

# Muropeptide Modification-Amidation of Peptidoglycan D-Glutamate Does Not Affect the Proinflammatory Activity of *Staphylococcus aureus*<sup>∇</sup>

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**Peptidoglycan muropeptides, potent proinflammatory components, are amidated in *Staphylococcus aureus* for unknown reasons. To study whether this modification may modulate proinflammatory capacity, cytokine induction by isogenic *S. aureus* strains with different amidation levels and by synthetic amidated/nonamidated muramyldipeptides was evaluated. However, amidation did not significantly affect cytokine induction. This finding contributes to defining peptidoglycan receptor specificities and indicates that further rationales for muropeptide amidation have to be considered.**

*Staphylococcus aureus* is a frequent constituent of human nasal microflora and a major cause of severe endogenous infections (11). The staphylococcal cell wall determines several key aspects of the infection process, such as adherence (11, 28), immune recognition (13), and resistance to host defenses (21, 22). While many adhesive surface proteins have been investigated (12), the structure, function, and variability of nonproteinaceous polymers, such as peptidoglycan (PG) or teichoic acids, have remained parts of a neglected field of research. *S. aureus* is known to modify the canonical PG structure by O acetylation of the glycan strand and by amidation of the  $\alpha$ -carboxyl group of the D-glutamate (D-Glu) residue in muropeptides, resulting in the formation of D-isoglutamine (16, 29). While O acetylation confers lysozyme resistance (3), the primary role of D-Glu amidation has remained unclear. Nevertheless, the latter modification is known to affect the level of methicillin resistance (4, 17) and to contribute to vancomycin susceptibility in *S. aureus* (8). The amino group for D-Glu amidation in *S. aureus* muropeptides is most probably derived from free glutamine (17, 23). However, the responsible transamidase enzyme has not yet been identified. Gustafson et al. described a *femC* mutant with 48% decreased muropeptide amidation (17, 26). The *femC* mutant has a disrupted *glnR* gene encoding the regulator of the glutamine synthetase (Fig. 1A), which results in reduced glutamine synthetase activity. As a consequence of lower amounts of the amino group donor, muropeptide amidation is reduced in this mutant (17).

PG has potent proinflammatory properties (5). It is sensed by the human innate immune system via NOD1 or NOD2 proteins (15, 18). An additional role of the TLR2 receptor in PG sensing has been described previously (10) but remains a

matter of debate (27). The minimal structure required for NOD1 recognition is a dipeptide consisting of D-Glu and meso-diaminopimelate (mesoDAP), which is mainly produced by gram-negative bacteria (14, 15). D-Glu amidation (leading to D-isoglutamine) strongly impairs PG recognition by NOD1 (7), and the amidation of mesoDAP completely abrogates the ability of NOD1 to detect PG (15). NOD2 has a different substrate specificity as it requires a muramyldipeptide (MDP) composed of *N*-acetylmuramic acid, L-alanine, and D-Glu (Fig. 1B) (19, 27). *S. aureus* PG is recognized by NOD2 but not by NOD1 since it contains L-lysine instead of meso-DAP (7, 15). However, the relevance of D-Glu amidation for the efficiency of NOD2 recognition has remained unclear. MDP amidation has a profound impact on the physicochemical properties of the molecule since it removes the negative charge from the D-Glu  $\alpha$ -carboxyl group (Fig. 1B). Accordingly, the net charge of MDP changes from  $-2$  to  $-1$  at physiological pH as a result of D-Glu amidation. In an attempt to study whether this structural difference has an impact on proinflammatory activity, we evaluated the tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-stimulating capacities of *S. aureus* strains with different levels of PG amidation and of synthetic MDPs with and without amidation.

**Proinflammatory capacities of *S. aureus* wild type and *femC* mutant.** *S. aureus* NCTC8325 wild type and the isogenic *femC* mutant (17) were grown overnight in glutamine-deficient, lipopolysaccharide-free RPMI 1640 medium (Sigma), harvested, and washed twice in phosphate-buffered saline (PBS). The mutant grew slower than the wild type did, but both strains reached similar bacterial densities. After heat inactivation at 90°C for 15 min, bacteria were washed again with PBS and adjusted to 10<sup>9</sup> bacteria/ml by using a Neubauer chamber. Endotoxin contamination of these preparations was below the detection limit of 10 pg/ml of the *Limulus* amoebocyte lysate test (Cambrex). A total of 360  $\mu$ l whole blood from healthy volunteers was incubated with 40  $\mu$ l of PBS containing increasing numbers of bacteria at 37°C for 8 h. Subsequently, samples

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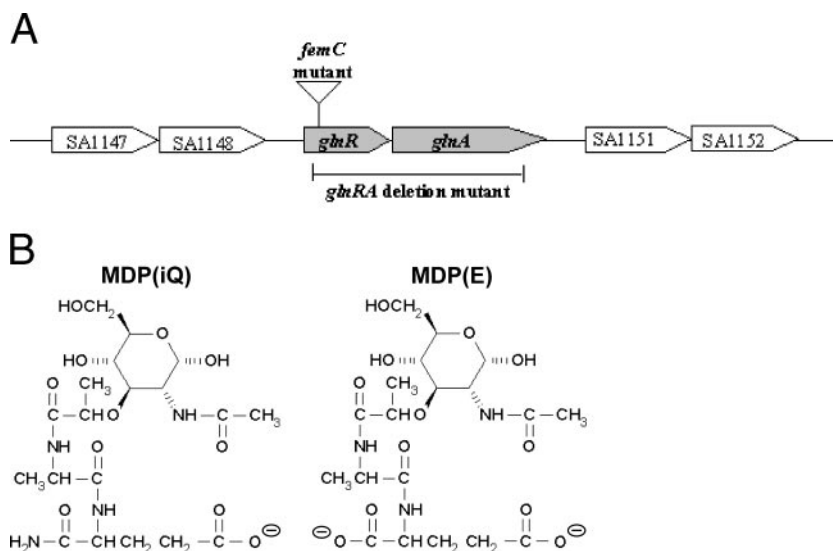


FIG. 1. The *S. aureus* glutamine synthetase operon, with the insertion site of Tn551 in the *femC* mutant and the deleted region in the *glnRA* mutant (A), and structures of amidated MDP(iQ) and nonamidated MDP(E) (B).

were centrifuged and TNF- $\alpha$  levels in the supernatant were determined by enzyme-linked immunosorbent assay (R&D Systems). Both bacterial strains stimulated TNF- $\alpha$  production in a dose-dependent fashion (Fig. 2A). However, there was no significant difference in TNF- $\alpha$  levels induced by wild-type or *femC* mutant bacteria, suggesting that the modulation of mucopeptide structure by D-Glu amidation may not affect the inflammatory activity of *S. aureus* cells.

In an attempt to obtain a mutant strain that completely lacks D-Glu amidation and might cause a stronger phenotypic difference, we deleted the entire operon composed of glutamine synthetase (*glnA*) and a corresponding regulator gene (*glnR*) (Fig. 1A). However, the resulting mutant grew only upon supplementation with significant amounts of glutamine and did not have any advantage relative to the *femC* mutant (data not shown).

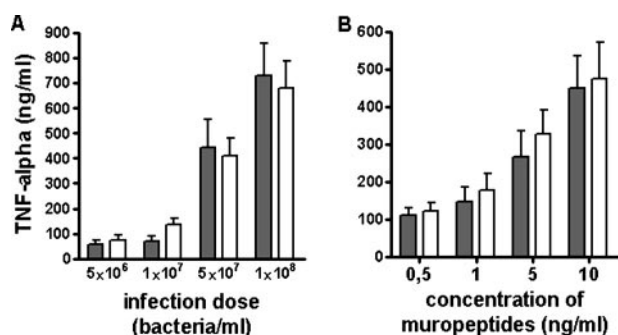


FIG. 2. TNF- $\alpha$  induction by *S. aureus* strains (A) and synthetic MDPs (B). (A) Increasing numbers of *S. aureus* wild-type (gray bars) or *femC* mutant bacteria (white bars) were incubated with whole human blood, and TNF- $\alpha$  levels were determined. (B) MDP(iQ) (gray bars) or MDP(E) (white bars) was incubated with whole human blood, and TNF- $\alpha$  levels were determined. The means and standard errors of the means (error bars) of four independent experiments, run in duplicates, are shown. The small differences between the strains in panel A are not significant as calculated by Student's *t* test.

**Proinflammatory capacities of synthetic MDPs with or without D-Glu amidation.** In order to study whether *S. aureus* MDPs with or without D-Glu amidation differ in their levels of proinflammatory activity when administered in the absence of other bacterial molecules, we chemically synthesized a nonamidated MDP with a D-Glu residue at position 2 of the peptide strand MDP(E), which is not commercially available. Synthesis of the acetylmuramyl-L-alanyl-D-glutamyl peptide was accomplished using Fmoc/tBu solid-phase peptide synthesis methodology (1) on acid-sensitive chlorotrityl chloride resin (2). Briefly, to 0.5 g 2-chlorotrityl chloride resin (0.6 mmol chloride/g resin) in 5 ml dichloromethane, 1 mmol of Fmoc-D-Glu(OBut)-OH and 2.5 mmol diisopropylethylamine (DIPEA) in 4 ml dichloromethane were added. After vigorous shaking for 60 min, the resin was filtered off, washed twice with dimethylformamide (DMF), and deactivated with a mixture of dichloromethane-methanol-DIPEA (80:15:5) for 15 min. After washing with DMF, the Fmoc group was deprotected with 5 ml of 20% piperidine in DMF for 5 min. After intensive washing with DMF, isopropanol, and diethyl ether, 1 mmol Fmoc-Ala-OH, 1 mmol diisopropylcarbodiimide, and 1 mmol 1-hydroxybenzotriazole in 5 ml DMF were added and incubated for 60 min. Deprotection with piperidine and further coupling with *N*-acetylmuramic acid yielded the protected peptide. Removal of the side chain-protecting group and detachment from the resin were accomplished by treatment with trifluoroacetic acid-water-phenol (90:5:5). The peptide was precipitated by the addition of diethyl ether and lyophilized. The crude peptide was purified by preparative, reversed-phase, high-performance liquid chromatography on a Reprosil C<sub>8</sub> column (150  $\times$  10 mm) using a flat gradient from 100% A to 40% B in 40 min (A, 0.055% trifluoroacetic acid; B, 80% acetonitrile-0.05% trifluoroacetic acid) while monitoring absorbance at 214 nm. Appropriate fractions were lyophilized, and the identity of the product was confirmed by electrospray ionization mass spectrometry (ESI-MS). Our synthetic MDP(E) and the amidated

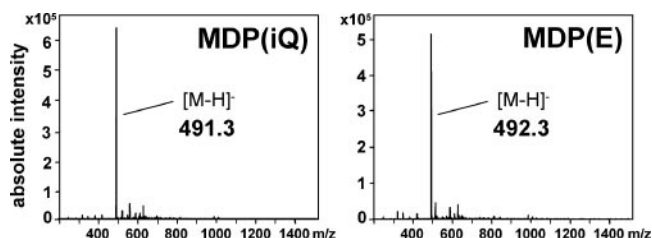


FIG. 3. Averaged negative ion mode ESI-MS of MDP(iQ) and MDP(E). Peaks with the expected masses for MDP(iQ) (491.3) or MDP(E) (492.3) are indicated.

MDP(iQ) purchased from Bachem were found to have the expected mass difference of 1 (Fig. 3).

MDP(E) and MDP(iQ) were dissolved in PBS. Again, the endotoxin contamination of the preparations was below the detection limit. Forty microliters of PBS containing increasing concentrations of these substances was incubated with 360  $\mu$ l whole human blood at 37°C for 8 h to stimulate TNF- $\alpha$  production. TNF- $\alpha$  was quantified as described above. Both compounds stimulated TNF- $\alpha$  production in a dose-dependent manner. However, amidated and nonamidated MDP did not differ in their potency levels (Fig. 2B), which is in accordance with the equal proinflammatory activities of *S. aureus* wild type and the *femC* mutant.

**Concluding comments.** The inflammation-eliciting capacity of *S. aureus* plays a crucial role in the host response to infections and contributes to life-threatening complications in septicemia (5, 13). However, the diversity, relative importance, and variability of proinflammatory staphylococcal molecules, such as peptidoglycan, lipopeptides, lipoteichoic acid, formylated peptides, and others have remained uncertain. While the relative importance of lipoproteins and formylated peptides has recently been confirmed using defined mutants lacking the corresponding classes of molecules (6, 9, 25), such a strategy is impossible for PG, which is essential for bacterial viability. However, bacterial mutants with altered PG structures might help to investigate the relative role of PG, along with the modulation of PG structure. This study demonstrates that the amidation of mucopeptides does not influence the capacity of *S. aureus* or its MDP to stimulate the production of TNF- $\alpha$ , which contributes to our understanding of the specificity of PG-sensing host receptors. Why *S. aureus* amidates its mucopeptides remains an open question. A possible reason may be the concomitant alteration of the net charge in the *S. aureus* cell envelope, which may play a role in the maintenance and turnover of the bacterial cell wall. Accordingly, mucopeptide amidation has been implicated in PG cross-linking efficiency (20, 24). Identifying the mucopeptide amidase gene would enable the construction of an *S. aureus* mutant lacking any mucopeptide amidation without affecting the intracellular glutamine pool. Such an approach represents an important challenge for future studies.

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