Identification and purification of bacterial lipids from *Enterococcus faecalis* 12030

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Background

Bacterial compounds from the outer cell membrane of bacteria can induce cytokine production and may play an important role in inflammatory immune responses. For gram-negative bacteria, lipid A is known as cytokine inducing factor. However, the equivalent components of gram-positive bacteria have not been well investigated. We therefore studied membrane lipids extracted from *Enterococcus faecalis* and from two isogenic targeted deletion mutants, and investigated these extracts regarding cytokine induction in cell cultures, and through different stainings (α-naphthol, Mo-staining and ninhydrin).

In mutant EF 2890 the gene *bgsB* is deleted, and therefore the synthesis of the two glycolipids monoglycosyl-diacylglycerol (MGlcDAG) and diglycosyl-diacylglycerol (DGlcDAG) is blocked (see Fig. 1). In the deletion mutant EF 2891 the gene *bgsA* is deleted, and therefore this mutant can not produce the glycolipid DGlcDAG.

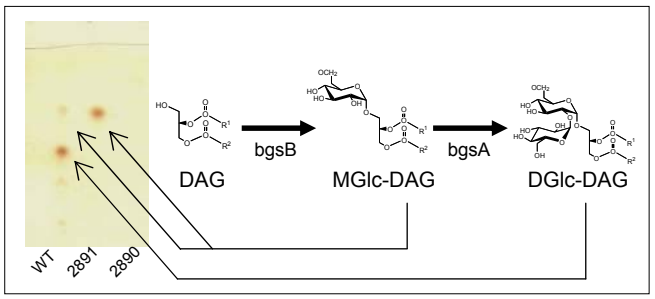

Fig. 1: Pathway of glycolipids in *E. faecalis*

Methods

Bacteria growth conditions:

We used an *Enterococcus faecalis* 12030 strain and two deletion mutants for identification and purification of bacterial lipids. 20 ml Caso Bouillon (ROTH) was inoculated with 1 colony from a plate and was grown at 37 °C for 8h. After that, 4 L Caso Bouillon (ROTH) was inoculated with the pre-incubated 20 ml culture and grown over night at 37°C (16h).

Purification:

The lipids were extracted by two different extraction procedures (Bligh-Dyer and butanol extraction). Preliminary data showed higher extraction yields by use of Bligh-Dyer and also distinct differences in the composition of the extracted lipid compounds.

Thin Layer Chromatography:

For TLC, different staining methods were tested to identify the functional lipid groups. In addition, the samples were separated one-dimensional and two-dimensional.

Preparative Layer Chromatography:

For the investigation of membrane lipids in cell culture, the lipids were separated on PLC plates (MERCK; 0,5mm). For the first experiments the whole separation distance was splitted in four large fractions. The fractions were removed from the plates and washed three times with chloroform-methanol mixtures (1:2, 1:1 and 2:1). The supernatants received by centrifugation were combined and filtered through a PTFE membrane filter (ROTH; 0,2 µm). After evaporation of the solvents the amount of lipids can be determined.

Cell Culture and ELISA:

For determination of cytokine formation, RAW264.7 mouse macrophages were seeded at a density of $1x10^5$ cells/well in 48-well dishes in DMEM with 10% FBS. The cells were stimulated with glycolipids which were previously extracted by the Bligh-Dyer method. We also studied the effect of the purified fractions of wild type and deletion mutants. Stimulated cells were incubated for 16 h at 37°C in a 5% humidified CO_2 environment. The production of cytokines was measured by ELISA using commercially available kits (TNF-α, IL-10 eBioscience).

Results

Thin Layer Chromatography:

Differences could not only be seen in glycolipids (Fig. 4) but also in lipoproteins and phospholipids (see Fig. 2, 3).

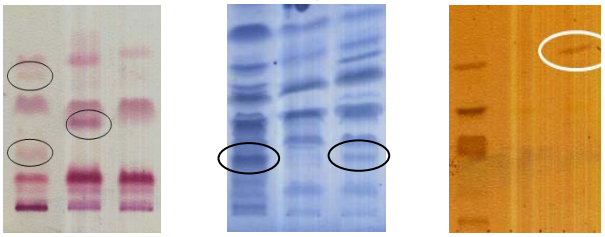

Fig. 2, 3 and 4: lane 1 Wildtype, lane 2 EF2890, lane 3 EF 2891
Fig. 2: stained with ninhydrine
Fig. 3: stained with Mo-Staining
Fig. 4: stained with α-naphthol

ELISA:

The analysis of glycolipids regarding cytokine induction in cell culture supernatants were showed an increased TNFα production by the non-purified samples of wild-type and mutant EF 2891. In the 4 fractions of the wild type this could not be shown. In the mutant one group was detected, which has a similar effect as the non-purified samples (Fig. 5). This is explored in more detail in further experiments.

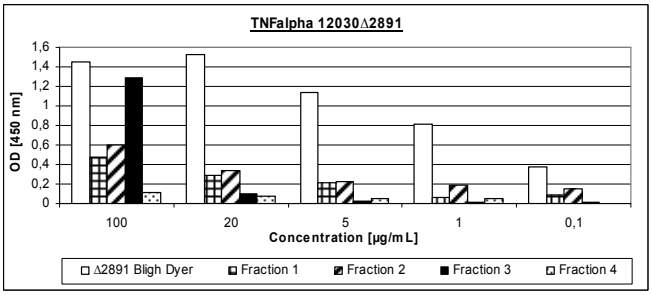

Fig. 5: TNFα-ELISA of the deletion mutant EF 2891

Conclusions and Perspectives

The best stain for the detection of phospholipids was Mo-Staining, for the visualization of glycolipids it was α-naphthol, and for the detection of lipoproteins ninhydrin. Orcinol was unsuitable for staining of glycolipids since the high concentration of sulfuric acid destroys the silica layer. When comparing the two-dimensional TLC with α-naphthol staining results in the literature only a rough classification of some glycolipids is possible, as for these lipids there are no standards available. A direct comparison can be assigned to MGlcDAG and DGlcDAG with high probability (5).

Furthermore, it is promising to purify certain fractions by PLC and to examine these in cell culture. In addition, the identification and quantification of individual lipids from wild type and mutants by TLC and coupled techniques are of interest.

The investigation of the individual fractions on TNFα production in cell culture supernatants also confirmed results of an animal test series showing increased lethality after injection of the mutant. This observed inflammatory effect of the purified fraction has to be further investigated.

References

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