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"Comparative proteome analyses of the cheese isolate Pseudomonas fluorescens 84095, grown in absence and presence of sub-lethal concentration of the antimicrobial peptide Lactoferricin B".

Materials and Methods

Bacterial growth conditions: Microtiter plates were inoculated from overnight LB-grown cultures diluted 1:100 in minimal M63 medium (5 mL) supplemented with glucose (0.2%), magnesium sulfate (1 mM), and Casamino Acids (CAA) (0.5%) as previously described (Kuchma, e t al., 2005) with or without pepsin digested bovine lactoferrin (HLF; 0.75 mg/mL). The microtiter plates were then incubated at 15°C for up to 72h . After removing planktonic cells from the wells and two times washing, biofilm formation was stained with 0.1% crystal violet, followed by the solubilization of the biofilm-associated crystal violet in 30% acetic acid and the measurement of their optical densities at 570 nm (O'Toole, G. A. 2011). Planktonic cells recovered from each samples were sent to the Institute of Microbiology, University of Greifswald (Germany) in order to perform proteomic analyses.

Whole protein exctraction: Cell pellets were washed twice with 1 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), resuspended in 1 mL TE buffer and transferred in a 2 mL screw cap micro tube containing 500 µL glass beads with a diameter of 0.1 mm. Mechanical disruption of the cells was achieved using a Precellys 24 homogenizator (PeqLab, Germany; 3 × 30 sec at 6,800 rpm). Cell debris and glass beads were separated from the proteins by centrifugation for 10 min at 4 °C at 15,000 rpm followed by a second centrifugation step to remove insoluble and aggregated proteins (30 min, 4 °C, 15,000 rpm). Determination of protein concentration by Roti-Nanoquant (Carl Roth, GmbH, Germany).

Protein expression and 1D gel-LCMS/MS analysis of protein: Amounts 25 µg were separated by 1D-SDS-PAGE using Criterion TGX Precast Gels (BioRad Laboratories, Hercules, CA, USA). for 15 min and 150 V for 1 h. Lanes were cut in ten equidistant pieces and subjected to trypsin digestion and identified as previously described (Grube et al., 2015). Database searches were performed using Sorcerer SEQUEST (Version v. 27 rev. 11, Thermo Scientific) and Scaffold 4.0.5 (Proteome Software, Portland, OR, USA).

Results and Discussion

Comparative proteomic analysis of planktonic cells, grown at 70 h of growth in M63 supplemented with 0.75 mg/mL of HLF allowed to steal a glance at some modified metabolic pathways by the application of the antimicrobial peptides.. Following a Ge-LC/MS (Piersma et al., 2013) approach, from a total of 584 proteins (cytoplasmatic and cell surface proteins) identified in at least 2 out of 3 biological replicates, 486 were identified in both samples while 21 and 77 were found to be exclusively expressed in treated and un-treated samples, respectively. In contrast 29 proteins changed their amount with the majority (22) of these proteins detected in higher amount in treated samples in comparison with untreated samples.

For the first time, all enzymes involved in biosynthesis of the pigment pyomelanin (PhhA/B, PhhC, Hpd) from chorismic acid were identified in *P. fluorescens* (Figure 11). Other enzymes, similar to that described for pyomelanogenic *P. areuginosa* were also found in all samples (PyrE, PyrC, PyrD, LolA, IspH; Hunter et al., 2010). In *hmgA*-mutant *P. areuginosa* strain, the synthesis of this pigment was found to be correlated with a reduced fitness in the chronic infection, and with an increased persistence during infection putatively due to its protective function against free-radicals (Rodrı´guez-Rojas et al., 2009).

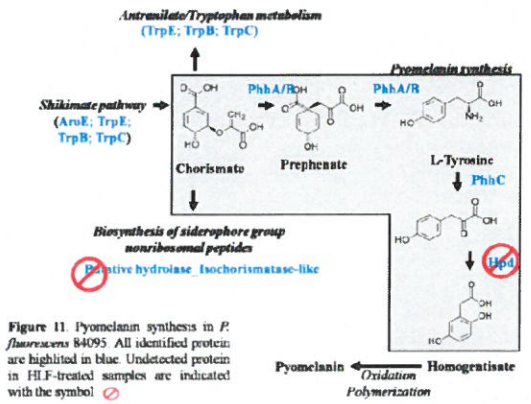

Figure 11. Pyomelanin synthesis in *P. fluorescens* 84095. All identified protein are highlited in blue. Undetected protein in HLF-treated samples are indicated with the symbol ⊘

According to the absence of grey/dark substrate discoloration in presence of BLF derived peptides, data

preliminary obtained from the analysis of *P. fluorescens* proteome showed that the HLF-treatment caused the inhibition of synthesis of 4-hydroxyphenylpyruvate dioxygenase (Hpd), involved in the production of homogentisate, rapidly oxidized and polymerized into pyomelanin once exported out from the cell (Figure 11). In addition, the metabolic pathways involved in the synthesis of tyrosine from aromatic precursor D-erytrose 4-phosphate (E4P; Gosset, 2009) was putatively inhibited as suggested by the absence of glucose-6-phosphate dehydrogenase (Zwf) and NAD^+ dependent 3-dehydroquinate synthase (AroB) in treated samples.
Besides the inhibition of pyomelanin, the BLF derived peptides putatively inhibited the synthesis of yellow-green fluorescent siderophores (such as vibriobactin, myxochelin, enterochelin etc) since the isochorismatase like hydrolase, was not found in treated samples in comparion with untreated culture (Figure 11). This data is worthy of interest since siderophore biosynthesis is becoming an increasingly appealing therapeutic target in the development of novel antibiotics against several pathogen strains (Stirrett et al., 2008; Sosnin et al., 2014).
Among siderophores responsible to acquire iron, more than 60 pyoverdins (PVDI) produced by diverse strains have been chemically characterized. All these siderophores are composed of three parts: a dihydroquinolinetype chromophore responsible for their fluorescence, a strain-specific peptide comprising 6–12 amino acids; and a side-chain (usually succinic, malic or a-ketoglutaric acid) bound to the nitrogen atom at position C-3 of the chromophore. The synthesis of pyoverdines begins in the cytoplasm and ends in the periplasm, from where they are secreted into the extracellular medium. Numerous different enzymes are involved, including non-ribosomal peptide synthetases (NRPSs) (Schalk and Guillon, 2013). It has been recently proposed that PVDI starts with the assembling of coenzyme A with a myristate fatty acid in an ATP-dependent reaction (Hannauer et al., 2012); aminoacids and 2,4-diaminobutyrate are incorporated by other enzymes in subsequent steps (Schalk and Guillon, 2013). Unfortunally, no enzymes correlated with PVDI were identified in *P. fluorescens* 84095 in the condition assayed; however, further analysis are required since the synthesis of these proteins have been strictly correlated with growth stage (Park et al., 2014). In spite of this, HLF putatively compromised pyoverdin synthesis by the inhibition of synthesis of fatty acid (FA; including myristic acid) as determined in treated samples. Moreover, the enzyme acetyl-CoA carboxylase (AccC) involved in the first step of FA biosynthesis, and BioD (ATP-dependent dethiobiotin synthetase BioD), involved in the synthesis of its cofactor (Cronan and Waldrop, 2002), were undetected in treated samples. This result need to be further investigated since in other bacteria the interruption of the gene encoding the biotin carboxylase subunit of acyl-CoA carboxylase also affected the synthesis of *quorum-sensing* signaling molecules (Hao et al., 2010).
According to these results, TonB-dependent receptor were not identified in treated samples in comparison with untreated ones. The protein TonB1 necessary for the transport and signaling cascade of Fe-PVD has been recently characterized. This receptor is member of a family of proteins responsible for the energy transduction required for the import of molecules via outer membrane proteins. Binding of TonB1 to the TonB box present in the cell surface receptor FpvA is essential for both the transport of Fe-PVD and the consequent signaling cascade controlled by this molecule (Jimenez et al., 2012).
Besides the effects on the FA-biosynthesis process, BLF derived peptides inhibited the synthesis of the enzyme involved in propanoate metabolism (2-methylisocitrate lyase, Acetyl-coenzyme A synthetase, 2-oxoisovalerate dehydrogenase beta subunit, 2-oxoisovalerate dehydrogenase alpha subunitacetyl-CoA acetyltransferase and Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex). Propanoate metabolism is correlated with the TCA cycle, similarly inibited at level of aconitate hydratase (AcnA), malate dehydrogenase (Mqo), fumarate hydratase, class II (FumC1).
The interruption both of FA synthesis and propanoate catabolism, were in turn attributed to the repression of GntR-family regulatory protein (Fad/GntR; Suvorova et al., 2012), previously reported to be involved in the production of signalling compounds, N-Acyl homoserine lactones (Hao et al., 2010).
Among 10 identified *quorum-sensing* regulated proteins, Acyl-CoA synthetase and spermidine/putrescine transport system were not expressed in treated samples.
Polyamins (putrescin and spermidin), play an important role in porines formation by reducing the permeability of outer membrane (Shah and Swatlo, 2008). In treated samples, the amount of ornithine decarboxylase (+ 2.2 fold), responsible for the synthesis of putrescine from L-ornithine was higher than that found in untreated samples; putatively due to the of the spermidine/putrescine transport system, involved in the uptake of extracellular polyamines.
It has been demonstrated that putrescine concentration is also correlated to the amount of superoxide dismutase as both molecoles exhibit a protective role against oxidative stress; in particular, in polyamine deficient mutant strains the absence of putrescine was balanced by an increase of superoxide dismutase (Jung

and Kim 2003). Therefore, in treated samples the repression of SOD synthesis could contribute to the compensative increase of putrescine amount.
The response to physiological stress, in HLF treated samples was also suggested, by an increased amount of catalase (+2.6 fold), and the finding of nicotinate-nucleotide pyrophosphorylase, dehydrogenase/oxidoreductase, NH(3)-dependent NAD(+) synthetase, NADH dehydrogenase I chain F, involved in NAD+ production, putatively to restore NADH/NAD+ ratio.
The effect of BLF derived peptides also effected the lipopolysaccharide (LPS) byosinthesis. In particular the LPS assembly protein LptC, required for the translocation of LPS from the inner membrane to the outer membrane, was not detected in treated samples.
The synthesis of several proteins (11 out of 39 identified) involved in ATP-binding cassette was inhibited in HLF-treated samples, while only the putative osmoprotectant transport system substrate-binding protein (OpuBC) was induced (+ 2.4 fold) in the same samples.
According to the microbiological data showing the occurring of flagella and pili, several proteins correlated with flagella were identified in all *P. fluorescens* 84095 cultures, grown at 15°C. Preliminary data did not show sidìgnificant differences in the amount of these proteins between HLF- and HLF –free *Pseudomonas* cells. However other experiments are required since the protein dipeptide ABC transport system, substrate-binding protein (DppA1) involved in bacterial chemotaxis (Abouhamad et al.,1991) was inhibited by the treatment with cationic peptides.

Conclusions

The comparative analysis of proteome in *P. fluorescens* under HLF treatment shows the ability of these peptides to inhibit the synthesis of pigment and other cellular pathways (e.g. fatty acids biosynthesis) involved in biofilm forming. Even though other analyses are required this study poses the basis for the application of BLF derived peptides in dairy industry to improve food quality.

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