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Subinhibitory Concentrations of Linezolid Reduce *Staphylococcus aureus* Virulence Factor Expression

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The influence of the antibiotic linezolid on the secretion of exotoxins by *Staphylococcus aureus* was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis combined with matrix-assisted laser desorption ionization–time of flight mass spectrometry and Western blot analysis. *S. aureus* suspensions were treated with grading subinhibitory concentrations of linezolid (12.5, 25, 50, and 90% of MIC) at different stages of bacterial growth (i.e., an optical density at 540 nm [OD_{540}] of 0.05 or 0.8). When added to *S. aureus* cultures at an OD_{540} of 0.05, linezolid reduced in a dose-dependent manner the secretion of specific virulence factors, including staphylococcal enterotoxin A (SEA) and SEB, bifunctional autolysin, autolysin, protein A, and alpha- and beta-hemolysins. In contrast, other presumably nontoxic exoproteins remained unchanged or even accumulated in supernatants in the presence of linezolid at a 90% MIC. Similarily, when added at OD_{540} of 0.8, that is, after quorum sensing, linezolid reduced the release of virulence factors, whereas the relative abundance of nontoxic exoproteins such as triacylglycerol lipase, glycerol ester hydrolase, DnaK, or translation elongation factor EF-Tu was found to be increased. Consistently, linezolid reduced in a dose-dependent manner the tumor necrosis factor-inducing activity secreted by *S. aureus* into the culture supernatants. The results of our study suggest that the expression of virulence factors in *S. aureus* is especially sensitive to the inhibition of protein synthesis by linezolid, which should be an advantage in the treatment of infections with toxin-producing *S. aureus*.

Linezolid is a member of the new synthetic class of antibacterial oxazolidinones that inhibit bacterial protein synthesis at the initiation step of protein biosynthesis. Because gram-positive bacteria increasingly develop resistance against currently available antibiotics, e.g., methicillin and vancomycin, linezolid has become a valuable antibiotic for the treatment of nosocomial and community-acquired pneumonia, complicated skin and soft tissue infections caused by methicillin-resistant *Staphylococcus aureus*, glycopeptide-intermediate *S. aureus*, methicillin-resistant *Staphylococcus epidermidis*, vancomycin-resistant enterococci, and penicillin-resistant strains of *Streptococcus pneumoniae* (11, 27, 33).

The clinical efficacy of antibiotics is not only determined by their respective bactericidal or bacteriostatic activity and pharmacokinetics but also by their action on bacterial virulence factor release, especially at suboptimal concentrations. In principle, antibiotics can both up- and downmodulate the synthesis and release of virulence factors. Protein synthesis-suppressing antibiotics such as clindamycin can induce a general inhibition of exoprotein expression, including virulence factors such as alpha-toxin (19, 24, 35, 40, 44). In contrast, subinhibitory concentrations of the commonly used cell wall affecting β-lactam antibiotics, such as methicillin, lead to an increase of alpha-toxin expression through a stimulatory effect on exoprotein synthesis (16, 17, 30, 39). Although the molecular mode of action of linezolid has been determined, little information was available about the effects of linezolid on bacterial virulence factor production.

The possible up- or downregulation of exoprotein release is especially important for *S. aureus* infections because *S. aureus* produces a wide array of toxins that determine, at least in part, the pathogenesis of infection. Thus, antibiotic-induced modulation of virulence factors could lead to either worsening or attenuation of the disease (12, 20, 25).

The production of staphylococcal exoproteins is regulated in a coordinated, growth-phase-dependent manner, occurring preferentially during the postexponential phase of growth (1, 5, 9). When *S. aureus* organisms reach high cell population densities, they sense a quorum through a cell-cell communication system. Cell-cell communication in bacteria is accomplished through the exchange of signaling molecules called autoinducers in a process referred to as quorum sensing (2, 8, 26, 31). Quorum sensing allows bacterial populations to coordinate gene expression and probably enhance the effectiveness of processes such as virulence factor expression, antibiotic production, and biofilm development (6, 7, 14).

During the postexponential phase of growth, the production of several exoproteins in *S. aureus* (e.g., alpha-toxin, enterotoxins, toxic shock syndrome toxin 1, and cell wall-associated proteins) is principally regulated by the *agr* (accessory gene regulator) locus (31, 32). *agr* acts at the transcriptional level and upregulates alpha-toxin, toxic shock syndrome toxin 1, and other extracellular proteins and downregulates cell wall-associated proteins (31, 32). Besides *agr*, other pleiotropic regulatory genes such as *sarA*, *sarS*, and *rot* have been identified that transcriptionally control not only virulence factor expression

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but also cytoplasmic proteins, including catabolic enzymes (6, 8, 10, 18, 42; for a review, see reference 28).

To obtain a comprehensive picture of the antibacterial effects of linezolid, we used the technology of proteomics to analyze the effects of linezolid on virulence factor production by *S. aureus.* To differentiate the modulation of virulence factor production from effects secondary to quorum-sensing phenomena, we investigated virulence factor secretion by *S. aureus* exposed to linezolid at distinct growth phases.

MATERIALS AND METHODS

Bacterial growth conditions. The methicillin-sensitive *S. aureus* strain ATCC 29213 was obtained from the American Type Culture Collection (ATCC) and used throughout this study. Bacteria were stored as a 20% glycerol stock at −80°C. Bacteria were precultured from the glycerol stock in Luria-Bertani (LB) broth at 37°C with constant shaking to log growth (i.e., an optical density at 540 nm [OD_{540}] of 2). An aliquot of the preculture was inoculated into 200 ml of LB broth to obtain a starting OD_{540} of 0.05. Bacteria were cultured at 37°C with constant shaking under aerobic conditions. The growth of cells was monitored by reading the OD_{540} values. The MIC in LB broth was determined by broth microdilution according to the NCCLS standard method M7-A2. The MIC for linezolid was 2.5 mg/liter, and the MIC for erythromycin was 0.15 mg/liter. Solubilized linezolid (2 mg/ml) was commercially obtained from Pharmacia (Pharmacia & Upjohn, Peapark, N.J.), and erythromycin was obtained from Sigma Aldrich (Taufkirchen, Germany).

Exoprotein preparation. After the indicated growth phases, bacteria cells were pelleted by centrifugation at 8,500 × *g* for 30 min at 4°C. The culture supernatant was precipitated by adding 100% trichloroacetic acid (Sigma) to a final concentration of 10%. After overnight incubation at 4°C, the precipitate was centrifuged at 8,500 × *g* for 70 min at 4°C and finally washed three times with ice-cold (−50°C) ethanol. The aggregated proteins were dried by using a Speed-Vac for a few minutes. The protein extracts were dissolved in 0.5 ml of 8 M urea for two-dimensional gel electrophoresis or in 0.5 ml of 0.1 M Tris containing 2 mM phenylmethylsulfonyl fluoride for one-dimensional applications as described previously (3).

The protein concentration was determined with a Bio-Rad (Munich, Germany) protein assay kit according to the instructions of the manufacturer.

SDS-PAGE. One-dimensional denaturating sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10% polyacrylamide gels according to the method of Schägger and Jagow (34) in a Bio-Rad Protean-II electrophoresis system. The gels were stained overnight in 0.2% Coomassie brilliant blue R250 with 45% ethanol and 15% acetic acid. Alternatively, silver staining was performed according to the method described by Shevchenko et al. (38).

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (29) by using the Multiphor II (Pharmacia-FRG) system according to the instructions of the manufacturer. Protein samples were separated by using immobilized pH gradient (IPG) strips in a nonlinear pH range of 3 to 10. Isoelectric focusing was performed as described by Görg et al. (15) with 8 M urea, 2 M thiourea, 2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 0.05% bromophenol blue. Isoelectric focusing was carried out with the same buffer with increasing voltage levels (i.e., 1 h at 100 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1,000 V, 1 h at 2,000 V, and 14 h at 3,500 V). Rod gels were soaked for 15 min at an ambient temperature in equilibration buffer (50 mM Tris-HCl [pH 8.8], 8 M urea, 2 M thiourea, 30% glycerol, 2% SDS, 0.05% bromophenol blue, 10 mg of dithiothreitol/ml). A second equilibration was performed for a further 15 min in equilibration buffer containing 25 mg of iodoacetamide/ml instead of dithiothreitol and applied to a second dimension by using a 12.5% Tris-glycine SDS gel (25 cm by 20 cm by 1.0 mm) with the Ettan Dalt II system (Pharmacia).

In-gel preparation of tryptic peptides. In-gel digestion with trypsin was performed according to standard protocols (21, 38) with minor modifications. Coomassie blue-stained protein bands were excised from the gel, washed three times for 10 min with water (high-pressure liquid chromatography grade; Merck, Darmstadt, Germany), equilibrated with 100 μl of 50 mM NH_4HCO_3 (pH 7.8), shrunk with acetonitrile, rehydrated with 100 μl of 50 mM NH_4HCO_3 (pH 7.8), and finally shrunk again with acetonitrile. The gel pieces were reswollen in a digestion buffer containing 50 mM NH_4HCO_3 and treated with 0.2 μg of trypsin (Promega) at 37°C for 16 h. Peptides were extracted as described previously (3). The pellet was dissolved in 10 μl of 0.1% trifluoroacetic acid (TFA).

MALDI-TOF/MS. Aliquots of 0.5 μl of the combined extract were used for matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF/MS) to obtain MS fingerprints. Mass spectra were obtained with the Bruker REFLEX IV mass spectrometer (Bruker-Daltonik, Leipzig, Germany). The validation of all data obtained, including averaging of the TOF data, recalibration on trypsin signals, and all further data processing, was carried out by using XMASS 5.1.1 postanalysis software.

LC-MS. Liquid chromatography (LC)-MS data were aquired on a Q-Tof II quadrupole-TOF mass spectrometer (Micromass, Manchester, United Kingdom) equipped with a Z spray source. Samples were introduced by using the Ultimate Nano-LC system (LC Packings, Amsterdam, The Netherlands) equipped with the Famos autosampler and the Switchos column switching module. The column setup comprises a 0.3-mm-by-1-mm trap column and a 0.075-by-150-mm analytical column, both packed with 3 μm PepMap C18 (LC Packings, Amsterdam, The Netherlands). Samples were diluted 1:10 in 0.1% TFA. A total of 10 μl was injected onto the trap column and desalted for 3 min with 0.1% TFA and a flow rate of 30 μl/min. The 10 port valve switched the trap column into the analytical flowpath, and the peptides were eluted onto the analytical column by using a gradient of 5% acetonitrile (ACN) in 0.1% TFA to 40% ACN in 0.1% TFA over 20 min and a column flow rate of ca. 200 nl/min, resulting from a 1:1,000 split of the 200 μl/min flow delivered by the pump. The electrospray ionization (ESI) interface comprised a metal-coated PicoTip spray emitter (New Objective, Woburn, Mass.) mounted onto the PicoTip holder assembly (New Objective). Stable nanospray was established by the application of 2.5 to 3.0 kV to the distal end of the PicoTip and a nitrogen counter flow rate of ~40 liter/min. The data-dependent acquisition of MS and tandem MS (MS/MS) spectra was controlled by the Masslynx software. Survey scans of 1 s covered the range from *m/z* 400 to 1,200. Doubly and triply charged ions rising above a given threshold were selected for MS/MS experiments. In MS/MS mode the mass range from *m/z* 40 to 1,400 was scanned in 1 s, and 10 scans were added up for each experiment. Micromass-formated peaklists were generated from the raw data by using the Proteinlynx software module.

Database searching. Proteins were identified from MALDI fingerprint data by using MASCOT for websearch (http://www.matrixscience.com) against a public database (National Center for Biotechnology Information) or a locally installed protein prospector algorithm (http://prospector.ucsf.edu) with sequence data obtained from The Institute for Genomic Research (http://www.tigr.org), Oklahoma University, or the N315 database (23). To identify proteins with the LC-MS peaklists, we performed MS/MS ion searches by using a local installation of Mascot 7.0 and the sequence databases mentioned above.

Western blot. Western blot analysis was performed under the conditions described by Towbin et al. (41). Antibodies to *S. aureus* enterotoxin A (SEA), SEB, and protein A were purchased from Sigma Aldrich.

Tumor necrosis factor (TNF) release assay. (i) Preparation of bacterial exotoxins. An overnight culture of ATCC 29213 in Dulbecco modified Eagle medium (DMEM; Biochrom AG, Berlin, Germany) was diluted 30-fold in 1,000 ml of prewarmed DMEM, incubated for 30 min at 37°C with constant shaking, and divided into aliquots of 200 ml. Graded concentrations of linezolid (12.5, 25, 50 and 90% MIC) were added to the diluted bacterial suspensions before incubation for further 4 h. *S. aureus* supernatants without antibiotic treatment served as controls. Proteins secreted into the supernatants were filtered through a 0.2-μm-pore-size filter (Braun Melsungen AG, Melsungen, Germany) and immediately analyzed as described below.

(ii) Preparation of spleen cells and macrophages. C57BL/6 mice were obtained from Charles River Wiga, Sulzfeld, Germany. Mice were kept under barrier conditions and used when 6 to 10 weeks old. Mice were euthanized by cervical dislocation. Resident peritoneal macrophages were harvested by rinsing the peritoneal cavity with chilled 0.9% NaCl. Single spleen cell suspensions were prepared by passing the spleen through cell strainer meshes of 100-μm pore size. Cells were seeded at a density of 10^6/ml in DMEM–5% fetal calf serum without antibiotics into 96-well flat tissue culture plates (Nunc, Kamstrup, Denmark) and incubated in 5% CO_2 at 37°C for 1 h to allow adherence. Nonadherent cells were removed by aspiration before bacterial filtrates were added to the adherent cells. After incubation for 16 h the supernatants were collected, centrifuged (1,000 × *g* for 5 min), and stored at −70°C until TNF was measured by enzyme-linked immunosorbent assay (ELISA).

(iii) ELISA. TNF in the supernatants was determined by using the Mouse TNF-α DuoSet ELISA (R&D Systems, Inc., Minneapolis, Minn.). The mininum detection level of the test was 31.25 pg/ml.

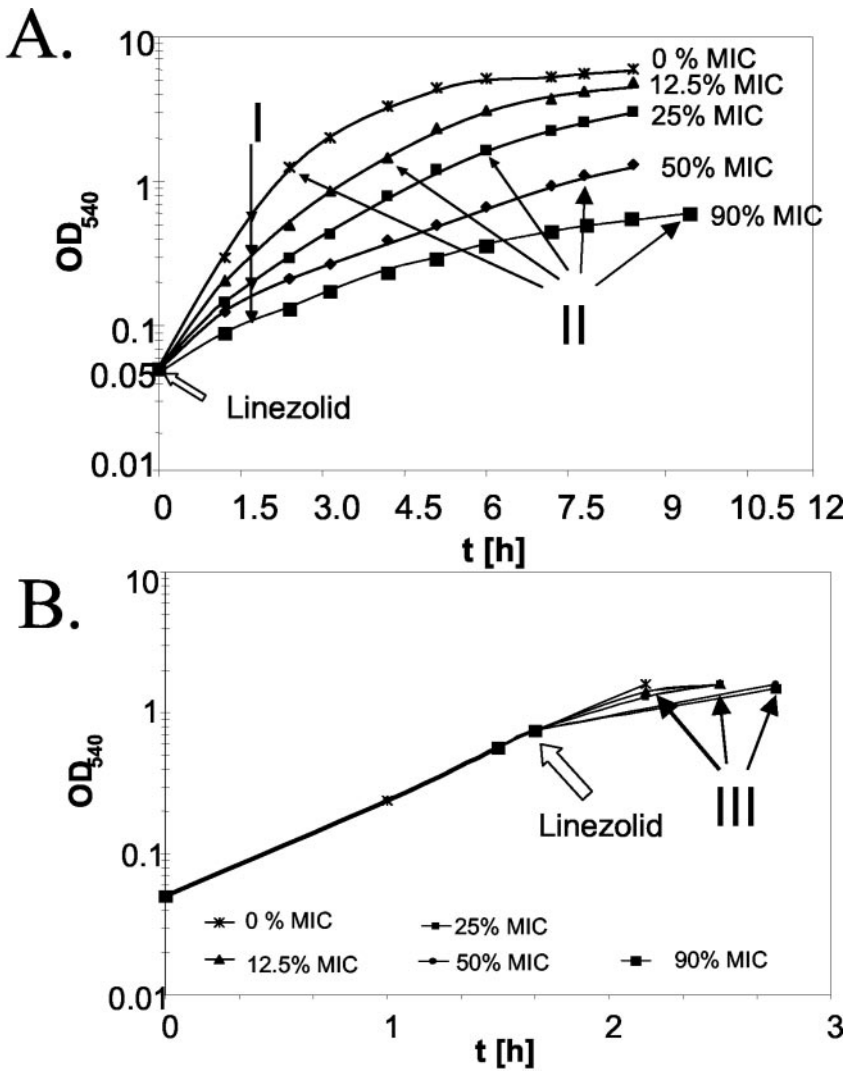

FIG. 1. Effect of linezolid on growth of *S. aureus*. *S. aureus* strain ATCC 29213 was grown at 37°C. At indicated time points, the OD_{540} was measured. (A) Bacteria were grown in the presence of grading concentrations of linezolid from the start of growth (OD_{540} ~0.05) and harvested after 2 h (I) or after they reached an OD_{540} of ~1.0 (II). (B) Bacteria were grown first to an OD_{540} of 0.8; thereafter, growth was continued in the presence of linezolid with increasing concentrations and harvested at an OD_{540} of ~1.0 (III). Arrows indicate the time point of harvest.

RESULTS

Effects of linezolid on *S. aureus* growth. To examine the influence of linezolid on the bacterial production of virulence factors prior to or after quorum sensing, *S. aureus* strain ATCC 29213 was treated with linezolid at different stages of growth. Treatment of *S. aureus* from the start of growth with subinhibitory concentrations of linezolid (e.g., 12.5, 25, 50, and 90% MIC) attenuated the growth rate in a dose-dependent manner (Fig. 1).

For the analysis of protein profiles, supernatants were harvested after 2 h (Fig. 1A, I), which reveals protein secretion within a constant period of time and yet at various densities. Alternatively, supernatants were harvested at similar OD values (Fig. 1A, II), which allows protein secretion at similar bacterial densities over different periods of time. When *S. aureus* was first cultured to an OD_{540} of 0.8 and then exposed to linezolid, only slightly retarded growth rates were observed (Fig. 1). Supernatants were harvested at an OD_{540} of ~1.3 (Fig. 1B, III), which was achieved within a narrow period of time. As depicted in Table 1, the overall secretion of exoproteins remained largely constant. As expected, a slight decrease of protein secretion was observed in the presence of linezolid at a concentration of 90% MIC.

TABLE 1. Protein contents in supernatants of *S. aureus*

Antibiotic[a]	Protein content (mg/ml) at antibiotic concn (% MIC) of:				
	0	12.5	25	50	90
Linezolid I	4.89	4.23	4.13	3.71	2.77
Linezolid II	4.33	3.92	3.57	3.14	2.71
Linezolid III	4.31	5.06	4.06	4.27	2.92
Erythromycin II	4.48	4.49	4.16	3.80	2.48

[a] I to III refer to the culture conditions described in the legend to Fig. 1.

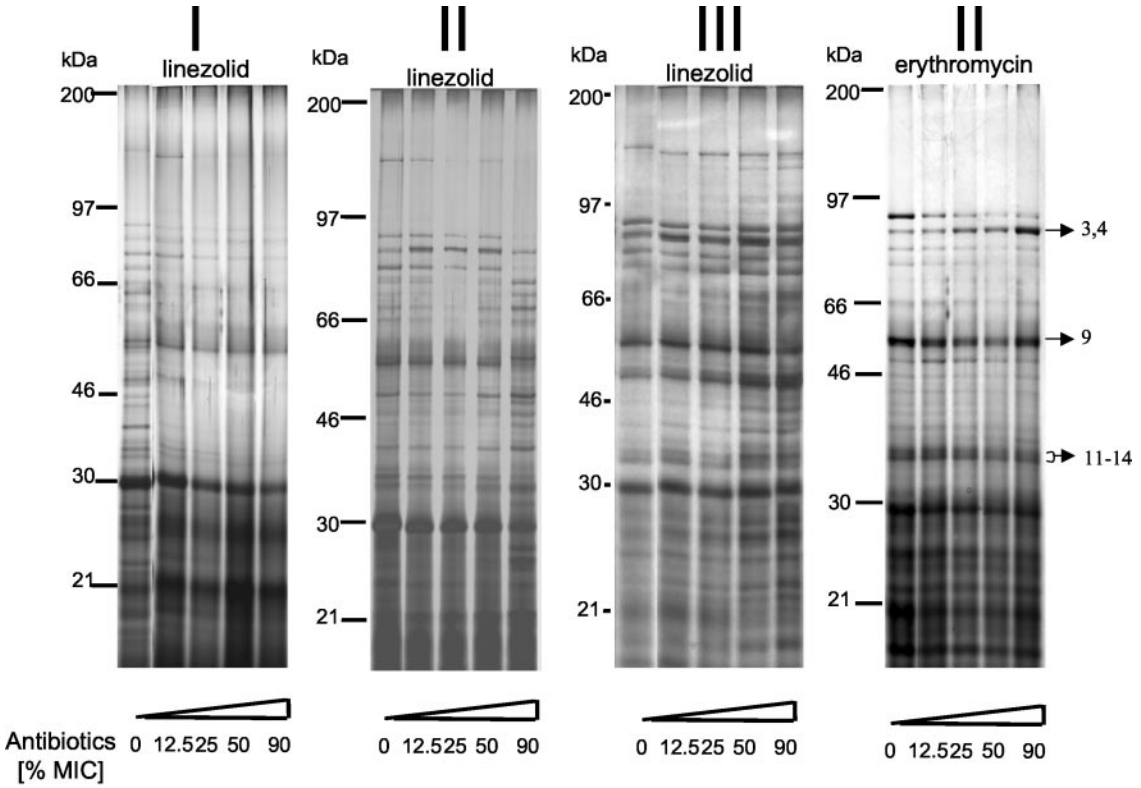

FIG. 2. SDS-PAGE of exotoxin production by *S. aureus* in the presence of antibiotics. Culture supernatants of *S. aureus* treated with different concentrations of linezolid or erythromycin were analyzed by SDS-PAGE as described in Materials and Methods. Protein bands were visualized by silver staining (sample of 10 μg of protein). Panels I to III correspond to the conditions described in the legend to Fig. 1. The arrows indicate the protein bands subjected to MALDI-TOF/MS and/or LC-MS analysis.

Effects of linezolid on protein secretion by *S. aureus*. To monitor the changes in the pattern of secreted proteins, constant amounts of proteins within culture supernatants of *S. aureus* treated with subinhibitory concentrations of linezolid were analyzed by SDS-PAGE.

When *S. aureus* was exposed to linezolid for 2 h, linezolid reduced the secretion of higher-molecular-weight proteins into the supernatants in a dose-dependent manner (Fig. 2I, linezolid). This result is not necessarily secondary to decreased bacterial cell densities because equal amounts of proteins were loaded onto the gels. When linezolid-treated *S. aureus* cultures were harvested at similar cell densities (OD_{540} of 0.8 to 1.0), significant changes of the exoprotein patterns were observed, especially when linezolid was added at a 90% MIC (Fig. 2II, linezolid).

The effects of linezolid on protein secretion by *S. aureus* were compared to the effects of erythromycin, a macrolide with protein inhibitory activity. *S. aureus* was grown from the beginning of the culture in the presence of grading subinhibitory concentrations of erythromycin and harvested at a cell OD_{540} of 1.3. Compared to linezolid, erythromycin induced less-pronounced changes in protein patterns. Notably lipase and glycerol ester hydrolase (Fig. 2, proteins "3,4") appeared slightly enhanced.

When linezolid was added to *S. aureus* at a density of OD_{540} of 0.8 and supernatants were harvested at an OD_{540} of ~1.3, a significant increase of select exoproteins was discovered (Fig. 2III, linezolid).

Because of the limitation in the resolution of the one-dimensional gel electrophoresis, secreted proteins were also analyzed by two-dimensional SDS-PAGE analysis.

A typical set of experiments is depicted in Fig. 3 (upper panels, A to F). *S. aureus* cultures were grown in the presence of grading concentrations of linezolid. At a cell OD_{540} of 1.0, supernatants were harvested and subjected to two-dimensional gel electrophoresis. As shown in Fig. 3, discrete changes in protein profiles can be detected, a finding consistent with the results obtained by one-dimensional SDS-PAGE (Fig. 2II, linezolid).

Identification of linezolid-sensitive proteins. To identify individual linezolid-sensitive proteins, both one- and two-dimensional gels were visualized by Coomassie blue staining, and stained bands were subjected to MALDI-TOF/MS and/or LC-MS/MS analysis as a complementary technique (Fig. 3 and 4). Protein profiles were subjected to the genome sequence databases as described in Materials and Methods. The peptide matches and the sequence coverage allowed the identification of 33 proteins. The changes in the secreted protein patterns of *S. aureus* treated with linezolid are illustrated in Tables 2 and 3.

At 90% MIC, linezolid enhanced the relative expression of lipases; these included triacylglycerol lipase and glycerol ester hydrolase (Fig. 4, proteins 3 and 4, and Table 3), glycerophosphoryl diester phosphodiesterase (Fig. 4, protein 14, and Table 3), and stress-induced proteins such as the DnaK (Fig. 4, protein 6, and Table 2); a hypothetical protein similar to SceD

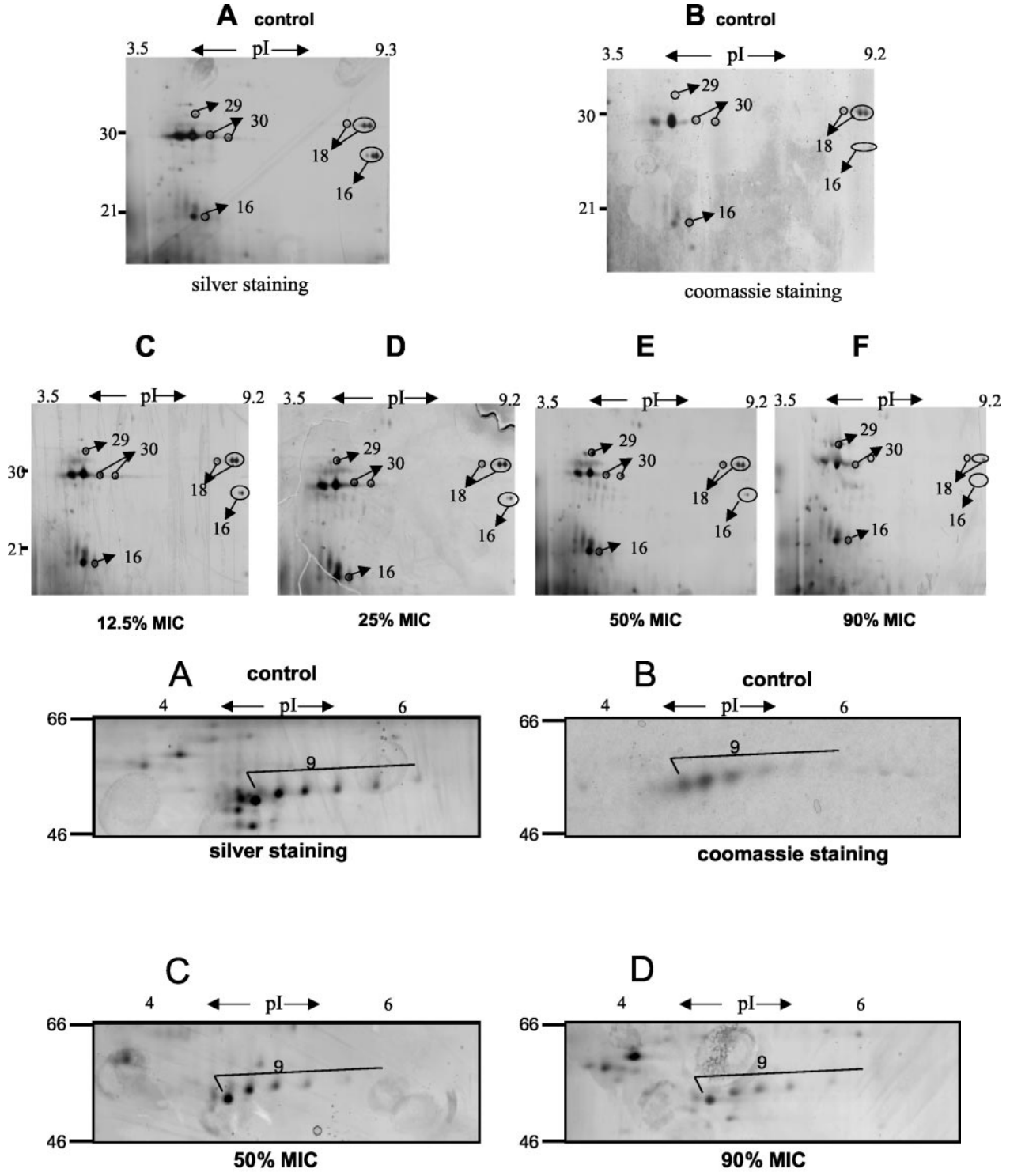

FIG. 3. Two-dimensional patterns of exoproteins in the presence of graded concentrations of linezolid. (Upper panels [A to F]) Partial view (pI 3 to 10 and 10 to 40 kDa); (lower panels [A to D]) partial view (pI 3.2 to 6.9 and 46 to 66 kDa). *S. aureus* ATCC 29213 was grown at 37°C with increasing concentrations of linezolid from the start of growth (OD_{540} ~0.05) and harvested at an OD_{540} of ~1.0. Supernatants of *S. aureus* cultures were subjected to two-dimensional gel electrophoresis. Lettered panels: A and B, untreated; C to F treated with linezolid at 12.5, 25, 50, and 90% MICs, respectively. Protein spots were visualized by silver staining (A and C to F [sample of 100 μg of protein]) or Coomassie blue stain (B [sample of 500 μg of protein]). The arrows indicate the protein bands subjected to MALDI-TOF/MS and/or LC-MS analysis.

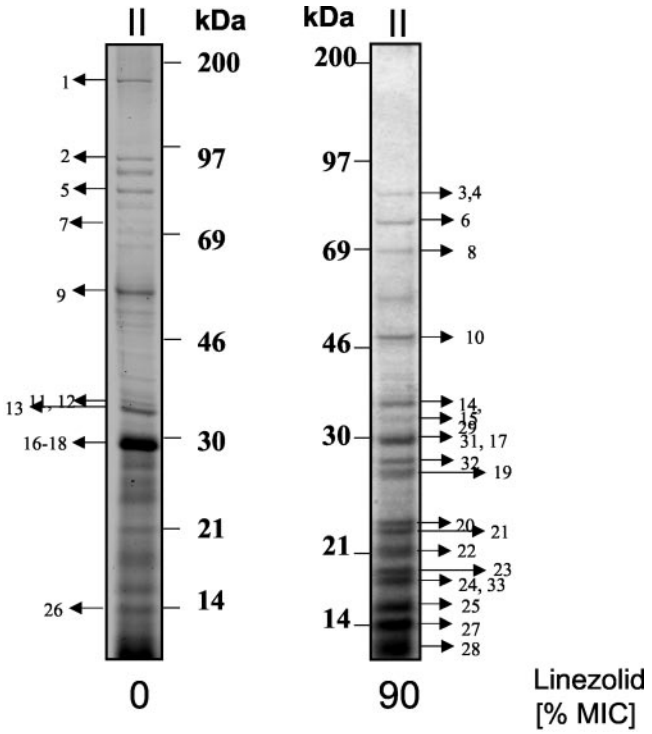

FIG. 4. Identification of exoproteins of *S. aureus* treated with linezolid. Supernatants of *S. aureus* cultures left untreated (left) or treated with linezolid at 90% MIC from the start of growth (OD_{540} ~0.05) (right) were harvested at an OD_{540} of ~0.8. Protein bands were visualized by coomassie staining. The arrows indicate the protein bands subjected to MALDI-TOF/MS or LC-MS/MS analysis.

(Fig. 4, protein 29 and Table 2); and proteins involved in the protein synthesis, such as the ribosomal proteins (Fig. 4, proteins 19 to 25 and 27 to 28, and Table 2) and the translational-elongation factor EF-TU (Fig. 4, protein 10, and Table 2).

In contrast, linezolid reduced the expression of virulence factors or proteins such as alpha- and beta-hemolysin (Fig. 4, proteins 11 and 13, and Table 2), members of the autolysin family (e.g., autolysin, bifunctional autolysin, and the hypothetical protein similar to autolysin) (Fig. 4, proteins 1, 2, 5, and 12, and Table 2), EDIN (Fig. 4, protein 16, and Table 3), and secretory antigen precursor SsaA homolog (Fig. 4, proteins 18 and 30, and Table 2). A decrease of protein A was also detected (Fig. 4, protein 9; Table 1; and Fig. 3, lower panels [A to D]). Although protein A appeared as a single band in one-dimensional gels, multiple protein A species were resolved by two-dimensional gel analysis (Fig. 3, lower panels [A to D]). Notably, the abundance of some proteins remained unchanged (e.g., the hypothetical protein, similar to cell surface protein Map-w [Fig. 4, protein 26, and Table 2]).

Due to their low concentration in the *S. aureus* supernatants, SEA and SEB could not be identified by SDS-PAGE and/or MALDI-TOF/MS (3). To visualize the effects of linezolid on SEA and SEB secretion, these virulence factors were analyzed by Western blotting. As shown in Fig. 5, linezolid reduced in a dose-dependent manner the secretion of virulence factors SEA and SEB.

Linezolid reduces the proinflammatory activity of *S. aureus* supernatants. To elucidate the biological relevance of changes in the protein profiles induced by linezolid, supernatants were added to murine splenic macrophages or peritoneal macrophages. Linezolid dissolved in DMEM alone did not induce TNF production by isolated peritoneal macrophage cells (data not shown). The culture supernatants of *S. aureus* previously treated with grading subinhibitory concentrations of linezolid (OD_{540} of 0.05) elicited significantly lower amounts of TNF generated by either spleen or peritoneal macrophages (Fig. 6). Apparently, linezolid reduced the TNF-inducing activity in a dose-dependent manner.

DISCUSSION

The numerous virulence factors produced by *S. aureus* play an important role for the pathogenesis of infection. Therefore, the clinical performance of antibiotics used for the treatment of *S. aureus* infections not only depends on the respective bacteriostatic or bactericidal effects but also on the ability to prevent virulence factor release by dying or stressed bacteria. We report here that linezolid at subinhibitory concentrations potently inhibits the secretion of *S. aureus* virulence factors. Notably, the prevention of virulence production was also observed when other strains of *S. aureus* were investigated (data not shown). Our observations suggest that adverse effects might not be expected during linezolid treatment at any stage of infection.

The effects of antibiotics on virulence factor synthesis by *S. aureus* have been intensively studied in the past. As a paradigm of a protein synthesis inhibitor targeting the 50S rRNA, the clindamycin effect on extracellular proteins has been well documented. Clindamycin at concentrations of 12.5% MIC has been shown to decrease the expression of virulence factors, such as alpha- and delta-hemolysin, and coagulase (35). Clindamycin also blocked production of several of the individual exoprotein genes (e.g., *spa*, *hla*, and *spr*), suggesting that the primary effect must be differential inhibition of the synthesis of one or more regulatory proteins (19).

Indeed, many genes encoding virulence factors are coordinately regulated in response to a variety of intra- and extracellular signals. Octapaptide signaling molecules reaching a threshold at increased cell densities mediate a transcriptional switch from genes encoding surface-expressed proteins such as adhesins to genes encoding exoproteins (21). The best-studied regulatory loci are the accessory gene regulator *agr* and staphylococcal accessory regulator *sarA* loci (7, 8, 18). It is tempting to speculate that linezolid-induced inhibition of global regulators might result in the decreased virulence factor secretion observed in our study. However, the pattern of individual exoproteins reduced in the presence of linezolid is not consistent with the pattern to be expected after inactivation of a specific global regulator. For example, protein A is a prototypical surface protein anchored into the cell wall via glycine cross-links (36). As shown in that study and recently by Gemmell and Ford (13), linezolid reduces protein A expression. If the primary effect of linezolid was to decrease expression of the octapeptide, then protein A expression was expected to be increased rather than decreased. Similarily, lipase (glycerol ester hydrolase) belongs to the group of *agr*- and *sarA*-upregulated genes (10, 45) The finding that lipase was increased in the supernatants of linezolid-treated *S. aureus* also indicates that

TABLE 2. *S. aureus* exoproteins identified by MALDI-MS fingerprints

Spot no.	GenBank accession no.	Z score[a]	Sequence coverage[b] (%)	Molecular mass (Da) measured	Molecular mass (Da)/pI (theoretical)	Linezolid change[c]	Putative identification
1	1703465	1.77e+007[A]	20	145,000	137,385/9.60	↓	Bifunctional autolysin
2	15924043	171[B]	56	97,000	102,656/9.65	↓ ↓	Autolysin
3	13702629	1.98e+023[A]	77	90,000	76,662/6.58	↑ ↑	Triacylglycerol lipase
4	13700235	7.16e+013[A]	34	90,000	76,543/8.99	↑ ↑	Glycerol ester hydrolase
5	15925634	101[B]	21	80,000	69,186/5.96	↓ ↓	Hypothetical protein ORFID:SA2437 (similar to autolysin)
6	15924570	122[B]	30	75,000	66,321/4.65	↑ ↑	DnaK protein
8	15926694	1.59e+003[A]	19	72,000	69,196/4.9	↑	GTP-binding elongation factor homolog ORFID:SA0959
9	225821	101[B]	49	60,000	57,939/5.48	↓ ↓	Protein A
10	15923538	175[B]	65	48,000	43,134/4.74	↑	Translational elongation factor TU
11	21283669	74[B]	41	36,000	31,350/7.68	↓ ↓	Truncated beta-hemolysin
12	15926142	72[B]	47	36,000	35,871/9.67	↓ ↓	Hypothetical protein ORFID:SA0423 (similar to autolysin)
13	15924153	6e+012[A]	34	33,000	35,953/8.70	↓ ↓	Alpha-hemolysin
14	15923949	1.31e+012[A]	50	33,000	35,289/8.67	↑ ↑	Glycerophosphoryl diester phosphodiesterase
16	119131	116[B]	45	28,000	27,663/9.15	↓ ↓	Epidermal cell differentiation inhibitor precursor (EDIN)
18	15925289	64[B]	51	31,000	29,366/8.96	↓ ↓	Secretory antigen precursor SsaA homolog ORFID:SA2093
19	15923528	122[B]	58	26,000	24,693/9.00	↑ ↑	50S ribosomal protein L1
20	15925225	243[B]	74	24,000	19,774/9.54	↑ ↑	50S ribosomal protein L6
21	15927829	112[B]	56	23,000	22,451/9.90	↑ ↑	50S ribosomal protein L4
22	15925208	180[B]	72	21,000	16,323/9.3	↑ ↑	50S ribosomal protein L13
23	2500269	106[B]	57	20,000	15,587/10.28	↑ ↑	50S ribosomal protein L15
24	15927797	80[B]	43	16,000	14,607/10.56	↑ ↑	30S ribosomal protein S9
25	15925235	65[B]	40	14,000	12,827/9.92	↑ ↑	50S ribosomal protein L22
26	15926570	3.56e+007[A]	84	12,000	15,898/9.28	+/−	Hypothetical protein (similar to cell surface protein Map-w)
27	15927227	74[B]	66	13,000	11,326/9.84	↑	50S ribosomal protein L21
28	14029570	54[B]	32	9,000	7,990/10.16	↑	50S ribosomal protein L27
29	15925085	101[B]	36	32,000	24,063/5.53	↑ ↑	Hypothetical protein (similar to SceD precursor ORFID:SA1898)
30	15923655	3.45e+004[A]	40	29,000	28,169/6.12	↓	Secretory antigen precursor SsaA homolog ORFID:SA0620

[a] Superscripts: A, Z-score value from Protein Prospector; B, Z-score value from Mascot.
[b] Sequence coverage of total amino acid numbers.
[c] ↑ ↑, Great accumulation; ↑, weak accumulation; ↓, weak inhibition; ↓ ↓, complete inhibition; +/−, no effect.

TABLE 3. *S. aureus* exoproteins identified by LC-MS/MS analysis

Spot no.	GenBank accession no.	Z score[a]	Molecular mass (Da) measured	Molecular mass (Da)/ pI (theoretical)	Linezolid change[b]	Putative identification
1	1703465	995	145,000	137,385/9.59	↓ ↓	Bifunctional autolysin precursor
2	15924043	1260	97,000	102,593/9.65	↓ ↓	Autolysin
3	15925661	1690	90,000	76,616/6.58	↑ ↑	Triacylglycerol lipase
4	15923310	1520	90,000	76,496/8.99	↑ ↑	Glycerol ester hydrolase
5	15925634	1150	80,000	69,186/5.96	↓ ↓	Hypothetical protein ORFID:SA2437 (similar to autolysin)
7	15923709	942	70,000	74,353/9.04	↓	Hypothetical protein ORFID:SA0674
9	225821	1130	60,000	57,939/5.48	↓ ↓	Protein A
13	15924153	1010	33,000	35,953/8.7	↓ ↓	Alpha-hemolysin precursor
14	15923949	759	33,000	35,289/8.67	↑ ↑	Glycerophosphoryl diester phosphodiesterase
15	15923085	387	32,000	37,064/7.71	ND	1-Phosphatidylinositol phosphodiesterase precursor
16	119131	550	32,000	27,663/9.15	↓ ↓	Epidermal cell differentiation inhibitor precursor (EDIN)
17	21284219	283	30,000	24,188/6.11	↓ ↓	Immunodominant antigen A
18	15925289	551	30,000	29,366/8.96	↓ ↓	Secretory antigen precursor SsaA homolog ORFID:SA2093
20	15925225	380	24,000	19,774/9.54	↑ ↑	50S ribosomal protein L6
25	15925235	206	14,000	12,827/9.92	↑ ↑	50S ribosomal protein L22
26	15926570	137	12,000	15,898/9.28	+/−	Hypothetical protein (similar to cell surface protein Map-w)
28	14029570	131	9,000	7,990/10.16	↑	50S ribosomal protein L27
29	15925085	192	32,000	24,063/5.53	ND	Hypothetical protein (similar to SceD precursor ORFID:SA1898)
31	15924246	424	29,000	29,133/5.44	↑ ↑	30S ribosomal protein S2
32	15927830	232	28,000	23,703/9.8	↑ ↑	50S ribosomal protein L3
33	3024540	199	16,000	14,922/9.04	↑ ↑	50S ribosomal protein L11

[a] Z-score value from Mascot.
[b] ↑ ↑, Great accumulation; ↑, weak accumulation; ↓, weak inhibition; ↓ ↓, complete inhibition; ND, not determined; +/−, no effect.

linezolid inhibits virulence factor expression in an *agr*- and *sarA*-independent manner.

Alternatively, the differential effects of linezolid on virulence factor secretion could be due to different half-lives of the respective protein and/or mRNA. Although 80% of all mRNAs had half-lives of between 3 and 8 min, a wide range of stabilities was reported for individual mRNAs of *Escherichia coli* (4, 37). It might be predicted that transcripts of housekeeping genes and proteins would have longer half-lives than transcripts and proteins synthesized in response to acute stimuli. A

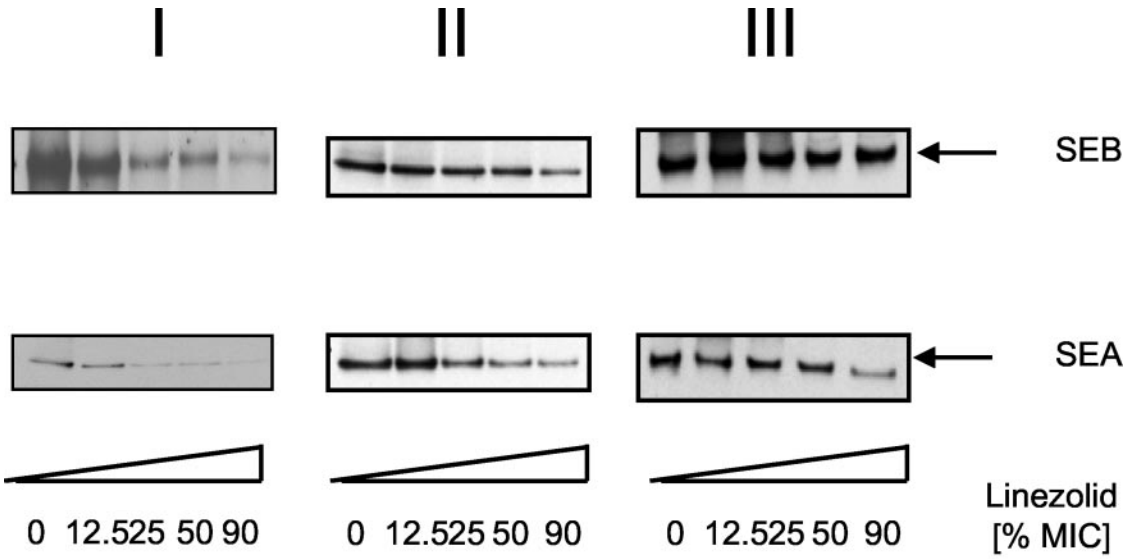

FIG. 5. Western blot analysis of exotoxins of *S. aureus* treated with linezolid. *S. aureus* ATCC 29213 was grown at 37°C and treated with linezolid at various growth phases. Panels I to III correspond to the conditions described in the legend to Fig. 1. Supernatants were subjected to SDS-PAGE. After transfer to nitrocellulose, proteins were stained specifically with the indicated antibodies against SEA and SEB. A horseradish peroxidase-conjugated goat anti-rabbit antibody was used as second reagent and visualized by using an enhanced chemiluminescence detection kit (Amersham-Pharmacia).

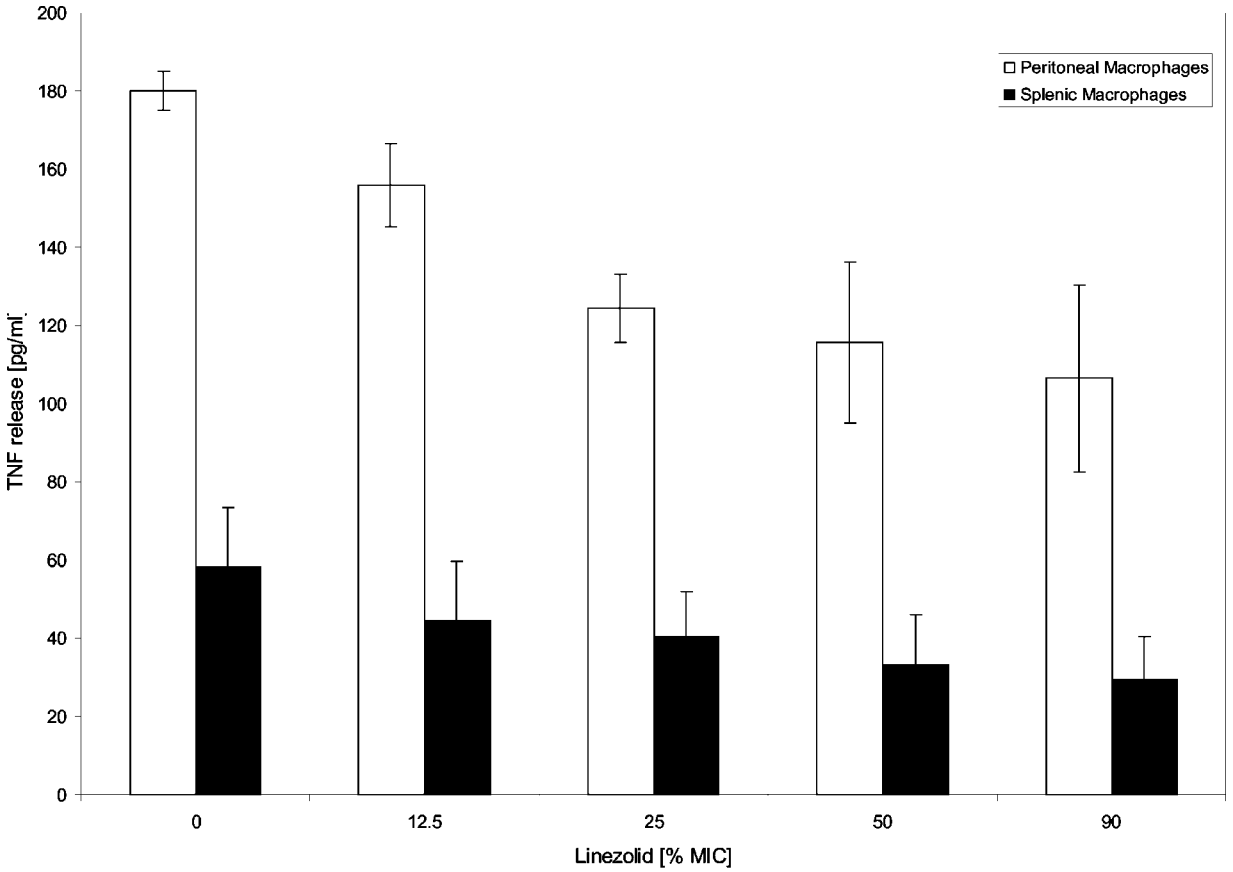

FIG. 6. TNF release by macrophages stimulated with supernatants of *S. aureus*. *S. aureus* strains were cultured in DMEM and treated with the indicated concentrations of linezolid as described in the text. Secreted proteins present in the supernatant were collected, followed by incubation for 20 h at 37°C with mouse splenic macrophages or peritoneal macrophages. The TNF levels were measured by ELISA. Linezolid itself (1× MIC and 2× MIC) did not induce TNF (data not shown).

shorter half-life of virulence factors would explain why virulence factors were more sensitive to linezolid than other proteins. However, little is known about possible relationships between gene function and protein or mRNA half-life or abundance, and the apparent sensitivity of virulence factors for the action of linezolid requires further investigations.

Remarkably, using oligonucleotide microarrays for the study of global RNA degradation in wild-type *E. coli*, Selinger et al. reported that a single operon (*tdcABCDEFG*) was relatively rifampin insensitive (37). All seven open reading frames of this operon that encode a pathway for the transport and anaerobic degradation of L-threonine were significantly upregulated at 2.5 min after rifampin addition. Although the precise mechanism of rifampin-induced upregulation of the *tdcABCDEFG* operon needs to be determined, differential sensitivity to rifampin can be brought about by specific structural features of the RNAP holoenzyme (43).

The unexpected detection of the rifampin-insensitive *tdc* operon underlines the necessity to study the effects of an antibiotic in a comprehensive manner. The high-resolution two-dimensional protein gel electrophoresis is a well-established technique for visualizing a very large set of proteins secreted by a bacterial cell (22, 45). A combination of two-dimensional gelelectrophoresis and MALDI/TOF analysis or N-terminal Edman sequencing analysis had already led to the identification of 18 differentially regulated exoproteins by SarA and σ^B mutants of *S. aureus* (45). In addition to two-dimensional analysis, we also used one-dimensional protein gels combined with the identification of protein by MALDI-TOF/MS, which allowed the assignment of 31 exoproteins, including basic proteins that were not resolved by conventional two-dimensional analysis. Using this methodology of proteomics, we detected some specific effects of linezolid that were not observed with other protein synthesis-inhibiting antibiotics. For example, linezolid induced the secretion of ribosomal proteins into bacterial supernatants (Fig. 2 and Table 2). In contrast, both erythromycin (Fig. 2) and tetracycline (data not shown) do not induce the accumulation of ribosomal proteins in *S. aureus* culture supernatants. Linezolid prevents the formation of the formylmethionyl-tRNA:mRNA:30S subunit ternary complex. Thus, the linezolid-induced failure of ribosomal assembly might promote the leakage of small-sized individual ribosomal proteins.

Taken together, the results of our study provide comprehensive analysis of the effects of linezolid on virulence factor release by *S. aureus*. The methods presented here establish a framework for further investigation of the mode of action of antibiotics on a proteome-wide basis.

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