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# Distinct Mode of Action of the Lipopeptide Antibiotic Friulimicin B and the Lipodepsipeptide Daptomycin: A Proteomic Study

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## Abstract

**Background:** Friulimicin B (FRI), a cyclic lipopeptide, is intended for the treatment of severe infections caused by Gram-positive pathogens. FRI shows structural similarities with daptomycin (DAP), both are acidic, amphipathic and require physiological calcium concentrations. We compared the mode of action of both compounds using a proteomic approach.

**Methods:** *B. subtilis* 168 wildtype was grown in Belitsky minimal medium and FRI / DAP was added (~1.3 and 3 x MIC for both compounds). 10 and 30 minutes after treatment the cells as well as untreated control cells were labelled with [$^{35}$S]-methionine for 5 minutes. Isolated protein extracts were separated in 2D gel electrophoresis. Proteins were visualized by autoradiography. Induction of the LiaRS two-component system for both antibiotics was investigated using a $P_{liaI}$-*lacZ* reporter system.

**Results:** Both antimicrobial compounds induced a set of proteins (including DltA, YceC, Ywfl, PtsH) that are also synthesized after addition of other cell envelope-damaging substances. The most prominent protein synthesized after DAP treatment was LiaH, known to be induced by compounds like bacitracin and vancomycin and other compounds involved in the lipid II cycle of cell wall synthesis, but not by other cell wall antibiotics, such as fosfomycin, D-cycloserine, or ß-lactams. FRI did not induce LiaH but other proteins (YceH, YoxD) described as marker proteins for cell envelope stress. In quantitative assays using $P_{liaI}$-*lacZ* reporter system, a strong LiaH induction was demonstrated for DAP at subinhibitory and MIC concentrations, but not for FRI in the concentration range from 0.01 - 50 µg/mL.

**Conclusions:** Although the proteomic induction patterns suggest some common response elements to disturbances of the cell envelope architecture, FRI and DAP have a different molecular mode of action.

## Introduction

Friulimicin B (FRI, Fig. 1) is a novel lipopeptide antibiotic that is produced by *Actinoplanes friuliensis*. FRI is structurally similar with the lipodepsipeptide daptomycin (DAP) and displays a similar antibacterial activity spectrum like DAP addressing all important Gram-positive pathogens such as staphylococci, enterococci, pneumococci (including multi-resistant and DAP-resistant strains)[1-3] and obligatory anaerobic bacteria[4].

The purpose of the studies presented here, was to compare the mode of action (MOA) of FRI and DAP using both, a proteomic approach and the antibiotic sensing *B. subtilis* LiaRS two-component system.

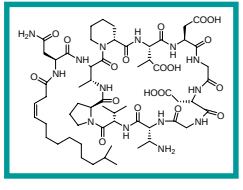

**Fig. 1** Friulimicin B

## Methods

**Proteomic study:**
*B. subtilis* 168 wildtype strain was grown aerobically at 37°C in Belitsky minimal medium[5] to mid-log phase. 10 and 30 minutes after addition of DAP or FRI (1.3 x and 3 x MIC, respectively) the cells as well as untreated control cells were labelled with 15 µCi/ml [$^{35}$S]-methionine. After 5 minutes incorporation of radioactive methionine was stopped by adding an excess of nonradioactive methionine and chloramphenicol to stop translation. Samples were taken and the harvested cells were sonicated to disrupt them. The isolated protein extracts were separated in 2D gel electrophoresis and radioactively labelled proteins were visualized by autoradiography. DECODON Delta2D software (DECODON GmbH, Greifswald, Germany) was used for warping and Dual Channel Imaging of autoradiographs.

**LiaRS two component system:**
Induction of the the *liaI* promoter, primary target of the *Bacillus subtilis* LiaRS two-component system, in the presence of FRI or DAP was quantified using $P_{liaI}$-*lacZ* reporter systems[6,7]. In semi-quantitative disk diffusion assays 100 µl mid-log cultures of two *B. subtilis* reporter strains HB0961 (*liaI::pMUTIN*) and TMB016 (*amyE*::$P_{liaI}$-*lacZ*) have been added to 3 ml of 0.7% soft LB agar and poured onto LB agar plates containing 40 µg XGal/plate and 50 mg $Ca^{2+}$/l. Filter paper disks carried 5 µl of FRI and DAP stock solution (10 mg/ml each) Quantitative activation was measured by β-galactosidase assays. *B. subtilis* HB0961 was grown to an $OD_{600}$ of 0.5 in 130 ml LB broth containing 50 mg $Ca^{2+}$/l and 1 mg/L of erythromycin. FRI and DAP in a concentration range of 0.01 and 50 mg/L were added to 6 ml aliquots of the culture to induce the system. For β-galactosidase assays 1 ml of each culture was harvested and analysed 30 min after induction[7].

## Results and Discussion

**Proteome analysis of *B. subtilis* in response to FRI and DAP**
Addition of FRI and DAP to growing *B. subtilis* cells lead to a change in protein synthesis (Fig. 2). Induced proteins can be classified into three categories (Table 1). Category A compiled a set of four proteins (DltA, PtsH, YceC and Ywfl) which are induced by both, FRI and DAP. These proteins are known to be induced by other cell wall damaging antibiotics, like bacitracin and vancomycin, indicating that both compounds, FRI and DAP might target the cell envelope. LiaH (category B) is the only protein exclusively induced by DAP. The induction of this marker enzyme known to be induced by lipid II cycle inhibitors was not expected, as DAP seems to interfere with the membrane[8]. Proteins NfrA, TrxA, YceH and YoxD represent category C proteins which are exclusively induced by FRI. Some of these proteins are marker enzymes for cell envelope stress coinduced by cell wall antibiotics, such as vancomycin and bacitracin.

**Analysis of LiaRS Two Component System in response to FRI and DAP**
The *B. subtilis* LiaRS system is known to be a antibiotic-sensing system coordinating the response to several cell wall active compounds, like bacitracin, vancomycin, nisin and ramoplanin activating the $P_{liaI}$ promoter[7]. Using a semi-quantitative disk diffusion assay (Fig. 3) and a quantitative β-galactosidase assay (Fig. 4), we could show that only DAP and not FRI is able to induce the $P_{liaI}$-*lacZ* reporter system. These results are in agreement with the proteomic response and underline that both related antibiotics have a distinct mode of action.

## Results and Discussion

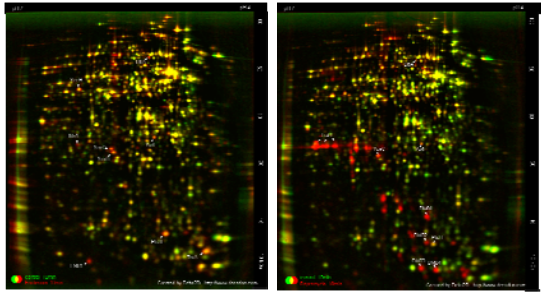

**Fig. 2** Dual channel images of radioactively labelled proteins of *B. subtilis* 168 synthesised before (green image) and 10 min after exposure (red image) to FRI (left) and DAP (right)

| A. Proteins induced by Friulimicin B and Daptomycin | | |
|---|---|---|
| **Protein** | **Function / Functional category** | **Known Inducer** |
| DltA | D-alanyl-D-alanine carrier protein ligase involved D-alanylation of teichoic acids[9] | |
| PtsH | histidine-containing phosphocarrier protein of the PTS (HPr protein)[10] | Bacitracin[10] |
| YceC | unknown; similar to tellurium resistance protein | Bacitracin, Vancomycin[11] |
| Ywfl | unknown; similar to unknown proteins | |
| **B. Proteins exclusively induced by Daptomycin** | | |
| **Protein** | **Function / Functional category** | **Known Inducer** |
| LiaH | Involved in maintenance of cell membrane integrity, induced by antibiotics that interfere with lipid II cycle[7] | Bacitracin[11] |
| **C. Proteins exclusively induced by Friulimicin B** | | |
| **Protein** | **Function / Functional category** | **Known Inducer** |
| NfrA | FMN-containing NADPH-linked nitro/flavin reductase | |
| TrxA | Thioredoxin involved in membrane bioenergetics | |
| YceH | unknown; similar to toxic anion resistance protein | Bacitracin, Vancomycin[11] |
| YoxD | unknown; similar to 3-oxoacyl-acyl-carrier protein reductase | |

**Table 1** Marker proteins induced by FRI and/or DAP

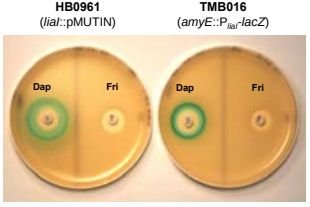


**Fig. 3**: Disk diffusion assay using $P_{liaI}$-*lacZ* reporter strains *B. subtilis* HB0961 and TMB016 after addition of DAP and FRI

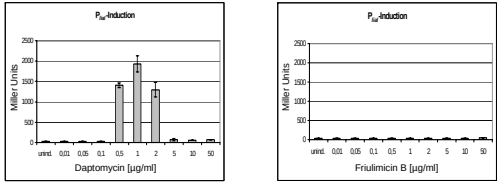


**Fig. 4** β-galactosidase assay[7] using $P_{liaI}$-*lacZ* reporter strains *B. subtilis* HB0961 after addition of DAP and FRI

## Conclusions

- Proteome induction patterns and the response of the $P_{liaI}$-lacZ reporter system suggest that both antibiotics, FRI and DAP, target the cell envelope but display a distinct molecular mode of action.
- These results are consistent with the described MOA of DAP (cell membrane interaction)[8] and the MOA of FRI (complex formation with bactoprenol-phosphate leading to the interruption of peptidoglycan and teichoic acid biosynthesis)[12].
- Based on the distinct molecular MOA for FRI and DAP one would not expect appearance of target based cross-resistance.

## Literature

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