

# Subinhibitory Concentrations of Protein Synthesis-Inhibiting Antibiotics Promote Increased Expression of the *agr* Virulence Regulator and Production of Phenol-Soluble Modulins Cytolysins in Community-Associated Methicillin-Resistant *Staphylococcus aureus*<sup>∇</sup>

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**Tetracycline, clindamycin, and other protein synthesis inhibitors at subinhibitory concentrations significantly increased the expression of the pivotal virulence regulator *agr* and production of the *agr*-regulated cytolytic phenol-soluble modulins in the community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strain USA300. Our results suggest that such protein synthesis inhibitors may exacerbate the progression of CA-MRSA disease when applied at concentrations that are too low or when treating infections caused by strains resistant to those antibiotics.**

Phenol-soluble modulins (PSMs) are key virulence factors of *Staphylococcus aureus* that lyse neutrophils and other cell types and elicit a series of proinflammatory responses (14). PSMs are produced by most *S. aureus* strains and contribute significantly to the progression of skin infection caused by epidemic community-associated methicillin-resistant *S. aureus* (CA-MRSA) (5, 14).

Subinhibitory concentrations of antibiotics may trigger differential expression of bacterial virulence determinants (4), which may lead to exacerbated disease, for example, when antibiotic regimes are not strictly followed by the patient. The effect of subinhibitory antibiotic concentrations on the expression of PSMs has not been investigated. Here, we analyzed PSM production under the influence of subinhibitory concentrations of several antibiotics in clinical use, focusing on antibiotics used for the treatment of hospital-associated MRSA (HA-MRSA) and CA-MRSA, including multidrug-resistant CA-MRSA (6) (Fig. 1A and C). Of note, “subinhibitory” is often defined solely as having no effect on overnight growth. However, growth patterns may be severely affected even if strains reach comparable optical density values overnight; in that case, observed effects may simply be due to growth inhibition. Therefore, we ascertained that the used antibiotic concentrations did not cause considerable growth defects over the entire growth curve (Fig. 1B and D). As test strains, we chose the CA-MRSA strain USA300 (clone LAC), which is by far the most frequent cause of CA-MRSA infections in the United States (7, 11), and strain Sanger 252, representing a prevalent hospital-associated MRSA clone (9). To select optimal subinhibitory antibiotic concentrations (SICs)

(Table 1), we first applied 1/10 of the published MICs. If under those conditions we found severe growth defects, SICs were gradually adjusted until only minimal effects on growth were observed. If no effect on PSM production was observed, we increased the SIC as much as possible without seeing a considerable growth defect. PSM concentrations were determined in triplicate by reversed-phase high-pressure chromatography/electrospray ionization mass spectrometry (RP-HPLC/ESI-MS) as described previously (14). One-way analysis of variances (ANOVAs) with Dunnett’s multiple comparison tests were used to evaluate differences in PSM expression. Increased PSM levels were seen with tetracycline, clindamycin, erythromycin, and linezolid, and decreased levels were seen especially with oxacillin (Fig. 1A). In strain Sanger 252, changes in PSM levels were similar to those in strain LAC but in general less pronounced (Fig. 1C). However, in contrast to strain LAC, no significant change in PSM levels by oxacillin were observed, although oxacillin was applied at much higher concentrations for strain Sanger 252 (Table 1). Furthermore, there was decreased PSM production with mupirocin, which was not observed in strain LAC.

PSM peptides of *S. aureus* include the PSM $\alpha$  peptides (PSM $\alpha$ 1, PSM $\alpha$ 2, PSM $\alpha$ 3, PSM $\alpha$ 4), which are encoded in the *psm* $\alpha$  operon, the PSM $\beta$  peptides (PSM $\beta$ 1, PSM $\beta$ 2), which are encoded in the *psm* $\beta$  operon, and the  $\delta$ -toxin, which is encoded within the region coding for RNAPIII, the intracellular effector molecule of *agr*-mediated quorum sensing (12–14). PSM $\alpha$  peptides and  $\delta$ -toxin are cytolytic, while PSM $\beta$  peptides lack that activity at physiological concentrations (14). In general, changes seen with antibiotics were most pronounced for PSM $\alpha$ 1 and PSM $\alpha$ 2. PSM $\alpha$ 3 and PSM $\alpha$ 4 were less affected, but the direction of changes was consistent with all PSM $\alpha$  peptides. Concentrations of the  $\delta$ -toxin were not considerably affected, except for decreased production under the influence of oxacillin in strain LAC and mupirocin in strain Sanger 252. Concentrations of PSM $\beta$  peptides were very low and only PSM $\beta$ 1 could

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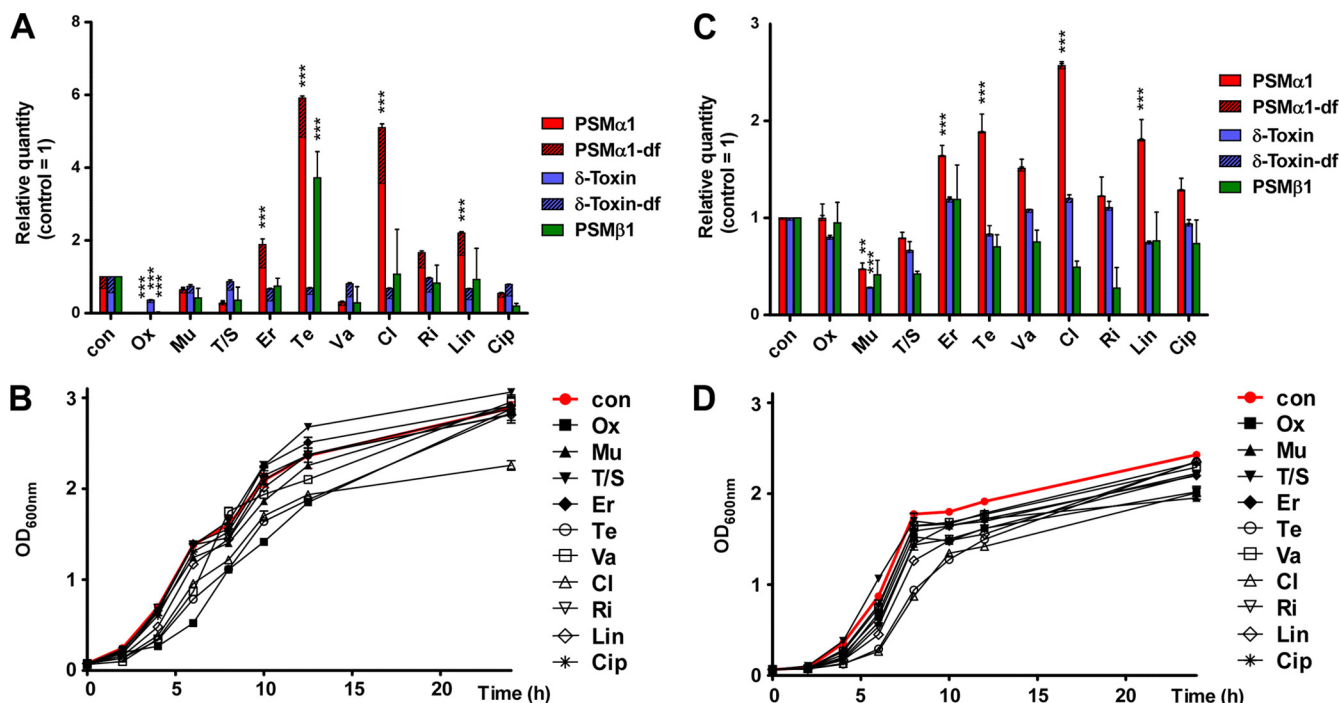


FIG. 1. Effects of antibiotics at subinhibitory concentrations on the production of PSMs. Different antibiotics, at the concentrations shown in Table 1, were added to cultures of *S. aureus* strains at the time of inoculation (1:100 from precultures) into 5-ml tubes filled with 1 ml of tryptic soy broth. Tubes were shaken for 24 h at 200 rpm, and PSM concentrations were determined in the culture filtrates using RP-HPLC/ESI-MS (14). Growth under the same conditions was monitored by determining the optical density at 600 nm ( $OD_{600}$ ). PSM concentrations in *S. aureus* LAC (USA300) (A), growth curves for *S. aureus* LAC (USA300) (B), PSM concentrations in *S. aureus* Sanger 252 (C), growth curves for *S. aureus* Sanger 252 (D). The parts of bars that are striped show the amount of N-deformylated (df) PSM among total PSM peptide concentration. Concentrations of PSM $\alpha$ 1 are shown as an example of PSM $\alpha$  peptides. Statistical analysis is shown for the effects and antibiotics described in the text (Er, Te, Cl, Lin for both strains, Ox for LAC, and Mu for Sanger 252); \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; con, control (without antibiotic). Error bars represent means  $\pm$  standard deviations.

be measured in most samples. Changes observed with PSM $\beta$ 1 were overall consistent with those observed for PSM $\alpha$  peptides.

Expression of the *psm $\alpha$*  and *psm $\beta$*  operons is under strict and direct control of the *agr* quorum-sensing system (13–14). In addition, expression of the  $\delta$ -toxin gene *hld* is directly linked to *agr*, as it is encoded within RNAPIII (10). Overall, changes in PSM production under antibiotic influence were consistent among the different PSM peptides, suggesting that the various

antibiotics may affect PSM production by an impact on *agr* activity. Thus, to evaluate whether *agr* contributes to the observed effects, we measured transcript levels of RNAPIII, using those antibiotics for which the most pronounced changes in PSM levels were observed. In addition, we determined whether amounts of *psm $\alpha$*  and *psm $\beta$*  transcripts were affected, as can be expected in the case of an *agr* effect given the strong impact of *agr* on PSM expression (13–14). This experiment demonstrated that *agr* activity was significantly affected by clindamycin and

TABLE 1. Subinhibitory concentrations of antibiotics used in this study<sup>a</sup>

Antibiotic	Abbreviation	LAC (USA300)		Sanger 252	
		MIC ( $\mu$ g/ml)	SIC ( $\mu$ g/ml)	MIC ( $\mu$ g/ml)	SIC ( $\mu$ g/ml)
Oxacillin	Ox	>16	0.4	>16	32
Mupirocin	Mu	0.12–0.25 (0.5)	0.03	0.25 (0.5)	0.06
Trimethoprim	T/S	0.25	0.1	0.25	0.2
Sulfamethoxazole		4.75	2	4.75	4
Erythromycin	Er	>8	0.8	>8	16
Tetracycline	Te	1 (1)	0.08	1 (1)	0.05
Vancomycin	Va	1	2	0.5	1
Clindamycin	CL	0.12	0.08	>4	0.3
Rifampin	Ri	0.004–0.016 (0.0625)	0.006	0.004–256 (0.03125)	0.006
Linezolid	Lin	2	0.2	1	0.2
Ciprofloxacin	Cip	2	3.2	>16	3.2

<sup>a</sup> All MICs are from the NARSA website (www.narsa.net), except those for mupirocin, tetracycline, and rifampin, for which general MICs for *S. aureus* were obtained from the literature (1–3) and MICs were determined for the Sanger 252 and LAC strains (shown in parentheses). SIC, subinhibitory concentration used.

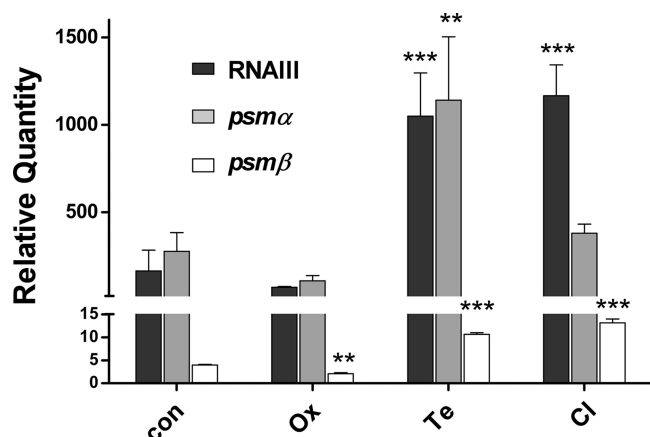


FIG. 2. Activity of *agr* in comparison to *psmα* and *psmβ* transcript levels under the influence of subinhibitory concentrations of tetracycline, clindamycin, and oxacillin. The activity of *agr* was determined by real-time quantitative reverse transcription-PCR (qRT-PCR) of RNAIII in strain LAC (USA300). The expression of *psm* operons was determined by qRT-PCR of *psmα* and *psmβ* transcripts under the same conditions (after 8 h of growth in shaken 125-ml flasks). Primers and conditions for qRT-PCR were as described, using the *gyrB* transcript as a control (13–14). Experiments were performed in triplicate. Error bars represent means  $\pm$  standard deviations. The antibiotic concentrations used are shown in Table 1. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

tetracycline, and *psmα* and *psmβ* transcript levels changed accordingly. This suggests that an effect of *agr* is likely involved in the increased PSM production with those antibiotics, while the decreased production of PSMs under the influence of oxacillin did not appear to be mediated through *agr* (Fig. 2). In support of the former, *agr* activity is greater in strain LAC than in strain Sanger 252 (14), which is in accordance with the more pronounced changes in PSM production in LAC than in Sanger 252.

To our knowledge, these results demonstrate for the first time that subinhibitory concentrations of tetracycline and clindamycin have a strong stimulatory effect on the activity of *agr*. This represents an important finding, as *agr* controls a series of key virulence determinants of *S. aureus*, and tetracycline and clindamycin are frequently used for the treatment of MRSA infections. These infections may thus be severely exacerbated when using tetracycline or clindamycin at subinhibitory concentrations, such as when not following regimes or when dealing with strains that are resistant to those antibiotics. We are aware that our results contradict those achieved by Herbert et al., who reported that subinhibitory concentrations of clindamycin have no or a negative effect on *agr* expression (8). However, those authors used laboratory strains of *S. aureus* that may differ in many aspects from the clinical strains investigated herein.

Intriguingly, the antibiotics that caused increased activity of *agr* and production of PSMs work through the inhibition of protein synthesis by binding to the ribosome complex. In addition, although the treatment of a MRSA infection with oxacillin is certainly clinically wrong, the mechanism by which

subinhibitory concentrations of oxacillin reduce PSM production in strain LAC is of great interest.

In conclusion, our study shows that the use of important antibiotics leads to increased expression of *agr*, PSMs, and possibly further virulence determinants and may thus exacerbate disease progression. This calls for rigid surveillance of regimes using these antibiotics and frequent susceptibility testing to avoid promoting virulence in bacterial strains resistant to those antibiotics.

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