Journal of Antimicrobial Chemotherapy (2006) **58**, 564–574
doi:10.1093/jac/dkl291

JAC

Subinhibitory quinupristin/dalfopristin attenuates virulence of *Staphylococcus aureus*

Carmen Koszczol, Katussevani Bernardo†, Martin Krönke and Oleg Krut*

[1]*Institute for Medical Microbiology, Immunology and Hygiene, Medical Center, University of Cologne, Goldenfelsstrasse 19-21, 50935 Köln, Germany*

Received 19 September 2005; returned 10 March 2006; revised 5 May 2006; accepted 23 June 2006

***Objectives*: The semi-synthetic streptogramin quinupristin/dalfopristin antibiotic exerts potent bactericidal activity against *Staphylococcus aureus*. We investigated whether, like other bactericidal antibiotics used at subinhibitory concentrations, quinupristin/dalfopristin enhances release of toxins by Gram-positive cocci.**

***Methods*: The activity of quinupristin/dalfopristin on exotoxin release by *S. aureus* was investigated by 2D SDS–PAGE combined with MALDI-TOF/MS analysis and by western blotting.**

***Results*: We show that quinupristin/dalfopristin at subinhibitory concentrations reduces the release of *S. aureus* factors that induce tumour necrosis factor secretion in macrophages. Furthermore, quinupristin/dalfopristin but not linezolid attenuated *S. aureus*-mediated killing of infected host cells. When added to *S. aureus* cultures at different stages of bacterial growth, quinupristin/dalfopristin reduced in a dose-dependent manner the release of specific virulence factors (e.g. autolysin, protein A, α- and β-haemolysins, lipases). In contrast, other presumably non-toxic exoproteins remained unchanged.**

***Conclusions*: The results of the present study suggest that subinhibitory quinupristin/dalfopristin inhibits virulence factor release by *S. aureus*, which might be especially helpful for the treatment of *S. aureus* infections, where both bactericidal as well as anti-toxin activity may be advantageous.**

Keywords: proteomics, exoproteins, mass spectrometry, antibiotics

Introduction

Staphylococcus aureus is one of the most common pathogens of community-acquired and nosocomial diseases with a significant morbidity and mortality. Infections due to this organism have a variety of clinical manifestations, ranging from localized skin infections to severe sepsis, including toxic-shock syndrome.[1] *S. aureus* produces a wide array of toxins that determine, at least in part, the pathogenesis of infection. This is exemplified by toxic-shock syndrome caused by toxic-shock syndrome toxin-1 (TSST1)-producing *S. aureus* or by necrotizing pneumonia and epidemic furunculosis caused by community-acquired methicillin-resistant *S. aureus* (cMRSA) that is often associated with Panton-Valentine leucocidin (PVL) production.[2] For the management of toxic *S. aureus* infections, β-lactam antibiotics proved unfavourable, because even subinhibitory concentrations (for example of methicillin) lead to an increase in α-toxin expression through a stimulatory effect on exoprotein synthesis.[3–5] Instead, protein-synthesis-suppressing antibiotics such as clindamycin and linezolid are recommended for the treatment of *S. aureus*-produced toxic syndromes as concentrations below the MIC have been shown to impair the expression of virulence factors of *S. aureus*.[6,7] Clindamycin at a concentration of 1/8 MIC inhibits the expression of α- and δ-haemolysin as well as coagulase.[8] In addition, the expression of protein A is reduced when *S. aureus* is exposed to clindamycin at concentrations below the MIC, leading to increased bacterial susceptibility to phagocytosis and suggesting additional therapeutic efficacy.[9] However, clindamycin cannot be used for toxic MRSA infections because MRSA are mostly resistant to clindamycin. For this reason, two new drugs, quinupristin/dalfopristin and linezolid, came into the clinics, which seem to be suitable for

*Corresponding author. Tel: +49-221-478 3018; Fax: +49-221-478-3067; E-mail: Oleg.Krut@uni-koeln.de
†Present address. BIOCRATES life sciences GmbH, Innrain 66, A-6020 Innsbruck, Austria

the treatment of infections caused by multidrug-resistant Gram-positive cocci.[10] Recently, we reported that subinhibitory concentrations of linezolid downmodulate virulence factor release by *S. aureus*.[7] It should be noted, however, that neither linezolid nor clindamycin is expected to be effective for the treatment of *S. aureus* infections in neutropenic patients, which call for bactericidal rather than bacteriostatic antibiotics. In contrast to linezolid, quinupristin/dalfopristin exerts bactericidal activity against *S. aureus*.[11] Although quinupristin/dalfopristin appears to be a valuable alternative for MRSA infections, the action of quinupristin/dalfopristin on virulence factor release by *S. aureus* has not yet been investigated.

Quinupristin/dalfopristin is the first injectable streptogramin; it belongs to the macrolide–lincosamide–streptogramin (MLS) family of antibiotics. It is composed of two semi-synthetic pristinamycin derivates, quinupristin (RP57669) and dalfopristin (RP54476), in a 30:70 ratio.[10] Quinupristin and dalfopristin bind to distinct sites on the 50S subunit of the bacterial ribosome, where they act synergically to inhibit protein synthesis and exert a bactericidal effect.[12] Quinupristin/dalfopristin is active against a broad spectrum of Gram-positive pathogens including methicillin-resistant staphylococci, penicillin/erythromycin-resistant streptococci and vancomycin-resistant *Enterococcus faecium* (VREF).[10] While the molecular mode of action of quinupristin/dalfopristin has been unravelled, little information was available about the effects of quinupristin/dalfopristin on bacterial virulence factor production.

S. aureus produces in a growth-phase-dependent manner, predominantly during the post-exponential phase of growth, a variety of different extracellular proteins including non-toxic enzymes, enzyme activators or toxins. The vast array of virulence factors produced by *S. aureus* include α-, β-, γ- and δ-haemolysins, protein A as well as leucocidin and coagulase.[1] The expression of staphylococcal genes is generally regulated in response to fluctuations in cell-population density through a process termed quorum sensing. Gram-positive bacteria use quorum-sensing communication circuits to regulate a diverse array of physiological activities, including symbiosis, competence, conjugation, antibiotic production, biofilm formation and virulence.[13] When a sufficient number of cells, a 'quorum', is present, the accumulation of released chemical signal molecules called autoinducer-peptide (AIP) leads to gene expression.

S. aureus virulence gene expression is mainly controlled by at least two global regulatory elements such as the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*).[14] The accessory gene regulator *agr* suppresses the post-exponential phase expression of cell surface binding proteins (thought to play a role in the early stages of colonization) and enhances the expression of secreted proteins (suggested to be involved in the long-term survival of the organism at the site of infection). Unlike *agr* the *sar* locus activates the synthesis of both extracellular and surface bound proteins in *S. aureus.*[15] Further global regulatory elements are *S. aureus* exoprotein expression (*sae*), repressor of toxin (*rot*) and sigma B factor (σ^B).[13]

In the present study we show that subinhibitory concentrations of quinupristin/dalfopristin reduce *S. aureus*-mediated toxicity towards infected host cells. In addition, the supernatants of quinupristin/dalfopristin-treated *S. aureus* elicited an attenuated inflammatory response of macrophages. By comprehensive investigations of toxin production using proteomic approaches we found that while subinhibitory quinupristin/dalfopristin efficiently impedes the secretion of most exotoxins by *S. aureus,* the release of few exoproteins increases.

Materials and methods

Bacterial strain and growth conditions

Quinupristin/dalfopristin was commercially obtained from Aventis (Rhône-Poulenc Rorer S.A., Madrid, Spain) as powder and diluted before the analysis with sterile water to yield a final concentration of 0.5 g/L. The methicillin-susceptible *S. aureus* strain ATCC 29213 was obtained from ATCC and used throughout the study. Bacteria were stored in Microbank® (Pro-Lab Diagnostics, Austin, USA) at –80°C. The MIC in LB broth was determined by broth microdilution according to the National Committee for Clinical Laboratory Standards (NCCLS) standard method M7-A3. For *S. aureus* ATCC 29213, the MIC of quinupristin/dalfopristin was 0.25 mg/L.

For each analysis bacteria were cultivated overnight at 37°C on blood agar plates, removed from the agar surface and suspended in Luria–Bertani (LB) broth and precultured at 37°C with constant shaking (200 rpm) to post-exponential growth phase (log growth OD_{540} of 2). To obtain a starting OD_{540} of 0.05 an aliquot of the preculture was inoculated into 200 mL of LB broth and when indicated supplemented with quinupristin/dalfopristin at concentrations of 12.5%, 25%, 50%, 75% and 90% MIC. Bacteria were further cultured at 37°C with constant shaking under aerobic conditions. The growth of cells was monitored by reading the OD_{540} values. Supernatants were harvested at four defined culture conditions: (I) quinupristin/dalfopristin was added from the beginning of culture and supernatants were harvested in the exponential phase of growth; (II) quinupristin/dalfopristin was added from the beginning of culture and supernatants were harvested in the post-exponential phase of growth; (III) bacteria were first grown to OD_{540} = 0.2, then quinupristin/dalfopristin was added and supernatants were harvested in the early stationary phase of growth; (IV) bacteria were first grown to OD_{540} = 0.4, then quinupristin/dalfopristin was added and supernatants were harvested in the early stationary phase of growth.

Exoprotein preparation

After indicated growth phases, supernatants of bacterial cultures were harvested. Bacteria were pelleted by centrifugation at 8500 **g** for 25 min at 4°C. The culture supernatant was precipitated by adding 100% trichloroacetic acid (TCA, Sigma) to a final concentration of 10%. After incubation at 4°C overnight, the precipitate was centrifuged at 8500 **g** for 70 min at 4°C and finally washed with ice-cold 100% ethanol three times and then with ice-cold 70% ethanol. The aggregated proteins were air-dried for 30 min and dissolved in 0.5 mL of 8 M urea. The protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad Corporation, Munich, Germany) according to the instructions of the manufacturer.

SDS–PAGE

One-dimensional denaturing SDS–PAGE was performed with 10% polyacrylamide gels in a Bio-Rad Protean-II electrophoresis system. The gels were stained overnight in Novex® Colloidal Blue staining kit (Invitrogen, Carlsbad, California, USA). Alternatively, silver staining was performed according to the method described by Shevchenko *et al.*[16] The slight decrease in overall protein secretion caused by quinupristin/dalfopristin was corrected by loading equal amounts of proteins.

Two-dimensional gel electrophoresis was performed according to O'Farrell[17] using the Multiphor II (Pharmacia-FRG) system

according to the instructions of the manufacturer. Protein samples were separated by using IPG strips in a non-linear pH range of 3–10. Isoelectric focusing was performed as described by Görg *et al.*[18] using 8 M urea, 2 M thiourea, 2% CHAPS and 0.05% DTT. Isoelectric focusing was carried out using the same buffer with increasing voltage (1 h 100 V, 1 h 200 V, 1 h 500 V, 1 h 1000 V, 1 h 2000 V and 14 h 3500 V). Rod gels were soaked for 15 min at ambient temperature in equilibration buffer (50 mM Tris–HCl, pH 8.8, 8 M urea, 2 M thiourea 30% glycerol, 2% SDS, 10 mg/mL DTT). A second equilibration was performed for a further 15 min in equilibration buffer containing 25 mg/mL iodoacetamide and 0.05% Bromophenol Blue instead of DTT and applied to a second dimension using a commercially available 12.5% Tris–Glycine SDS gel (25 × 20 cm × 1.0 mm) (Pharmacia, Uppsala, Sweden) with an Etan Dalt II System (Pharmacia, Uppsala, Sweden).

In-gel-preparation of tryptic peptides

In-gel-digestion with trypsin was performed according to standard protocols[16,19] with minor modifications. Coomassie-stained protein bands were excised from the gel and washed three times for 10 min with water (HPLC grade, Merck Darmstadt Germany). Reduction was performed with 0.1 M Tris (pH 8.5), 0.01 M EDTA, 6 M GuHCL and 25 mM DTT for 30 min at 37°C, and the proteins were subsequently alkylated with 125 mM iodoacetamide in the dark for an additional 1 h at 37°C. Gel pieces were equilibrated twice with 100 μL of 50 mM NH_4HCO_3 (pH 7.8) for 10 min, shrunk with 100 μL of acetonitrile, rehydrated with 100 μL of 50 mM NH_4HCO_3 (pH 7.8) and finally shrunk again with acetonitrile. After air-drying, gel pieces were reswollen in a digestion buffer, containing 20 μL of 50 mM NH_4HCO_3, and 0.05 μg of trypsin (Promega, Madison, USA) at 37°C for 16 h. Peptides were extracted by subsequent incubation for 20 min at room temperature with 50 mM NH_4HCO_3, 0.1% TFA and finally 0.1% TFA/acetonitrile (2:3, v/v). The pellet was dissolved in 10 μL of 0.1% TFA.

MALDI-TOF/mass spectrometry

Aliquots of 0.5 μL of the combined extract were used for MALDI mass spectrometry to obtain MS-fingerprints. Mass spectra were obtained with the Bruker REFLEX IV mass spectrometer (Bruker-Daltonik, Bremen, Germany). The validation of all data obtained, including averaging of the time-of-flight data, recalibration on trypsin signals, as well as all further data processing, was carried out using the XMASS 5.1.5 post-analysis software.

Database searching

Proteins were identified from MALDI-fingerprint data using a locally installed MASCOT for web search (Matrix Science, Boston, USA) against *S. aureus* proteins extracted from public database (NCBI) or using the data obtained from *S. aureus* genome sequencing projects (The Institute for Genomic Research, Oklahoma University and Sanger Centre).

Western-blot

Western-blot analysis was performed as described previously.[7] *Antibodies to S. aureus* enterotoxins A (SEA) and B (SEB), α-haemolysin and protein A were purchased from Sigma Aldrich.

Tumour necrosis factor (TNF) release assay

An overnight culture of ATCC 29213 in DMEM (Biochrom) was diluted 30-fold in pre-warmed 1000 mL DMEM, incubated for 30 min at 37°C under constant shaking and divided into aliquots of 200 mL. Graded concentrations of quinupristin/dalfopristin (12.5%, 25%, 50% and 90% MIC) were added to the diluted bacterial suspensions before incubation for a further 4 h. *S. aureus* supernatants without antibiotic treatment and uninoculated medium served as controls. Proteins secreted into the supernatants were filtered through a 0.2 μm pore-size-filter (Braun Melsungen AG, Germany) and immediately analysed as described below.

C57BL/6 mice (Charles River Wiga, Sulzfeld, Germany) were kept under specific pathogens free conditions and used when 6–10 weeks old. Animal experiments were approved by the Ethics Committee of Cologne Government and were performed according to German law. Resident peritoneal macrophages were harvested by rinsing the peritoneal cavity with chilled 0.9% NaCl. Cells were seeded at a density of 10^6/mL in DMEM, 5% FCS 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. Non-adherent cells were removed by aspiration before bacterial filtrates were added to the adherent cells. After incubation for 6 h the supernatants were collected, centrifuged (1000 **g** for 5 min) and stored at –70°C. TNF concentration in the supernatants was determined using Mouse TNF-α DuoSet ELISA (R&D Systems Inc., Minneapolis, MN, USA) according to the instructions of the manufacturer.

Cytotoxicity assay for S. aureus-infected host cells

The mouse fibroblast mKSA cell line was cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% heat-inactivated fetal calf serum, 10^5 U/L penicillin G and 100 mg/L streptomycin sulphate (all from Biochrom, Berlin, Germany), and 18 h prior to infection, 5×10^5 cells were seeded in 6-well plates (total volume = 3 mL) (Nunc, Wiesbaden, Germany). Viability was monitored using Trypan Blue (Biochrom) exclusion. Prior to infection, cells were washed with growth medium without antibiotics and kept for 1 h at 37°C. *S. aureus* (1×10^7 cfu) grown in the presence of quinupristin/dalfopristin at 12.5%, 50%, 75% or 90% MIC were added to produce an m.o.i. of 50. After 1 h of incubation cells were washed with growth medium. Extracellular staphylococci were killed by incubation with 100 mg/L lysostaphin (Sigma) for 7 min at 37°C. This treatment was found to be effective in eradicating all extracellular *S. aureus*. Cells were washed with growth medium complemented with penicillin/streptomycin and reseeded in new 2 mL cultures. After 24 h of incubation, cells were harvested and analysed by microscopy. Host cell viability was measured using Trypan Blue exclusion.

Statistical analysis

Experimental data were assessed with a two-tailed unpaired Student's *t*-test for comparison between means. P values of <0.01 were considered to be statistically significant.

Results

Reduced inflammatory potential of S. aureus supernatants treated with subinhibitory concentrations of quinupristin/dalfopristin

Secreted proteins of *S. aureus* are known to stimulate cells of the immune system such as macrophages resulting in the release of TNF and other proinflammatory cytokines. In addition, bactericidal antibiotics such as penicillins induce release of endogenous factors by *Staphylococci*[20] that activate macrophages via stimulation of toll-like receptors (TLRs). To address the proinflammatory potential of quinupristin/dalfopristin we studied TNF release from peritoneal macrophages incubated with *S. aureus* culture

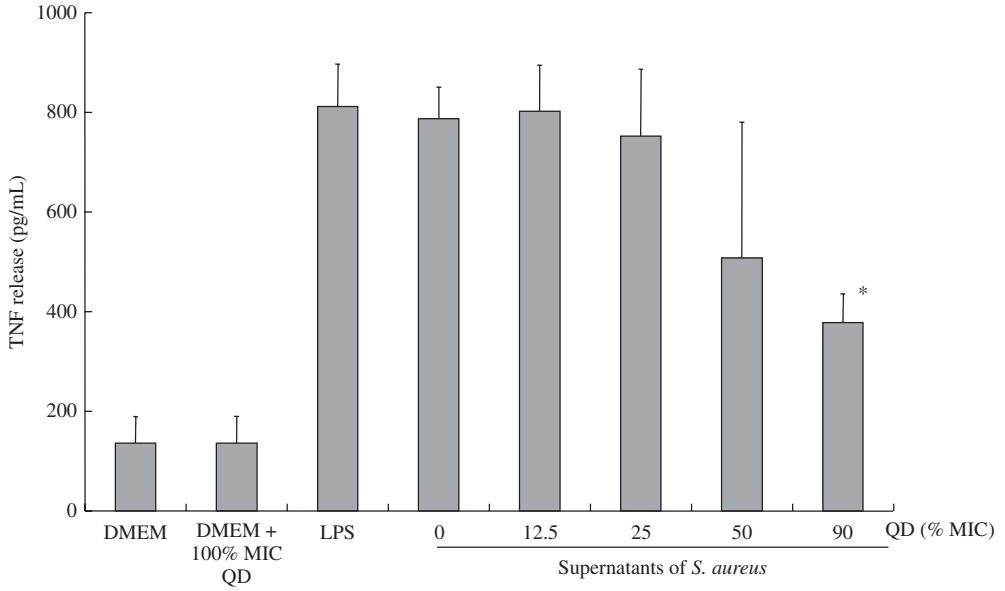

Figure 1. TNF release from murine macrophages stimulated with exoproteins of *S. aureus* grown in the presence of subinhibitory concentrations of quinupristin/dalfopristin (QD). Murine peritoneal macrophages were stimulated with supernatants of *S. aureus* previously grown to an OD_{540} of 0.8 in the absence or presence of quinupristin/dalfopristin at concentrations of 12.5%, 25%, 50% and 90% MIC. The amount of TNF released after 6 h of the stimulation was measured by ELISA. The cell culture medium alone or supplemented with 100% MIC of quinupristin/dalfopristin served as negative controls. The TNF release after LPS stimulation was included as a positive control. Asterisk (∗) indicates statistically significant reduction in TNF release ($P < 0.01$).

supernatants previously treated with graded concentrations of quinupristin/dalfopristin. As shown in Figure 1, the supernatant of untreated *S. aureus* induced a high level of TNF secretion, which was comparable to the TNF release induced by lipopolysaccharide (LPS) from *Salmonella enterica* serovar Typhimurium, a strong agonist of TLR4. Treatment of macrophages with the supernatants of *S. aureus* grown with quinupristin/dalfopristin at concentrations of 50% and 90% of the MIC inhibited the release of TNF in a dose-dependent manner.

Reduction of host cell cytotoxicity of intracellular S. aureus by quinupristin/dalfopristin

S. aureus is able to enter, survive and induce cell death in several non-professional phagocytes.[21] The killing of host cells by intracellular *S. aureus* involves presumably one or several exoproteins secreted in the host phagosome. To address the question of whether quinupristin/dalfopristin can modulate *S. aureus*-mediated cytotoxicity we used as a eukaryotic host cell a murine fibroblast cell line, mKSA, that has been previously successfully employed to analyse the intracellular activity of *S. aureus*.[22] Infection of mKSA cells with untreated *S. aureus* resulted in death of ∼80% of the host cells within 24 h after infection (Figure 2). When mKSA cells were infected with *S. aureus* cultured in the presence of graded subinhibitory concentrations of quinupristin/dalfopristin, the rate of host cell death dramatically decreased from 78% to 36% at a quinupristin/dalfopristin concentration of 50% MIC. Similar effects on cytotoxicity were observed for clindamycin, which is known to inhibit virulence factor production at sub-MIC concentrations.[6,8,23] In contrast, host cell viability was not significantly improved when *S. aureus* was previously cultured in the presence of linezolid at concentrations up to 90% MIC prior to infection.

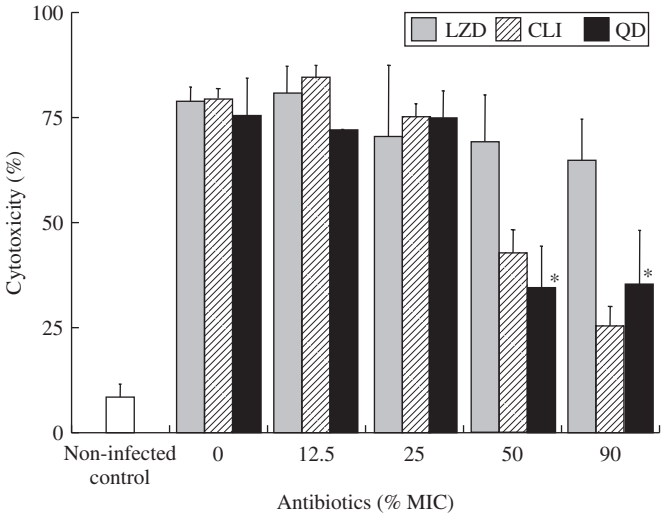

Figure 2. Host cell toxicity of intracellular *S. aureus* grown in the presence of subinhibitory concentrations of quinupristin/dalfopristin in comparison with linezolid and clindamycin. The murine fibroblast cell line mKSA was infected *in vitro* with *S. aureus* grown to an OD_{540} of 0.3 in the absence or in the presence of quinupristin/dalfopristin (QD, black bars), linezolid (LZD or, grey bars) or clindamycin (CLI, hatched bars) at the following concentrations: 12.5%, 25%, 50% and 90% MIC. The percentage of dead host cells was measured 24 h after infection. Asterisk (∗) indicates statistically significant inhibition of cytotoxicity ($P < 0.01$).

Effects of sub-MIC quinupristin/dalfopristin on protein secretion by S. aureus and protein identification

The decreased TNF-releasing activity found in the supernatants and the decreased cytotoxicity of *S. aureus* previously treated

with subinhibitory concentrations of quinupristin/dalfopristin suggested that quinupristin/dalfopristin down-regulates the production and/or release of *S. aureus* virulence factors. To differentiate quinupristin/dalfopristin-related modulation of virulence factor production from effects secondary to growth arrest or quorum-sensing phenomena, we addressed quinupristin/dalfopristin action on *S. aureus* at distinct growth phases. *S. aureus* ATCC 29213 cultures were treated from the start of growth (OD_{540} = 0.05) with graded concentrations of quinupristin/dalfopristin (0%, 12.5%, 25%, 50% and 90% MIC). Bacterial growth was monitored for 8.5 h. As expected a dose-dependent attenuation of bacterial growth was observed (Figure 3a). When *S. aureus* was exposed to subinhibitory concentrations of quinupristin/dalfopristin from the start of growth and harvested

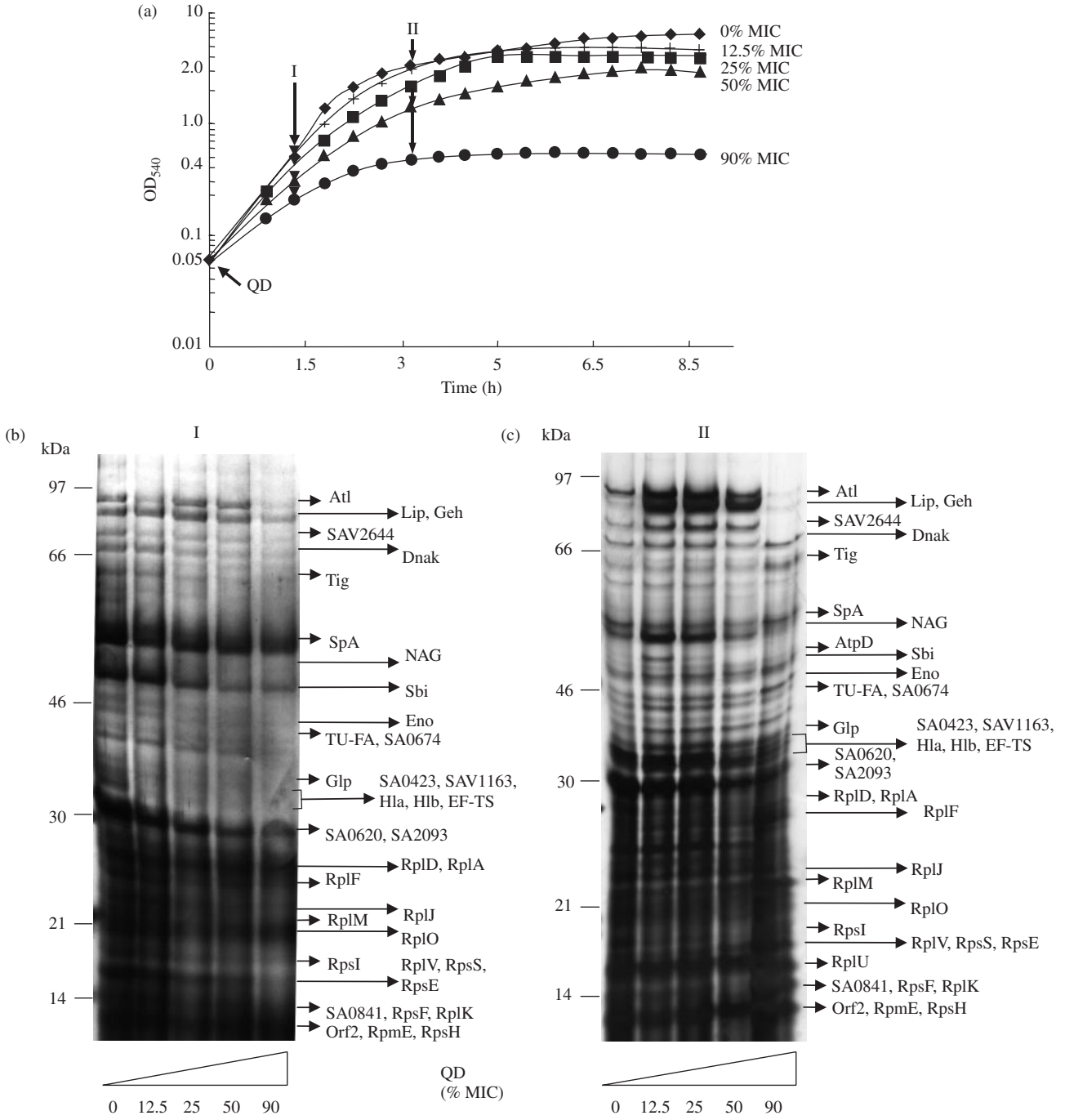

Figure 3. Effect of quinupristin/dalfopristin (QD) on exoprotein expression of *S. aureus*, present from the start of growth. (a) Growth curves of *S. aureus* in the presence of graded concentrations of quinupristin/dalfopristin (OD_{540}). Arrows indicate time points of exoprotein harvesting condition (I) and (II). (b and c) SDS–PAGE and identification of proteins secreted by *S. aureus* in the presence of quinupristin/dalfopristin. Protein bands were visualized by Coomassie staining. Panel I and II correspond to the harvesting conditions above. The arrows indicate the protein bands identified by MALDI-TOF/MS analysis (Table 2).

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

	OD_{540} at QD addition	Time of culture in the presence of QD (h)	Protein concentration (g/L)[a] at QD (% MIC)					
			0	12.5	25	50	75	90
I[b]	0.05	1.3	1.15 ± 0.64	0.85 ± 0.49	0.35 ± 0.35	0.50 ± 0.14	ND	0.65 ± 0.07
II	0.05	3.1	2.75 ± 0.49	2.90 ± 1.27	2.40 ± 0.28	1.25 ± 0.91	ND	2.35 ± 0.21
III	0.2	1.5	6.35 ± 0.35	ND	6.45 ± 0.21	5.00 ± 1.09	4.35 ± 1.20	4.15 ± 1.20
IV	0.4	1.4	4.75 ± 0.21	ND	4.55 ± 0.77	4.35 ± 0.21	3.80 ± 0.98	3.30 ± 0.42

ND, not determined.
[a]Mean values from three independent experiments ± SD.
[b]I–IV refer to the harvesting conditions described in the Materials and methods section.

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

Protein	Accession no.	Mascot *Z* score	Sequence coverage (%)	MW (Da) observed	MW (Da)/ pI theoretical	Putative identification
Atl	15924043	171	56	97 000	102 656/9.65	autolysin
Lip	15928257	234	44	90 000	76 662/6.58	triacylglycerol lipase
Geh	13700235	98	20	90 000	76 543/8.99	glycerol ester hydrolase
SAV2644	15925634	137	48	75 000	69 186/5.96	hypothetical protein ORFID: SA2437 (similar to autolysin)
Dnak	15924570	110	38	70 000	66 321/4.65	Dnak protein
SpA	225821	61	21	57 000	53 178/5.46	protein A
SA0674	15926396	204	42	46 000	74 353/9.04	hypothetical protein (similar to anion binding protein)
TU-FA	15923538	217	70	46 000	43 134/4.74	translational elongation factor TU
Glp	15923949	130	62	36 000	35 289/8.67	glycerophosphoryl diester phosphodiesterase
SA0423	15926142	89	64	35 000	35 871/9.67	hypothetical protein ORFID:SA0423 (similar to autolysin)
SAV1163	15924153	147	42	35 000	35 953/8.70	α-haemolysin precusor
Hla	2914570	99	33	33 000	33 227/7.94	α-haemolysin
Hlb	21283669	89	30	33 000	31 350/7.68	truncated β-haemolysin
SA2093	15925289	103	55	30 000	29 366/8.96	secretory antigen precursor SsaA homolog ORFID: SA2093
SA0620	15923655	54	28	30 000	28 169/6.12	Secretory antigen precursor SsaA homolog ORFID: SA0620
RplA	15923528	122	58	27 000	24 693/9.00	50S ribosomal protein L1
RplD	15927829	78	64	27 000	22 451/9.90	50S ribosomal protein L4
RplF	15927815	74	53	26 000	19 774/9.54	50S ribosomal protein L6
RplM	15927798	137	84	23 000	16 323/9.3	50S ribosomal protein L13
RplO	2500269	163	56	21 000	15 587/10.28	50S ribosomal protein L15
RpsI	15927797	76	67	18 000	14 607/10.56	30S ribosomal protein S9
RplV	15927825	81	59	16 500	12 827/9.92	50S ribosomal protein L22
RplU	15927227	69	81	15 000	11 326/9.84	50S ribosomal protein L21
SA0841	15926570	42	45	14 000	15 898/9.28	hypothetical protein (similar to cell surface protein Map-w)
RplK	15926215	199	40	14 000	14 922/9.04	50S ribosomal protein L11
Orf2	14029570	51	60	13 000	7990/10.16	50S ribosomal protein L27
Tig	15927254	70	42	62 000	48 579/4.34	trigger factor
NAG	2506027	95	58	53 000	53 620/9.67	NAG
AtpD	15927677	90	55	51 000	51 368/4.68	ATP synthase β-chain
Sbi	15927996	73	40	50 000	50 012/9.38	IgG binding protein SBI
Eno	15926453	117	31	47 000	47 145/4.55	enolase
Gap	15923762	109	35	37 000	36 372/4.89	glyceraldehyde-3-phosphate dehydrogenase
EF-TS	15926840	64	44	35 000	32 587/5.15	translational elongation factor TS
RplJ	5926217	51	63	24 000	17 699/4.82	50S ribosomal protein L10
RpsE	15927813	93	65	16 500	17 732/9.88	30S ribosomal protein S5
RpsS	18311383	48	45	16 500	10 634/10.29	30S ribosomal protein S19
RpsF	15926066	58	69	14 000	11 588/5.07	30S ribosomal protein S6
RpsH	15927816	87	65	13 000	14 822/9.33	30S ribosomal protein S8
RpmE	15927694	48	76	13 000	9717/8.04	50S ribosomal protein L31

before quorum sensing, that is at OD_{540} < 0.8, quinupristin/dalfopristin slightly reduced the secretion of proteins into the supernatants in a dose-dependent manner (Table 1 and Figure 3b). To examine possible effects of quinupristin/dalfopristin on the bacterial release of proteins, supernatants of bacterial cultures were harvested at the exponential and the post-exponential phase of bacterial growth. Coomassie Blue stained bands were subjected to MALDI-TOF/MS analysis and subsequent identification. The matches of peptide masses and the sequence coverage allowed the identification of 39 proteins including the 33 proteins previously described by Bernardo *et al.*[7] (Table 2).

When the supernatants of *S. aureus* cultures were harvested in the post-exponential phase of bacterial growth, a significant accumulation of low molecular weight proteins was observed with quinupristin/dalfopristin at a concentration of 90% MIC (Figure 3b and c). These proteins were identified as ribosomal proteins (Rpl, Rpm, Rps families). Furthermore the secretion of the stress-inducible protein DnaK was enhanced. In contrast, the release of virulence factors α- and β-haemolysins (Hla, Hlb), proteins of the autolysin family (Atl) and lipases (Lip, Geh), and the secretory antigen precursor SsaA homologue was reduced, while the secretion of the protein A (SpA) remained unchanged. Interestingly, the secretion of autolysin, lipases and enolase (Eno) showed a biphasic response, in that these proteins were increased by quinupristin/dalfopristin at concentrations below 50% MIC, but were down-regulated by quinupristin/dalfopristin at 90% MIC.

With the exception of prophylactic antibiotic therapy, antibiotics are administered when bacterial infections produce clinical symptoms. It was, therefore, important to investigate the effects of quinupristin/dalfopristin added to *S. aureus* cultures at a later stage of bacterial growth (OD_{540} = 0.2 and OD_{540} = 0.4).

As shown in (Figure 4a) the anti-proliferative effects of quinupristin/dalfopristin on *S. aureus* growth were less pronounced. When added at early log phase (OD_{540} of 0.2) quinupristin/dalfopristin affected the release of some exoproteins more than others (Figure 4b). For example, the secretion of α- and β-haemolysins (Hla and Hlb) is more impaired than that of protein A (SpA), lipases (Lip, Geh) or mature autolysin (Atl).

We next employed 2D gel electrophoresis to confirm the changes of protein profiles at higher resolution. Approximately 100 protein spots were separated by 2D gel electrophoresis in a molecular range between 10 and 80 kDa and a pI range between 3.5 and 10. In general, the protein profiles obtained by 2D gel analysis corresponded to those obtained by 1D SDS–PAGE yet allowed us to distinguish changes of expression of proteins with similar molecular weight.

For example, when the same protein samples previously separated by 1D SDS–PAGE were analysed by 2D gels, the changes in the protein profiles of Figure 5 (a–e) are comparable to the changes in the protein bands of Figure 4(b). For some proteins such as triacylglycerol lipase (Lip) and DnaK a biphasic response was observed. Intermediary concentrations of quinupristin/dalfopristin (25–50% MIC) increased, while quinupristin/dalfopristin at 90% MIC diminished triacylglycerol lipase (Lip) and DnaK expression (Figure 5a–e). In contrast, the production of the α- and β-haemolysins (Hla, Hlb) and to a lesser extent protein A (Spa) is dose-dependently reduced.

Addition of quinupristin/dalfopristin in the mid-log phase (OD_{540} of 0.4) also resulted in the attenuation of *S. aureus* growth accompanied by a decrease in exoprotein release in a similar pattern (Figure 6). However the impact of both growth rate and the release of extracellular proteins was significantly less pronounced compared with harvesting conditions III. 2D gel analysis revealed the absence of the biphasic response of Lip and DnaK, which was observed previously (Figure 7a–e). Instead,

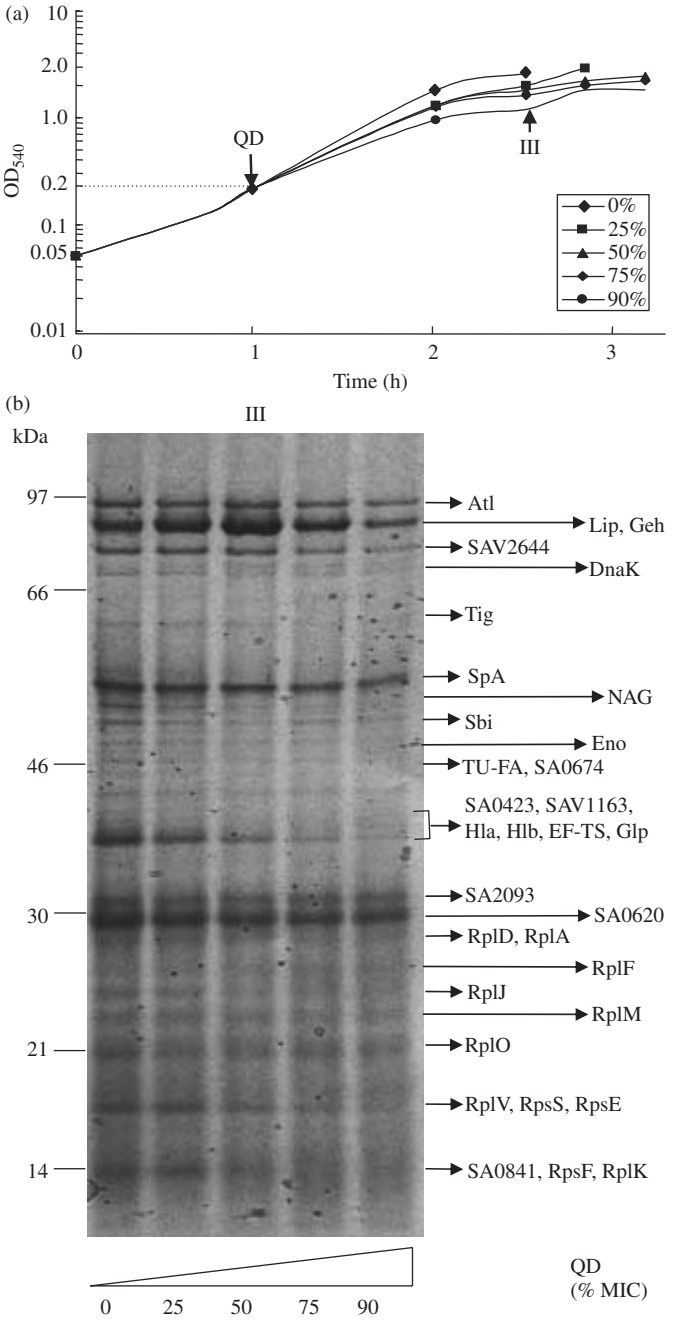

Figure 4. Effect of quinupristin/dalfopristin (QD) on exoprotein expression of *S. aureus*, present from early logarithmic growth phase. (a) Growth curves of *S. aureus* in the presence of graded concentrations of quinupristin/dalfopristin (OD_{540}): bacteria were grown first to an OD_{540} of 0.2 and thereafter growth was continued in the presence of graded subinhibitory concentrations of quinupristin/dalfopristin. Arrow indicates time point of exoprotein harvest (condition III). (b) Coomassie-stained SDS–PAGE of secreted *S. aureus* proteins. Arrows indicate proteins bands identified by MALDI-TOF/MS.

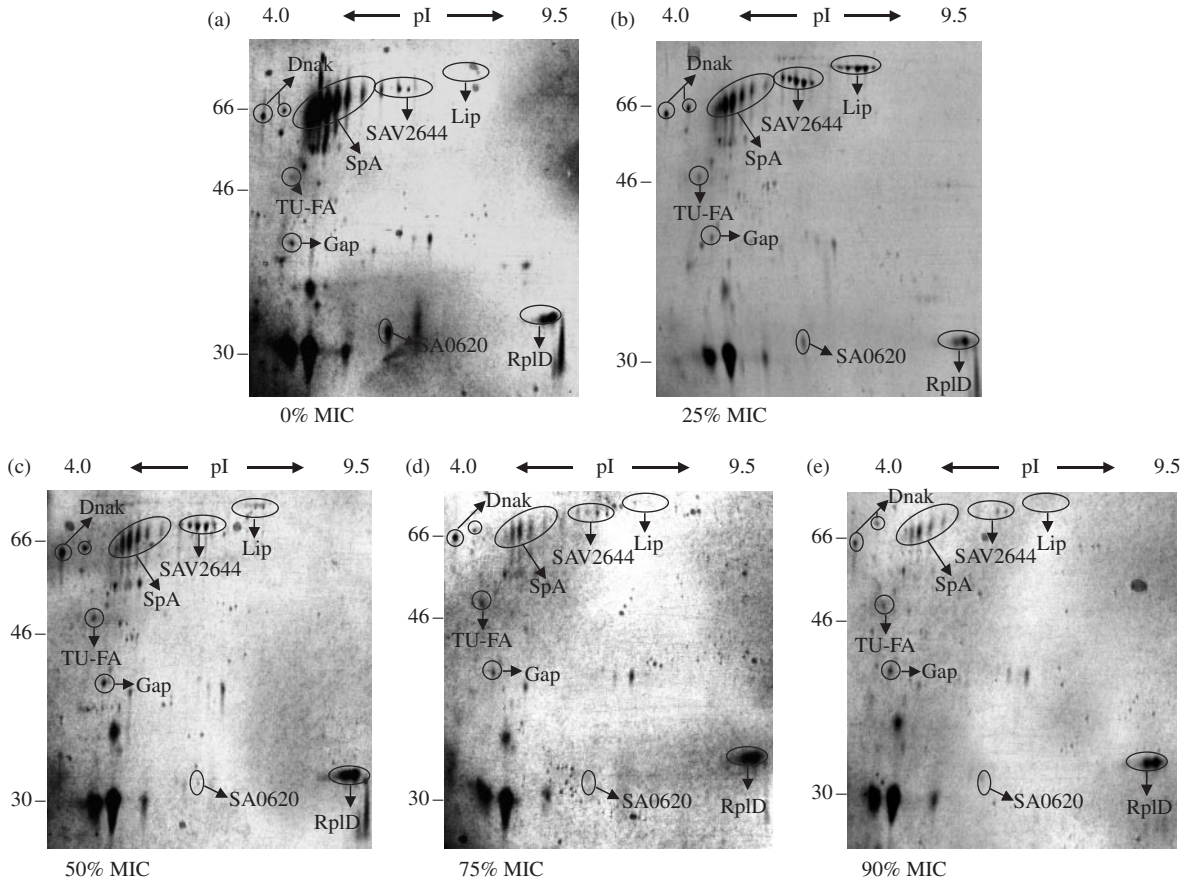

Figure 5. 2D patterns of exoprotein secretion in *S. aureus* in the presence of graded subinhibitory concentrations of quinupristin/dalfopristin from early log growth phase. Supernatants of *S. aureus* cultures previously grown in the presence of quinupristin/dalfopristin were harvested corresponding to condition III and subjected to 2D gel electrophoresis. (a) In the absence of quinupristin/dalfopristin. (b–e) In the presence of quinupristin/dalfopristin at concentrations of 25%, 50%, 75% and 90% MIC, respectively. Protein spots were visualized by Coomassie staining. Partial view of 2D gel is shown (pI 4.0–9.5 and 10–80 kDa). The arrows indicate the protein bands identified by MALDI-TOF/MS analysis (Table 2).

a release profile of these proteins showed dose-dependent down-regulation. In addition, inhibition of the release of other exoproteins required a higher quinupristin/dalfopristin dose, for example the same level of SpA down-regulation by 50% MIC of quinupristin/dalfopristin (Figure 5c) was first observed at 75% of quinupristin/dalfopristin (Figure 7d).

SEA and SEB could not be identified unambiguously by 2D SDS–PAGE/MALDI-TOF/MS probably due to their low concentrations in *S. aureus* supernatants. In order to visualize the effects of quinupristin/dalfopristin on SEA and SEB secretion, western-blot analysis was performed. As shown in Figure 8 (a–c), addition of quinupristin/dalfopristin before quorum sensing resulted in the inhibition of α-haemolysin, SEA, SEB and protein A in a dose-dependent manner. Addition of quinupristin/dalfopristin at later time points in the mid-logarithmic phase caused reduced release of α-haemolysin, SEA and SEB (Figure 8d).

Discussion

The pathogenicity of *S. aureus*, like that of other Gram-positive bacteria, depends largely on extracellular virulence factors. The clinical efficacy of antibiotics is not only determined by their respective bactericidal or bacteriostatic activity and pharmacokinetics but also by their action on virulence factor release. In general, antibiotic-induced release of exotoxins can enhance *S. aureus*-related toxic syndromes as well as proinflammatory host responses, while down-regulation of exotoxins expression causes a significant attenuation of virulence. In particular, cell-wall-affecting bactericidal antibiotics not only increase the release of endotoxin and exotoxin by destroying bacterial integrity but also stimulate exotoxins to be released at subinhibitory concentrations.[24] For example, β-lactams and glycopeptides induce alpha toxin expression through a stimulatory effect on exoprotein synthesis.[3] On the other hand, the synthesis of many staphylococcal exoproteins, including virulence factors, is inhibited by subinhibitory concentrations of antibiotics whose mode of action is to block protein synthesis. The fact that quinupristin/dalfopristin inhibits protein synthesis yet exerts bactericidal activity towards *S. aureus* raised the question about its possible action on virulence factor release by *S. aureus*.

In the present study we demonstrate that quinupristin/dalfopristin dose-dependently reduces both the induction of TNF-releasing activity by, and the toxicity of, *S. aureus* towards host cells. These two phenotypic consequences of quinupristin/dalfopristin action on *S. aureus* indicate the overall down-regulation of *S. aureus* virulence, because (i) the TNF-releasing

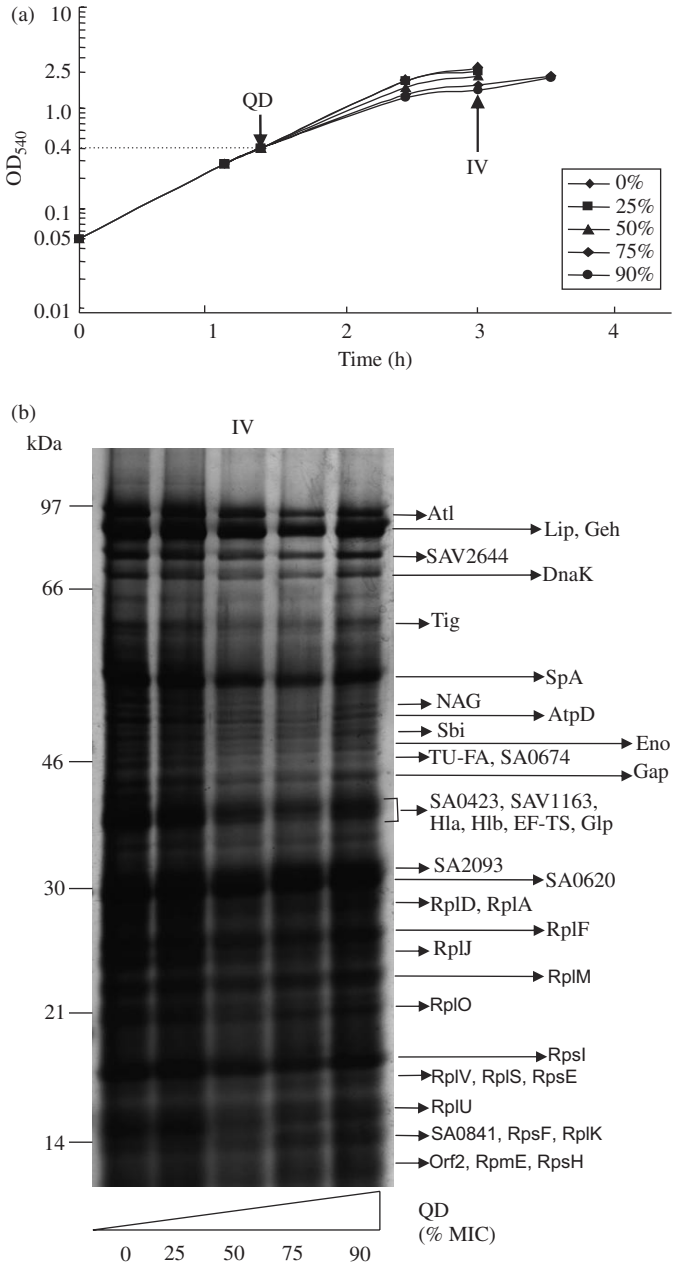

Figure 6. Effect of quinupristin/dalfopristin (QD) on exoprotein expression of *S. aureus* present from mid-logarithmic growth phase. (a) Growth curves of *S. aureus* in the presence of graded concentrations of quinupristin/dalfopristin (OD_{540}): bacteria were grown first to an OD_{540} of 0.4 and thereafter growth was continued in the presence of graded subinhibitory concentrations of quinupristin/dalfopristin. Arrow indicates time point of exoprotein harvest (IV). (b) Coomassie-stained SDS–PAGE of secreted *S. aureus* proteins. Arrows indicate proteins bands identified by MALDI-TOF/MS.

activity represents the sum of *S. aureus* proinflammatory exotoxins able to stimulate macrophages and (ii) host cell toxicity has been previously associated with tissue penetration and invasiveness of *S. aureus*. It is important to note that the TNF release assay and the host cell toxicity assay, respectively, seem to represent two functionally distinct, only partially overlapping sets of exotoxins. In this respect the comparison between quinupristin/dalfopristin and linezolid is instructive. Linezolid was recently found to inhibit the production of TNF-releasing activity by *S. aureus* at concentrations of 12.5% MIC[7] and hence is more effective than quinupristin/dalfopristin that down-regulated TNF-releasing activity only at 50% MIC. In contrast, linezolid was essentially ineffective in reducing the cytotoxicity of *S. aureus*, while quinupristin/dalfopristin decreased *S. aureus*-mediated cytotoxicity at 50% MIC. These findings suggest that like linezolid subinhibitory quinupristin/dalfopristin attenuates the inflammatory host response, yet in addition impairs *S. aureus* cytotoxicity, which might be of particular importance for the treatment of invasive *S. aureus* infections.

The proteomic analysis of quinupristin/dalfopristin effects on exotoxin release revealed a non-uniform response. When added from the start of culture, quinupristin/dalfopristin dose-dependently reduced the expression of most exotoxins within a culture period of 1.5 h. However, when cultured for 3 h, some exoproteins showed a biphasic response. For example, autolysin, triacylglycerol lipase and NAG were induced at 12.5%, 25% and 50% MIC quinupristin/dalfopristin and repressed at 90% MIC quinupristin/dalfopristin. This can be explained by secondary effects caused by growth retardation in the presence of 90% MIC quinupristin/dalfopristin, by enhanced transcriptional activity, as shown for clindamycin,[25] or by quinupristin/dalfopristin consumption by *S. aureus* during prolonged culture, which might become evident only when quinupristin/dalfopristin is added at low concentrations. At any rate, the biphasic induction of select exotoxins obviously did not impact on the overall TNF-releasing activity or cytotoxicity produced by *S. aureus*. The phenotypic reduction of *S. aureus* virulence, in fact, corresponded well with the dose-dependent down-regulation of established virulence factors such as haemolysin-α, SEA, SEB and protein A. When added at later growth phases, quinupristin/dalfopristin did not significantly influence the growth rate of *S. aureus*. At 90% MIC, quinupristin/dalfopristin reduced the expression of select exotoxins, whereas a number of exoproteins remained unchanged and biphasic responses were hardly detectable.

We have recently reported on reduction of *S. aureus* virulence factor expression by subinhibitory concentrations of linezolid.[7] As shown by proteomic analysis linezolid reduced in a dose-dependent manner the secretion of specific virulence factors, including SEA, SEB, autolysin, protein A, and α- and β-haemolysins. In contrast, other presumably non-toxic exoproteins remained unchanged or even accumulated in supernatants in the presence of linezolid at 90% MIC. While the overall response to linezolid seems to be similar to that observed with quinupristin/dalfopristin, distinct differences of exoprotein expression could be discerned. For example, triacylglycerol lipase, glycerol ester hydrolase, glycerophosphoryl diester phosphodiesterase and glyceraldehyde-3-phosphate dehydrogenase were up-regulated by subinhibitory linezolid but down-regulated by subinhibitory quinupristin/dalfopristin.

Herbert *et al.*[25] reported that subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *S. aureus*. In conjunction with our own observations with linezolid, the mainly inhibitory action of subinhibitory quinupristin/dalfopristin on exoprotein release was not expected. With regard to clindamycin, the differential inhibitory effects on gene transcription were suggested to be secondary to the inhibition of translation of one or more global regulatory mRNAs.[25] Indeed, many genes encoding virulence genes are coordinately regulated in response to a variety of intracellular

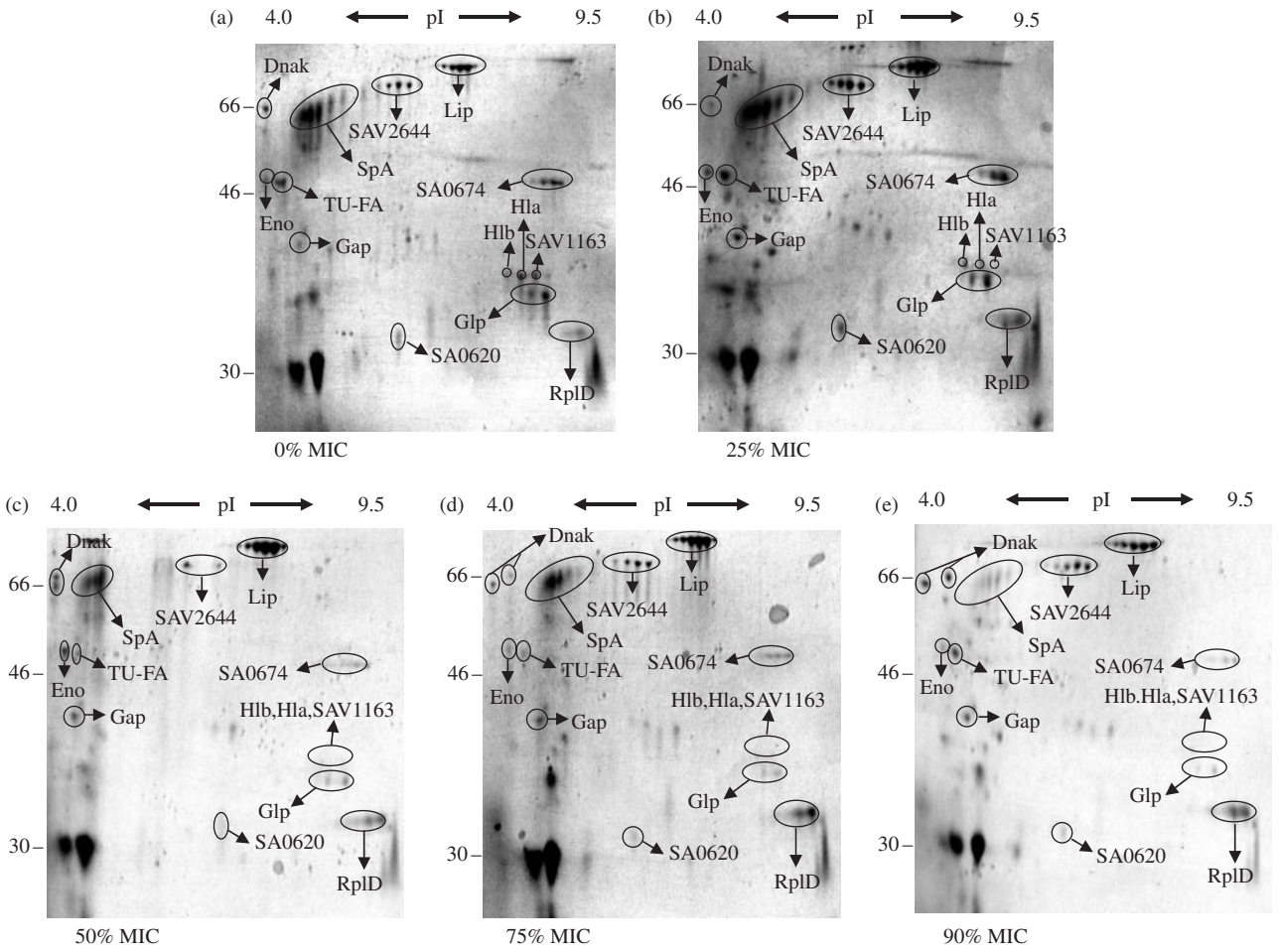

Figure 7. 2D patterns of exoprotein secretion in *S. aureus* in the presence of graded subinhibitory concentrations of quinupristin/dalfopristin from mid-log growth phase. Supernatants of *S. aureus* cultures previously grown in the presence of quinupristin/dalfopristin were harvested corresponding to condition IV and subjected to 2D gel electrophoresis. (a) In the absence of quinupristin/dalfopristin. (b–e) In the presence of quinupristin/dalfopristin at concentrations of 25%, 50%, 75% and 90% MIC, respectively. Protein spots were visualized by Coomassie staining. Partial view of 2D gel is shown (pI 4.0–9.5 and 10–80 kDa). The arrows indicate the protein bands identified by MALDI-TOF/MS analysis (Table 2).

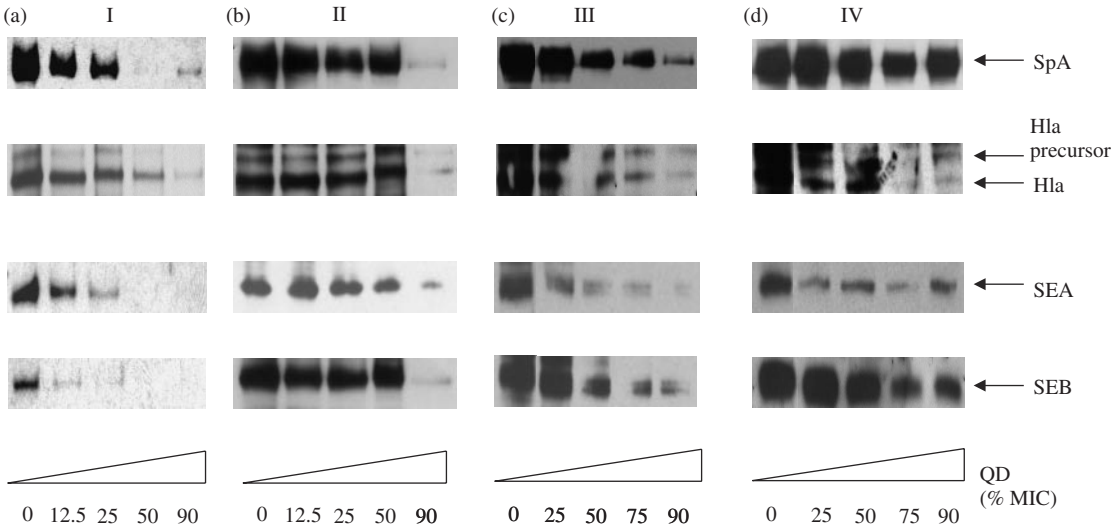

Figure 8. Western-blot analysis of exotoxins of *S. aureus* treated with quinupristin/dalfopristin (QD). I–IV indicate harvesting conditions. *S. aureus* culture supernatants were subjected to SDS–PAGE. After transfer to nitrocellulose, proteins were stained specifically with indicated antibodies against (a) protein A, (b) α-haemolysin, (c) enterotoxin A (SEA) and (d) enterotoxin B (SEB).

and extracellular signals. It is well established that antibiotics of different structure and known inhibitory activity are able to stimulate different promoters when bacteria are exposed to subinhibitory concentrations of the drugs.[3,25,26] A variety of chromosomal gene promoters are activated, including those involved in virulence, metabolic and adaptive functions. The extent and magnitude of the effects observed suggest that the transcriptional modulation by commonly used antibiotics could lead to negative consequences during the treatment of bacterial infections in human hosts. The up-regulation of global virulence regulators by low concentrations of antibiotics would lead to the activation of bacterial regulons, including the production of virulence factors in pathogens.

It is tempting to speculate that quinupristin/dalfopristin-induced inhibition of global regulators such as *agr* or *sarA* might result in the decreased virulence factor secretion. However, the pattern of individual exotoxins reduced in the presence of quinupristin/dalfopristin is not consistent with the pattern to be expected after inactivation of specific global regulator(s). For example, quinupristin/dalfopristin reduced protein A expression at any growth phase tested. If the primary effect of quinupristin/dalfopristin was to decrease expression of the octapeptide regulating *agr* expression, then protein A expression would be expected to be increased rather than decreased. Taken together, the proteomic analysis of *S. aureus* exotoxins revealed a robust down-regulation of release of exotoxins by subinhibitory quinupristin/dalfopristin, which explains the downmodulating action of subinhibitory quinupristin/dalfopristin on *S. aureus* virulence *in vitro*.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 589) and the 'Frau Maria Pesch' Stiftung, Cologne, Germany.

Transparency declarations

None to declare.

References

1. Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* 1998; **339**: 520–32.

2. Zetola N, Francis JS, Nuermberger EL *et al*. Community-acquired meticillin-resistant *Staphylococcus aureus*: an emerging threat. *Lancet Infect Dis* 2005; **5**: 275–86.

3. Ohlsen K, Ziebuhr W, Koller KP *et al*. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla*) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* 1998; **42**: 2817–23.

4. Shibl AM. Effect of antibiotics on production of enzymes and toxins by microorganisms. *Rev Infect Dis* 1983; **5**: 865–75.

5. Hallander HO, Laurell G, Lofstrom G. Enhancement of staphylococcal pathogenicity in the presence of penicillin. *Acta Pathol Microbiol Scand* 1966; **68**: 463–4.

6. Schlievert PM, Kelly JA. Clindamycin-induced suppression of toxic-shock syndrome—associated exotoxin production. *J Infect Dis* 1984; **149**: 471.

7. Bernardo K, Pakulat N, Fleer S *et al*. Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression. *Antimicrob Agents Chemother* 2004; **48**: 546–55.

8. Gemmell CG, Shibl AM. The control of toxin and enzyme biosynthesis in staphylococci by antibiotics. In: Jeljaszewicz J, ed. *International Symposium on Staphylococci and Staphylococcal Infections*. 3rd edn. Stuttgart, Germany: Gustav Fischer, 1976; 657–64.

9. Veringa EM, Verhoef J. Influence of subinhibitory concentrations of clindamycin on opsonophagocytosis of *Staphylococcus aureus*, a protein-A-dependent process. *Antimicrob Agents Chemother* 1986; **30**: 796–7.

10. Eliopoulos GM. Quinupristin-dalfopristin and linezolid: evidence and opinion. *Clin Infect Dis* 2003; **36**: 473–81.

11. Chant C, Rybak MJ. Quinupristin/dalfopristin (RP 59500): a new streptogramin antibiotic. *Ann Pharmacother* 1995; **29**: 1022–7.

12. Cocito C, Di Giambattista M, Nyssen E *et al*. Inhibition of protein synthesis by streptogramins and related antibiotics. *J Antimicrob Chemother* 1997; **39** Suppl A: 7–13.

13. Yarwood JM, Schlievert PM. Quorum sensing in *Staphylococcus* infections. *J Clin Invest* 2003; **112**: 1620–5.

14. Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* 2003; **48**: 1429–49.

15. Cheung AL, Zhang G. Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. *Front Biosci* 2002; **7**: d1825–42.

16. Shevchenko A, Wilm M, Vorm O *et al*. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996; **68**: 850–8.

17. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 1975; **250**: 4007–21.

18. Görg A, Postel W, Gunther S. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 1988; **9**: 531–46.

19. Bernardo K, Fleer S, Pakulat N *et al*. Identification of *Staphylococcus aureus* exotoxins by combined sodium dodecyl sulfate gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *Proteomics* 2002; **2**: 740–6.

20. Mattsson E, Van Dijk H, Verhoef J *et al*. Supernatants from *Staphylococcus epidermidis* grown in the presence of different antibiotics induce differential release of tumor necrosis factor alpha from human monocytes. *Infect Immun* 1996; **64**: 4351–5.

21. Lowy FD. Is *Staphylococcus aureus* an intracellular pathogen? *Trends Microbiol* 2000; **8**: 341–3.

22. Krut O, Utermöhlen O, Schlossherr X *et al*. Strain-specific association of cytotoxic activity and virulence of clinical *Staphylococcus aureus* isolates. *Infect Immun* 2003; **71**: 2716–23.

23. Blickwede M, Wolz C, Valentin-Weigand P *et al*. Influence of clindamycin on the stability of *coa* and *fnbB* transcripts and adherence properties of *Staphylococcus aures* Newman. *FEMS Microbiol Lett* 2005; **252**: 73–8.

24. Gemmell CG. Antibiotics and expression of microbial virulence factors: implications for host defense. *J Chemother* 1991; **3** Suppl 1: 105–11.

25. Herbert S, Barry P, Novick RP. Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. *Infect Immun* 2001; **69**: 2996–3003.

26. Bisognano C, Vaudaux PE, Lew DP *et al*. Increased expression of fibronectin-binding proteins by fluoroquinolone-resistant Staphylococcus aureus exposed to subinhibitory levels of ciprofloxacin. *Antimicrob Agents Chemother* 1997; **41**: 906–13.