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Transcriptional and Biochemical Characterization of Two *Azotobacter vinelandii* FKBP Family Members

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Peptidyl-prolyl *cis/trans* isomerases (PPIases, EC: 5.2.1.8), a class of enzymes that catalyse the rate-limiting step of the *cis/trans* isomerization in protein folding, are divided into three structurally unrelated families: cyclophilins, FK506-binding proteins (FKBPs), and parvulins. Two recombinant FKBPs from the soil nitrogen-fixing bacterium *Azotobacter vinelandii*, designated as *Av*fkbX and *Av*fkbB, have been purified and their peptidyl-prolyl *cis/trans* isomerase activity against Suc-Ala-Xaa-Pro-Phe-pNA synthetic peptides characterised. The substrate specificity of both enzymes is typical for bacterial FKBPs, with Suc-Ala-Phe-Pro-Phe-pNA being the most rapidly catalysed substrate by *Av*fkbX and Suc-Ala-Leu-Pro-Phe-pNA by *Av*fkbB. Both FKBPs display chaperone activity as well in the citrate synthase thermal aggregation assay. Furthermore, using real-time RT-qPCR, we demonstrated that both genes were expressed during the exponential growth phase on glucose minimal medium, while their expression declined dramatically during the stationary growth phase as well as when the growth medium was supplied exogenously with ammonium.

***Keywords*:** *A. vinelandii*, chaperone, FKBP, peptidyl-prolyl *cis/trans* isomerase, RT-qPCR

During protein folding, several potentially rate-limiting steps can occur, one of which is the *cis/trans* isomerization of proline peptide bonds, catalysed by a ubiquitously expressed group of peptidyl prolyl *cis/trans* isomerases (PPIases; EC: 5.2.1.8) (Schmid *et al*., 1993). Distinct, structurally unrelated families of PPIases exist, such as cyclophilins, FK-506 binding proteins (FKBPs), and parvulins, which can be distinguished by being inhibited by cyclosporin A, FK-506, and 5-hydroxy-1, 4-naphthoquinone, respectively (Galat, 2003). Due to their ability to catalyze peptidyl-prolyl bond isomerization *in situ*, PPIases have been implicated in signal transduction, protein assembly, and cell cycle regulation (Gothel and Marahiel, 1999; Fischer and Aumüller, 2003; Weiwad *et al*., 2004).

FKBPs are defined by the presence of at least one FK506-binding domain, which is a conserved sequence of approximately 110 amino acids (Galat, 2003; Somarelli and Herrera, 2007). However, despite strict FK506-binding domain conservation, numerous FKBPs demonstrate low or no PPIase activity (Kamphausen *et al*., 2002; Sinars *et al*., 2003; Lima *et al*., 2006) as they operate primarily as chaperones, with the FK506-binding domain providing a binding site for protein interaction (Galigniana *et al*., 2001; Riggs *et al*., 2007). Additionally, bacterial SlyD (Scholz *et al*., 2006); FkpA (Ramm and Plückthun, 2000, 2001; Saul *et al*., 2004); trigger factor (Stoller *et al*., 1995; Scholz *et al*., 1997); and archaeal *Mt*FKBP17 (Suzuki *et al*., 2003) exhibit both prolyl isomerase and chaperone activity. In most of these proteins, enzymatic and chaperone-like activities are localised to separate domains.

In the absence of immunosuppressant drugs, FKBP12, the most extensively characterised single-domain FKBP, binds to and regulates the activity of calcium-release channels (Timerman *et al*., 1993) or associates with transforming growth factor (Wang *et al*., 1994). Additional domains in the multidomain FKBPs are often tetratricopeptide repeat regions, additional FKBP domains, or calcium- or calmodulin-binding domains (Galat, 2003). The most prominent representative of multidomain FKBPs is the mammalian FKBP52, which is associated with the native glucocorticoid receptor complex. FKBP52 can substitute for FKBP51, resulting in a switching of the cytoplasmic glucocorticoid receptor into a higher affinity hormone-binding state (Davies and Sánchez, 2005). Following the substitution, FKBP52 promotes translocation of the receptor to the nucleus by linking the receptor via tetratricopeptide repeat region-bound HSP90 to the dynein motor that binds to the PPIase domain of FKBP52 (Davies and Sánchez, 2005).

Here we report the expression and purification of the recombinant *Av*fkbX and *Av*fkbB from the soil nitrogen-fixing *Azotobacter vinelandii* and analyze their biochemical properties *in vitro*. We also use RT-qPCR to study the effect of growth phase and the presence of ammonium in the growth medium on the expression of both genes.

Materials and Methods

Bacterial strains and growth conditions

E. coli strain XL-Blue1 (Invitrogen, USA) was used for the propagation of recombinant forms of the plasmid pET28a (Novagen, Germany). *E. coli* strain BL21 (DE3) (Novagen) was used for the expression of recombinant proteins. All *E. coli* strains were grown in LB medium supplemented with kanamycin. *A. vinelandii* was grown at 30°C in Burk's nitrogen-free salts, supplemented with glucose at 1% (BG) and in Burk's salts supplemented with ammonium chloride at 0.1% and glucose at 1% (BNG). *A. vinelandii* was grown at 30°C in LB medium.

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Heterologous expression of *Av*fkbX1 and *Av*fkbB in *E. coli* and purification of recombinant proteins

The coding sequence of *AvfkbX* (YP_002798384) and of *AvfkbB* (YP_002801192) were amplified using PCR with *A. vinelandii* genomic DNA as a template. The primers used were *AvfkbX*-F: 5′-AAACCATGGCT GAGCTGCGCATCGGTCCCGATC-3′ with *AvfkbX*-R: 5′-TTTAAG CTTGACGGCCCTGACCTCGAGGATTTC-3′ and *AvfkbB*-F: 5′-AA ACCATGGGCGAACTCAACCTTTCCACCG-3′ with *AvfkbB*-R: 5′-AAAAAGCTTTTACAGCACGTCCAGCAGTTCG-3′, carrying restriction sites for ligation to the pET28a expression vector. The underlined nucleotides at each primer represent *Nco*I, *Hin*dIII, *Nco*I, and *Hin*dIII, respectively. The fragments excised from amplified *AvfkbA1* and *AvfkbA2* sequences were cloned between the corresponding sites of pET28a, resulting in *Av*fkbX-pET28a and *Av*fkbB-pET28a. The absence of undesired alterations was checked by nucleotide sequencing. Synthesis of recombinant proteins in *E. coli* BL21 (DE3) cells was initiated by addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside when the culture reached A_{600} of 0.6 followed by continued cultivation for an additional 4 h at 30°C. Cells were harvested by centrifugation and disrupted by sonication in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole supplemented with 1 mg/ml lysozyme). Cellular lysates were centrifuged and the supernatants were used for protein purification. Recombinant proteins were purified with Ni-NTA chromatography (Ni^{2+}-nitrilotriacetate, QIAGEN, Germany) according to the manufacturer's instructions. To remove any imidazole and salts in the collected fractions, they were pooled and dialysed against 35 mM Hepes buffer pH: 8.0 and 70 mM NaCl, for 12 h. The purity of the purified proteins was analysed by 15% SDS-PAGE electrophoresis.

Peptidyl-prolyl *cis/trans* isomerase enzymatic assay

PPIase activity was tested with a chymotrypsin-coupled PPIase assay (Kofron *et al*., 1991), which is rate-limited by the *cis*/*trans* isomerisation of the Xaa-Pro peptide bond of synthetic Suc-AXaaPF-pNA (Bachem, Germany). The assay mixture contained 50 mM Hepes buffer pH: 8.0 and 100 mM NaCl, 50 μg α-chymotrypsin (dissolved in 1 mM HCl) (Fluka, Germany), 25 μM Suc-AXaaPF-pNA (5 mM stock dissolved in trifluoroethanol supplemented with 0.45 M LiCl) and the appropriate amount of the PPIase. The assay buffer was mixed with α-chymotrypsin and subsequently with the PPIase. The reaction was initiated inside the cuvette with the addition of the peptide, and the increase in absorbance at 390 nm was monitored at 4°C using a HITACHI U-2800 spectrophotometer.

Citrate synthase thermal aggregation assay

Chaperone activity was tested as previously described (Buchner *et al*., 1998). Thermal denaturation of citrate synthase (0.25 μM final concentration, Sigma, Germany) was achieved by incubation at 45°C in 40 mM Hepes pH: 7.5, for 15-20 min, in the absence or in the presence of additional proteins. Aggregation of citrate synthase was measured by monitoring the increase in turbidity at 500 nm in a HITACHI U-2800 spectrophotometer equipped with a thermostated cell holder. The absorbance change recorded is due to the increase in light scattering upon aggregation of citrate synthase. Protein disulfide isomerase (Sigma) was used as a positive control and albumin (Research Organics) as a negative.

RT-qPCR

Total RNA was isolated using a hot SDS/hot phenol method (Jahn *et al*., 2008) and quantified as previously described (Dimou *et al*., 2010). First-strand cDNA synthesis was performed using Superscript II RT (Invitrogen). The oligonucleotide primers used for RT-qPCR were designed using Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and optimised to an equal annealing temperature of 60°C (Table 1). Each primer pair was further assessed for specificity with melting curve analysis and gel electrophoresis of amplification products.

Table 1. Primers used in RT-qPCR reactions

Primer name	Nucleotide sequence
Avin_11800-qPCR-F	5′-GATCGGGAAGTCACCCTGCATTTCG-3′
Avin_11800-qPCR-R	5′-ATTGCCATCGCCAACCCGGAAG-3′
Avin_40820-qPCR-F	5′-TTACAGCACGTCCAGCAGTTCGAC-3′
Avin_40820-qPCR-R	5′-GCGGCAGCAAATGGCGTCTTT-3′
Avin_38560-qPCR-F	5′-TCCGGGTTGCCGAACATCACA-3′
Avin_38560-qPCR-R	5′-TGCGCAAGATCACCGGCAACA-3′

Reactions were run as previously described (Dimou *et al*., 2010). The efficiency of each RT-qPCR reaction was calculated using LinRegPCR software (Ruijter *et al*., 2009). Relative expression of the target gene in the various Burk's minimal media versus LB full medium was calculated using the following equation, described by Pfaffl (Pfaffl, 2001). Relative expression ratio per gene: $R = E_{GOI} \,\hat{}\, (C_{t,\ CONTROL} - C_{t,\ SAMPLE})_{GOI} / E_{REF} \,\hat{}\, (C_{t,\ CONTROL} - C_{t,\ SAMPLE})_{REF}$. recA (Avin_38560) was used as internal control. Statistical significance was determined by ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism v5.0 (GraphPad Software, USA).

Results and Discussion

Recombinant *Av*fkbX and *Av*fkbB have PPIase activity

Analysis of the fully sequenced genome of *A. vinelandii* (Setubal *et al*., 2009) revealed the existence of six members of the FKBP family with two designated as *Av*fkbX (Avin_11800) and *Av*fkbB (Avin_40820), showing higher homology to FkpX and FkbB from *E. coli*, respectively. Both proteins consist of a conserved C-terminal FKBP domain (cl11587), while *Av*fkbB contains an additional N-terminal domain of unknown function (cl03173) as revealed by CDD searches (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Figure 1 shows a CLUSTAL W multiple protein alignment among the six members of the *A. vinelandii* FKBP family and other characterised FKBPs.

Initially, we examined whether *Av*fkbX and *Av*fkbB exhibit the functional characteristics of PPIases. To that purpose, both genes were expressed as His-tagged proteins, and the purified fractions were applied to the standard PPIase assay utilising isomer-specific proteolysis of succinyl-Ala-Xaa-Pro-Phe-4-nitroanilides by chymotrypsin (Kofron *et al*., 1991). In the presence of PPIases, the Xaa-Pro bond is more rapidly converted to the *trans* conformation, which is readily cleaved by chymotrypsin leading to the formation of the colored product 4-nitroaniline. Kinetic data were obtained in the presence of increasing amounts of *Av*fkbX or *Av*fkbB and the differences between the isomerization rate constants k_{obs} and k_o, referring to the catalysed and uncatalysed reactions respectively, were plotted as a function of protein concentration (data not shown). The slope of the resultant line is equivalent to the

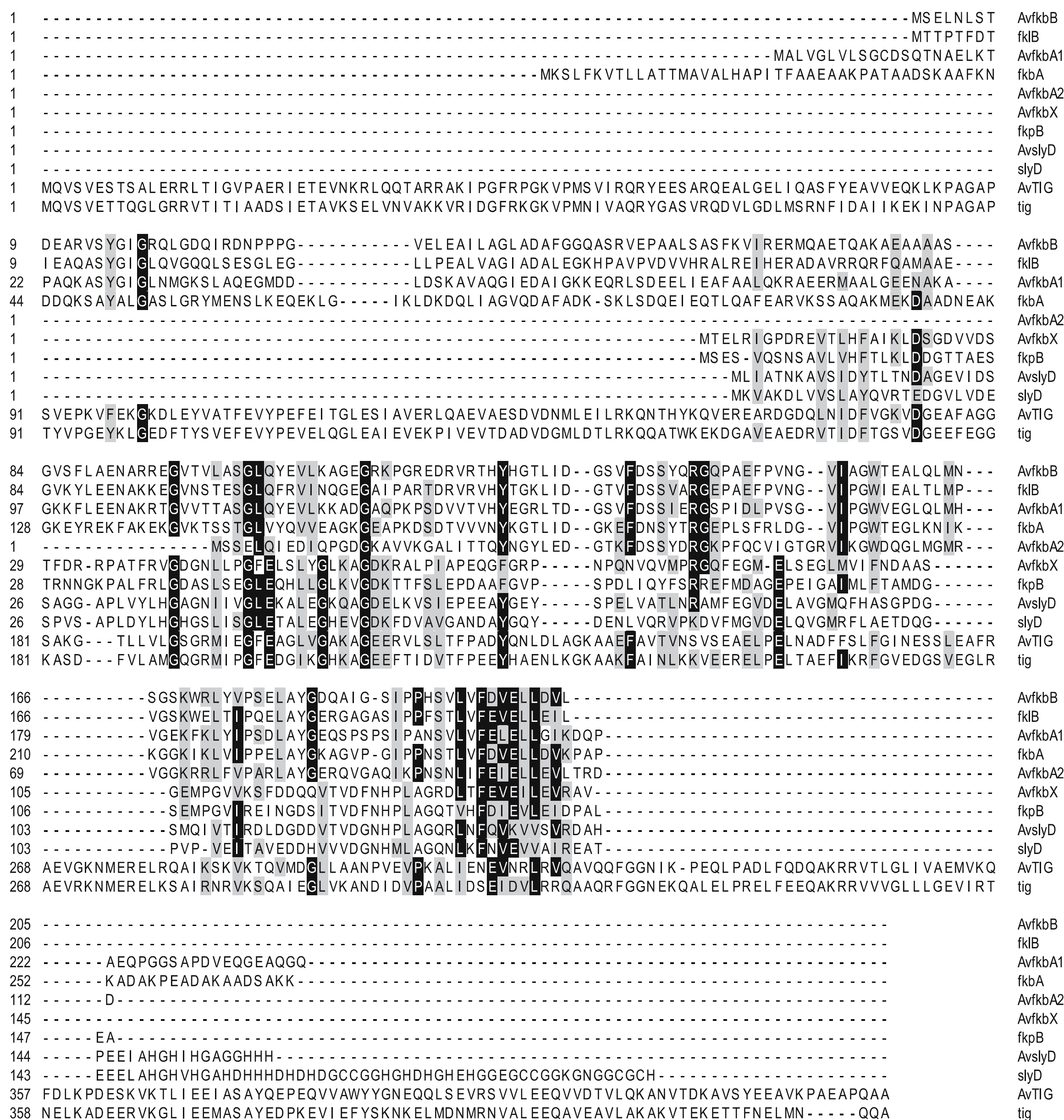

Fig. 1. Sequence alignment of *A. vinelandii* *Av*fkbB (YP_002798384), *Av*fkbX (YP_002801192), *Av*fkbA1 (YP_002800645), *Av*fkbA2 (YP_002801518), *Av*slyD (YP_002801219), *Av*TIG (YP_002799518), and related enzymes. The sequences included are *E. coli* fkpB (NP_414569), fklB (NP_418628), fkpA (NP_417806), slyD (NP_417808), and tig (NP_414970). Black boxes indicate identical amino acids while grey boxes indicate similar. Multiple sequence alignment was performed using CLUSTAL W (Chenna *et al*., 2003).

specificity constant k_{cat}/K_m (Table 2).

Both enzymes showed enzymatic activities characteristic of FKBPs, with the highest K_{cat}/K_m for *Av*fkbX observed for the substrate peptide where Phe preceded Pro, while for *Av*fkbB the amino acid that preceded Pro was Leu (Table 2). In addition, both FKBPs showed specificity for Ala, although at lower levels (Table 2). However, even if both enzymes showed distinct enzymatic activities, the K_{cat}/K_m values would not necessarily indicate their physiological properties since artificial substrates were used to determine these catalytic parameters. The identification of natural substrates for these enzymes awaits further studies and will shed light on the physiological role of bacterial FKBPs.

Table 2. Substrate specificities of *Av*fkbX and *Av*fkbB. -Xaa- represents various amino acids of substrates with the general sequence suc-A-Xaa-PF-pNA. nd, not detected

Substrate (-Xaa-)	*Av*fkbX	*Av*fkbB
	K_{cat}/K_m (mM^{-1}s^{-1})	
-Leu-	nd	29.55±4.18
-Ala-	21.70±5.63	1.14±0.52
-Phe-	123.17±41.82	7.39±1.57

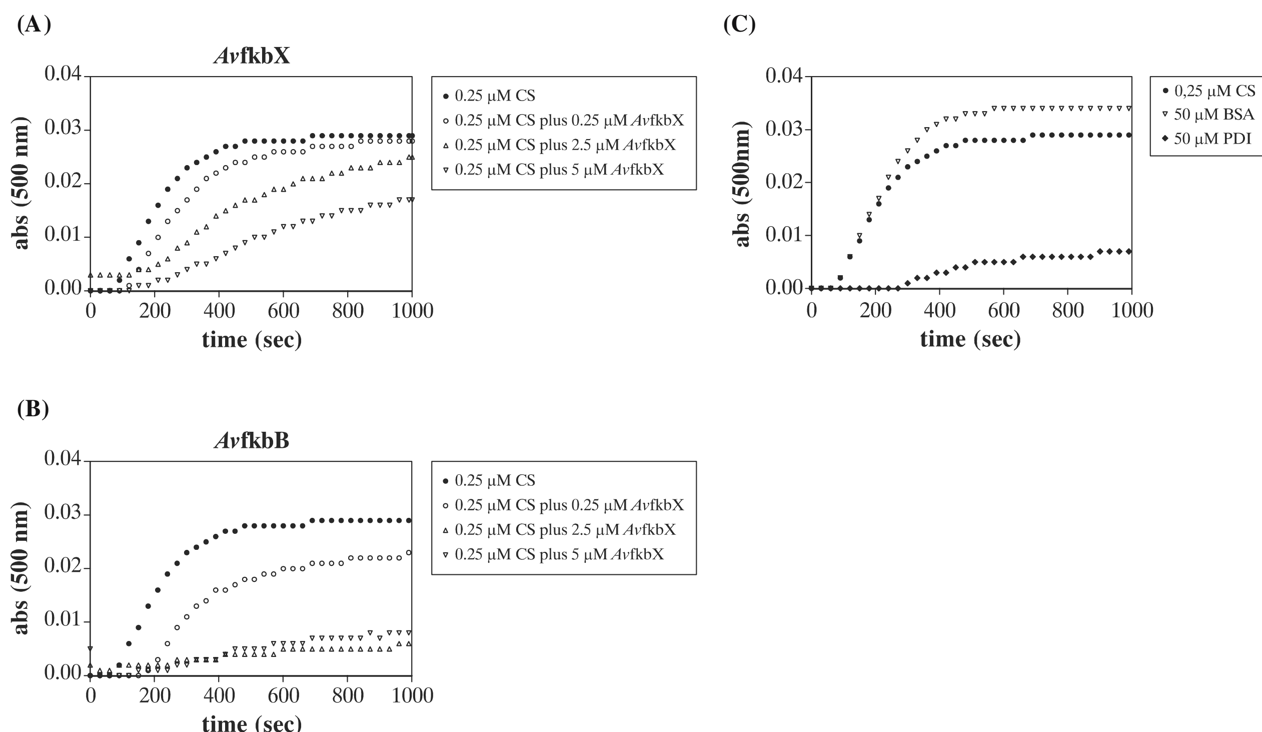

Fig. 2. Influence of *Av*fkbX and *Av*fkbB on the thermal aggregation of citrate synthase. Citrate synthase was incubated at 45°C. Aggregation was monitored by measuring the turbidity of the solution at 500 nm in the absence and presence of additional components. (A) Aggregation of 0.25 µM citrate synthase in the absence of additional components (●) and in the presence of 0.25 µM *Av*fkbX (○), 2.5 µM *Av*fkbX (▲), and 5 µM *Av*fkbX (▼). (B) Aggregation of 0.25 µM citrate synthase in the absence of additional components (●) and in the presence of 0.25 µM *Av*fkbB (○), 2.5 µM *Av*fkbB (▲), and 5 µM *Av*fkbB (▼). The results are representative of three series of measurements performed with different preparations of *Av*fkbX and *Av*fkbB. (C) Aggregation of 0.25 µM citrate synthase in the absence of additional components (●) and in the presence of 50 µM BSA (▼) and 50 µM PDI (▼).

Recombinant *Av*fkbX and *Av*fkbB have chaperone activity

The possible chaperone activity of *Av*fkbX and *Av*fkbB was monitored using the citrate synthase thermal aggregation assay (Buchner *et al*., 1998). Both proteins were able to partially suppress thermal aggregation of citrate synthase when added in 10×to 20×molar concentration to the assay mixture (Figs. 2A and B). In control reactions, addition of 50 µM of PDI efficiently prevents citrate synthase aggregation while addition of up to 50 µM of BSA has no effect (Fig. 2C). Furthermore, under the same assay conditions but without citrate synthase addition, both proteins remained stable (data not shown).

Examples have demonstrated a chaperoning function for classical PPIases, such as the eukaryotic Cyp-40 (Freeman *et al*., 1996) and the prokaryotic SurA (Behrens *et al*., 2001); SlyD (Weininger *et al*., 2009); and FkpA (Ramm and Plückthun, 2000). All these are multidomain proteins with most of the additional domains acting as chaperone and/or mediating the oligomerisation of the protein (Saul *et al*., 2004). The artificial introduction of the chaperone domain of SlyD into human FKBP12, which binds both peptides and protein substrates with a low affinity but a very high specificity with regard to the amino acid before the proline, created a folding enzyme that is virtually indistinguishable from trigger factor with regard to substrate specificity and catalytic efficiency. Binding to the chaperone domains apparently overrides the inherent sequence specificity of the PPIase site and thus solves the problem of generic substrate recognition during protein folding (Jakob *et al*., 2009). However, a single domain cyclophilin from *Leishmania donovani* has been shown to have an isomerase independent chaperone function (Chakraborty *et al*., 2002).

Interestingly, although *Av*fkbX, does not possess additional domains, like *Av*fkbB, it shows chaperone activity as well. Thus the chaperone function of both *A. vinelandii* enzymes probably indicates a broader range of substrate proteins on which they could act as molecular chaperones and/or PPIases. It remains to be clarified, using PPIase active site mutants and expression and biochemical characterisation of separate domains, whether the chaperoning site of these enzymes is functionally linked to their PPIase site.

AvfkbX and *AvfkbB* expression under various growth conditions

We studied the influence of growth medium and developmental phase on the relative expression levels of *AvfkbX* and *AvfkbB* using real-time RT-qPCR. Since *A. vinelandii* is a nitrogen-fixing soil bacterium that can convert atmospheric nitrogen to ammonium aerobically, we tested the influence of growth phase (exponentially or stationary grown cells) and source of fixed nitrogen (ammonium supplied either endogenously by

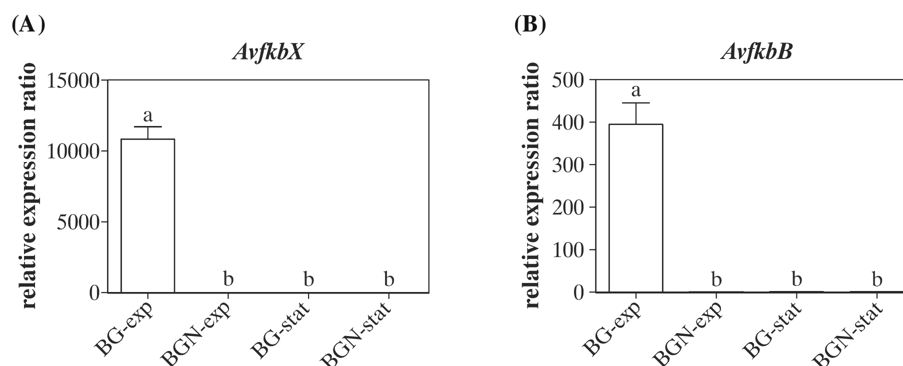

Fig. 3. Relative expression levels of *AvfkbX* and *AvfkbB* in *A. vinelandii* cultures grown in Burk's minimal media with glucose (BG) and in Burk's minimal media with glucose and ammonium (BGN) versus *A. vinelandii* cultures grown in LB full medium as determined by RT-qPCR. Fold change was calculated according to the equation described in the 'Materials and Methods', with normalisation against recA. The data are the mean of three biological replications, while the bars represent standard errors. 'a' represents non-significant difference ($p>0.05$) and 'b' represents significant difference ($p<0.05$) calculated by ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism v5.0 (GraphPad Software, USA).

nitrogen fixation or exogenously to the medium) on the relative expression levels of the two genes. Cells from the exponential and the stationary phases were harvested and the relative gene expression levels were calculated against cells grown in LB full medium from the same developmental phases. For normalisation, recA (Avin_38560) was used as a reference gene.

Both genes were expressed during exponential growth phase on glucose minimal medium; their expression declined dramatically during stationary growth phase and when the growth medium was supplied exogenously with ammonium ($p<0.05$) (Fig. 3). Nitrogen starvation is possibly a demanding condition for diazotrophic *A. vinelandii*, one that requires the presence of stress-related proteins such as FKBPs, which may have roles in stabilising and refolding proteins during exposure of cells to stress.

In conclusion, we biochemically characterised two members of the *A. vinelandii* FKBP family, *Av*fkbX and *Av*fkbB that show *in vitro* PPIase and chaperone activities. Furthermore, using real-time RT-qPCR, we demonstrated that both genes are mainly up-regulated during exponential growth on nitrogen-depleted medium, indicating that both proteins are required under nitrogen starvation conditions. However, further structural and functional studies are necessary to understand the exact function of these enzymes in bacterial physiology.

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