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

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The availability of a silkworm larva infection model to evaluate the therapeutic effectiveness of antibiotics was examined. The 50% effective doses (ED_{50}) 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 ED_{50} 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 (ED_{50}) 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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TABLE 1. MICs of antibiotics against *S. aureus ddlA* mutants[a]

Strain	MIC (μg ml^{-1}) of antibiotic:					
	D-Cycloserine	Vancomycin	Flomoxef	Tetracycline	Chloramphenicol	Norfloxacin
RN4220	100	0.4	0.4	0.063	6.3	0.63
TS2921	12.5	0.4	0.2	0.063	3.1	0.63
TS5337	25	0.4	0.2	0.063	3.1	0.63
TS10007	50	0.4	0.4	0.063	3.1	0.63
TS2921/pS*ddlA*[b]	>100	0.4	0.4	0.063	ND[d]	0.63
TS2921 + D-Ala-D-Ala[c]	50	1.6	0.4	0.031	3.1	0.63

[a] 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.
[b] pS*ddlA*, 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.
[c] MICs were determined in the medium containing 100 mM D-Ala-D-Ala, which could complement the temperature sensitivity of these *ddlA* mutants.
[d] ND, not determined because of the chloramphenicol resistance of the plasmid.

nificant. The TS2921 phenotype of high susceptibility to D-cycloserine was reversed by introduction of the pS*ddlA* plasmid or by addition of D-Ala-D-Ala to the medium (Table 1), suggesting that the susceptible phenotype is caused by a reduced ability to produce the D-Ala-D-Ala dimer of the mutant enzyme. MICs of vancomycin, tetracycline, and norfloxacin against these *ddlA* mutants were the same as those of these drugs against the parent strain. Also, the differences between the MICs of flomoxef or chloramphenicol against the parent strain and the MICs against the *ddlA* mutants were not significant (Table 1). Therefore, these results suggest that the *S. aureus ddlA* TS2921 and TS5337 mutants were susceptible to D-cycloserine and that the high sensitivity of the TS2921 mutant to D-cycloserine is antibiotic specific.

Next, we determined the therapeutic effectiveness of several antibiotics against *S. aureus* parent- or *ddlA* mutant-mediated larval death according to our previously established method (3, 7), except that bacterial cells were suspended in 0.9% NaCl, and we compared the values between the parent strain and the mutants. When silkworm larvae were inoculated with 3×10^7 CFU of *S. aureus* parental and mutant cells, their 50% lethal times, the times at which half of larvae were dead, were much the same between the parent strain and the three *ddlA* mutants (Table 2), suggesting that they have comparable lethal effects on silkworm larvae. The ED_{50} of D-cycloserine against *ddlA* mutant-mediated larval death, which was determined from survival curves, was 12.5% or <5.6% of that against larval death mediated by the parent strain (Table 2). In all *ddlA* mutants tested, the decrease in ED_{50} of D-cycloserine compared with that of the parent starin was more noticeable than the decrease in the MIC. In contrast, the difference between the parent and the *ddlA* mutants with respect to the ED_{50} of vancomycin, flomoxef, tetracycline, or chloramphenicol was within twofold, suggesting a selective effectiveness of D-cycloserine against all the *ddlA* mutants tested. These results suggest that the silkworm model is useful to examine the target-mediated antibacterial and therapeutic actions of the compounds. Differences between the *S. aureus ddlA* mutants were observed with regard to MIC and ED_{50}. Therefore, using multiple mutants that have distinct mutations makes it possible to identify a wider variety of candidate compounds during screening.

We questioned why the decreases in ED_{50} were larger than the decreases in MIC against the *ddlA* mutants. Two explanations can be proposed. One is that the innate immunity of the silkworm larvae suppressed bacterial cell growth. The other is that it is not bacterial cell growth itself but rather host-killing ability that is being evaluated in the silkworm model.

In conclusion, we report here that comparison studies of therapeutic effectiveness of the compounds against parental and mutant bacterial strains can be helpful for evaluating their target-specific actions. These merits make this model worthy of attention as a secondary screening method for candidate antibacterial agents identified through target-based high-throughput screening.

TABLE 2. Therapeutic effectiveness of antibiotics against *S. aureus ddlA* mutants in a silkworm larva infection model[a]

Strain	LT_{50}[b] (h)	ED_{50} (μg/g of larva) of drug:				
		D-Cycloserine	Vancomycin	Flomoxef	Tetracycline	Chloramphenicol
RN4220	28.8 ± 2.1	40 ± 17	0.53 ± 0.15	0.054 ± 0.022	0.68 ± 0.21	4.9 ± 0.1
TS2921	29.3 ± 0.4	<2.2	0.43 ± 0.04	0.031 ± 0.012	0.70 ± 0.08	5.3 ± 0.4
TS5337	27.4 ± 2.3	5.4 ± 0.6	0.45 ± 0.08	0.031 ± 0.001	0.75 ± 0.12	
TS10007	27.5 ± 1.7	<2.2	0.25 ± 0.11	0.048 ± 0.021	0.35 ± 0.06	

[a] *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 ED_{50} were calculated. The ED_{50} was determined as the amount of drug per 1 g of silkworm 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.
[b] LT_{50} 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.

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