Evaluation of Target Specificity of Antibacterial Agents Using *Staphylococcus aureus* ddlA Mutants and d-Cycloserine in a Silkworm Infection Model

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Received 13 February 2009/Returned for modification 12 March 2009/Accepted 10 June 2009

The availability of a silkworm larva infection model to evaluate the therapeutic effectiveness of antibiotics was examined. The 50% effective doses (ED50) of d-cycloserine against the *Staphylococcus aureus* ddlA mutant-mediated killing of larvae were remarkably lower than those against the parental strain-mediated killing of larvae. Changes in MICs and ED50 of other antibiotics were negligible, suggesting that these alterations are d-cycloserine selective. Therefore, this model is useful for selecting desired compounds based on their therapeutic effectiveness during antibiotic development.

The spread of multidrug-resistant strains continues to cause serious clinical problems (2, 4). Due to the expansion of the compound library in recent years, high-throughput screening of these compounds for their effectiveness against defined target enzymes has been performed as the first step in antibacterial agent development (1, 9). While many inhibitory compounds are discovered by this approach, further analysis of the compounds for structure, antibacterial effect, and therapeutic effectiveness in animal models is important for identifying the most promising candidates. Among them, evaluation of a compound’s therapeutic effectiveness in mammal models is usually carried out for only a limited number of candidates because of cost and ethical concerns. However, it is extremely significant, because pharmacological actions of drugs are affected by various factors, such as absorption, distribution, metabolism, excretion, and drug interactions in the body.

We previously reported a silkworm larva infection model in which inoculation of pathogenic bacteria or true fungi into larval hemolymph leads to larval death (6, 7) but in which death can be prevented by coinjection of antibiotics or antifungal agents (3). Fifty percent effective doses (ED50) for antibiotics tested on silkworm larvae were similar to those reported for antibiotics tested on mice. The large size of silkworm larvae and their slow movement allow quantitative evaluation of pathogens and drugs by injection (such as of 50 μl) into the hemolymph. In addition, the midgut or the fat body (equivalent to the liver) can be removed and used for pharmacological experiments. Owing to these characteristics, the therapeutic effectiveness of candidates can be evaluated using the silkworm larva model (3).

We have identified *Staphylococcus aureus* genes essential for cell growth or viability by isolating temperature-sensitive *S. aureus* mutants (12). *S. aureus* is a gram-positive pathogenic bacterium that causes abscesses, pneumonia, endocarditis, and food poisoning and has received increasing attention because it has rapidly gained resistance to various currently available antibiotics. Because temperature-sensitive mutants often have altered sensitivity to antibiotics, these alterations can be exploited for evaluation of the target specificity of antibacterial agents. In this study, we examined the antibacterial effect and therapeutic effectiveness of d-cycloserine against ddlA mutants of *S. aureus*. The d-Ala-d-Ala ligase encoded by the ddlA gene synthesizes a d-Ala-d-Ala dimer, which is then added to UDP-N-acetylmuramyl-tripeptide (10) and is thereby an essential intermediate during peptidoglycan biosynthesis. d-Cycloserine inhibits both d-Ala-d-Ala ligase and alanine racemase and thus attenuates peptidoglycan biosynthesis (8, 11).

First, we examined whether ddlA mutations altered the MIC of d-cycloserine selectively or not. The three temperature-sensitive *S. aureus* ddlA mutants that we used here were newly acquired by a previously described method (5); each of the mutants had a single transition mutation (G304A, C532T, and G115A), resulting in single amino acid substitutions (Asp102Asn, Pro178Ser, and Asp39Asn) in the TS2921, TS5337, and TS10007 strains, respectively. Although the MIC of d-cycloserine against the parent RN4220 strain was 100 μg/ml, the MIC for TS2921 decreased to one-eighth of that value, 12.5 μg/ml (Table 1). Decrease in the MIC of d-cycloserine for TS5337 was also evident, while that of the MIC for TS10007 was not sig-

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Published ahead of print on 22 June 2009.
The MIC was defined as the lowest concentration of the drug that completely inhibited the growth of the strain with Mueller-Hinton medium (100 μl) at 30°C after 20 h of incubation. Values were those obtained from at least two separate experiments, which represented the same values.

pSddlA, which could complement the temperature sensitivity of these ddlA mutants, is a plasmid harboring a 1,630-bp ddlA gene-containing fragment at the SmaI site of pND50.

MICs were determined in the medium containing 100 mM D-Ala-D-Ala, which could complement the temperature sensitivity of these ddlA mutants.

ND, not determined because of the chloramphenicol resistance of the plasmid.

Table 1. MICs of antibiotics against S. aureus ddlA mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>d-Cycloserine (μg/ml)</th>
<th>Vancomycin (μg/ml)</th>
<th>Flomoxef (μg/ml)</th>
<th>Tetracycline (μg/ml)</th>
<th>Chloramphenicol (μg/ml)</th>
<th>Norfloxacin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220</td>
<td>100</td>
<td>0.4</td>
<td>0.4</td>
<td>0.063</td>
<td>6.3</td>
<td>0.63</td>
</tr>
<tr>
<td>TS2921</td>
<td>12.5</td>
<td>0.4</td>
<td>0.2</td>
<td>0.063</td>
<td>3.1</td>
<td>0.63</td>
</tr>
<tr>
<td>TS5337</td>
<td>25</td>
<td>0.4</td>
<td>0.2</td>
<td>0.063</td>
<td>3.1</td>
<td>0.63</td>
</tr>
<tr>
<td>TS10007</td>
<td>50</td>
<td>0.4</td>
<td>0.4</td>
<td>0.063</td>
<td>3.1</td>
<td>0.63</td>
</tr>
<tr>
<td>TS2921/pSddlA</td>
<td>≥100</td>
<td>0.4</td>
<td>0.4</td>
<td>0.063</td>
<td>ND</td>
<td>0.63</td>
</tr>
<tr>
<td>TS2921 + d-Ala-d-Ala</td>
<td>≥100</td>
<td>1.6</td>
<td>0.4</td>
<td>0.031</td>
<td>3.1</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Table 2. Therapeutic effectiveness of antibiotics against S. aureus ddlA mutants in a silkworm larva infection model

<table>
<thead>
<tr>
<th>Strain</th>
<th>LT50 (h)</th>
<th>d-Cycloserine (μg/g)</th>
<th>Vancomycin (μg/g)</th>
<th>Flomoxef (μg/g)</th>
<th>Tetracycline (μg/g)</th>
<th>Chloramphenicol (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220</td>
<td>28.8 ± 2.1</td>
<td>40 ± 17</td>
<td>0.53 ± 0.15</td>
<td>0.054 ± 0.022</td>
<td>0.68 ± 0.21</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>TS2921</td>
<td>29.3 ± 0.4</td>
<td>&lt;2.2</td>
<td>0.43 ± 0.04</td>
<td>0.031 ± 0.012</td>
<td>0.70 ± 0.08</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>TS5337</td>
<td>27.4 ± 2.3</td>
<td>5.4 ± 0.6</td>
<td>0.45 ± 0.08</td>
<td>0.031 ± 0.001</td>
<td>0.75 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>TS10007</td>
<td>27.5 ± 1.7</td>
<td>&lt;2.2</td>
<td>0.25 ± 0.11</td>
<td>0.046 ± 0.021</td>
<td>0.35 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

S. aureus cells (50 μl) in 0.9% NaCl (3 × 10^7 cells) were injected into the hemolymph of fifth-instar silkworm larvae, followed by injection of antibiotics (50 μl in 0.9% NaCl). More than 10 larvae were used for each dose of antibiotics. Survival of larvae incubated at 27°C was determined 48 h after injection, and ED50 was calculated. The ED50 was determined as the amount of drug per larva required for 50% survival under conditions in which more than 90% of silkworm larvae were killed by S. aureus cells. Data show the means ± standard deviations of at least four independent experiments.

LT50 represents the time at which half of larvae were dead after injection of each S. aureus strain in the absence of antibiotics. Data show the means ± standard deviations obtained from three independent experiments.
We thank Makiko Miyatani, Hiromi Komaki, Kozue Saito, Kaori Hayasaka, Aya Yoshino, Yumiko Matsuzawa, and Kiyomi Kyogoku for their technical assistance.

This work was supported in part by Grants-in-Aid for Scientific Research from the JSPS, by the Industrial Technology Research Grant Program in 2004 from NEDO of Japan, by the Program for Promotion of Fundamental Studies in Health Sciences of NIBIO, and by grants from Genome Pharmaceuticals Institute Co. Ltd.

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