Muropeptide signature of inhibitors of protein synthesis correlates with β -lactam synergism against methicillin-resistant *Staphylococcus aureus* [★]

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ABSTRACT

Quinupristin/dalfopristin and β -lactams interact positively against methicillin-resistant *Staphylococcus aureus* (MRSA). The effect extends to other inhibitors of protein synthesis, but not to inhibitors of polynucleotide synthesis or assembly, or to Q/D plus non- β -lactam cell wall inhibitors. Moreover, electron microscopy studies have correlated this effect with a thickened cell wall. In this study, we sought to determine whether inhibitors of protein synthesis might produce a specific peptidoglycan muropeptide signature that would correlate with their positive β -lactam interaction. The muropeptides of six *S. aureus* isolates (three methicillin-susceptible and three MRSA) were analysed using high-performance liquid chromatography and mass spectrometry. Exposure to 0.25 $\times$ the minimum inhibitory concentration of inhibitors of protein synthesis consistently produced three main alterations irrespective of methicillin resistance: (i) an increase in peak 12 (a cyclic dimer of glycine-containing disaccharide–tetrapeptide); (ii) an increase in poorly resolved late-eluting materials; and (iii) a decrease in peak 1 (a disaccharide–pentapeptide). Eventually, the rate of autolysis was also decreased, supporting the structural alteration of the peptidoglycan. Other drug classes did not produce these anomalies. An increase in peak 12 was also observed in staphylococci treated with fosfomycin, which decreases expression of the native penicillin-binding protein (PBP) 2 and 4. Parallel blockage of normal PBPs with β -lactams abolished the anomalies, indicating that they resulted from altered function of native PBPs. This underlines the potential of inhibiting both protein synthesis and transpeptidation simultaneously and suggests that such a drug combination strategy might be efficaciously exploited.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are intrinsically resistant to β -lactams by virtue of newly acquired low-affinity penicillin-binding protein (PBP) 2A, which can build a wall when other PBPs are blocked by β -lactams [1]. Vancomycin is still the preferred treatment for MRSA, but it fails against some vancomycin-intermediate strains [2]. New agents, including linezolid and tigecycline, are available for treatment of MRSA, but they are only bacteriostatic against *S. aureus* and are not approved for invasive difficult-to-treat infections such as endocarditis. Moreover, resistance to these drugs is emerging [3,4].

A better understanding of the mechanism of PBP2A-mediated resistance to β -lactams has allowed for the development of β -lactams with improved anti-PBP2A affinity, such as ceftobiprole medocaril [1]. Alternatively, exploring combinations of existing drugs can be useful because they may act synergistically or their resistance mechanisms may be mutually exclusive. Although combining existing drugs is advantageous because their intrinsic toxicity is usually known, drug combinations exert their effects through complex, often incompletely understood, interactions.

Quinupristin/dalfopristin (Q/D) and β -lactams act synergistically against MRSA both in vitro and in rats with experimental endocarditis [5–7]. Recently, we demonstrated that this synergism extends to other inhibitors of protein synthesis (erythromycin, chloramphenicol, tetracycline, linezolid and fusidic acid), but not to inhibitors of

polynucleotide synthesis or assembly [ciprofloxacin and trimethoprim/sulfamethoxazole SXT]) or to Q/D plus non- β -lactam cell wall inhibitors, such as D-cycloserine, vancomycin and teicoplanin [8]. The synergism is specific to MRSA and does not extend to methicillin-susceptible *S. aureus* (MSSA).

This MRSA specificity suggests that protein synthesis inhibitors either directly or indirectly affect PBP2A functionality. A decrease of PBP2A activity would be the simplest explanation for the restoration of β -lactam susceptibility. However, decreased PBP2 activity would also explain the MRSA-specific synergism. PBP2 is the only bifunctional PBP of *S. aureus*, providing both transglycosylase and transpeptidase activities. Hence, PBP2 is indispensable for the functionality of purely transpeptidase PBPs, including low-affinity PBP2A [9]. When MRSA are exposed to β -lactams, their transpeptidase sites are blocked, except for low-affinity PBP2A, and the indispensable PBP2-transglycosylase site remains functional even if the PBP2-transpeptidase site is inhibited by β -lactams [9]. If this transglycosylase is further inhibited, for instance by decreasing its expression or by drugs such as moenomycin [10], PBP2A can no longer assemble the wall and β -lactams regain their antibacterial activity. The situation differs in MSSA, which lacks PBP2A. Here, blockage of the native high-affinity transpeptidases with low concentrations of β -lactams is enough to block peptidoglycan synthesis overall, and additional inhibition of transglycosylase provides no supplementary efficacy. Although MGT, a monofunctional transglycosylase recently reported in *S. aureus*, can assume transglycosylation if the PBP2-transglycosylase domain is inactivated, it cannot

replace it in the presence of β -lactams, suggesting that only PBP2 can collaborate with PBP2A [11].

If protein synthesis inhibition alters PBP expression, one might expect alterations in the peptidoglycan itself. Cell wall changes have indeed been observed by electron microscopy in *S. aureus* treated with subinhibitory concentrations of Q/D [12]. However, a muropeptide signature that is specific to protein synthesis inhibitors and that could correlate with β -lactam synergism against MRSA was not described.

Here we further examined peptidoglycan alterations that might occur both in MSSA and MRSA isolates grown in subinhibitory concentrations of various inhibitors of protein synthesis and inhibitors of polynucleotide synthesis or assembly. The former produced specific muropeptide anomalies previously reported in *S. aureus* with altered native PBP2 [13], whereas the latter, which do not synergise with β -lactams, induced no changes [5,8]. Importantly, the specific muropeptide alterations caused by inhibitors of protein synthesis were abrogated by blocking native PBPs with β -lactams, which supports the theory that these alterations result from a drug-induced imbalance in native PBP functionality.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The test organisms included three MSSA and three MRSA isolates with various antibiotic susceptibility profiles (Table 1) [5,14–17]. Bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit, MI) at 37 °C with aeration in a rotating incubator (120 rpm). Growth was followed with a photometer (Sequoia-Turner, Mountain View, CA) by measuring the optical density at 620 nm (OD_{620}). When necessary, the growth medium was supplemented with subinhibitory concentrations of various antibiotics. Subinhibitory concentrations used corresponded to 0.25× the minimum inhibitory concentration (MIC) as in our previous experiments [5,8]. Bacterial cells were stored at –70 °C in tryptic soy broth containing 10% (v/v) glycerol.

2.2. Antibiotics and chemicals

Antibiotics included: (i) the β -lactam cefepime; (ii) the protein synthesis inhibitors Q/D, erythromycin, chloramphenicol, tetracycline, linezolid and fusidic acid; (iii) the nucleic acid synthesis or assembly inhibitors ciprofloxacin and SXT; and (iv) the non- β -lactam cell wall inhibitors β -chloro-D-alanine, D-cycloserine, bacitracin, fosfomycin and vancomycin. All drugs and chemicals were commercially available.

2.3. Cell wall preparation and purification

Cell walls were prepared as previously described [18]. Briefly, 2 L of early log-phase culture ($OD_{620} = 0.2$) were rapidly chilled in an ice/ethanol bath and were harvested by centrifugation ($16\,000 \times g$, 15 min, 4 °C). Cells were re-suspended in boiling sodium dodecyl sulphate (SDS) (4% final concentration) for 15 min to deactivate intrinsic autolytic enzymes, were washed extensively with water to remove SDS and were broken open with a FastPrep® FP120 apparatus (Bio 101; Savant Instruments, Holbrook, NY). Intact cells were removed by centrifugation ($1500 \times g$, 5 min, 4 °C). Cell walls were then pelleted by centrifugation ($40\,000 \times g$, 20 min, 4 °C), were re-suspended in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.05% sodium azide and were treated with DNase, RNase and trypsin as described previously [18]. Peptides obtained from protease digestion were extracted by boiling for 10 min with SDS (1% final concentration) and the walls were incubated with 8 M lithium chloride followed by 100 mM ethylene diamine tetra-acetic acid (EDTA) to remove ionically bound material. Teichoic acid was removed by stirring the disrupted cell walls in 0.5 mL of hydrofluoric acid (48%) per milligram of walls for 48 h at 4°C. The insoluble preparation was extensively washed, re-suspended in water and dried by rotary evaporation.

2.4. Cell wall digestion

The bonds between *N*-acetylmuramoyl and *N*-acetylglucosamine residues were hydrolysed with mutanolysin from *Streptomyces globisporus* (Sigma-Aldrich, Buchs, Switzerland; EC 3.2.1.17), which was added to a final concentration of 500 U/mL and 1 mg/mL substrate in 12.5 mM potassium phosphate buffer (pH 5.5). The mixture was

incubated for 18 h with shaking at 37 °C and was then boiled to remove the enzyme as described above; insoluble material was pelleted by centrifugation as described previously [18]. The supernatant was reduced with sodium borohydride, was adjusted to pH 2 with *o*-phosphoric acid and was stored at –20 °C [18].

2.5. Digested peptidoglycan separation by high-performance liquid chromatography (HPLC)

Muropeptides were separated by reverse-phase HPLC essentially as previously described [18]. The HPLC system (Hitachi Instruments, Ichige, Hitachinaka, Japan) consisted of an L-7200 Autosampler, an L-7100 gradient pump with low-pressure mixing and an L-7400 UV detector. The column temperature was maintained at 34 °C using an L-7360 column oven. The results were analysed using the D-7000 HPLC System Manager program (Hitachi Instruments).

A 100 µL sample containing 100 µg of wall digest was injected into a C18 reverse-phase column (ODS-Hypersil, 3 µm, 4.6 × 250 mm; Keystone Scientific, Bellefonte, PA) protected with a guard C18 cartridge (5 µm, 4 × 10 mm; Keystone Scientific). The mixture was separated using a linear gradient of 5% (v/v) methanol in 0.1 M NaH₂PO₄ (pH 2.5) to 30% (v/v) methanol in 0.1 M NaH₂PO₄ (pH 2.8) over 150 min at a flow rate of 0.5 mL/min. Detection was at 210 nm, the areas of the peaks of interest were added, and individual peaks were expressed as percentages of this total.

2.6. Peak identification

Fractions were collected at 0.5-min intervals. The solvent was removed by rotary evaporation. Fractions corresponding to the same peak were pooled and desalted (Zip-tips; Millipore, Billerica, MA). The content of specific peaks was analysed as follows.

First, the amino acid content was quantified. Samples were hydrolysed with 6 N sequanal-grade HCl vapour (Pierce, Rockford, IL) containing 1% phenol for 18 h at 105 °C (Hydrolysis/Derivatization Station; Eldex, Napa, CA). Glutamate, glycine, alanine and lysine were determined by 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate precolumn derivatisation, followed by HPLC on a C18 reverse-phase column (Waters AccQ•Tag; Millipore). The HPLC system (Hitachi Instruments) consisted of the L-7250 Autosampler, L-7100 gradient pump with low-pressure mixing and L-7480 fluorescence detector. The column temperature was maintained at 37 °C using an L-7360 column oven. The results were analysed using the D-7000 HPLC System Manager program (Hitachi Instruments). Separation was performed following the instructions of the derivatisation/separation kit manufacturer. An amino acid hydrolysate standard solution (Pierce) was used for calibration, with α -aminobutyric acid as an internal standard. To prevent external amino acid contamination, all glassware was pyrolysed at 500 °C for 4 h (Nabertherm GmbH, Lilienthal, Germany), and organic trace-free water (Fluka, Buchs, Switzerland) was used.

Second, the molecular mass of specific mucopeptide peaks was measured using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS)

(AutoflexTM; Bruker Daltonics, Bremen, Germany) in positive-ion mode with a 5-chloro-2-mercaptobenzothiazole matrix in water, tetrahydrofuran and ethanol (1:1:1). Post-source decay (PSD) analysis was performed on certain fractions. *N*-acetyl-D-glucosaminyI-(14)-*N*-acetylmuramoyl-L-alanyl-D-isoglutamine (Calbiochem[®]; EMD Biosciences, San Diego, CA) was used as a standard (*Mr* 694.7).

2.7. Autolysis assay

The assay was performed as described previously [19]. Briefly, 100 mL of early log-phase culture ($OD_{620} = 0.2$) was rapidly cooled in an ice/ethanol bath and was centrifuged ($16\,000 \times g$, 15 min, 4 °C). The pelleted cells were washed once with ice-cold water and were suspended to $OD_{620} = 1.0$ in 50 mM glycine–Triton X-100 (0.01%) buffer (pH 8). The autolysis rate, measured during incubation with aeration at 37 °C, was defined as the time (per hour) needed for a 50% decrease in OD_{620} .

2.8. Statistical analysis

HPLC data obtained after wall preparations are expressed as the mean $\pm$ standard deviation of the six test isolates. Means in experiments with subinhibitory antibiotic concentrations and in experiments without antibiotic were compared using Student's *t*-test for paired samples. Statistical analysis was performed using IBM SPSS Statistics for Windows v.22.0 (IBM Corp., Armonk, NY).

3. Results

3.1. Muropeptide patterns of MSSA and MRSA grown with or without subinhibitory concentrations of protein or polynucleotide synthesis inhibitors

Fig. 1 depicts the muropeptide patterns of MSSA AW26 and MRSA P8 grown either without antibiotic (Fig. 1A,B) or with 0.25× MIC of Q/D (Fig. 1C,D), tetracycline (Fig. 1E,F) or ciprofloxacin (Fig. 1G,H). All patterns were reproducible in more than three independent wall preparations and independent HPLC runs.

The muropeptide pattern of untreated bacteria was comparable with that of other *S. aureus* walls described in the literature [18]. Conversely, the muropeptide pattern of staphylococci grown with Q/D (Fig. 1C,D) or tetracycline (Fig. 1E,F) showed specific alterations, mostly in the form of a modified peak 12/peak 11 ratio and a global increase in the final hump that has been shown to contain a complex of poorly resolved materials [13,18]. These alterations were observed both in the MSSA and MRSA isolates and were also reproduced in HPLC analysis of all six test isolates exposed to each protein synthesis inhibitor in Table 1 (muropeptide patterns not presented), but not after exposure to 0.25× MIC of ciprofloxacin (Fig. 1G,H) or SXT (muropeptide patterns not shown on Fig. 1). Peak 1 drastically decreased after exposure to protein synthesis inhibitors, but not to ciprofloxacin or SXT. This third alteration was visually evident readily only with MRSA COL (Fig. 2) because of the high naturally present peak 1, but also occurred in the other organisms.

Table 2 presents peak 12/peak 11 ratios and the proportions of both the final hump and peak 1 relative to the sum of all the peaks on the chromatogram. All of the tested protein synthesis inhibitors significantly increased the peak 12/peak 11 ratio and the final hump ($P < 0.05$ or 0.01) and decreased peak 1 (not significant). As mentioned above, the peak 1 decrease was visually evident readily only with MRSA COL, with ratios of 0.20, 0.55, 0.38, 0.30, 0.15 and 0.13 with subinhibitory concentrations of Q/D, erythromycin, chloramphenicol, tetracycline, linezolid and fusidic acid, respectively. These alterations were not observed after exposure to $0.25\times$ MIC of ciprofloxacin or SXT.

3.2. Muropeptide pattern of Staphylococcus aureus grown in subinhibitory concentrations of non- β -lactam cell wall inhibitors

Only experiments with MSSA AW26 are presented here (Fig. 3). Some of the drugs that induce specific muropeptide anomalies have been described previously. Bacitracin had little effect (Fig. 3B). Conversely, β -chloro-D-alanine and D-cycloserine, which block D-alanine-D-alanine addition to wall precursors, increased an early peak that contained only a disaccharide–tripeptide (Fig. 3C and 3D, respectively), and vancomycin increased peak 5, which contained a disaccharide–pentapeptide, as described previously (Fig. 3E) [6,20]. None of these non- β -lactam cell wall inhibitors induced the three specific alterations observed in the muropeptide pattern after treatment with protein synthesis inhibitors. Only dual-acting fosfomycin, which interferes early in 5'-pyrophosphate-*N*-acetylmuramic acid synthesis and decreases expression of native PBP2 and 4 as well as PBP2A [21,22], increased the peak 12/peak 11 ratio, as with

Q/D, by a ratio of 1.7 relative to the results without antibiotic, but did not increase the final hump (Fig. 3F). These experiments were reproduced with all other test isolates (muropeptide patterns not presented).

3.3. Molecular structures contained in altered HPLC peaks

The structures of the muropeptides in peaks 1, 11 and 12 were as previously identified (Fig. 4) [13,18]. The corresponding peaks observed in the present experiments were identical to those described above, as determined by both amino acid analysis and MALDI-PSD. The muropeptide fractions corresponding to peaks 1, 11 and 12 were first collected from bacteria grown without antibiotic. Their composition corresponds to their described homologues [13,18]. To ensure that the peak 12 increase due to the protein synthesis inhibitors was not due to an overlapping muropeptide of different composition, the muropeptide in the increased peak 12 of bacteria exposed to Q/D and other protein synthesis inhibitors was re-analysed. Both the amino acid composition and the PSD fragmentation spectrum confirmed the identity of this peak to the previously described cyclic muropeptide peak 12 [13].

3.4. Effect of blocking native penicillin-binding proteins on quinupristin/dalfopristin-induced muropeptide anomalies

If protein synthesis inhibitors affect the normal function of native PBPs, their related muropeptide signature should be abrogated by blocking the normal staphylococcal transpeptidases. The experiments were run with MRSA isolates that could grow despite

high concentrations of cefepime (>4 mg/L) owing to PBP2A and unblocked PBP2-transglycosylase site. Only experiments with MRSA P8 are presented here (Fig. 5). Exposure to 0.25× MIC of Q/D alone resulted in the expected mucopeptide signature due to protein synthesis inhibitors (Fig. 5B). Conversely, exposure to 0.125× MIC of cefepime alone produced a simplified mucopeptide pattern typical of β -lactam-treated MRSA (Fig. 5C), consistent with a blockage of native PBPs [23]. This pattern includes the disappearance of the final hump and an increase in the monomeric and dimeric pentaglycine-decorated mucopeptides (peaks 5 and 11, respectively), which are believed to express the activity of uninhibited PBP2A [23]. However, when Q/D and cefepime were applied together, at 0.25× and 0.125× MIC respectively, the PBP2A-pattern prevailed and the Q/D-specific anomalies disappeared (Fig. 5D). Because they vanished after blockage of the transpeptidase function of high-affinity transpeptidases, these anomalies likely resulted from a Q/D-induced imbalance related to native PBP functionality. These experiments were reproduced with the other two MRSA isolates and with other β -lactamase-resistant β -lactams (e.g. amoxicillin/clavulanic acid, flucloxacillin, cefuroxime, cefpirome and imipenem) (mucopeptide patterns not presented).

3.5. Antibiotic impact on bacterial lysis

Abnormally thickened walls have been observed in staphylococci exposed to Q/D [12]. Therefore, we sought to determine whether the drug also affected spontaneous cell wall turnover. Bacterial autolysis is a complex phenomenon that involves both the susceptibility of the wall to its intrinsic autolysins and the amount of autolysins present in

the wall. Hence, to some extent this reflects spontaneous wall turnover. Autolysis was measured in early log-phase MSSA AW26 and MRSA P8 ($OD_{620} = 0.2$) with or without subinhibitory concentrations of various antibiotics, followed by exposure to Triton X-100 as described previously [19]. In both organisms, protein synthesis inhibitors drastically decreased spontaneous autolysis (Fig. 6). Tetracycline, linezolid, fusidic acid and Q/D had the most marked effects, prolonging the time to 50% lysis by 5×, 6×, 20× and >40×, respectively. In contrast, ciprofloxacin and SXT did not affect time to 50% lysis. Thus, muropeptide anomalies also correlated with altered autolysis.

4. Discussion

We recently observed that synergism between Q/D and β -lactams against MRSA extends to β -lactams plus other inhibitors of protein synthesis, but not to β -lactams plus inhibitors of polynucleotide synthesis or assembly, or to Q/D plus non- β -lactam cell wall inhibitors [8]. Another study reported a positive anti-MRSA interaction between β -lactams plus Q/D or erythromycin, but not between β -lactams plus tetracycline, chloramphenicol or gentamicin [6]. This discordance might result from the utilisation of resistant organisms in which higher concentrations of antibiotics are needed to detect the beneficial effect. Therefore, in our present and preceding experiments [8], antimicrobials were always used at concentrations that were multiples of their MIC, thus avoiding the problem of susceptibility or resistance as defined by clinical breakpoints.

Subinhibitory concentrations of Q/D alter the cell wall structure [12]. Therefore, we tested whether different protein synthesis inhibitors affected the staphylococcal muropeptide and provided a signature correlating with their ability to synergise with β -lactams against MRSA. Indeed, we identified such a signature. All of the protein synthesis inhibitors tested produced the same statistically significant altered muropeptide pattern on the three MRSA and the three MSSA isolates: an increase in the peak 12/peak 11 ratio; an increase in the poorly resolved late-eluting final hump; and a decrease in peak 1, which was visually evident readily only with MRSA COL because of the high naturally present peak 1. This latter alteration was also observed in the other organisms, although it was not statistically significant, probably because of the small peak 1, for which differences of amount were difficult to detect. As a control, ciprofloxacin, SXT, β -lactams and most non- β -lactam cell wall inhibitors (i.e. bacitracin, β -chloro-D-alanine, D-cycloserine and vancomycin) produced no such effect. Only fosfomycin detectably increased the peak 12/peak 11 ratio, but not the final hump. Aside from inhibiting early steps in *N*-acetylmuramic acid synthesis, fosfomycin also decreased expression of native PBP2, PBP4 and low-affinity PBP2A [22], which could be involved in the increased production of the abnormal peak 12 cyclic structure.

Changes in peaks 1 and 12 may provide a clue to the abnormalities underlying this signature. An increase in the peak 12/peak 11 ratio was originally described in an MSSA isolate selected for decreased susceptibility to cefotaxime and methicillin by serial exposures to the drugs [13]. The parent lacked PBP2A, and the major change associated with the increased MIC, and hence the muropeptide anomalies, was

decreased β -lactam affinity of native PBP2. Therefore, linking abnormal activity of PBP2 with alterations in the peak 12/peak 11 ratio and maybe peak 1 is tempting. A specific decrease in the β -lactam affinity of PBP2 would imply altered transpeptidation in the resistant mutants, whereas a global decrease in PBP2 expression would imply decreases in both transpeptidation and transglycosylation in Q/D-treated staphylococci. Since fosfomycin also decreases PBP2 expression, the altered peak 12/peak 11 ratio observed after drug exposure supports this possibility.

The mechanisms of the alterations in the peak 12/peak 11 ratio and peak 1 are unknown. The Q/D-induced peak 12 disappeared in MRSA after native high-affinity transpeptidases were blocked with β -lactams. Therefore, peak 12 likely resulted from abnormal action of native PBPs, rather than from abnormal action of PBP2A (which is not inhibited by β -lactams).

Peak 12 is a cyclic structure produced by two consecutive transpeptidase reactions on two pentaglycine-decorated monomeric components that act as both donor and acceptor in the reactions [13]. Its resulting structure (Fig. 4) creates a 'dead-end' for further transpeptidation, especially for PBP2A, which requires a pentaglycine-decorated stem peptide to be operational. The concomitant decreases in peaks 1 and 11 may contribute to worsening wall polymerisation. Peak 1, a disaccharide–pentapeptide, is likely to be a poor acceptor because it lacks the pentaglycine side chain (Fig. 4). Conversely, it might act as a donor in normal peptidoglycan cross-linking. In symmetry,

peak 11 is a disaccharide–pentapeptide–pentaglycine dimer, which may act as both donor and acceptor (Fig. 4) [18,23]. Its decrease may further deteriorate PBP2A activity.

Eventually, the poorly resolved final hump may arise as another consequence of abnormal cell wall assembly by native PBPs. Like peak 12, it was abrogated by blocking native PBPs in MRSA with β -lactams. Moreover, it was associated with a drastic decrease in cell wall autolysis, which was specific to protein synthesis inhibitors. Altered autolysis can arise from altered autolysin substrates, decreased autolysin amounts, or both. That the final hump disappeared after PBP blockage, but not after blockage of other synthetic processes such as that of polynucleotides, suggests that altered autolysis was due to substrate alteration rather than to decreased autolysins.

In contrast to the situation with protein synthesis inhibitors, the final hump was absent in the low-affinity PBP2 mutants described previously [13]. Moreover, autolysis and cell wall turnover in these mutants increased, whilst they were decreased after exposure to protein synthesis inhibitors. These unexplained discrepancies could result from differences in the two ways of wall modification, i.e. selection for methicillin-resistant mutations and treatment with protein synthesis inhibitors.

As discussed earlier [8], protein synthesis inhibitors may induce complex stress responses, including the stringent response [24–26], which affects physiological features that may affect cell wall metabolism. Likewise, exposure to β -lactams—and selection for resistance—are associated with a global stress response referred to as the

'cell-wall-stress stimulon' [27,28]. The triggering of both systems may be an additional burden for the bacterium.

These experiments reveal a unique mucopeptide signature in *S. aureus* exposed to subinhibitory concentrations of protein synthesis inhibitors. This signature may have resulted from altered expression of native PBPs, possibly PBP2, which provides indispensable transglycosylase activity for assembling a coherent peptidoglycan. Further investigations, including simultaneous titration of PBPs (mainly PBP2 and 2A) in the presence or absence of Q/D or other protein synthesis inhibitors at subinhibitory concentrations, would be required. While this finding may explain the positive interaction between inhibitors of protein synthesis and β -lactams against MRSA, it underlines the potential of combining existing drugs in these classes.

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Fig. 1. Separation of staphylococcal cell wall muropeptides by reverse-phase high-performance liquid chromatography. Muropeptides were obtained after peptidoglycan purification and hydrolysis by mutanolysin from methicillin-susceptible *Staphylococcus aureus* AW26 and methicillin-resistant *S. aureus* P8 grown (A,B) without antibiotic or (C–H) in the presence of 0.25× MIC of quinupristin/dalfopristin (C,D), tetracycline (E,F) or ciprofloxacin (G,H). Chemical structures of the known muropeptides 11 and 12 (as marked on the chromatogram) are presented in Fig. 4 [13,18]. MIC, minimum inhibitory concentration.

Fig. 2. Separation of cell wall muropeptides of methicillin-resistant *Staphylococcus aureus* COL. Cells were grown (A) without antibiotic or (B–D) in the presence of 0.25× MIC of quinupristin/dalfopristin (B), tetracycline (C) or ciprofloxacin (D). The chemical structure of the known muropeptide monomer 1 (as marked on the chromatogram) is presented in Fig. 4 [18]. MIC, minimum inhibitory concentration.

Fig. 3. Separation of cell wall muropeptides of methicillin-susceptible *Staphylococcus aureus* AW26 grown in the absence or presence of 0.25× MIC of non-β-lactam cell wall inhibitors. Bacteria were grown (A) without antibiotic or (B–F) in the presence of bacitracin (B), β-chloro-D-alanine (C), D-cycloserine (D), vancomycin (E) or fosfomycin (F). Peptidoglycan was purified and analysed by high-performance liquid chromatography as described. MIC, minimum inhibitory concentration.

Fig. 4. Cartoon depicting the mucopeptide structures present in peaks 1, 11 and 12 of the *Staphylococcus aureus* peptidoglycan (adapted from [13,18]). GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid, Ala, L- or D-alanine; Glx, D-isoglutamine or D-isoglutamate; Lys, L-lysine; and G, glycine.

Fig. 5. Separation of mucopeptides from methicillin-resistant *Staphylococcus aureus* P8 grown in subinhibitory concentrations of various antibiotic combinations. Cells were grown (A) without antibiotic or (B–D) in the presence of 0.25× MIC of quinupristin/dalfopristin (B), 0.125× MIC of cefepime (C) or 0.25× MIC of quinupristin/dalfopristin and 0.125× MIC of cefepime in association (D). MIC, minimum inhibitory concentration.

Fig. 6. Detergent-induced autolysis of *Staphylococcus aureus* grown in the absence or presence of various antibiotics. (A) Methicillin-susceptible *Staphylococcus aureus* AW26 and (B) methicillin-resistant *S. aureus* P8 were grown in the absence (×) or presence of 0.25× MIC of inhibitors of protein synthesis such as quinupristin/dalfopristin (■), erythromycin (□), chloramphenicol (▲), tetracycline (△), linezolid (●) and fusidic acid (○), or of polynucleotide synthesis or assembly such as ciprofloxacin (◆) and trimethoprim/sulfamethoxazole (◇). Bacteria were recovered in early log-phase of growth ($OD_{620} = 0.2$), were treated with Triton X-100 and the loss of OD_{620} was followed. MIC, minimum inhibitory concentration; OD_{620} , optical density at 620 nm.

Table 1

Staphylococcus aureus isolates and minimum inhibitory concentrations (MICs) of various antibiotics

Antibiotic	MIC (mg/L)					
	MSSA			MRSA		
	AW26	Newman	P7142	P8	AW7	COL
β -Lactams						
Cefepime	4	2	2	32	64	1024
Protein inhibitors						
Quinupristin/dalfopristin	0.5	0.5	0.25	0.125	0.5	0.25
Erythromycin	0.5	0.25	1024	1024	512	0.5
Chloramphenicol	8	8	64	16	32	8
Tetracycline	0.25	0.5	256	32	32	128
Linezolid	2	2	4	2	2	2
Fusidic acid	0.12	0.12	0.25	0.25	0.25	0.125
Polynucleotide inhibitors						
Ciprofloxacin	0.06	0.25	0.25	0.25	0.25	0.125
Trimethoprim/sulfamethoxazole	0.25	2	0.25	1	2	1
Reference	This study	[14]	[15]	[16]	[5]	[17]

MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*.

Table 2

Proportion of specific mucopeptide HPLC peaks of the three methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates and three methicillin-resistant *S. aureus* (MRSA) isolates exposed or not to 0.25× MIC of various antibiotics

	Peak 12/Peak 11 ^a				Final hump ^b				Peak 1 ^c			
	Mean d	S.D.	Ratio e	P- value f	Mean d	S.D.	Ratio e	P- value f	Mean d	S.D.	Ratio e	P- value f
No antibiotic	0.38	0.06			18%	13%			0.50%	0.60%		
Quinupristin/dalfopristin	0.62	0.19	1.7	<0.01	39%	18%	2.1	<0.05	0.14%	0.10%	0.3	0.07
Erythromycin	0.45	0.15	1.2	<0.05	31%	8%	1.7	<0.01	0.36%	0.30%	0.7	0.17
Chloramphenicol	0.52	0.18	1.4	<0.05	35%	6%	1.9	<0.05	0.22%	0.21%	0.4	0.08
Tetracycline	0.55	0.24	1.5	<0.05	35%	10%	2.0	<0.05	0.19%	0.18%	0.4	0.08
Linezolid	0.61	0.16	1.6	<0.01	38%	5%	2.1	<0.05	0.14%	0.11%	0.3	0.09
Fusidic acid	0.60	0.20	1.6	<0.05	41%	11%	2.3	<0.01	0.18%	0.09%	0.4	0.12
Ciprofloxacin	0.33	0.07	0.9	<0.05	21%	7%	1.1	0.32	0.64%	0.95%	1.3	0.20
Trimethoprim/sulfamethoxazole	0.39	0.06	1.0	0.13	16%	11%	0.9	0.11	0.47%	0.59%	0.9	0.15

HPLC, high-performance liquid chromatography; MIC, minimum inhibitory concentration; S.D., standard deviation.

^a Ratio of peak 12/peak 11 area.

^b Final hump of chromatograph containing poorly resolved materials presented as area percentage of the sum of all peaks of the chromatograph.

^c Peak 1 presented as area percentage of the sum of all peaks of the chromatograph.

^d Mean of the experiments on three MRSA strains (P8, AW7 and COL) and three MSSA strains (AW26, Newman and P7142).

^e Ratio of the results with subinhibitory concentrations of antibiotics relative to the results without antibiotic.

^f Means in experiments with subinhibitory antibiotic concentrations and in experiments without antibiotic were compared using Student *t*-tests for paired samples.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6