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Daptomycin versus Friulimicin B: In-Depth Profiling of *Bacillus subtilis* Cell Envelope Stress Responses[▽]†

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The related lipo(depsi)peptide antibiotics daptomycin and friulimicin B show great potential in the treatment of multiply resistant gram-positive pathogens. Applying genome-wide in-depth expression profiling, we compared the respective stress responses of *Bacillus subtilis*. Both antibiotics target envelope integrity, based on the strong induction of extracytoplasmic function σ factor-dependent gene expression. The cell envelope stress-sensing two-component system LiaRS is exclusively and strongly induced by daptomycin, indicative of different mechanisms of action in the two compounds.

Staphylococcus aureus is a leading cause of nosocomial infections, especially in mechanically ventilated patients. Its remarkable potential to acquire and accumulate high-level resistance against most of the classical antibiotics (including vancomycin) used for the treatment of gram-positive infections is one of the reasons for the ongoing mortality caused by hospital-acquired *S. aureus* infections (7, 17).

Daptomycin is the first of a new class of cyclic lipodepsipeptide antibiotics (Fig. 1A) with strong bactericidal activities against gram-positive pathogens (2). It interferes with cell envelope integrity, and cell death occurs presumably by either membrane depolarization or membrane perforation (19, 20). Friulimicin B, an acidic, cyclic lipopeptide produced by *Actinoplanes friuliensis*, shows structural similarities to daptomycin (Fig. 1B) and is also active against multidrug-resistant gram-positive bacteria (1, 22).

As part of a coordinated effort to study and characterize its mode of action, we have performed comparative in-depth expression profiling for both antibiotics. This technique is a powerful approach to elucidate the inhibitory mechanisms of novel antimicrobial compounds (4, 9) and has been successfully applied to characterize and differentiate antimicrobial actions, often using *Bacillus subtilis* as a model organism (3, 10). *B. subtilis* is particularly well suited for studying cell wall antibiotics, since the regulatory network orchestrating its cell envelope stress response (CESR) is well characterized. It consists of four two-component systems and at least four extracytoplasmic function (ECF) σ factors (11).

Here, we present results from an in-depth analysis of the expression signature provoked by the treatment of *B. subtilis* with sublethal amounts of daptomycin and friulimicin B. Our data show that both antibiotics specifically target cell envelope integrity. But significant differences in the corresponding

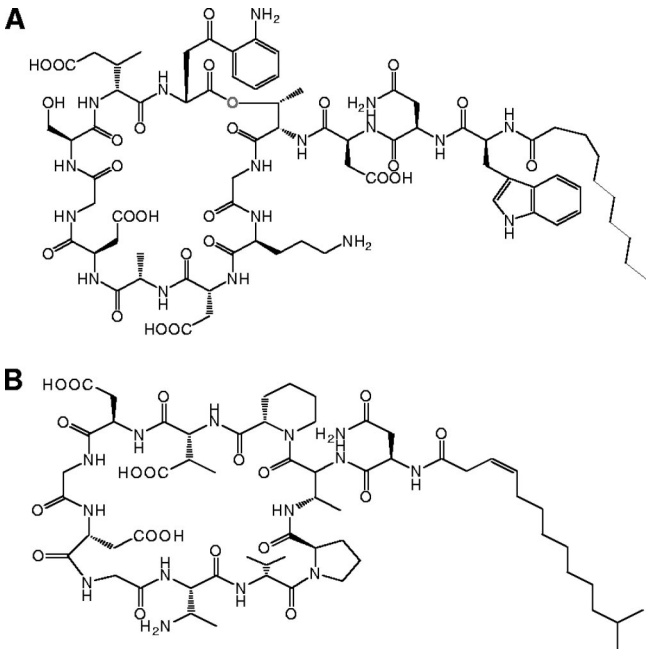

FIG. 1. Chemical structures of the lipodepsipeptide antibiotic daptomycin (A) and the lipopeptide antibiotic friulimicin B (B).

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TABLE 1. Marker genes induced by daptomycin and/or friulimicin B

Gene(s)[a]	Induction by:[b]		Regulator(s)[c]	Localization (putative)[d]	Homology, function, remarks[e]
	DAP	FRI			
ywaC	4.5 ± 4.3	8.7 ± 3.8	σ^V, σ^M, σ^W	C	Putative GTP-pyrophosphokinase
mreBH	3.9 ± 1.9	3.1 ± 1.2		C	Control of cell shape; membrane-associated
ydaH	3.3 ± 0.3	9.1 ± 2.4	σ^M	M	Conserved membrane protein
yqjL	3.3 ± 0.3	8.9 ± 1.6	σ^V, σ^M	C	Putative hydrolase
bcrC	3.3 ± 1.0	8.2 ± 2.8	σ^V, σ^M, σ^W, σ^X	M	Undecaprenyl pyrophosphate phosphatase
yrhH	3.1 ± 1.3	8.5 ± 3.4	σ^V, σ^M, σ^W	C	Putative methyltransferase
liaIH(GFSR)	429 ± 53	−	LiaRS	M, S	Conserved membrane protein; phage-shock protein A homolog (three-component regulatory system)
gerAAABAC	15 ± 2.9	−	(LiaRS)	M, S	Downstream *lia* operon, known polar effect from P_{liaI}
ybeF	4.6 ± 0.9	−		M	Conserved membrane protein
sigM-yhdLK	−	7.4 ± 4.0	σ^M	C, M	ECF σ factor
yjbC-spx	−	7.2 ± 1.7	σ^V, σ^M, σ^W	C	Glutaredoxin family; transcriptional regulator Spx
sms-yacKL	−	7.1 ± 0.5	σ^M	C, C, M	DNA repair/binding proteins; membrane protein
radC	−	6.9 ± 2.1	σ^M	C	DNA repair protein
ypuA	−	6.5 ± 2.3	σ^M	S	Conserved hypothetical
ypbG	−	6.4 ± 1.0	σ^M	S	Putative phosphoesterase
ypuD	−	6.2 ± 0.7	σ^M	S	Unknown
ycgRQ	−	5.9 ± 0.6	σ^V, σ^M	M	Conserved membrane protein; permease
yrhIJ	−	5.7 ± 0.8	σ^M	C,	Cytochrome P450; transcriptional repressor BscR
sigV-yrhM	−	5.1 ± 2.0	σ^V	C, M	ECF σ factor
yfnI	−	4.7 ± 2.0	σ^M	M (S)[f]	Similar to phosphoglycerol transferases
yebC	−	4.1 ± 0.6	σ^M	M	Unknown
yppC	−	4.1 ± 0.4		C	Conserved hypothetical
ywnJ	−	4.1 ± 0.1	σ^M, σ^X	M	Unknown
ywtF	−	3.9 ± 0.6	σ^M	C (S)[f]	Putative transcriptional regulator
pbpI	−	3.8 ± 1.3		M	Class B penicillin-binding protein
rodA	−	3.8 ± 0.9	σ^M	M	Control of cell shape and elongation
ylxW	−	3.5 ± 0.3	σ^M	M	Unknown
yoxD	−	3.7 ± 0.2		C	Putative 3-oxoacyl-acyl-carrier protein
yqiG	−	3.4 ± 0.4		C	Putative NADH-dependent flavin oxidoreductase
yjbQ	−	3.4 ± 0.2		M	Putative Na^+/H^+ antiporter

[a] Only genes that were induced ≥threefold in three independent experiments by daptomycin and/or friulimicin B are shown. The proteins corresponding to the underlined genes were also significantly upregulated in the cytoplasmic proteome (Fig. 2).

[b] Average induction ratio of the highest value for each locus (usually the first gene in an operon) and the corresponding standard deviation are given. DAP, daptomycin; FRI, friulimicin B; −, no significant induction.

[c] Assignment of regulators is based on the corresponding regulon papers: LiaRS (12), σ^M (8), σ^V (24), σ^W (6), and σ^X (5).

[d] Localization of the corresponding proteins is based on the presence of transmembrane regions (membrane proteins) and signal peptides (secreted proteins) detected with SMART. C, cytoplasmic proteins; M, membrane proteins; S, secreted proteins.

[e] Putative function is derived from BSORF/Subtilist entries (at http://bacillus.genome.ad.jp/ and http://genolist.pasteur.fr/SubtiList/genome.cgi, respectively), NCBI blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), or SMART (http://smart.embl-heidelberg.de/) analysis.

[f] YfnI and YwtF are assigned to secreted proteins based on experimental evidence (21).

CESRs, as clearly documented by transcriptomics, proteomics, and detailed gene expression profiling, strongly suggest different modes of action of the two structurally related antibiotics.

(This study was presented in part at the 47th International Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 17 to 20 September 2007 [25]).

Transcriptomics and proteomics. For microarray experiments, midlogarithmic cultures of *B. subtilis* were challenged with 1 μg/ml (sublethal amounts) of either daptomycin or friulimicin B. The cells were harvested 10 min postinduction, and cell pellets were directly snap-frozen in liquid nitrogen. RNA preparation and microarray experiments were performed essentially as described previously (13, 23). To validate the gene expression profiles, we also performed two-dimensional gel electrophoresis of the cytoplasmic proteome of *B. subtilis* cells, quantifying de novo protein synthesis after the addition of daptomycin or friulimicin B by incubating the cultures in the presence of L-[^{35}S]methionine, as described previously (3). The results are summarized in Table 1 and Fig. 2. The complete microarray data sets can be found in the supplemental material and, together with additional supporting information, at http://microbial-stress.iab.kit.edu/87.php.

Both antibiotics induced a limited number of genes, most of which could be assigned to known CESR regulons. Daptomycin specifically and strongly activated the LiaRS two-component system, with more than 200-fold induction of its primary target genes, *liaIH*. This induction has also been observed recently in an independent study (9a) and is in good agreement with data from the orthologous VraSR system of *S. aureus* which was also induced by daptomycin (16). Moreover, a strong LiaH induction was also observed with proteomics analysis, where it was identified in three strong neighboring spots (differing in their isoelectric points), indicative of posttranslational modifications (Fig. 2).

Both compounds induced numerous genes known to be regulated by ECF σ factors. This ECF-dependent response was much stronger for friulimicin B (Table 1). In addition, only seven genes/proteins of unknown regulation were differentially expressed (Table 1 and Fig. 2), including the actin homolog *mreBH*, which was induced about three- to fourfold by both

TABLE 2. Strains and oligonucleotides used in this study

Strain or oligonucleotide	Relevant genotype or fragment amplified	Source, construction, reference, or sequence[a]
B. subtilis strains		
W168	Wild-type strain; *trpC2*	Lab stock
BFS2470	W168 *liaI*::pMUTIN	Zoltan Pragai (Harwood lab); 14, 15
TMB389	W168 *liaIH*::Tet^r	W168, transformed with chromosomal DNA of strain HB0935; 14
Oligonucleotides		
822/823	*sigY*-RT	Fwd, ACAAGAAGAACAGCGGCTGA; rev, TTCCTGAACAAGTTCCTCGC
824/825	*sigX*-RT	Fwd, ACAGAAGACCTTCTTCAAGAG; rev, CGCTGTCTGATTGTTTGCTG
826/827	*sigM*-RT	Fwd, GTTTACAGGTTCCTGCTCTC; rev, ATGAAGGCGTTTCGCGCCA
828/829	*sigW*-RT	Fwd, TCAGCTTTGCTACCGTATGC; rev, TTGCGAATGCGGTCAATGGT
830/831	*sigV*-RT	Fwd, AAGCGTTGCTTGTCACATGC; rev, GTTCCTGACCGTTTCAACTG
832/833	*sigZ*-RT	Fwd, GCATCTCCCAAATCTGATCG; rev, TTTTCTTCCTCTGCACTGTCA
834/835	*ylaC*-RT	Fwd, TTGAGGACTTGTATCGGCAG; rev, GAGCCATGTTCTGATGGAAG
125/126	*liaH*-RT	Fwd, TGAAACAGCACACGATTGCC; rev, GTTTGCCTGTTCATAGGAAGC
156/157	*rpsI*-RT	Fwd, GAAACGGCAAAACGTTCTGG; rev, GTGTTGGGTTCACAATGTCG
158/159	*rpsE*-RT	Fwd, GCGTCGTATTGACCCAAGC; rev, TACCAGTACCGAATCCTACG

[a] Fwd, forward; rev, reverse.

FIG. 2. Synthesis patterns of marker proteins after induction with daptomycin (DAP) or friulimicin B (FRI) compared to patterns of untreated control cells. Details from the two-dimensional gels of the cytoplasmic proteome ("spot albums" of marker proteins) are shown for two time points postinduction with each of two compounds. (A) Proteins induced by both antibiotics. (B) Daptomycin-specific spots. (C) Friulimicin B-specific spots. See text for details.

TABLE 3. Induction of ECF σ factors and *liaH* by daptomycin and friulimicin B

Gene	Induction by:[a]	
	DAP	FRI
sigM	2.4 ± 0.1	8.7 ± 3.8
sigV	2.4 ± 0.7	7.4 ± 2.3
sigW	1.4 ± 0.3	0.9 ± 0.0
sigX	0.8 ± 0.2	0.8 ± 0.0
sigY	0.9 ± 0.1	1.8 ± 0.3
sigZ	1.0 ± 0.0	1.2 ± 0.1
ylaC	1.0 ± 0.0	2.9 ± 0.3
liaH	1170 ± 426	0.9 ± 0.0

[a] Levels of change given are the average ± standard deviation of the results of two independent real-time RT-PCR experiments, performed essentially as previously described (23), using an iScript one-step RT-PCR kit with Sybr green (Bio-Rad) according to the manufacturer's recommended procedure. DAP, daptomycin; FRI, friulimicin B.

compounds. Five more genes without known regulator, some of which are potentially involved in cell envelope biogenesis, specifically responded to friulimicin B (Table 1). All genes identified in our analysis have been linked to CESR of *B. subtilis* previously (data not shown). While no expression signature available so far resembles that of friulimicin B, both the transcriptome and the proteome profile for daptomycin closely resemble those of bacitracin (3, 14).

In-depth gene expression profiling. The results of our microarray study led to three follow-up analyses on the specificity of the corresponding CESR. (i) We analyzed the induction of all seven ECF σ factors by quantitative real-time reverse transcriptase PCR (RT-PCR), based on the known and highly ECF-specific autoregulation of their own genes, to determine the respective inducer spectrum and strength. The primers used for amplification are listed in Table 2. Both antibiotics activate σ^M and σ^V, with friulimicin B provoking a significantly stronger response. In addition, friulimicin B also induced the uncharacterized ECF σ factor σ^{YlaC} (Table 3).

(ii) The much stronger activation of ECF target genes by friulimicin B was not due to the corresponding lack of *liaIH* induction, as demonstrated by the induction values of ECF genes in the *liaIH* mutant strain TMB0389, which were identical to those in the wild type (data not shown). The stronger ECF response to friulimicin B is therefore LiaIH independent and a true antibiotic-specific difference in the corresponding gene induction profiles.

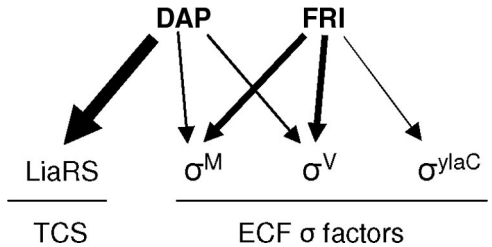

FIG. 3. Schematic representation of the regulatory networks orchestrating the daptomycin (DAP) and friulimicin B (FRI) stress responses. The thickness of the arrows corresponds to the strength of induction of the given regulators (see text and Table 1 for details). TCS, two-component system.

(iii) We also quantified the activity of the LiaR target promoter P_{liaI} as a function of the daptomycin/friulimicin B concentrations over a range of 4 orders of magnitude by performing a β-galactosidase assay (using strain BFS2470 as described previously) (15). P_{liaI} induction was indeed only observed in the presence of daptomycin and in a very narrow window of antibiotic concentrations (between 0.5 and 2 μg/ml) (data not shown). These results strongly suggest different modes of action for daptomycin and friulimicin B.

Conclusions. Our data clearly allowed the identification of cell envelope integrity as the site of daptomycin and friulimicin B action, but the results strongly suggest mechanistic differences between the two compounds. This assumption is primarily based on the dramatic differences in the LiaRS response. Moreover, friulimicin B activates both σ^M and σ^V more strongly than daptomycin and, additionally, induces σ^{YlaC} expression (summarized in Fig. 3). The strong similarities of CESR between daptomycin and bacitracin were initially viewed as an indication that daptomycin might interfere with the lipid II cycle of cell wall biosynthesis. But a detailed biochemical mechanism of action study revealed that friulimicin B, like amphomycin but in contrast to the membrane-interfering daptomycin, inhibits cell wall biosynthesis by binding bactoprenol phosphate (18).

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