WT: 1529 wild-type strain,ΔlytR: 1529 *lytR*-deficient strain. **: $p < 0.01$.

Figure 3 Biofilm formation ability

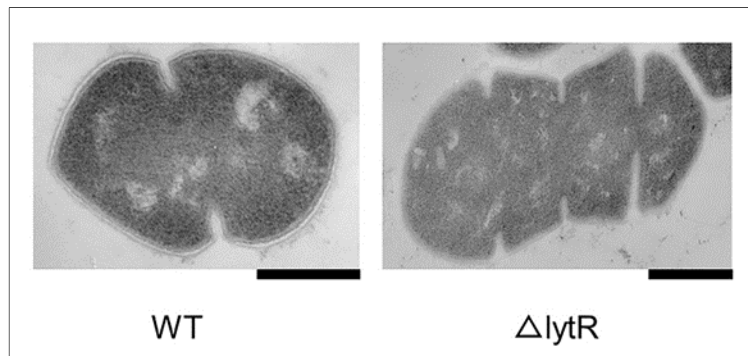


Figure 4 Morphological changes of bacteria. Bacterial morphology was captured by transmission electron microscopy. WT: 1529 wild-type strain,ΔlytR: 1529 *lytR*-deficient strain. **: $p < 0.01$. Bars represent 0.5 µm.

When the expression of penicillin-binding proteins involved in cell wall synthesis was examined by northern blotting, the expression of the *pbp2b* gene was stronger in the mutant strain than in the wild-type strain (Figure 5).

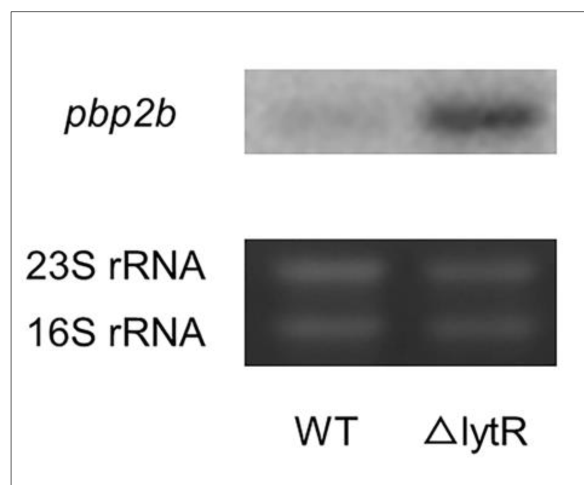


Figure 5 Expression *of pbp2b* mRNA. Expression of *pbp2b* mRNA was analyzed by northern blotting analysis. Expression levels of 23S rRNA and 16S rRNA were used as internal controls to evaluate the results of the Northern blotting analysis. WT: 1529 wild-type strain,ΔlytR: 1529 *lytR*-deficient strain.

4. Discussion

Our results showed that LytR-deficient strains had reduced bacterial linkage chaining formation and increased sensitivity to the cell wall synthesis inhibitor penicillin G, accompanied by increased expression of the *pbp2b* gene. The ability to form biofilms was also reduced, and electron microscopic analysis showed that the gene-deficient strains had an irregular morphology with abnormal bacterial septa. These results indicate that the *lytR* gene is involved in cell wall synthesis in *S. pyogenes*.

There are few reports on LytR in *S. pyogenes* [3], but there have been several reports on LytR in streptococci. In a study of the effect of LytR on the cell morphology of *S. suis*, transmission electron microscopy analysis showed that LytR mutants exhibit an abnormal septal arrangement with no obvious differences in capsular thickness. Crystal violet staining and laser scanning confocal microscopy also revealed that LytR contributes to *S. suis* biofilm formation; LytR mutant strains had reduced survival in whole human blood and were more sensitive to death by polymorphonuclear leukocytes (PMN). In addition, in a mouse infection model, the LytR mutant strain also showed significantly attenuated virulence and was more readily eliminated in blood [7].

In an examination of *S. pneumoniae* LytR, the LytR mutant showed abnormal cell septum formation [8]. The LytR mutant also showed reduced bacterial growth compared to the wild-type strain. Compared to the wild-type strain, the LytR mutant was more susceptible to phagocytosis. In animal studies, the ability to colonize rhinocytes and the virulence of *S. pneumoniae* were also reduced by disruption of the *lytR* gene [9]. In an examination of LytR in *S. mutans*. *lytR* mutants grew in long chains compared to wild strains, which may indicate a defect in cell division. *lytR* mutants showed increased bacterial autodegradation activity, and LytR attenuated bacterial autodegradation activity through regulation of autodegradation enzyme expression. No reduction in cell surface adhesion or biofilm growth was seen in the *lytR* mutant. However, there was an association between the cell proliferation phase and *lytR* transcription [10]. Since our results agree with several of the above previously reported results, LytR seems to be deeply involved in bacterial virulence in streptococci in general.

We mention here the limitations of this study. We have limited our experiments to 1529 strains in this study. In general, it would be acceptable to study only one strain, but we have not been able to confirm this with other *S. pyogenes* strains, and we have not performed any gene complementation experiments. However, we do not expect any problems with strains deficient in the *lytR* gene, based on our previously reported experimental results [3]. Since we have focused our study on the relationship between bacterial morphology and LytR, we have not conducted phagocytosis ability of immunocompetent cells and other aspects between LytR and infection immunity. Also, we did not use scanning electron microscopy to analyze the fine structure of biofilms or to evaluate the ratio and distribution of living and dead cells in biofilms. Although the expression of cell wall synthesis genes has only been confirmed for *pbp2b*, it is highly likely that the expression of other cell wall synthesis genes is also enhanced, since the expression of not only one gene but many genes often form an interactive network and are upregulated during cell wall synthesis activity. These studies, however, are topics for future investigation and are currently underway.

5. Conclusion

In this study, we have reported a role for LytR about the morphology of *S. pyogenes*, suggesting that LytR is involved in septum arrangement and biofilm formation, which is necessary for full pathogenicity of *S. pyogenes* during infection. However, further mechanistic elucidation is warranted.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All authors declare no conflict of interest of regarding the publication of this paper.

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