Mutations improving production and secretion of extracellular lipase by *Burkholderia glumae* PG1

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Abstract *Burkholderia glumae* is a Gram-negative phytopathogenic bacterium known as the causative agent of rice panicle blight. Strain *B. glumae* PG1 is used for the production of a biotechnologically relevant lipase, which is secreted into the culture supernatant via a type II secretion pathway. We have comparatively analyzed the genome sequences of *B. glumae* PG1 wild type and a lipase overproducing strain obtained by classical strain mutagenesis. Among a total number of 72 single nucleotide polymorphisms (SNPs) identified in the genome of the production strain, two were localized in front of the *lipAB* operon and were analyzed in detail. Both mutations contribute to a 100-fold overproduction of extracellular lipase in *B. glumae* PG1 by affecting transcription of the *lipAB* operon and efficiency of lipase secretion. We analyzed each of the two SNPs separately and observed a stronger influence of the promoter mutation than of the signal peptide modification but also a cumulative effect of both mutations. Furthermore, fusion of the mutated LipA signal peptide resulted in a 2-fold increase in secretion of the heterologous reporter alkaline phosphatase from *Escherichia coli*.

Keywords *Burkholderia glumae*, · Lipase, · Secretion, · Enzyme production

Introduction

*Burkholderia glumae* (formerly known as *Pseudomonas glumae*) belongs to the genus *Burkholderia* within the subphylum of the β-proteobacteria (Yabuuchi et al. 1992). *B. glumae* is a moderate rice pathogen (Ham et al. 2011), which also affects several other plants (Jeong et al. 2003). Until now, just a single case of *B. glumae* isolated from an immunodeficient patient was reported (Weinberg et al. 2007). All *B. glumae* strains studied so far infect rice panicles and produce a phytotoxin called toxoflavin (Jung et al. 2011; Kim

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et al. 2004; Suzuki et al. 2004; Vial et al. 2007), whose production is regulated by a LuxR-LuxI-type quorum sensing (QS) system (Chun et al. 2009; Chung et al. 2011; Goo et al. 2010; Kang et al. 2008; Kim et al. 2012; Kim et al. 2007). Biotechnological applications of Burkholderia species mainly comprise their use as biofertilizers or bioremediation agents (Chiarini et al. 2006; Paganin et al. 2011; Suarez-Moreno et al. 2012). Furthermore, the production of an extracellular lipase (Boekema et al. 2007; Santambrogio et al. 2013) and of rhamnolipid biosurfactants (Costa et al. 2011) were described, but detailed studies regarding further biotechnological applications are missing.

Lipases represent the third largest group within the worldwide enzyme market, which is estimated to increase with a 6.3% rate per year to reach US$6.9 billion in 2017 (Casas-Godoy et al. 2012; Freedonia 2014; Hasan et al. 2006). The production of biotechnologically relevant lipases is a well-known feature of bacteria belonging to the genera Pseudomonas and Burkholderia. These and other microbial lipases (triacylglycerol hydrolases, EC 3.1.1.3) belong to the family of \( \alpha \beta \) hydrolases and catalyze the hydrolysis of triglycerides to glycerol and fatty acids. They are the most frequently used biocatalysts in organic chemistry (Jaeger et al. 1999; Sharma and Kanwar 2014) as they are readily available at low production costs, do not require cofactors, and usually show a broad substrate specificity and high enantioselectivity as well as high stability in non-aqueous media such as ionic liquids, supercritical fluids, and organic solvents. Under non-aqueous reaction conditions, lipases can catalyze the synthesis of various esters by esterification, interesterification, and transesterification (Aravindan et al. 2007; Gandhi et al. 2000; Gupta et al. 2004; Jaeger et al. 1999; Jaeger and Eggert 2002; Jaeger et al. 1994; Jaeger and Reetz 1998; Krishna and Karanth 2002; Nagarajan 2012; Yahya et al. 1998). Additional fields of lipase application include the production of food and feed ingredients as well as intermediates for the synthesis of pharmaceuticals (Balkenhohl et al. 1997; Boekema et al. 2007). Classical mutagenesis methods were applied to construct the lipase overproducing strain \( B. \text{glumae} \) LU8093 derived from the PG1 wild type (R. Braatz, R. Kurth, E. Menkerl-Conen, H. Rettenmaier, T. Friedrich, and T. Subkowski, 1992, patent application WO93/00924 A1 and (Boekema et al. 2007)). Further mutagenesis resulted in strain \( B. \text{glumae} \) LU2023, which showed improved activity of another biotechnologically relevant enzyme, a butyneol I esterase (T. Friedrich, B. Hauer, C. Nuebling, R. Stuermer, 2001, patent application WO2002018560 A2).

The extracellular lipase LipA is encoded in an operon together with a second gene \( lipB \) (or \( lif \)) encoding a lipase-specific foldase (Frenken et al. 1993a; Frenken et al. 1993b; Frenken et al. 1992). The N-terminal signal peptide of LipA mediates its transport through the inner membrane via the Sec secretion system (Frenken et al. 1992). In the periplasm, the steric chaperone LipB interacts with the lipase (Frenken et al. 1993b; Rosenau and Jaeger 2000) facilitating downstream processing and lowering costs. The specific foldase (Frenken et al. 1993a; Frenken et al. 1993b; Frenken et al. 1992) encodes a lipase-lif (or lif) of the general secretory pathway (Filloux 2004). In this study, two mutations in the lipase overproducing strain \( B. \text{glumae} \) LU8093, one inside and one in front of the lipase operon \( lipAB \), were studied in detail to unravel their contribution to lipase overproduction. Furthermore, we demonstrated that increased secretion by the modified LipA signal peptide can be transferred to the secretion of the reporter enzyme PhoA.

Material and methods

Bacterial strains and growth conditions

\( E. \text{coli} \) strains DH5\( \alpha \) (Grant et al. 1990) and S17-1 (Simon et al. 1983) were cultivated in LB medium (Carl Roth, Karlsruhe, Germany) at 37 °C. \( B. \text{glumae} \) LU8093 (Balkenhohl et al. 1997) and R. Braatz, R. Kurth, E.
Menkerl-Conen, H. Rettemaier, T. Friedrich, and T. Subkowski, 1992, patent application WO 93/00924 A1), *B. glumae* PG1 wild type ([Frenken et al. 1992]), and its *lipAB* deficient derive *B. glumae* PG1Δ*lipAB* (Knorr 2010) were cultivated in LB medium at 30 °C. For analysis of lipase activities and transcript-level determination, *B. glumae* strains were cultivated for 14 h at 150 rpm. Standard cloning experiments were performed in *E. coli* DH5α. Plasmids were stabilized by using appropriate concentrations of chloramphenicol (50 μg/ml for *E. coli* and 200 μg/ml for *B. glumae*). Expression of the *lipAB* operon from plasmid pBBR-*lipAB* harboring its natural promoter was defined as native expression level. The wild type strain *B. glumae* PG1 is deposited as strain no. CBS 322.89 at the Centraalbureau voor Schimmelcultures, P.O.Box 85167, NL-3508 AD Utrecht, The Netherlands, and the closed genome sequence of *B. glumae* PG1 was deposited at the DDBJ/EMBL/GenBank under the accession CP002580 (chromosome 1) and CP002581 (chromosome 2) (Voget et al. 2015).

**Genome sequencing and SNP analysis of *B. glumae* LU8093**

Genomic DNA of *B. glumae* LU8093 was isolated with the Masterpure DNA purification Kit (Epicentre, Madison, USA). Genome sequencing was carried out with a hybrid approach using the 454 GS-FLX system with Titanium chemistry (Roche Life Science, Mannheim, Germany) and the Genome Analyzer IIx (Illumina, San Diego, CA). Sequencing results in 437,363 and 3,998,786 reads, respectively. In order to identify SNPs, sequence reads of LU8093 were mapped onto the *B. glumae* PG1 reference genome (Voget et al. 2015) with the GS Reference Mapper (Roche Life Science, Mannheim, Germany). All candidate SNP positions were then manually verified by PCR-amplifying corresponding genome regions and re-sequencing these fragments. Manual editing steps were performed using the GAP4 software package v4.6 (Staden 1996).

**Recombinant DNA techniques**

Standard DNA techniques were performed as described (Sambrook et al. 1989). PCR Extender System (5 Prime, Hilden, Germany) was used for amplification of DNA fragments. Other DNA-modifying enzymes were obtained from Thermo Scientific (St. Leon-Rot, Germany) using the manufacturer’s instructions. Plasmid isolation from *E. coli* DH5α was performed with innuPREP Plasmid Mini Kit (Analytic Jena, Jena, Germany). Genomic DNA from *B. glumae* PG1 (wild type) and *B. glumae* LU8093 was isolated using DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany).

The *lipAB* wild type operon (GenBank accession number: AJK49931.1 and AJK49932.1) and the *lipAB* operon that harbors the mutations in the promoter region and the region coding for the LipA signal peptide were amplified using the isolated genomic DNAs from both strains as template and the primer pair “PG1 *lipAB* up/dn” (5’-ATA TAT ATC TAG ATT TCA CCG GAT CGA TCG-3’/5’-ATA TAT AAG CTT ACC CGT TCG AAG CAC T-3’). The PCR products include 249 bp upstream of the *lipA* startcodon with the predicted promoter sequence. The resulting DNA fragments harboring primer introduced restriction sites were hydrolyzed with Xbal and HindIII, and the resulting 2444-bp fragments were ligated into Xbal-HindIII-treated plasmid pBBR1-MCS (Kovach et al. 1994). The resulting plasmids were named pBBR-*lipAB* and pBBR-*lipAB*-3, respectively. Plasmid pBBR-*lipAB*-3 was used as template for overlap-extension PCRs (Higuchi et al. 1988) to introduce single mutations. For the mutation in the promoter region, the primer pair “OLE PCR 1/2” (5’-CCT GTC TAC ATT CAG ACC GCC G-3’/5’-CGG CCG TCT GAT TGT AGA CAG G-3’) was used whereas the pair “OLE PCR 3/4” (5’-GGA ACG CAT CAA TCT GAC CAT G-3’/5’-CAT GGT CAG ATT GAT GCC TTC C-3’) was used for the mutation in the region coding for the signal peptide. The primer pair “PG1 *lipAB* up/dn” was used as flanking primers, and the resulting 2463 bp amplicon was then treated as described above. The resulting plasmids were named pBBR-*lipAB*-1 (mutation in the promoter region) and pBBR-*lipAB*-2 (mutation in the signal sequence).

**Transformation and conjugation**

*E. coli* strains were transformed with plasmid DNA by heat shock transformation (Hanahan 1983). *B. glumae* strains were transformed by biparental mating with *E. coli* S17-1 as follows: For conjugation, 1 ml overnight culture of *B. glumae* was mixed with 2 ml of *E. coli* S17-1 in the exponential growth phase (O.D.580nm = 0.6–0.8) containing the plasmid of interest. After centrifugation (1 min, 21,000×g), the cell pellet was washed with 0.5 ml LB medium, resuspended in 50 μl LB medium and dropped onto a membrane filter (M24, Whatman) placed on an LB agar-plate. Cells were washed off from the filter with LB medium after 6 h at 30 °C, and the cell suspension was plated in appropriate dilutions on MME (Vogel and Bonner 1956) agar plates containing antibiotics and 0.5 % (w/v) glucose.

**Western blot analysis**

Proteins from cell-free supernatants were precipitated with sodium deoxycholate and trichloroacetic acid (TCA) as described (Peterson 1977). After washing with 1/2 volume 80 % (v/v) acetone, the pellet was suspended with 2× SDS-sample puffer (50 mM Tris-HCl, 4 % (w/v) SDS, 10 % (v/v)
glycerol, 10 % (v/v) 2-mercaptoethanol, 0.03 % (w/v) bromophenol blue). Proteins were separated by SDS-PAGE with a 12 % polyacrylamide gel (Laemmli 1970). Western blot analysis of LipA and LipB was performed using specific antibodies (kindly provided by Jan Tommassen, University of Utrecht, The Netherlands). A goat-anti-rabbit IgG (H + L)-HRP conjugate (BioRad, Munich, Germany) was used as secondary antibody. Specific antibody-protein interactions were detected using the ECL Western Blotting Detection system (Amersham Pharmacia, Buckinghamshire, GB) and the luminescence detector Stella (raytest, Straubenhhardt, Germany).

**Lipase assay**

Lipase activity in whole cell extracts and supernatants was measured with para-nitrophenyl palmitate (p-NPP) as the substrate (Winkler and Stuckmann 1979) at 410 nm in microtiter plates using a SpectraMax 250 photometer (Molecular Devices, Ismaning/München, Germany). Relative lipase activity was correlated to cell density (O.D.<sub>550nm</sub>) and calculated as U/ml, with 1 U (unit) defined as the amount of lipase that releases 1 mmol of para-nitrophenol per minute (molar absorption coefficient 15 μMol<sup>-1</sup> × cm<sup>-1</sup>).

**Transcript level determination**

Two milliliters of culture were centrifuged (1 min, 21,000×g) and washed once with TE buffer (100 mM Tris-HCl pH 7.5, 20 mM EDTA). The cell pellet was then treated with RNeasy Mini Kit (Qiagen) according to the protocol for the isolation of bacterial RNA. DNaseI digestion was performed both, with a 12 % polyacrylamide gel (Laemmli 1970). Western blot analyses were detected using the ECL Western Blotting Detection system (Amersham Pharmacia, Buckinghamshire, GB) and the luminescence detector Stella (raytest, Straubenhhardt, Germany).

**Comparison of B. glumae wild type and the lipase production strain LU8093**

The extracellular lipase LipA produced by B. glumae PG1 is used by BASF SE for the production of enantiopure building blocks (Liese et al. 2006). Therefore, the lipase production strain B. glumae LU8093 was constructed from B. glumae PG1 by repeated rounds of random mutagenesis and subsequent assays for increased extracellular lipase production R. Braatz, R. Kurth, E. Menkerl-Conen, H. Rettenmaier, T. Friedrich, and T. Subkowski, 1992, patent application WO 93/00924 A1). We compared the genome sequences of B. glumae PG1 (Vogel et al. 2015) and B. glumae LU8093 and identified in the production strain 72 SNPs of which 51 were located on chromosome 1, with 29 non-synonymous, 16 synonymous and six intergenic ones. From 21 SNPs found on chromosome 2, 13 were non-synonymous, five synonymous and three intergenic. Among the 72 SNPs identified in the B. glumae LU8093 chromosomes, two were localized within the lipase operon on chromosome 2; one in the putative promoter region and the second in the region encoding the LipA signal peptide (Fig. 1).

We first determined lipase activity and protein amount in cell extracts and culture supernatants obtained from B. glumae PG1 wild type and the production strain LU8093 demonstrating that the production strain produced more lipase than the wild type (Fig. 2a). Previous studies using fusions of the lipase promoter with GFP indicated an increased transcription rate of lipA in the production strain B. glumae LU8093 (Boekema et al. 2007). Thus, we quantified the transcription levels of lipA by qPCR and determined a 100-fold increase in the production strain (Fig. 2b).
Two mutations localized within the lipase operon lipAB increase lipase production and secretion

The mutations identified in front of the lipAB operon were analyzed both separately and in combination by expression of the respective genes in a lipAB-deficient B. glumae PG1 strain (PG1ΔlipAB) to avoid basal expression of genome-encoded lipAB. To ensure that extracellular lipase activities were not caused by cell lysis, we determined cytoplasmic β-lactamase activities in cell-free culture supernatants. These activities were always less than 10% of the overall activities for all strains tested (Fig. S1) indicating that the observed effects of the mutations on extracellular lipase levels were not caused by significant cell lysis. As shown in Fig. 3a, the mutation in the promoter region of lipAB (lipAB-1) resulted in a 38-fold increased lipase activity in the supernatant (~2.68 compared to ~0.07 U/ml) and 42-fold in the cell extract (~0.168 compared to ~0.004 U/ml). The mutation in the signal peptide (lipAB-2) led to a slight (~4–7-fold) increase of lipase activity in the supernatant and the cell extract, whereas the combination of both mutations (lipAB-3) resulted in ~100-fold increased activity in the supernatant (~6.87 U/ml) and ~140-fold increased activity (~0.57 U/ml) in the whole cell extract. It should be noted here that lower lipase activities of B. glumae PG1 wild type and B. glumae LU8093 as shown in Fig. 2a can be attributed to the fact that these strains harbor just one chromosomal copy of the lipAB operon. The increased lipolytic activity of B. glumae PG1ΔlipAB expressing plasmid-encoded lipase variants corresponded to increased production and secretion as determined by Western blot analysis of LipA in cell-free supernatants (Fig. 3a, bottom). Remarkably, a significantly increased...
amount of LipB was detected only in the strains harboring the promoter mutation (lipAB-1 and lipAB-3).

A mutation in the lipAB promoter increases the transcript level of lipA

Next, we analyzed the influence of these mutations on the lipA transcript level by qPCR (see Fig. 3b). Whereas the mutation in the signal sequence (lipAB-2) exhibited just a slight effect at the transcript level, the promoter mutation (lipAB-1 and lipAB-3) led to an increase by a factor of 16. Interestingly, we observed that the transcript levels of lipA and lipB were differentially affected by the two mutations. While the lipA transcript level was increased by the promoter mutation, the amount of lipB transcript remained unaffected (data not shown). This may be explained by a faster degradation of the lipB transcript as already suggested by Frenken et al. (Frenken et al. 1993a). This assumption is further supported by the observation that more LipB was detected by Western blotting in strains harboring a lipase operon with the promoter mutation than in strains with the wild type operon (see Fig. 3a, bottom). Apparently, more lipB transcript could be produced and translated, but may be degraded faster than lipA transcript.

A signal peptide mutation in LipA improves secretion in B. glumae PG1

The second mutation identified in the lipAB operon results in an exchange of serine to leucine at position 4 of the LipA signal peptide. This mutation has almost no effect on lipA transcription rate, but caused a remarkable increase of extracellular lipase amount (see lipAB-2 in Fig. 3). The replacement of a polar serine by a hydrophobic leucine residue increases the hydrophobicity of the LipA signal peptide and may thus facilitate its interaction with the Sec-machinery thereby accelerating transport of LipA through the bacterial inner membrane (Driessen and Nouwen 2008). This hypothesis was tested by construction of alkaline phosphatase PhoA fusions to wild type and mutant LipA signal peptides and determination of PhoA activities in B. glumae PG1 and PG1ΔlipAB. PhoA shows enzymatic activity only after transport across the inner membrane and is therefore used as secretion reporter (Manoil et al. 1990). The LipA signal peptide derived from B. glumae LU8093 carrying mutation S4L resulted in a 2-fold increased PhoA activity (Fig. S2).

In summary, these results indicate that the combination of both mutations in the lipAB operon (see lipAB-3 in Fig. 3) results in an increased transcription rate as well as in increased lipase secretion. In addition, we did not observe any growth defects of B. glumae PG1ΔlipAB expressing plasmid-encoded lipAB-1 or lipAB-2 compared to the wild type lipAB operon or the empty vector control, respectively. Expression of lipAB-3 led to a slightly decreased cell density in the stationary growth phase after 24 h (O.D.580nm = 1.2 compared to 1.7 for wild type lipAB), which was, however, not observed upon comparing growth of B. glumae LU8093 with the wild type strain PG1 (data not shown).

Discussion

In this study, we analyzed the increased lipase production of the industrial production strain B. glumae LU8093 (Fig. 2) which is a derivate of the wild type strain B. glumae PG1. The comparison of the genome sequences revealed 72 SNPs
introduced in the production strain by classical mutagenesis methods with two of them being mainly responsible for increased lipase production and secretion (Fig. 3). One of these two mutations is located in the putative lip$A$ promoter region (Fig. 1). A previous study determined the transcriptional start site located 78 bases upstream of the lip$A$ start codon and the presence of two putative $\delta^{54}$-dependent promoters (Beselin 2005) with the first one located at a conserved distance of $\sim$24/12 bp upstream of the transcriptional start (Barrios et al. 1999), and the second one in a distance of $\sim$63/51 bp. This second putative promoter site fits perfectly with the $\delta^{54}$ consensus motif GG-N$_8$-TTGC (Barrios et al. 1999). The promoter mutation analyzed in this study changes this motif from $\sim$TGCCCC to $\sim$TGCT (see Fig. 1). One would expect that this C-to-T transition decreases the lip$A$ transcription rate, but surprisingly, it causes an increase in lip$A$ transcript level. The reasons are presently unknown; however, Boekema et al. have demonstrated that lipase expression in B. glumae PG1 but not in L$.\text{U}8093$ is prone to catabolite repression (Boekema et al. 2007). The promoter mutation may thus affect binding of not only $\delta^{54}$ but also of other, so far unknown, transcriptional regulators. A likely candidate could be the cAMP receptor protein (CRP) which was shown to repress the P. putida $\delta^{54}$-promoter Pu in a cAMP-dependent manner (Zhang et al. 2014). In B. glumae LU8093, CRP binding affinity to the mutated promoter may be diminished resulting in missing catabolic repression and correspondingly in an overall increased transcription rate. As lipase expression in B. glumae is also quorum sensing regulated (Devescovi et al. 2007), the promoter SNP may additionally uncouple lipase gene expression from quorum sensing regulation. The second mutation is located in the lip$A$ sequence coding for the 32 amino acid long signal peptide of LipA and changes the polar residue serine with a hydropathy value of $-0.8$ to a more hydrophobic leucine with a hydropathy value of $+3.8$ (Kyte and Doolittle 1982) thereby increasing lipase secretion (Fig. 3). This observation agrees with prior studies that showed improved protein secretion by signal peptide modifications (Yoon et al. 2010). Additive effects of additional modifications, as for example observed for heterologous protein secretion in Lactococcus lactis (Ng and Sarkar 2013), could further increase LipA secretion in B. glumae. Notably, we could demonstrate that this same mutated signal peptide also resulted in a 2-fold increased secretion of PhoA (Fig. S2), a well-established secretion reporter protein (Manoil et al. 1990). Nevertheless, it should be noted that additional 70 SNPs were identified in the production strain B. glumae LU8093 which may also contribute to and further increase lipase production. Interestingly, none of the SNPs is located within or close to genes known to be involved in quorum sensing or lipase secretion.

The fact that B. glumae PG1 secretes a lipase of biotechnological interest which is indeed used in industrial applications (Balkenhohl et al. 1997; Liese et al. 2006) raises the question if this strain possesses additional features which could be interesting for biotechnological applications. A very recent study dealing with the capacity of Burkholderia to adapt to different environments revealed certain differences between B. glumae PG1 and other members of the plant pathogenic Burkholderia group (Seo et al. 2015). The most striking difference is the absence of the toxoflavin biosynthesis and transport gene cluster in B. glumae PG1. Toxoflavin is a phytotoxin and a major virulence factor for phytopathogenic B. glumae strains in rice (Ham et al. 2011; Jeong et al. 2003). B. glumae PG1 could serve as an alternative host for the production of biotechnological relevant compounds like rhamnolipids (Costa et al. 2011). We also identified 25 putative secondary metabolite clusters in the genome of B. glumae PG1 (see Table S1). This further underlines the biotechnological potential of B. glumae PG1 not only as a lipase producer, which was demonstrated in this study, but also as a prolific source for known and new secondary metabolites.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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