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Inhibitory Role for D-Alanylation of Wall Teichoic Acid in Activation of Insect Toll Pathway by Peptidoglycan of *Staphylococcus aureus*

Yukichika Tabuchi,* Akiko Shiratsuchi,* Kenji Kurokawa,†‡ Ji Hee Gong,† Kazuhisa Sekimizu,‡ Bok Luel Lee,† and Yoshinobu Nakanishi*

Pathogenic bacteria mitigate host immunity to establish infections, but the mechanism of this bacterial action has not been fully elucidated. To search for cell wall components that modulate innate immune responses in host organisms, we examined *Staphylococcus aureus* mutants, which were deficient in components of the cell wall, for pathogenicity in *Drosophila*. A mutation of dltA, which is responsible for the D-alanylation of teichoic acids, brought about an increase in the survival rate of adult flies that had received a septic infection with the bacteria. The growth of dltA-deficient *S. aureus* in adult flies was less efficient than that of the parental strain. The level of mRNA of Toll pathway-dependent antimicrobial peptides was higher in flies infected with the dltA mutant than that observed after the infection with the parental strain. The defective phenotype associated with the mutation of dltA, reduced pathogenicity and growth, was not evident in flies lacking the Toll pathway. Finally, a fraction of peptidoglycan prepared from the dltA mutant induced the expression of mRNA of a Toll-dependent antimicrobial peptide in flies and was bound by peptidoglycan recognition protein-SA in vitro more effectively than that obtained from the parental strain, and this difference was lost after the removal of wall teichoic acid from peptidoglycan.

Taken together, we conclude that D-alanylated wall teichoic acid of *S. aureus* mitigates a Toll-mediated humoral response in *Drosophila* interfering with the recognition of peptidoglycan by a pattern recognition receptor. The Journal of Immunology, 2010, 185: 2424–2431.

Microbial pathogens that have invaded host organisms, either invertebrates or vertebrates, are recognized primarily by the innate immune system, and this recognition evokes a variety of humoral and cellular immune reactions that are either cytostatic or cytotoxic to the invaders. Microbes seem to be equipped with strategies for circumventing such innate immune responses to achieve a successful infection and prolonged survival in host organisms. This microbial action has been studied mainly with mammals, and several mechanisms have been proposed (1–7): a change to the structure of the cell surface to avoid recognition by sentinel immune cells or soluble factors; interference with signaling pathways for the production of proinflammatory cytokines and antimicrobial substances; inhibition of phagocytosis; and regulation of the maturation of phagosomes. Moreover, some microbes highjack the host innate immune system; *Staphylococcus aureus* exploits TLR2 to inhibit the production of superoxide in macrophages and evade killing after phagocytosis (8, 9). This suggests that pattern recognition receptors and microbial components as their ligands operate two different reactions, one enhancing and the other mitigating host immunity.

*Drosophila* is a genetically tractable animal useful for investigating innate immune responses (10). Previous studies have provided an outline of the mechanism whereby the fat body, equivalent to the liver, and hemocytes, leukocytes or blood cells of mammals, cooperate to induce humoral and cellular immune responses upon a microbial infection (10–12). The use of this model may help us to identify molecules responsible for the regulation of host immunity with the aid of genetics applied to both host organisms and microbial pathogens. In addition, we may combine genetics with biochemistry using a cell-free system with purified immune proteins of another insect *Tenebrio molitor*, which constitutes a proteolytic cascade for the activation of a humoral response (13, 14). We previously screened mutant strains of *S. aureus* that lacked the expression of genes responsible for the synthesis of cell wall components with regard to their susceptibility to phagocytosis by *Drosophila* hemocytes and identified lipoteichoic acid (LTA) as a ligand for Draper, a phagocytosis receptor expressed in hemocytes (15). In the current study, a similar screening of the mutant strains was carried out to examine their pathogenic effects on *Drosophila*, and we found that D-alanylated wall teichoic acid (WTA) of *S. aureus* affected a humoral innate immune response in flies that had been infected with the bacterium.

### Materials and Methods

**Bacterial strains and fly stocks**

The strains of *S. aureus* and the plasmids used in this study are listed in Table I. The mutant strains deficient in the expression of genes responsible...
for the synthesis of cell wall components were derivatives of the strain RN4220. In the strains M0614 and M0615, SA0614 and SA0615 genes, which encode the two-component gnuRS system required for the enhancement of transcription of genes including dltA, have been disrupted by homologous recombination. The strain M0793 is a mutant of the dltA gene that codes for α-alanine ligase. M0702 and M0875 are mutants of tagO and yfp genes, respectively, which are responsible for the synthesis of WTA and glycolipids. The strains N143 and JT1304 are mutants of sle1 and atg genes, respectively, which code for amidasase and an amidase–glucosaminidase fusion protein involved in the degradation of peptidoglycan. T013 is a mutant of the igt gene coding for lipoprotein diacylglycerol transferase and thus deficient in the lipidation of lipoproteins. CK1001 is a mutant of the mpvF gene essential for the synthesis of lypsyly phosphatidylglycerol. The strain M0107 is a mutant of the spa gene coding for protein A. All of the bacterial strains were cultured at 37˚C with Luria-Bertani medium supplemented with antibiotics wherever required. When the cultures reached full growth, the bacteria were harvested, washed with ice-cold PBS, and used for the experiments. The following lines of Drosophila were used as wild-type flies: Canton S (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN), Oregon R (Kyotor-Fly, Kyorion University, Tokyo, Japan), Oregon RS (Drosophila Genetic Resource Center, Kyoto Institute of Technology, Kyoto, Japan), Oregon RP2 (Drosophila Genetic Resource Center, Kyoto Institute of Technology), and Oregon RC (Drosophila Genetic Resource Center, Kyoto Institute of Technology). The fly lines Tt/cd/TM1, Su(Pv)16 and Tt/cd/Tm1 Ser (16) were used to generate Tt/cd/Tt4α, ru h s e/TM1 Ser (16) was used as a null mutant for toll.

**Bacterial infection and assays for fly survival, colony formation of bacteria, and level of mRNA of antimicrobial peptides**

Male adult flies received an injection of given numbers of live bacteria (indicated in the figures or corresponding figure legends) at the abdomen, reminiscent of a septic infection, as described previously (15). One to three vials (15–20 flies per vial) were used in each experiment, except for the determination of CFU values, where three to four vials (each containing five flies) were analyzed. Assays for fly survival, colony formation of the injected bacteria using an agar-solidified selection medium, and quantitative RT-PCR of antimicrobial peptide (AMP) mRNA were conducted as described previously (15). The DNA oligomers used as primers in PCR were: 5’-CGTGAGAAGGCTTTCTCCAAATGATG-3’ (forward) and 5’-CTCCAGGACCAGCAGCAT-3’ (reverse) for drosomycin; 5’-AACCTAATCTTTGAGGAGCA-3’ (forward) and 5’-CGTCTTGTGTTGGTTCGAG-3’ (reverse) for metchnikowin; 5’-CGGTCTTGGTTGGTTAG-3’ (forward) and 5’-GGGACAGTATCTG-3’ (reverse) for drosomycin; and 5’-AACTTAATC-3’ (forward) and 5’-GTTCTTCGTTCTCGTGG-3’ (reverse) for defensin.

**Assay for peptidoglycan activity inducing drosomycin mRNA expression in flies,** linking to peptidoglycan recognition protein-SA, and activating protease cascade

A fraction of the cell wall rich in lysine-type peptidoglycan with WTA attached was prepared from S. aureus strains according to a published method (17) with some modifications. In brief, S. aureus cells were disrupted using glass beads and centrifuged at 800 × g for 10 min. The supernatants were recentrifuged at 20,000 × g for 10 min, and the precipitates were suspended in 20 mM sodium citrate (pH 4.7) containing 0.5% (w/v) SDS, heated at 60˚C for 30 min, and centrifuged at 20,000 × g for 10 min. The resulting precipitates were used as WTA-attached peptidoglycan. To remove WTA from peptidoglycan, this fraction was incubated in 40% (v/v) hydrofluoric acid at 4˚C for 18 h, and WTA-removed peptidoglycan was separated by centrifugation. WTA of S. aureus that retained α-alanine was prepared as described previously (9). Immune proteins of T. molitor and Tribolium castaneum were obtained as follows: Gram-negative bacteria-binding protein 1 (GNBP-1), a zymogen form of modular serine protease (MSP), and a zymogen form Spätzle-processing enzyme (SPE) were partially purified from the hemolymph of T. molitor larvae; T. molitor peptidoglycan recognition protein (PGRP)-SA, a zymogen form of T. molitor SPE-activating enzyme (SAE), and a proform of T. castaneum Spätzle were prepared as recombinant proteins using a baculovirus-based DNA vector and the insect cell line S9, as described previously (13). To determine the activity of peptidoglycan for inducing AMP production, adult flies were injected at the abdomen with preparations of peptidoglycan, and the level of drosomycin mRNA was determined as done for flies infected with bacteria. Cell-free assays for the binding of T. molitor PGRP-SA to peptidoglycan (17) and the peptidoglycan-induced processing of zymogen or proforms of T. molitor immune proteins (13) were carried out as described previously.

**Data processing and statistical analysis**

Results from quantitative analyses are expressed as mean ± SD of the data from at least three independent experiments unless otherwise stated in the text. Other data are representative of at least three independent experiments that yielded similar results. The number of independent experiments performed is indicated in the figure legends. Statistical analyses were performed using the two-tailed Student t test. The number of groups examined for the significance of difference was two (test sample versus control), and means were evaluated statistically without considering an error within each experiment, which was no greater than 25%. All p < 0.05 were considered significant, and all of the data significantly different from control values are marked with asterisks. p values are shown in the figures.

**Results**

Identification of dltA required for pathogenic effects of S. aureus on Drosophila

To identify bacterial genes responsible for the modulation of innate immune responses in Drosophila, we screened 10 mutant strains of S. aureus lacking the expression of genes required for the synthesis and metabolism of cell wall components. Adult flies of the wild-type line Canton S were injected at the abdomen with the mutant bacteria or a parental strain as a control, and the survival rate of the flies was determined for the following days. The rate of fly survival varied with the strains of injected bacteria, although the survival ratio of flies decreased as bacterial doses increased. We compared the ratio of fly survival 8 d after the injection of 1000 bacteria at a dose of which the parental strain killed >90% of flies in 8 d (Fig. 1A). Six of the 10 mutant strains showed decreased pathogenicity, and the others were, more or less, as

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**Table I. S. aureus strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Names</th>
<th>Genotypes and Characteristics</th>
<th>References</th>
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<tbody>
<tr>
<td>RN4220</td>
<td>NCTC8325-4, restriction mutant</td>
<td>(33)</td>
</tr>
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<td>M0614</td>
<td>RN4220 SA0614::pSFSA0614</td>
<td>(9)</td>
</tr>
<tr>
<td>M0615</td>
<td>RN4220 SA0615::pSFSA0615</td>
<td>(9)</td>
</tr>
<tr>
<td>M0702</td>
<td>RN4220 tagO::pT0702</td>
<td>(34)</td>
</tr>
<tr>
<td>M0793</td>
<td>RN4220 dltA::pT0793</td>
<td>(34)</td>
</tr>
<tr>
<td>M0875</td>
<td>RN4220 yfp::pT0875</td>
<td>(34)</td>
</tr>
<tr>
<td>N143</td>
<td>RN4220 sle1::pMSle1</td>
<td>(35)</td>
</tr>
<tr>
<td>JT1304</td>
<td>RN4220 alt::cat</td>
<td>(36)</td>
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<td>RN4220 lgt::pMIgt</td>
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</tr>
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</tr>
<tr>
<td>M0107</td>
<td>RN4220 spa::phleo</td>
<td>(38)</td>
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<td>pH300PLK</td>
<td>Escherichia coli-S. aureus shuttle vector; Amp’ Tel’</td>
<td>Purchased from Takara-Bio (Otsu, Japan)</td>
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<tr>
<td>p0793</td>
<td>pH300 with intact dltABCD from RN4220</td>
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pathogenic as the parental strain (Fig. 1A). Three of the six genes were related to the D-alanylation of teichoic acids: dltA codes for D-alanine ligase (18), and the products of SA0614, SA0615, ypfP, sle1, tagO, spa, lgt) independent experiments with similar results are presented. B, Adult flies of the indicated wild-type fly lines as well as w1118 were injected with the dltA mutant or parental S. aureus strain (1 × 10^5 bacteria per fly), and the survival rate of flies was determined at the indicated time points. Data from one of two independent experiments with similar results are presented. C, Adult flies of Oregon R received an injection of the dltA mutant (1 × 10^5 bacteria per fly) that had been transformed with the plasmid pHY300PLK carrying the dltABCD operon (dltABCD) or the vector alone (vector), and the survival rates of flies were determined at the indicated time points. Data from one of three independent experiments with similar results are presented.

We next examined how the growth of S. aureus in flies was altered with a loss of dltA expression. Adult flies 15 h after an injection of bacteria at the abdomen were homogenized and plated on a selection culture medium, and the number of S. aureus colonies was determined. We found that the parental strain exhibited an increase in CFU values, whereas the dltA mutant did not (Fig. 2A, left panel). This seemed not due to a delay in the growth of the dltA mutant, because the level of colony-forming bacteria remained almost the same for a day and decreased thereafter (Fig. 2A, right panel). This defect in the growth of the dltA mutant also was recovered by the forced expression of the dltABCD operon (Fig. 2B). The pathogenicity of S. aureus is therefore likely that S. aureus requires D-alanylated WTA to be effectively pathogenic to Drosophila.
of a strain lacking entire WTA due to a mutation of tagO, whose product is involved in the transfer of N-acetylglucosamine to a lipid carrier as the first biosynthesis step of WTA (18), was comparable to that of the parental strain (Fig. 1A). We thus examined the growth of this mutant strain in flies and found that it grew as efficiently as the parental strain (Fig. 2C). This could be due to a complex effect of WTA on both the modulation of host immunity by bacteria and the susceptibility of bacteria to host immune reactions (see Discussion).

These results indicated that the expression of dltA is required for S. aureus to effectively grow in adult flies and suggested that a reduced growth rate resulted in a decrease in the pathogenicity of the dltA mutant.

Toll dependence of decreased pathogenicity of dltA-deficient S. aureus

We next examined a mechanism underlying the reduced pathogenicity and growth of the dltA mutant in Drosophila. We previously reported that the level of the phagocytosis of the same dltA mutant strain by Drosophila hemocytes was equivalent to that of the parental strain (15). It was thus presumable that the defective phenotypes of the dltA mutant were caused by an increase either in the level of the host humoral immunity or in the susceptibility of the bacterium to a humoral response. There is a report showing that a loss of D-alanine from teichoic acids makes S. aureus more sensitive to cationic AMP of mammals (21). Because it was not easy for us to examine the susceptibility of S. aureus to fly AMP, we first determined the level of a humoral immune response (i.e., the production of AMP) in flies infected with the dltA mutant. There are two signaling pathways for the induction of humoral immune responses in Drosophila: the Toll pathway, which responds mainly to Gram-positive bacteria and fungi, and the Immunodeficiency pathway, which is activated upon infection with Gram-negative bacteria (11). Both pathways are initiated when peptidoglycan of bacteria or β-1,3-glucan of fungi is sensed by immune receptors called PGRP and GNBP (11). In Drosophila infected with S. aureus, soluble sentinel immune proteins, including PGRP-SA, sense lysine-type peptidoglycan and induce the partial cleavage of pro-Spätzle, which is expressed in and secreted from hemocytes (22). The processed Spätzle in turn serves as a cytokine to activate the membrane receptor Toll in fat body cells so that it transmits a signal leading to the production of AMP-like drosomycin (11).

The level of drosomycin mRNA thus was determined in flies injected with the parental and dltA mutant strains. The septic infection with the parental strain brought about an increase of drosomycin mRNA in a manner dependent on the Toll pathway, and its level was higher in flies infected with the dltA mutant than that in flies infected with the parental strain (Fig. 3A, left and middle panels). In addition, flies infected with the tagO mutant, which lacks entire WTA but was as pathogenic as the parental strain (Fig. 1A), produced a higher level of drosomycin mRNA than those infected with the parental strain (Fig. 3A, right panel). Moreover, a higher level of mRNA in flies infected with the dltA mutant also was observed for metchnikowin and defensin, other Toll-dependent AMPs (Fig. 3B). These results indicated that S. aureus without α-alanlated WTA more effectively induces the production of mRNA of Toll-dependent AMP in adult flies. Flies deficient in the Toll pathway died of a septic infection with the parental S. aureus strain at a dose not pathogenic to wild-type flies (Fig. 3C). Furthermore, the dltA mutant was not less pathogenic than the parental strain and grew as effectively as the parental strain in Toll-deficient flies (Fig. 3D). Taken together, the decreased pathogenicity of S. aureus caused by a lack of dltA expression is due, if not exclusively, to enhancement of the Toll-mediated humoral immune response.

Inhibition of peptidoglycan-dependent, PGRP-SA-mediated activation of the Toll pathway by α-alanlated WTA

The data obtained so far suggested that α-alanlated WTA of S. aureus weakens the action of peptidoglycan to activate the Toll pathway for the production of AMP. To directly examine this possibility, we prepared a fraction of the cell wall rich in WTA-attached peptidoglycan from the parental and dltA mutant strains, injected it into adult flies, and determined the level of drosomycin mRNA. A peptidoglycan preparation of the parental strain induced drosomycin mRNA expression in a dose-dependent manner (Fig. 4A). We found that the level of drosomycin mRNA was higher in flies injected with a peptidoglycan fraction obtained from the dltA mutant than that in flies injected with a fraction prepared from the parental strain (Fig. 4B, left panel). In contrast, this difference was lost when the same peptidoglycan preparations were used after a chemical treatment to remove WTA (Fig. 4B, right panel). This indicated that the presence of WTA, most probably in the α-alanlated form, reduced the activity of peptidoglycan to induce drosomycin expression in adult flies. We then tried to reconstitute...
FIGURE 3. Toll dependence of phenotypes of the dltA mutant. A, Adult flies of Oregon R and a toll null mutant (Tl) were injected with the parental (parent), the dltA mutant (∆dltA), or the tagO mutant (∆tagO) S. aureus strain (1 × 10⁶ bacteria per fly) or PBS. After 3 h, the level of drosomycin mRNA (Drs) was determined and shown as a ratio to that of mRNA of ribosomal protein 49 (Rp49). Data are expressed as the mean ± SD of the results from three (left and right panels) and four (middle panel) independent experiments. B, Adult flies of Oregon R were injected with the parental (parent) or the dltA mutant (∆dltA) S. aureus strain (1 × 10⁶ bacteria per fly) or PBS. After 3 h, the levels of metchnikowin (Mtk) and defensin (Def) mRNA were determined and shown as a ratio to that of mRNA of ribosomal protein 49 (Rp49). Data are expressed as the mean ± SD of the results from eight (left panel) and nine (right panel) independent experiments. C, Adult flies of Oregon R (wt) or a toll null mutant (Tl) received an injection of the parental S. aureus strain (1 × 10⁶ bacteria per fly) or PBS, and the survival rates of flies were determined at the indicated time points. Data from one of four independent experiments with similar results are presented. D, Adult flies of a toll null mutant (Tl) were injected with the parental or dltA mutant strain (1 × 10⁶ [left panel] or 1 × 10⁵ [right panel] bacteria per fly) or PBS, and the survival rates of flies were determined. Data from one of three independent experiments with similar results (left panel) or the mean ± SD of the results from three independent experiments (right panel).

the effect of WTA by injecting flies separately with a WTA-free peptidoglycan preparation and D-alanylated WTA isolated from a cell wall fraction of the parental strain, but the level of drosomycin mRNA was almost the same as that in flies injected with WTA-free peptidoglycan alone (Fig. 4C). This suggested that direct association with peptidoglycan is needed for D-alanylated WTA to inhibit the action of peptidoglycan.

We next asked how D-alanylated WTA inhibits peptidoglycan activation of the Toll pathway. Lysine-type peptidoglycan of S. aureus is first recognized by a complex of proteins including PGRP-SA (11). We thus conducted an assay for the binding of PGRP-SA and a cell wall fraction of the parental strain after a cleavage of WTA (lanes 1 and 2). Under the conditions adopted in this study, PGRP-SA incubated with peptidoglycan of the parental strain was mostly recovered in the supernatant (lanes 1 and 2), indicating that it did not bind effectively to peptidoglycan containing D-alanylated WTA. In contrast, a majority of PGRP-SA was detectable in the precipitate in a reaction using a peptidoglycan preparation of the dltA mutant (lanes 3 and 4). Furthermore, PGRP-SA became able to bind to peptidoglycan of the parental strain after a cleavage of WTA (lanes 3 and 6) and seemed to bind to peptidoglycan of the tagO mutant, which lacked entire WTA, as effectively as to that of the dltA mutant (lanes 7 and 8). These results indicated that the association with D-alanylated WTA weakens the ability of peptidoglycan to bind to PGRP-SA. We next tested the functional consequence of this effect of D-alanylated WTA on the binding between peptidoglycan and PGRP-SA. The recognition of peptidoglycan by a complex including PGRP-SA culminates in the activation of a cascade of serine protease reactions that leads to the production of cleaved and thus activated Spätzle, a ligand for Toll (11). We previously succeeded in reconstituting this cascade in a cell-free system using peptidoglycan of S. aureus and six immune proteins of T. molitor (13). Taking advantage of this experimental system, we determined the activity of peptidoglycan fractions prepared from the parental, dltA mutant, and tagO mutant strains. A mixture containing peptidoglycan and T. molitor proteins was incubated and centrifuged, and the resulting supernatants were subjected to Western blotting with Abs recognizing MSP, SAE, and Spätzle to examine if zymogen MSP, SAE, and Spätzle were detected. In contrast, in a reaction with peptidoglycan obtained from the dltA (lanes 2, 3, and 8) or tagO (lanes 3, 6, and 9) mutant, cleaved products of zymogen MSP and SAE as well as pro-Spätzle were detected. The results described above collectively suggested that D-alanylated WTA interferes with the recognition of S. aureus peptidoglycan by PGRP-SA, resulting in reduced production of the processed Spätzle, a ligand for Toll.
by the host immune system: that is, D-alanylated WTA affects the cell wall component interferes with the sensing of another component in *Drosophila*. A strategy to reduce the level of a humoral innate immune response not only to the complexity of its structure but also to its association with another cell wall component. The molecular basis underlying the action of D-alanylated WTA remains to be elucidated. We showed that D-alanylated WTA by itself did not exhibit an inhibitory action when injected into flies together with WTA-free peptidoglycan. This could deny a direct action of D-alanine or its chemical modification, such as O-acetylation and attachment of WTA and then of WTA-containing peptidoglycan so that peptidoglycan becomes less effective in binding to and thus activating its receptor PGRP-SA. O-Acetylation of peptidoglycan at the C6 position of muramic acid confers peptidoglycan resistance to lysozyme (24), and WTA is linked to peptidoglycan at the same site. This suggests that muramic acid plays an important role in the recognition of peptidoglycan by lysozyme and PGRP-SA and that its receptor PGRP-SA. O-Acetylation of peptidoglycan at the C6 position of muramic acid confers peptidoglycan resistance to lysozyme (24), and WTA is linked to peptidoglycan at the same site. This suggests that muramic acid plays an important role in the recognition of peptidoglycan by lysozyme and PGRP-SA and that its chemical modification, such as O-acetylation and attachment of D-alanylated WTA, serves as a steric hindrance. In the case of D-alanylated teichoic acid-mediated resistance of *S. aureus* to mammalian cationic AMP, a role for D-alanine to introduce a positive charge has been suggested (18, 21, 25). At the present time, it is unclear if the same mechanism can be applied to the D-alanylated WTA-mediated inhibition of the recognition of peptidoglycan by PGRP-SA.

**Discussion**

In the current study, we showed that *S. aureus* is armed with a strategy to reduce the level of a humoral innate immune response in *Drosophila*. This type of bacterial action is unique in that a cell wall component interferes with the sensing of another component by the host immune system: that is, D-alanylated WTA affects the recognition of peptidoglycan, to which it is covalently attached, by the pattern recognition receptor PGRP-SA. A consequence of this mechanism was inhibition of the production of activated Spätzle, a ligand for Toll, and thus a lower level of mRNA of AMP-like drosomycin. Previous reports showed that partial degradation by lysozyme is required for peptidoglycan of *S. aureus* to be more effectively recognized by the immune system of host organisms (17, 23). Our finding suggests that the inefficiency of lysine-type peptidoglycan as a pathogen-associated molecular pattern is due not only to the complexity of its structure but also to its association with another cell wall component. The molecular basis

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**FIGURE 4.** Increased level of drosomycin mRNA in flies injected with peptidoglycan of the *ΔdltA* mutant. A, Adult flies of Oregon *R* were injected with the indicated amounts of a peptidoglycan fraction prepared from the parental *S. aureus* strain (PGN), and the level of drosomycin mRNA was determined after 6 h. Data from one of four independent experiments with similar results are presented. B, Adult flies of Oregon *R* received an injection of a peptidoglycan fraction (5 ng per fly) prepared from the parental or *ΔdltA* mutant strain before (left panel) or after (right panel) the removal of WTA, and the level of drosomycin mRNA was determined after 6 h. Data are expressed as the mean ± SD of the results from four (left panel) or three (right panel) independent experiments. C, Adult flies of Oregon *R* were injected with WTA-removed peptidoglycan (PGN) (0.5 ng per fly) or a mixture of WTA-removed peptidoglycan and isolated WTA (0.5 ng per fly), and the level of drosomycin mRNA was determined after 6 h. Data are expressed as the mean ± SD of the results from three independent experiments.

**FIGURE 5.** Increased recognition by PGRP-SA of peptidoglycan prepared from the *ΔdltA* mutant. A, Peptidoglycan preparations (PGN) obtained from the indicated *S. aureus* strains (50 μg) and *T. molitor* PGRP-SA (0.25 μg) were incubated for 1 h at 30˚C and centrifuged, and the resulting precipitates (P) (containing peptidoglycan) and supernatants (S) were subjected to SDS-PAGE followed by staining with Coomassie brilliant blue. Lane 9 was loaded with PGRP-SA alone (0.25 μg) as a positive control, and the furthest left lane (M) contained molecular mass markers (molecular masses are shown in kiloDaltons). Data from one of three independent experiments with similar results are presented. B, Peptidoglycan fractions (PGN) prepared from the indicated *S. aureus* strains were incubated with a mixture of *T. molitor* proteins including PGRP-SA, GNB-1, MSP, SAE, SPE, and Spätzle for 1 h at 30˚C. The reaction mixtures then were centrifuged, and the supernatants were analyzed by Western blotting with anti-MSP, anti-SAE, and anti-Spätzle Abs. The arrowheads point to the positions of the uncleaved and cleaved forms of MSP, SAE, and Spätzle, and the positions of markers are indicated with molecular masses in kiloDaltons. Data from one of three independent experiments with similar results are presented.
Our data showed that a mutation of tagO, which leads to a loss of WTA, did not bring about a change in the pathogenicity of *S. aureus* to *Drosophila* (Fig. 1A) despite the level of AMP mRNA being augmented (Fig. 3A). This seemingly contradicts our conclusion as to the action of WTA. In our previous study, tagO appeared to be required for the effective phagocytosis of *S. aureus* by *Drosophila* hemocytes (15). This means that WTA exerts two apparently opposing effects on the survival of the bacterium in host organisms: reducing a humoral response and enhancing a cellular response. In fact, the tagO mutant grew in adult flies as efficiently as the parental strain. That the tagO mutant was as pathogenic as the parental strain thus could be an offset of the negative and positive effects of WTA on host immunity. However, the interpretation of the outcome from an assay like the one carried out in this study might not be so simple. A loss of WTA seems to confer on *S. aureus* either sensitivity (21) or resistance (26) to antimicrobial substances in mammals, although it is not known if this is also the case in *Drosophila*. Further investigation will be required to fully understand the role of WTA in the interaction between *S. aureus* and the immune system of host organisms.

There is more evidence, mostly obtained with mammals, for the importance of the α-alanylation of teichoic acids for *S. aureus* to evade host immunity: the resistance to antibiotics (27, 28), an increment in the pathogenicity of *S. aureus* to lymphocytes or other tissues (31). More recently, we reported that D-alanylated WTA of *S. aureus* avoids the activation of the insect Toll pathway induced by the peptidoglycan of *Staphylococcus aureus* by *Drosophila* hemocytes. *J. Immunol.* 183: 7451–7460.

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Disclosures

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