Gene Expression and Biochemical Characterization of *Azotobacter vinelandii* Cyclophilins and Protein Interaction Studies of the Cytoplasmic Isoform with dnaK and lpxH

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Key Words

Azotobacter vinelandii • Chaperone • Cyclophilin • dnaK • Peptidyl-prolyl *cis/trans* isomerase • Protein interaction • RT-qPCR • UDP-2, 3-diacetylglucosamine hydrolase

Abstract

The soil nitrogen-fixing bacterium *Azotobacter vinelandii* possesses two cyclophilins, comprising putative cytoplasmic and periplasmic isoforms, designated as AvPPIB and AvPPIA, respectively. Both recombinant cyclophilins have been purified and their peptidyl-prolyl *cis/trans* isomerase activity against Suc-Ala-Xaa-Pro-Phe-pNA synthetic peptides has been characterized. The substrate specificity of both cyclophilins is typical for bacterial cyclophilins, with Suc-Ala-Ala-Pro-Phe-pNA being the most rapidly catalyzed substrate. The cytoplasmic cyclophilin also displays a chaperone function in the citrate synthase thermal aggregation assay. Using real-time quantitative RT-PCR, we demonstrate that *AvppiB* is expressed under various physiological and growth conditions, mainly upregulated by acetate and downregulated by the stationary growth state, while *AvppiA* shows a tendency for downregulation under the tested conditions. Further, we identified chaperone protein dnaK and UDP-2, 3-diacetylglucosamine hydrolase lpxH as probable

interacting partners of AvPPIB and we demonstrate their physical interaction by coexpression studies. An increase in AvPPIB PPIase activity in the presence of AvdnaK and a decrease in the presence of AvlpxH further confirms each interaction. However, the PPIase activity does not seem to be essential for those interactions since AvPPIB active site mutants still interact with dnaK and lpxH, while their minor PPIase activity cannot be modulated by the interaction.

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Introduction

Amino acids are mainly connected in proteins via the *trans* peptide bond. The propensity for a *cis* peptide bond to exist in native proteins is <0.1%. However, in the case of the peptidyl-prolyl bond, the *cis* conformation increases from 5 to 6% in the native state [Jabs et al., 1999]. Spontaneous isomerization of peptidyl-prolyl bonds requires free energy and is a slow process which constitutes a rate-limiting step in protein folding. Peptidyl-prolyl *cis/trans* isomerases (PPIases, EC 5.2.1.8) catalyze this interconversion of peptidyl-prolyl bonds [Schmid, 1993] while they can also act on polypeptides as folding helper enzymes [Freeman et al., 1996; Rassow et al., 1995; Zarnt et

al., 1997]. The first type of this enzyme found proved to be the protein cyclophilin which binds the immunosuppressant cyclosporin A [Fischer et al., 1984]. Other distinct families of the enzyme exist, one being FK-506-binding proteins and another that includes parvulins. These families are structurally unrelated and can be distinguished by being inhibited by cyclosporin A, FK-506 and 5-hydroxy-1, 4-naphthoquinone, respectively [Galat, 2003].

All cyclophilins share a common domain of approximately 109 amino acids, the cyclophilin-like domain, and are surrounded by domains unique to each member of the family that are associated with subcellular compartmentalization and functional specialization [Marks, 1996]. When the interaction with other proteins is not transient, these additional domains mediate a stable interaction [Schiene-Fischer and Yu, 2001]. Besides mediating the cyclosporin A-induced immunosuppression, cyclophilins also participate in various cellular processes ranging from cell division, protein folding and regulation of HIV infection to several other important cellular steps [Baker et al., 1994; Baum et al., 2009; Braaten and Luban, 2001; Rassow et al., 1995]. Activities of various enzymes, transcription factors and heat shock proteins have also been shown to be modulated by binding with cyclophilins [Carrello et al., 2004; Jaschke et al., 1998; Lee et al., 2001].

Cyclophilins are ubiquitously distributed proteins, highly conserved during evolution and present in almost all organisms analyzed to date, with particular exceptions in bacteria such as *Mycoplasma genitalium* and some archaea [Göthel and Marahiel, 1999]. Although many of the eukaryotic cyclophilins have been studied extensively, few examples of functional characterization of these enzymes exist in prokaryotes, except *Escherichia coli* [Compton et al., 1992; Hayano et al., 1991; Liu and Walsh, 1990], *Bacillus subtilis* [Göthel et al., 1998], *Streptomyces chrysomallus* [Pahl et al., 1992; Pahl et al., 1997], *Streptomyces antibioticus* [Manteca et al., 2004], *Legionella pneumophila* [Schmidt et al., 1996] and *Erwinia chrysanthemi* [Pissavin and Hugouvieux-Cotte-Pattat, 1997]. The most obvious characteristic of bacterial cyclophilins is a lower affinity for cyclosporin A compared to human cyclophilin 18 (hCyp18) [Schönbrunner et al., 1991]. The cellular function of the bacterial homologues is still, however, poorly understood.

In this study, we describe the expression and purification of the recombinant *Azotobacter vinelandii*'s cytoplasmic and periplasmic cyclophilins in *E. coli* and analyze their biochemical properties in vitro. We study the

effect of different carbon sources, as well as the presence of ammonium in the growth medium, on both cyclophilins' gene expression, using RT-qPCR. Since it has been proposed that cyclophilins facilitate the peptidyl-prolyl isomerization of a *trans*-isomer to a *cis*-isomer in the refolding process of unfolded proteins [Konno et al., 2004], we considered proteins with *cis*-prolines to be some of the interacting candidates for them. We suggest the *A. vinelandii* Hsp70 chaperone protein dnaK (EC 3.6.3.51) to be such a protein and we demonstrate a stable in vivo interaction with the cytoplasmic cyclophilin AvPPIB. Further, we identified UDP-2, 3-diacetylglucosamine hydrolase lpxH (EC 3.6.1.B2) as a potential interacting partner of AvPPIB and we demonstrate their physical interaction by coexpression studies, as well. An increase in AvPPIB PPIase activity, in the presence of AvdnaK and a decrease in the presence of AvlpxH, further confirms each interaction. Finally, we demonstrate that the PPIase activity does not seem to be essential for these interactions since AvPPIB active site mutants still interact with dnaK and lpxH, while their minor PPIase activity cannot be modulated by the interaction.

Results

AvppiB and AvppiA Encode Putative Cytoplasmic and Periplasmic Cyclophilins, Respectively

Using sequence homology searches, we identified two putative cyclophilins from *A. vinelandii*, AvPPIB (ACO78539) and AvPPIA (ACO78121). A search for recognized protein motifs, using the Prosite database, revealed a cyclophilin-type peptidyl-prolyl *cis/trans* isomerase signature at amino acids 36–53 of AvPPIB and at amino acids 60–77 of AvPPIA. Comparison of the predicted amino acid sequences of both proteins with other proteins in the public databases revealed that they show similarity to other cyclophilins from different organisms. AvPPIB shows an overall 64% identity with cytoplasmic ppiB from *E. coli*, while AvPPIA shows an overall 66% identity with periplasmic ppiA from *E. coli* and is predicted to have a periplasmic localization using the PSORT-B program (<http://www.psort.org/psortb/>). Further, SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>) predicted a signal peptide probably consisting of the first 33 amino acids of AvPPIA. Figure 1 shows the amino acid sequence alignment of AvPPIB and AvPPIA with representative cyclophilins from bacteria as well as with cypA from *Homo sapiens*.

Fig. 1. Sequence alignment of AvPPIB (ACO78539), AvPPIA (ACO78121) and related cyclophilins. The sequences included are *H. sapiens* cypA (P05092), *Acinetobacter calcoaceticus* rotA (CAA52834), *E. chrysanthemi* rotA (CAA70935), *S. antibioticus* cyp18 (AAQ88274), *E. coli* ppiB (P23869) and *E. coli* ppiA

(P0AFL3). Multiple sequence alignment was performed using ClustalW [Chenna et al., 2003]. Black boxes indicate identical amino acids while grey boxes indicate similar ones. Amino acid residues involved in substrate binding of *H. sapiens* cypA (black dots) [Kallen and Walkinshaw, 1992] are indicated.

Recombinant AvPPIB and AvPPIA Have Peptidyl-Prolyl Cis/Trans Isomerase Activity

To elucidate whether AvPPIB and AvPPIA are active as peptidyl-prolyl *cis/trans* isomerases, both genes were cloned from *A. vinelandii* and fused to an expression vector to obtain the corresponding His-tagged proteins, which were overproduced in *E. coli* BL21 (DE3) cells and purified using Ni-NTA chromatography (fig. 2c, 3c). The purified fractions were applied to the standard PPIase assay utilizing isomer-specific proteolysis by chymotrypsin. The elution fraction from Ni-NTA purification, using *E. coli* BL21 (DE3) cells overexpressing pET28a vector only, was used in control reactions.

The assay for PPIase activity is based on the conformational specificity of chymotrypsin, which cleaves the 4-nitroanilide moiety from succinyl-Ala-Xaa-Pro-Phe-4-nitroanilides only when the Xaa-Pro peptide bond is in the *trans* conformation. 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 [Kofron et al., 1991]. Kinetic data were obtained in the presence of increasing amounts of (His)₆-AvPPIB and (His)₆-AvPPIA, and the differences between

the isomerization rate constants k_{obs} and k_0 (referring to the catalyzed and uncatalyzed reactions, respectively) were plotted as a function of protein concentration (fig. 2a, 3a). The slope of the resultant line is equivalent to the specificity constant k_{cat}/K_m (fig. 2b, 3b).

The substrate specificity for both proteins was determined. The highest K_{cat}/K_m for both enzymes was demonstrated for the substrate peptide where alanine preceded the proline residue (table 1). In addition, the substrate specificity was not restricted to alanine preceding the proline residue since both cyclophilins showed specificity for phenylalanine and leucine as well, although in lower levels (table 1).

Recombinant AvPPIB Has Chaperone Activity

To monitor the possible chaperone activity of AvPPIB and AvPPIA, the citrate synthase aggregation assay was used [Buchner et al., 1998]. At temperatures above 37°C, citrate synthase loses its native conformation and undergoes spontaneous aggregation. AvPPIB was able to suppress thermal aggregation of citrate synthase when added in an equimolar concentration to the assay mixture (fig. 4). On the contrary, AvPPIA does not seem to be a chaperone because citrate synthase aggregation could not

Fig. 2. Enzymatic measurements of the PPIase activity of the purified AvPPIB. **a** Hydrolysis of the peptide Suc-AAPF-pNA by α -chymotrypsin in the absence and presence of AvPPIB at 1 nM, 2.5 nM, 5 nM, 7.5 nM and 10 nM concentrations. The background absorbance of *p*-nitroaniline release is due to the initial presence of the *trans* form of Suc-AAPF-pNA. The results are representative of three series of measurements. **b** Dependence of the isomerization rate constant on the AvPPIB concentration. Each point is represented as the mean $\pm$ SE of three determinations. **c** SDS-PAGE analysis of the soluble cytoplasmic fraction from *E. coli* BL21 (DE3) cells overexpressing AvPPIB (lane a), the flow through fraction after the Ni-NTA purification (lane b) and the elution fraction after the Ni-NTA purification of the recombinant AvPPIB (lane c).

Table 1. Two substrate specificities of AvPPIB and AvPPIA

Substrate (-Xaa-)	K_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)	
	AvPPIB	AvPPIA
-Ala-	1.201 ± 0.064	0.554 ± 0.028
-Phe-	0.272 ± 0.018	0.053 ± 0.005
-Leu-	0.016 ± 0.001	0.052 ± 0.004

PPIase activity was determined at 390 nm with the isomer specific proteolytic assay at 4°C in 50 mM Hepes pH (8.0), 70 mM NaCl. K_{cat}/K_m is given in $\mu\text{M}^{-1}\text{s}^{-1}$.

-Xaa- represents various amino acids of substrates with the general sequence suc-A-Xaa-PF-pNA.

be suppressed, even when AvPPIA was added in high excess (fig. 4; data not shown). Under the same assay conditions but without citrate synthase addition, AvPPIB remained stable while AvPPIA lost its conformation and aggregated as well, indicating the need for further studies before a final conclusion of the enzyme characteristics is considered. At 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. Further, there was no effect with addition of the elution fraction after the Ni-NTA purification using *E. coli* BL21 (DE3) cells overexpressing pET28a vector only.

Oligomeric State Analysis of Recombinant AvPPIB and AvPPIA

In order to gain insight into the oligomeric state of recombinant AvPPIB and AvPPIA, we performed gel filtration analysis. The recombinant AvPPIB was estimated as an approximate 18-kDa peak being close to the theoretical molecular mass of AvPPIB, which is approximately 21 kDa. Similarly, recombinant AvPPIA was estimated as an approximate 18-kDa peak being close to the theoretical molecular mass of AvPPIA, which is approximately 19 kDa.

Effect of Carbon and Ammonium Source and Growth Phase on AvppiB and AvppiA Expression

The influence of growth phase (exponentially or stationary grown cells), carbon source (acetate or glucose) and source of fixed nitrogen (ammonium supplied either by nitrogen fixation or exogenously supplied to the medium) on the relative expression levels of the two cy-

Fig. 3. Enzymatic measurements of the PPIase activity of the purified AvPPIA. **a** Hydrolysis of the peptide Suc-AAPF-pNA by α -chymotrypsin in the absence and presence of AvPPIA at 6 nM, 12 nM, 17.5 nM, 24 nM, 29 nM and 35 nM concentrations. The background absorbance of *p*-nitroaniline release is due to the initial presence of the *trans* form of Suc-AAPF-pNA. The results are representative of three series of measurements. **b** Dependence of the isomerization rate constant on the AvPPIA concentration. Each point is represented as the mean $\pm$ SE of three determinations. **c** SDS-PAGE analysis of the soluble cytoplasmic fraction from *E. coli* BL21 (DE3) cells overexpressing AvPPIA (lane a), the flow through fraction after the Ni-NTA purification (lane b) and the elution fraction after the Ni-NTA purification of the recombinant AvPPIA (lane c).

Fig. 4. Influence of AvPPIB and AvPPIA 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 the presence of additional components. Aggregation of 0.25 μM citrate synthase in the absence of additional components and in the presence of 0.25 μM AvPPIB and 0.25 μM AvPPIA. Control reactions were performed in the presence of 50 μM PDI and in the presence of 50 μM BSA. The results are representative of three series of measurements performed with different preparations of AvPPIB and AvPPIA.

clophilin genes was examined by measuring the accumulation of their gene transcripts using real-time RT-qPCR. Cells from the exponential and the stationary phases were harvested and the relative gene expression levels were calculated against LB full medium grown cells from the same developmental phases. For normalization, *recA* (Avin_38560) was used as a reference gene.

The presence of ammonium in the acetate-containing medium increased about threefold the relative expression ratio of *AvppiB*, while in all other tested conditions, *AvppiB* was downregulated or not significantly upregulated (fig. 5a). *AvppiA*, on the other hand, shows a tendency for downregulation, with no significant differences among the exponential and the stationary phase, in both nitrogen-fixing and nonfixing conditions (fig. 5b).

Fig. 5. Relative expression levels of *AvppiB* and *AvppiA* in *A. vinelandii* cultures grown in various Burk's minimal media (BG: Burk's with glucose, BGN: Burk's with glucose and ammonium, BA: Burk's with acetate, BAN: Burk's with acetate and ammonium) vs. *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 normal-

ization against *recA*. The data are the mean of three biological replications while the bars represent standard errors. ^aNonsignificant difference ($p > 0.05$); ^bsignificant difference ($p < 0.05$) calculated by ANOVA followed by Tukey's multiple comparison test using GraphPad Prism v5.0 (GraphPad Software, San Diego, Calif., USA).

AvdnaK and *AvlpxH* as Probable Interacting Candidate Proteins for *AvPPIB*

The application of high-throughput technologies, such as large-scale yeast two-hybrid or affinity purification analyses, has generated a vast amount of data that have allowed the construction of many protein-protein interaction databases. In an attempt to perform a predictive analysis of the interaction network for the *A. vinelandii* cyclophilins, we searched the IntAct database [Hermjakob et al., 2004] and particularly the *E. coli* protein interaction data sets [Arifuzzaman et al., 2006; Butland et al., 2005]. Among all the probable preys, we inferred proteins with *cis*-prolines to be the most evident ones and considered them as the initial base of our study.

Searching the literature as well as the PDB database for homologous structures with *cis*-prolines, we noticed that in 1bpr and 1dg4 entries, representing structures of the substrate binding domain of the Hsp70 chaperone protein dnaK from *E. coli*, Pro⁴¹⁹ adopts the *cis* configuration [Wang et al., 1998]. Further, Vogel et al. [2006] proposed that Pro¹⁴³ in the ATPase domain constitutes a switch in mutual allosteric control between ATPase domain and substrate binding domain of dnaK, likely involving a *cis/trans* transition. These two proline residues remain conserved in *A. vinelandii* and other bacterial dnaKs; there-

fore, we considered *AvdnaK* as a good interacting candidate protein for *AvPPIB*.

Further, using Pfam searches, we found that the Avin_23500 gene coding for a putative UDP-2, 3-diacylglucosamine hydrolase, located adjacent to Avin_23510 coding for *AvPPIB*, belongs to the calcineurin-like phosphoesterase family (PF00149). Since immunophilins are able to interact with calcineurin in the absence of exogenous immunosuppressive ligands and modulate its phosphatase activity [Cardenas et al., 1994], we hypothesized that such an interaction might be conserved among other members of the calcineurin-like phosphoesterase family as well. Therefore, we considered UDP-2, 3-diacylglucosamine hydrolase *AvlpxH* as a potential interacting candidate protein for *AvPPIB*.

AvPPIB Physically Interacts with *AvdnaK* or *AvlpxH*

In order to confirm our hypotheses, we tested whether *AvPPIB* interacts with *AvdnaK* or *AvlpxH* in vivo. We independently coexpressed *AvPPIB* with *AvdnaK* or *AvlpxH*, as two separate polypeptides, with a (His)₆-tag fused at the N-terminus of *AvPPIB* and an S-tag fused at the C-terminus of *AvdnaK* or *AvlpxH*, using the pCDF-Duet-1 vector. The soluble coexpressed proteins were purified under native conditions using Ni-resin affinity chromatography, indicating that each protein pair forms

Fig. 6. Molecular interaction between AvPPIB and AvdnaK or AvlpxH. **a** SDS-PAGE analysis of the soluble cytoplasmic fraction from *E. coli* BL21 (DE3) cells overexpressing AvPPIB-AvdnaK-pCDFDuet1 (lane 1) and the elution fraction after the Ni-NTA purification of the AvPPIB-AvdnaK protein complex (lane 2). Western blot analysis using His-tag specific antibodies of the elution fraction after the Ni-NTA purification of the AvPPIB-AvdnaK protein complex (lane 3). Western blot analysis using S-tag specific antibodies of the elution fraction after the Ni-NTA purification of the recombinant AvPPIB-AvdnaK protein com-

plex (lane 4). **b** SDS-PAGE analysis of the soluble cytoplasmic fraction from *E. coli* BL21 (DE3) cells overexpressing AvPPIB-AvlpxH-pCDFDuet1 (lane 1) and the elution fraction after the Ni-NTA purification of the AvPPIB-AvlpxH protein complex (lane 2). Western blot analysis using His-tag specific antibodies of the elution fraction after the Ni-NTA purification of the AvPPIB-AvlpxH protein complex (lane 3). Western blot analysis using S-tag specific antibodies of the elution fraction after the Ni-NTA purification of the recombinant AvPPIB-AvlpxH protein complex (lane 4).

a stable complex. The existence of both polypeptides was confirmed by SDS-PAGE and immunoblotting with antibodies against the His and S-tags (fig. 6). Control experiments were performed where just S-tag fused AvdnaK or AvlpxH was not retained by the Ni-NTA agarose column. Any minor fractions of other molecular weights represent *E. coli* proteins retained by the Ni-agarose since the elution fraction after the Ni-NTA purification using *E. coli* BL21 (DE3) cells overexpressing pCDFDuet-1 vector only was used as a control.

When we coexpressed the two protein pairs from pACYCDuet-1, a lower copy number vector, the respective proteins continued to interact. Furthermore, in order to further lower the concentration of the coexpressed proteins, demonstrating the specificity of the binding assay, we decreased the final isopropyl 1-thio- β -D-galactopyranoside concentration to 0.1 mM than the previously

used 0.5 mM. In all cases, the respective protein pairs continued to interact.

AvdnaK and AvlpxH Modulate AvPPIB Activity

To verify the binding of AvPPIB to AvdnaK or AvlpxH, the effect of AvdnaK or AvlpxH on the PPIase activity of AvPPIB was determined using the standard PPIase assay utilizing isomer-specific proteolysis by chymotrypsin [Kofron et al., 1991]. Both *AvdnaK* and *AvlpxH* genes were overexpressed in *E. coli* BL21 (DE3) cells producing His-tagged recombinant proteins which were purified using Ni-NTA chromatography. Kinetic data were obtained in the presence of AvPPIB and the difference between the isomerization rate constants k_{obs} and k_o , referring to the catalyzed and uncatalyzed reactions respectively, was determined.

Fig. 7. Effect of AvdnaK and AvlpxH on AvPPIB PPIase activity. **a** PPIase activity of 60 nM AvPPIB in the presence of 60 nM and 600 nM AvdnaK. **b** PPIase activity of 60 nM AvPPIB in the presence of 60 nM, 600 nM and 1.2 μ M AvlpxH. Mean values were obtained from three independent replicates and error bars represent standard errors. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks indicate a statistically significant difference ($p < 0.05$).

Fig. 8. Influence of AvPPIB_{F99A} and AvPPIB_{R43A} 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 μ M citrate synthase in the absence of additional components and in the presence of 0.25 μ M AvPPIB_{F99A}, 0.75 μ M AvPPIB_{F99A} and 2 μ M AvPPIB_{F99A}. **b** Aggregation of 0.25 μ M citrate synthase in the absence of additional components and in the presence of 0.25 μ M AvPPIB_{R43A}, 0.75 μ M AvPPIB_{R43A} and 2 μ M AvPPIB_{R43A}. The results are representative of three series of measurements performed with different preparations of AvPPIB_{F99A} and AvPPIB_{R43A}.

When we added AvdnaK to the reaction containing 60 nM AvPPIB, we observed an increase in activity, up to approximately 255% of the control reaction when AvdnaK was added in 1× molar excess over AvPPIB ($p < 0.05$; fig. 7a). When we added AvlpxH to the reaction containing 60 nM AvPPIB, we observed a concentration-dependent decrease in activity, up to approximately 64% of the control reaction when AvPTA-1 was added in 20× molar excess over AvPPIB ($p < 0.05$; fig. 7b). Addition of BSA

had no effect on PPIase activity, while AvdnaK or AvlpxH alone did not show any PPIase activity.

AvPPIB_{F99A} and AvPPIB_{R43A} Point Mutants Lack PPIase Activity but Retain Some Chaperone Activity

To examine the role of PPIase activity in AvPPIB function, we introduced active-site mutations and addressed the in vitro enzymatic activities of the mutant enzymes. Phe⁹⁹ and Arg⁴³ residues (*A. vinelandii* numbering) in-

Fig. 9. Molecular interaction between AvPPIB_{F99A} or AvPPIB_{R43A} and AvdnaK or AvlpxH. SDS-PAGE analysis of the elution fraction after the Ni-NTA purification of the AvPPIB_{F99A}-AvdnaK protein complex from *E. coli* BL21 (DE3) cells overexpressing AvPPIB_{F99A}-AvdnaK-pCDFDuet1 (lane 1). SDS-PAGE analysis of the elution fraction after the Ni-NTA purification of the AvPPIB_{R43A}-AvdnaK protein complex from *E. coli* BL21 (DE3) cells overexpressing AvPPIB_{R43A}-AvdnaK-pCDFDuet1 (lane 2). SDS-PAGE analysis of the elution fraction after the Ni-NTA purification of the AvPPIB_{F99A}-AvlpxH protein complex from *E. coli* BL21 (DE3) cells overexpressing AvPPIB_{F99A}-AvlpxH-pCDFDuet1 (lane 3). SDS-PAGE analysis of the elution fraction after the Ni-NTA purification of the AvPPIB_{R43A}-AvlpxH protein complex from *E. coli* BL21 (DE3) cells overexpressing AvPPIB_{R43A}-AvlpxH-pCDFDuet1 (lane 4).

involved in substrate binding of *H. sapiens* cypA [Kallen and Walkinshaw, 1992] were independently altered to Ala. The corresponding His-tagged proteins were overproduced in *E. coli* BL21 (DE3) cells and purified using Ni-NTA chromatography. AvPPIB_{F99A} and AvPPIB_{R43A} mutant proteins retained 1.7 and 1% of the catalytic efficiency of the wild-type enzyme, respectively, using the standard PPIase assay utilizing isomer-specific proteolysis by chymotrypsin [Kofron et al., 1991]. Further, both mutant proteins were not able to suppress thermal aggregation of citrate synthase when added in equimolar concentration to the assay mixture. However, they showed chaperone activity when they were added at 3- to 8-fold molar excess over citrate synthase (fig. 8a, b).

AvPPIB_{F99A} and AvPPIB_{R43A} Point Mutants Still Interact with AvdnaK or AvlpxH while Their Remaining PPIase Activity Cannot Be Modulated by the Interaction

In order to clarify the role of the PPIase activity on the binding of AvPPIB to AvdnaK or AvlpxH, we tested whether AvPPIB_{F99A} and AvPPIB_{R43A} PPIase active site mutant proteins still interact with AvdnaK or AvlpxH in vivo. We independently coexpressed AvPPIB_{F99A} or AvPPIB_{R43A} with AvdnaK or AvlpxH, as two separate polypeptides, with a (His)₆-tag fused at the N-terminus of AvPPIB and an S-tag fused at the C-terminus of AvdnaK or AvlpxH using the pCDFDuet-1 vector. The soluble coexpressed proteins were purified under native conditions using Ni-resin affinity chromatography, indicating that each protein pair yet again forms a stable complex. The existence of both polypeptides was confirmed by SDS-PAGE and immunoblotting with antibodies against the His and S-tags (fig. 9). Control experiments were performed where just S-tag fused AvdnaK or AvlpxH were not retained by Ni-NTA agarose column. Any minor fractions of other molecular weights represent *E. coli* proteins retained by the Ni-agarose since the elution fraction after the Ni-NTA purification using *E. coli* BL21 (DE3) cells overexpressing pCDFDuet-1 vector only was used as a control.

When we added 1× and 10× molar concentrations of AvdnaK or AvlpxH to the PPIase reaction containing AvPPIB_{F99A} or AvPPIB_{R43A}, we did not observe a significant modulation of the already very low remaining activity of the active site mutants.

Discussion

Cyclophilins are characterized by PPIase activity that is implicated in protein folding and may function as molecular chaperones as well. To analyze whether *A. vinelandii* cyclophilins exhibit the functional characteristics of a cyclophilin, we determined the kinetic parameters of the two enzymes using Succinyl-Ala-Xaa-Pro-Phe-pNA tetrapeptides as substrates at 4°C. Both enzymes are characterized by kinetic parameters which are very similar. Compton et al. [1992] and Hayano et al. [1991] have also indicated that the activities of the two enzymes from *E. coli* are almost identical. Comparison of the catalytic efficiencies (K_{cat}/K_m) of AvPPIB and AvPPIA with other PPIases revealed that the K_{cat}/K_m of *A. vinelandii* cyclophilins shows high similarity to other previously characterized cyclophilins [Compton et al., 1992; Hayano et al.,

1991]. Accordingly, it may be stated that both enzymes show enzymatic activities that are characteristic of cyclophilins. However, since the experiments for the determination of these catalytic parameters were performed at lower than physiological temperatures with artificial substrates, the K_{cat}/K_m values would not necessarily indicate the physiological properties of these PPIases.

There have been examples demonstrating a chaperoning function for classical PPIases, such as the eukaryotic Cyp-40 [Freeman et al., 1996], Cyp-20 [Rassow et al., 1995] and the prokaryotic trigger factor [Zarnt et al., 1997], SurA [Behrens et al., 2001], SlyD [Weininger et al., 2009], FkpA [Ramm and Plückthun, 2000], and Par27 [Hodak et al., 2008]. All these are multidomain proteins with most of the additional domains acting as chaperones and/or mediating the oligomerization 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 the 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] and human single domain cyp-18 holds chaperone activity as well [Moparthi et al., 2010]. Interestingly, *A. vinelandii* cytoplasmic cyclophilin, although not possessing additional chaperone domains, shows chaperone activity. Although AvPPIB active site mutants exhibit significantly reduced PPIase activity, they retain a considerable part of their chaperone activity implying that AvPPIB's chaperoning site is at least partially functionally linked to its PPIase site.

Our gene expression analyses showed that *AvppiB* is expressed under various physiological and growth conditions, and is downregulated mainly by the stationary growth state. The growth on acetate increased about 1- to 3-fold the relative expression ratio of *AvppiB*, according to the source of ammonium supplied (endogenously or exogenously, respectively). Taken together, these data suggest that enzymes, directly or indirectly involved in acetate metabolism, may require the cyclophilin for modulation of their activity. *AvppiA*, on the other hand, shows a tendency for downregulation, indicating the limited necessity of the protein under the tested conditions and/or

the probable requirement of the protein under specific but not yet identified physiological conditions. Knockout of the cytoplasmic *ppiB* caused strongly repressed swarming as revealed in a genome-wide screening of genes required for swarming motility in *E. coli* [Inoue et al., 2007]. Inactivation of the periplasmic PpiA from *E. coli* had no evident phenotype, significantly hindering the ability to identify specific protein substrates [Kleerebezem et al., 1995]. However, *ppiB*, a novel secreted PPIase from *L. pneumophila*, is necessary for optimal growth at low temperatures [Söderberg and Cianciotto, 2008], while in *E. coli* several molecular chaperones including *ppiA* were transiently induced upon cold shock [Kandror and Goldberg, 1997; Phadtare and Inouye, 2004], indicating a previously unidentified role of secreted peptidyl-prolyl *cis-trans* isomerases in low temperature growth. Furthermore, *ppiA* is induced in response to periplasmic stresses such as unfolded proteins or elevated pH [Pogliano et al., 1997].

In an attempt to find natural substrates for the *A. vinelandii* cyclophilins, we performed a predictive analysis by searching the IntAct database for such protein interactions in various bacterial species, considering the proteins with *cis*-prolines to be a good starting point for our study. Searching the literature as well as the PDB database for homologous structures with *cis*-prolines, we noticed that in the structure of the substrate binding domain of dnaK from *E. coli*, Pro⁴¹⁹ adopts the *cis* configuration [Wang et al., 1998]. Further, Vogel et al. [2006] proposed that Pro¹⁴³ in the ATPase domain constitutes a switch in mutual allosteric control between ATPase domain and the substrate binding domain of dnaK, likely involving a *cis/trans* transition. Therefore, we considered AvdnaK as a good interacting candidate protein for AvPPIB, and by coexpression of the two proteins we showed that they physically interact. This interaction was further verified by the observed stimulatory effect of AvdnaK on the PPIase activity of AvPPIB. Eukaryotic Cyp40 appears to have an accessory role with heat shock protein Hsp90 in chaperone protein-folding machinery, essential for the activity of diverse intracellular signaling molecules [Duina et al., 1996; Nair et al., 1996], while it interacts with Hsc70 as well [Carrello et al., 2004]. It is important to further verify whether bacterial AvPPIB regulates AvdnaK in vivo and how this mechanism is used in conjunction with other known regulatory mechanisms or whether the two molecules act synergistically on protein folding.

Further, we found that the gene coding for a putative UDP-2, 3-diacetylglucosamine hydrolase, located adjacent to *Avppib*, belongs to the calcineurin-like phosphoester-

ase family (PF00149). Since immunophilins are able to interact with calcineurin in the absence of exogenous immunosuppressive ligands and modulate its phosphatase activity [Cardenas et al., 1994], we hypothesized that such an interaction might be conserved among other members of the calcineurin-like phosphoesterase family as well. Considering *AvlpxH* as a probable interacting candidate protein for *AvPPIB*, we coexpressed the two proteins and showed that they physically interact. UDP-2, 3-diacylglucosamine hydrolase is involved in lipid A biosynthesis catalyzing the pyrophosphate bond hydrolysis of the precursor UDP-2, 3-diacylglucosamine to yield 2, 3-diacylglucosamine 1-phosphate and UMP [Babinski et al., 2002]. This binding was further verified by the inhibitory effect of *AvlpxH* on the PPIase activity of *AvPPIB*, implying that *AvlpxH* binds to the active site of *AvPPIB*. The physiological meaning as well as the mechanism of such an interaction remains to be clarified.

In an attempt to clarify whether the PPIase activity of *AvPPIB* is required for these interactions, we separately introduced two active site point mutations to the enzyme which previously resulted in substantial reduction of enzymatic activity of human *cypA* as well [Zydowsky et al., 1992]. Both *AvPPIB* mutant proteins lacked significant PPIase activity towards a synthetic tetrapeptide while they retained reduced levels of chaperone activity. When we coexpressed these mutant proteins with each of the interacting partners, we still observed an *in vivo* interaction probably due to the remaining chaperone activity of the active site mutant proteins. Their remaining minor PPIase activity could not be modulated by the interaction, indicating that the *AvPPIB* PPIase activity may not be sufficient for *dnaK* or *lpxH* protein-protein interactions. Therefore, we speculate that the PPIase activity is at least partially involved in *AvPPIB* *in vivo* function. Similarly, *Drosophila* main rhodopsin forms protein complexes rather than catalytic enzyme-substrate interactions with cyclophilin [Baker et al., 1994].

In summary, our study reveals that cytoplasmic and periplasmic *A. vinelandii* cyclophilins show *in vitro* PPIase activity, and cytoplasmic cyclophilin exhibits chaperone activity as well. Both proteins are monomeric based on gel filtration analyses. Using real-time RT-qPCR, we demonstrate that *AvppiB* is expressed under various physiological and growth conditions: mainly upregulated by the presence of acetate and downregulated on the stationary growth phase, while *AvppiA* shows a tendency for downregulation under the tested conditions. Further, we identified *hsp70* chaperone protein *dnaK* and UDP-2,

3-diacylglucosamine hydrolase *lpxH* as potential interacting partners of the cytoplasmic cyclophilin and demonstrated their physical interaction by coexpression studies. An increase in *AvPPIB* PPIase activity, in the presence of *AvdnaK* and a decrease in the presence of *AvlpxH*, further confirms each interaction. However, the PPIase function does not seem to be essential for those interactions since *AvPPIB* active site mutants still interact with *dnaK* and *lpxH*. Nevertheless, the exact function of cyclophilins in the bacterial physiology remains elusive, indicating the need for further structural and functional studies. Clearly, the mechanism as well as the physiological significance of the interaction of *AvPPIB* with *AvdnaK* and *AvlpxH* needs to be determined while the identification of more natural cyclophilin substrates would link the catalytic properties of these enzymes with their physiological functions. At present, work towards these goals is under way.

Experimental Procedures

Bacterial Strains and Growth Conditions

E. coli XL-Blue1 strain (Invitrogen) was used for the propagation of recombinant forms of the plasmid pET28a and pCDFDuet-1 (Novagen). *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 or streptomycin. *A. vinelandii* was grown at 30°C in Burk's nitrogen-free salts, supplemented with either glucose at 1% (BG) or sodium acetate at 0.3% (BA) and in Burk's salts supplemented with ammonium chloride at 0.1% and either glucose at 1% (BNG) or sodium acetate at 0.3% (BNA). Further, *A. vinelandii* was grown at 30°C in LB medium.

Heterologous Expression of *AvPPIB* and *AvPPIA* in *E. coli* and Purification of Recombinant Proteins

The coding sequence of *AvppiB* (YP_002799514) and the coding sequence of *AvppiA* (YP_002799096) without the first 33 amino acids which comprise the probable leader peptide were amplified using PCR with *A. vinelandii* genomic DNA as a template. The primers used were *AvPPIB*-F: 5'-AAAGGATCCATCAAGCTGCAAACCAACCACG-3' with *AvPPIB*-R: 5'-AAACTCGAGTTATTTCGACGATCTCGGCCTTC-3' and *AvPPIA*-F: 5'-AAAACATGTTAAATCCCATGTCCTGCTGG-3' with *AvPPIA*-R: 5'-TTTCTCGAGGAGCTTTTTCGCCGAGGTGATC-3', carrying restriction sites for ligation to the pET28a expression vector. The underlined nucleotides at each primer represent *Bam*HI, *Xho*I, *Pst*I and *Xho*I, respectively. The fragments excised from amplified *AvppiB* and *AvppiA* sequences were cloned between the *Bam*HI and *Xho*I sites of pET28a, resulting in *AvPPIB*-pET28a, and between *Nco*I and *Xho*I sites of pET28a, resulting in *AvPPIA*-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

Table 2. Primers used in RT-qPCR reactions

Primer name	Nucleotide sequence
Avin_23510-qPCR-F	5'-ATCGGTGCCCTCGACCACTTCA-3'
Avin_23510-qPCR-R	5'-ACGCCTTTCTCGACCACACTGC-3'
Avin_19090-qPCR-F	5'-ATGAAGCCGGGGATGACCCGAT-3'
Avin_19090-qPCR-R	5'-AGGCGCCGATCAGCGTGAAA-3'
Avin_38560-qPCR-F	5'-TCCGGGTGCGGAACATCACA-3'
Avin_38560-qPCR-R	5'-TGCGCAAGATCACCGGCAACA-3'

1-thio- β -D-galactopyranoside when the culture reached A_{600} of 0.6 and continued cultivation for an additional 4 h at 30°C. Cells were harvested by centrifugation and were 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) according to the manufacturer's instructions. To remove any imidazole and salts in the collected fractions, fractions were pooled accordingly and dialyzed against 35 mM Hepes buffer pH (8.0) and 70 mM NaCl, for 12 h. The purity of the purified proteins was analyzed by 15% SDS-PAGE electrophoresis.

Size Exclusion Chromatography

Size exclusion chromatography was performed on a Prominence liquid chromatography system (Shimadzu Co., Tokyo, Japan) equipped with a ZORBAX GF-250 4- μm column (4.6 $\times$ 250 mm, Agilent Technologies, Palo Alto, Calif., USA) and a SPD 20A diode array detector. Aliquots of 20 μl of protein samples were injected and eluted isocratically with 20 mM Na_2HPO_4 (pH 7.0), 130 mM NaCl at a flow rate of 1 ml/min. Molecular weight calibration was performed using bovine thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa) mixture, all components of the Bio-Rad gel filtration standard kit. Chromatograms were analyzed at 280 nm.

Peptidyl-Prolyl *Cis/Trans* Isomerase Enzymatic Assay

To test whether AvPPIB and AvPPIA have PPIase activity, recombinant $(\text{His})_6$ -AvPPIB and $(\text{His})_6$ -AvPPIA were expressed in and purified from *E. coli* BL21 (DE3), as described above. PPIase activity was tested with a chymotrypsin-coupled PPIase assay [Kofron et al., 1991], which is rate-limited by the *cis/trans* isomerization of the Xaa-Pro peptide bond of synthetic Suc-AXaaPF-pNA (Bachem). 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), 25 μM Suc-AXaaPF-pNA (5 mM stock dissolved in trifluoroethanol supplemented with 0.45 M LiCl), and the appropriate amount of enzyme. The assay buffer was mixed with α -chymotrypsin and subsequently with the enzyme. 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 equipped with a thermostated cell holder.

Citrate Synthase Thermal Aggregation Assay

Thermal denaturation of citrate synthase (0.25 μM final concentration, Sigma) 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, as previously described [Buchner et al., 1998]. 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 recorded change in absorbance was due to the increase in light scattering upon aggregation of citrate synthase. Protein disulfide isomerase (Sigma) was used in positive control reactions and albumin (Research Organics) was used in negative control reactions.

RNA Isolation

Total RNA was isolated using a hot SDS/hot phenol method [Jahn et al., 2008]. Briefly, bacterial cultures were added to a 1/10 volume of 95% ethanol plus 5% saturated phenol to stabilize cellular RNA and the cells were then harvested for 5 min at 6,000 rpm centrifugation at 4°C. The supernatant was aspirated and the pellets were kept at –80°C for no longer than 1 week. Pellets were resuspended in 800 μl of lysis buffer (10 mM Tris-HCl (pH 8.0) 1 mM EDTA) and 3 mg/ml lysozyme. After the pellets were resuspended, 80 μl of a 10% SDS solution was added and the lysate was mixed and incubated at 64°C for 2 min. After incubation, 88 μl of 1 M NaOAc (pH 5.2) was mixed with the lysate. To this solution, an equal volume of water-saturated phenol was added, mixed and incubated at 64°C for 6 min inverting the tubes several times. The samples were chilled on ice and centrifuged at 13,000 rpm for 10 min at 4°C. The aqueous layer was transferred to a tube with equal volume of chloroform and centrifuged at 13,000 rpm for 5 min at 4°C. The aqueous layer was ethanol precipitated by adding 1/10 volume of 3 M NaOAc (pH: 5.2), 1 mM EDTA and 2 volumes cold EtOH. The samples were incubated at –80°C overnight. The RNA was pelleted by centrifugation at 13,000 rpm for 25 min at 4°C. Pellets were washed with ice cold 80% EtOH and centrifuged at 13,000 rpm for 5 min at 4°C. The pellets were resuspended in a total of 100 μl of RNase-free water.

Total RNA was quantified using microspectrophotometry (NanoDrop Technologies Inc.) and its quality was assessed by agarose gel electrophoresis. DNA was removed with DNase I (Promega). Removal of DNA from the RNA samples was confirmed by performing real-time PCR on 100 ng of total RNA using the Avin_38560-qPCR primer set (table 2), but without a reverse transcriptase step and those RNA samples found to yield C_t values larger than 35 were judged to be sufficiently free of contaminating DNA for further analysis. Once more, total RNA was quantified using microspectrophotometry (NanoDrop Technologies) while the A_{260}/A_{280} ratio was always above 2. Purified RNA was converted to cDNA immediately or stored frozen at –80°C.

cDNA Synthesis

First-strand cDNA synthesis was performed using Superscript II RT (Invitrogen). Briefly, 1.5 μg of each RNA sample was reverse transcribed into cDNA. RNA was added to 1 μl of 250 ng/ μl random hexamer primers, 1 μl of 10 mM dNTP mix (Finnzymes) and RNase-free water to make 12 μl . The mixture was denatured at 65°C for 5 min and immediately chilled. 4 μl of 5 $\times$ first-strand buffer, 2 μl of 0.1 M DTT, 1 μl of 40 units/ μl RNaseOUT (Invitrogen) and 1 μl of 200 units/ μl SuperScript II (Invitrogen) was

then added and the volume was adjusted to 20 μ l. The mixture was incubated at 25°C for 10 min, 42°C for 50 min and finally 70°C for 15 min to inactivate the reaction.

RT-qPCR

The oligonucleotide primers used for RT-qPCR are listed in table 2. All primers were supplied by Invitrogen. The primers were designed using Primer-BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and optimized to an equal annealing temperature of 60°C. Each primer pair was further assessed for specificity with melting curve analysis and gel electrophoresis of amplification products.

One microliter of diluted cDNA was added to the primer pair mix and SYBR Green Master Mix (Applied Biosystems) in each well. RT-qPCR was conducted on an MxPro Mx3005P PCR system and analyzed with MxPro v4.01 software (Stratagene). The PCR cycling conditions were 95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 1 min; and 95°C for 1 min, 60°C for 30 s and 95°C for 30 s. Plates were run for 40 cycles and fluorescence intensity was measured after every cycle. For each target sequence, the average cycle number at which fluorescence was detected above background in the exponential phase of amplification was obtained. No reverse transcription controls were included. The efficiency of each RT-qPCR reaction was calculated using the 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 [2001]. Relative expression ratio per gene: $R = E_{GOI}^{\Delta(C_t, CONTROL - C_t, SAMPLE)} / E_{REF}^{\Delta(C_t, CONTROL - C_t, SAMPLE)}$. *recA* (Avin_38560) was used as internal control. Statistical significance was obtained by ANOVA followed by Tukey's multiple comparison test using GraphPad Prism v5.0 (GraphPad Software, San Diego, Calif., USA).

Heterologous Coexpression of AvPPIB with AvdnaK or AvlpxH in E. coli and Purification of Protein Complexes

AvPPIB was coexpressed with AvdnaK or AvlpxH as two separate polypeptides with a (His)₆ tag fused at the N-terminus of AvPPIB and an S tag fused at the C-terminus of AvdnaK or AvlpxH, using the pCDFDuet-1 vector (Novagen). The primers used were AvPPIB.H.Duet-F: 5'-AAAGGATCCAATCAAGC-TGCAAACCAACACG-3' with AvPPIB.H.Duet-R: 5'-AAA-GCGGCCGCTTATTCGACGATCTCGGCCTTC-3', and Avdna K.S.Duet-F: 5'-GGGCATATGGGAAAAATCATCGGTATCG-ATC-3' with AvdnaK.S.Duet-R: 5'-AAACTCGAGCTTGTTGCCCTTGCTGTCTTG-3', or AvlpxH.S.Duet-F: 5'-GGA-CATATGACCACACTGCTGATCTCGGATC-3' with AvlpxH.S.Duet-R: 5'-AAAGGTACCGTCGATGGGAAAGGGTGTGACG-3', carrying restriction sites for ligation to the pCDFDuet-1 expression vector. The underlined nucleotides at each primer represent *Bam*HI, *Not*I, *Nde*I, *Xho*I, *Nde*I and *Kpn*I, respectively. The fragments excised from amplified *AvppiB* and *AvdnaK* or *AvlpxH* sequences were cloned between the corresponding sites of pCDFDuet-1 or pACYCDuet-1, resulting in AvPPIB-AvdnaK-pCDFDuet-1/pACYCDuet-1 and AvPPIB-AvlpxH-pCDFDuet-1/pACYCDuet-1. The absence of undesired alterations was checked by nucleotide sequencing. *E. coli* BL21 (DE3) cells harboring the resulting vectors were grown at 30°C and induced with isopropyl 1-thio- β -D-galactopyranoside for 4 h. The soluble coexpressed proteins were purified to high purity under native conditions by

taking advantage of the His tag fused to AvPPIB employing a Ni-resin column (Pierce). The purity of the protein complex was verified, and existence of both polypeptides was confirmed by SDS-PAGE and immunoblotting with antibodies against the His and S tags (Novagen), respectively.

Heterologous Expression of AvdnaK or AvlpxH in E. coli and Purification of Recombinant Proteins

The coding sequence of *AvdnaK* (YP_002801394) and the coding sequence of *AvlpxH* (YP_002799513) were amplified using PCR with *A. vinelandii* genomic DNA as a template. Both proteins were expressed with a (His)₆ tag fused at their N-terminus. The primers used were *AvdnaK*.H.Duet-F: 5'-AAAGGATCCGGGAAAAATCATCGGTATCGATCTG-3' with *Avdna K*.H.Duet-R: 5'-AAAAAGCTTCTTGTTGCCCTTGCTGTCTTG ACC-3', or *AvlpxH*.H.Duet-F: 5'-AAAGGATCCGACCACACTGCTGATCTCGGATCTG-3' with *AvlpxH*.H.Duet-R: 5'-AAGAAGCTTGTCGATGGGAAAGGGTGTGACGGTGG-3', carrying restriction sites for ligation to the pCDFDuet-1 expression vector. The underlined nucleotides represent *Bam*HI at each forward primer and *Hind*III at each reverse primer. The fragments excised from amplified *AvdnaK* and *AvlpxH* sequences were cloned between the corresponding sites of pCDFDuet-1, resulting in *AvdnaK*-pCDFDuet-1 and *AvlpxH*-pCDFDuet-1. The absence of undesired alterations was checked by nucleotide sequencing. Protein expression and purification was performed as described above.

Site-Directed Mutagenesis of AvPPIB

F99A and R43A point mutations were engineered using the gene-SOEing method described by Horton [1995]. The primers used were AvPPIB.Duet-F with AvPPIB_{F99A}-R: 5'-GTCCTTGACGTTGATGCGAACTGCGCGCTGG-3' or AvPPIB_{R43A}-R: 5'-GAAGCCGTCGATCACTGCGTGGAAGATGGTGC-3' and AvPPIB_{F99A}-F: 5'-CCAGCGCGCAGTTCGCAATCAACGTCAAGGAC-3' or AvPPIB_{R43A}-F: 5'-GCACCATCTTCCACGCA-GTGATCGACGGCTTC-3' with AvPPIB.Duet-R. Nucleotides encoding mutated amino acids are underlined. The primary PCR products were purified and then used as templates for the second round of PCR. The final products were digested with restriction enzymes corresponding to the cleavage sites introduced via PCR and ligated into appropriately digested pCDFDuet-1 expression vector, resulting in AvPPIB_{F99A}-pCDFDuet-1 and AvPPIB_{R43A}-pCDFDuet-1. The absence of undesired alterations was checked by nucleotide sequencing. Protein expression and purification was performed as described above.

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