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Comparative transcriptome analysis of the biocontrol strain *Bacillus amyloliquefaciens* FZB42 as response to biofilm formation analyzed by RNA sequencing

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ABSTRACT

The strain *Bacillus amyloliquefaciens* FZB42 is a plant growth promoting rhizobacterium (PGPR) and biocontrol agent known to keep infections of lettuce (*Lactuca sativa*) by the phytopathogen *Rhizoctonia solani* down. Several mechanisms, including the production of secondary metabolites possessing antimicrobial properties and induction of the host plant's systemic resistance (ISR), were proposed to explain the biocontrol effect of the strain. *B. amyloliquefaciens* FZB42 is able to form plaques (biofilm-like structures) on plant roots and this feature was discussed to be associated with its biocontrol properties. For this reason, formation of *B. amyloliquefaciens* biofilms was studied at the transcriptional level using high-throughput sequencing of whole transcriptome cDNA libraries from cells grown under biofilm-forming conditions *vs.* planktonic growth. Comparison of the transcriptional profiles of *B. amyloliquefaciens* FZB42 under these growth conditions revealed a common set of highly transcribed genes mostly associated with basic cellular functions. The *lci* gene, encoding an antimicrobial peptide (AMP), was among the most highly transcribed genes of cells under both growth conditions suggesting that AMP production may contribute to biocontrol. In contrast, gene clusters coding for synthesis of secondary metabolites with antimicrobial properties were only moderately transcribed and not induced in biofilm-forming cells. Differential gene expression revealed that 331 genes were significantly up-regulated and 230 genes were down-regulated in the transcriptome of *B. amyloliquefaciens* FZB42 under biofilm-forming conditions in comparison to planktonic cells. Among the most highly up-regulated genes, the *yvq*HI operon, coding for products involved in nisin (class I bacteriocin) resistance, was identified. In addition, an operon whose products play a role in fructosamine metabolism was enhanced in its transcription. Moreover, genes involved in the production of the extracellular biofilm matrix including exopolysaccharide genes (*eps*) and the *yqx*M-*tas*A-*sip*W operon encoding amyloid fiber synthesis were up-regulated in the *B. amyloliquefaciens* FZB42 biofilm. On the other hand, highly down-regulated genes in biofilms are associated with synthesis, assembly and regulation of the flagellar apparatus, the degradation of aromatic compounds and the export of copper. The obtained transcriptional profile for *B. amyloliquefaciens* biofilm cells uncovered genes involved in its development and enabled the assessment that synthesis of secondary metabolites among other factors may contribute to the biocontrol properties of the strain.

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1. Introduction

Infections of crop plants and harvested vegetables by phytopathogenic microorganisms have an economically relevant impact on food production. Nowadays, biological control, defined as the prevention of infections caused by phytopathogenic organisms by application of natural antagonistic microorganisms (Duffy et al., 2003), is considered a suitable alternative to conventional methods like crop rotation and treatments involving chemical pesticides (Mathre et al., 1999; Asaka and Shoda, 1996). Several different bacteria and fungi with biocontrol properties are known. Efficiency of biocontrol strains used in agriculture relies on different mechanisms. Many strains colonize the root system of the host plant and promote plant growth. Several biocontrol strains are able to trigger the systemic resistance, a mechanism known as

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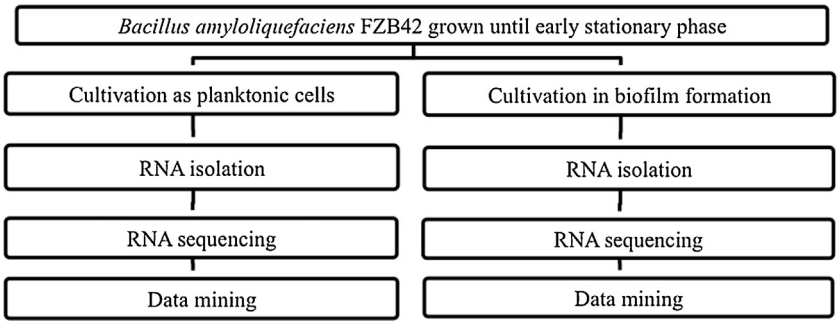

Fig. 1. Overview of the workflow to determine changes within the transcriptomes of *B. amyloliquefaciens* FZB42 grown either as planktonic cells or in biofilm formation in MgSS culture medium. All *B. amyloliquefaciens* FZB42 cultures were grown as planktonic cells until the stationary phase. Subsequently, in half of the cultures biofilm formation was induced. 24 h after biofilm induction, RNA was isolated and sequenced. Using the ReadXplorer software package, RPKM values were calculated to reveal insights into the most highly transcribed genes within both transcriptomes, whereas a DESeq analysis was performed to reveal differential gene transcription between the transcriptomes of *B. amyloliquefaciens* FZB42 in biofilm formation in comparison to planktonic cells.

rhizobacteria-induced systemic resistance (RISR) (Arguelles-Arias et al., 2009). Furthermore, the secretion of different secondary metabolites with antibacterial or antifungal properties by biocontrol strains has an impact on phytopathogenic organisms. Another factor relevant for the efficiency of biocontrol and therefore the protection of the host plant is the competition for nutrients and especially iron between biocontrol strains and phytopathogens (Arguelles-Arias et al., 2009).

The strain *Bacillus amyloliquefaciens* FZB42 is a Gram-positive member of the phylum *Firmicutes*. The Plant-Growth-Promoting-Rhizobacterium (PGPR) lives in association with plants and due to its biocontrol properties is able to stimulate their growth and protect them from infections (Koumoutsi et al., 2004; Chen et al., 2009a, 2009b) among others by suppressing different plant pathogens (Idriss et al., 2002). Pot and field experiments proved that *B. amyloliquefaciens* FZB42 colonizes the rhizosphere of lettuce during host plant cultivation and promotes significant suppression of bottom rot disease caused by the phytopathogen *Rhizoctonia solani* (Chowdhury et al., 2013). Colonizing *B. amyloliquefaciens* cells form plaques representing biofilm-like structures on the plant root surface. Accordingly, biofilms of biocontrol strains may be considered as a barrier against potential phytopathogens trying to access the plant's root system and additionally they provide the necessary proximity to the plant so that secretion of secondary metabolites featuring antimicrobial activity from biofilm-forming cells may directly counteract attacking pathogens.

The ability to form biofilms is an interesting phenomenon that was among others already studied in the closely related model organism *B. subtilis* 168 (Vlamakis et al., 2008). Biofilms are created by a heterogeneous population of motile, matrix-producing and sporulating cells. One characteristic feature of biofilms is the extracellular matrix built from exopolysaccharides (EPS) combined with macromolecules like proteins and nucleic acids (Czaczyk and Myszka, 2007; Marlow et al., 2014; Vlamakis et al., 2013). Gene products of the *epsA-O* operon involved in EPS synthesis and the *yqxM-tasA-sipW* gene cluster for production of amyloid fibers are of importance regarding the development of the extracellular biofilm matrix (Branda et al., 2005; Lei et al., 2013; Lemon et al., 2008; Vlamakis et al., 2013). While the genes involved in production of the extracellular matrix are mainly up-regulated during biofilm formation, genes involved in chemotaxis and flagella-based motility are down-regulated in contrast to planktonic cells (Lemon et al., 2008; Vlamakis et al., 2013).

Transcriptional profiling of *B. amyloliquefaciens* FZB42 in biofilm formation and grown as planktonic cells has not been addressed previously. This study therefore focuses on the analysis of changes regarding gene transcription of cells in corresponding growth states. RNA-Sequencing (RNA-Seq) featuring a high dynamic range and resolution at the nucleotide sequence level was applied to reveal the impact of the growth state of the biocontrol strain on its gene expression patterns. Additionally, RNA-Seq provides the advantage of measuring absolute and not only relative abundances of transcripts. This advantage was exploited not only to identify the most affected transcripts, but also to reveal the genes featuring the highest transcription rates in *B. amyloliquefaciens* FZB42 cells forming biofilms *vs.* planktonic cells.

2. Material and methods

2.1. *Cultivation of* B. amyloliquefaciens *FZB42 as planktonic cells and under biofilm-forming conditions*

B. amyloliquefaciens FZB42 was grown in three test tubes for each of the two replicates containing 10 ml biofilm inducing MgSS medium (5 mM K_2HPO_4 (pH 7), 100 mM MOPS (pH 7), 2 mM $MgCl_2$, 700 μM $MnCl_2$, 50 μM $FeCl_2$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 50 μg/ml tryptophan, 50 μg/ml phenylalanine) with an inoculation density of 0.01 OD_{600}. After 22.5 h, cell growth reached the early stationary phase and six of the test tubes were transferred from the shaker to a test tube rack to induce biofilm formation on the surface of the medium. Remaining test tubes were kept rotating to sustain the planktonic growth state. Cultivations were stopped 24 h after induction of biofilm formation. A thick white, wrinkled biofilm had formed then on the surface (Fig. 1).

2.2. *RNA isolation*

For the isolation of RNA from cells in biofilm formation the biofilm of three test tubes was combined in 1.5 ml RLT buffer (provided in RNeasy Mini Kit; Qiagen) per replicate. Biofilm was harvested by holding a pipette tip on the biofilm on the surface and fishing the whole biofilm out before resuspending it in RLT buffer. For the isolation of RNA from planktonic cells, 1.5 ml cell culture of three test tubes per replicate were centrifuged and the resulting pellet was resuspended in 1.5 ml RLT buffer. Resuspended cells were disrupted by ribolysation (30s; speed 6.5, repeated after 1 min on ice) in 2 ml tubes with Lysing Matrix B (MPbiomedical). Subsequently, the RNA was isolated according to the published protocol by (Rüberg et al. 2003) using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

Enrichment of the mRNA (depletion of rRNA) was performed applying the RiboZero rRNA Removal Kit for Bacteria according to the manufacture's protocol using 3 μg of RNA per sample. Library preparations for paired-end sequencing on the Illumina HiSeq 1500 platform were performed according to protocols provided by Illumina (Eikmeyer et al., 2015) (Fig. 1).

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