UNIVERSIDADE FEDERAL DO PARANÁ

ERICK PARIZE

RELAÇÃO FUNCIONAL ENTRE AS PROTEÍNAS PII E AS PROTEÍNAS ALVO: FATOR DE TERMINAÇÃO DE TRANSCRIÇÃO RHO, DIGUANILATO CICLASE, FOSFODIESTERASE E UMP FOSFATASE

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Orientadora: Prof. Dr. Maria Berenice Reynaud Steffens
Co-orientador: Prof. Dr. Luciano Fernandes Huergo
Co-orientadora: Dra. Edileusa Cristina Marques Gerhardt

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **ERICK PARIZE** intitulada: **RELAÇÃO FUNCIONAL ENTRE AS PROTEÍNAS PII E AS PROTEÍNAS ALVO: FATOR DE TERMINAÇÃO DE TRANSCRIÇÃO RHO, DIGUANILATO CICLASE, FOSFODIESTERASE E UMP FOSFATASE**, sob orientação da Profa. Dra. MARIA BERENICE REYNAUD STEFFENS, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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RESUMO

As proteínas PII são amplamente distribuídas em bactérias, arqueias e plantas. PII atua como transdutora de sinais e realiza a regulação do metabolismo nesses organismos via interação proteína-proteína. Ensaios com PII de *Azospirillum brasilense* e *Escherichia coli* indicaram que essas proteínas possuem uma gama de possíveis novos alvos, estendendo sua importância e atuação no metabolismo. Quatro desses possíveis novos alvos são o Fator de terminação de Transcrição Rho, proteína conhecida por estar envolvida em pelo menos metade dos processos de liberação de RNA pós transcrição em bactérias, uma Diguanilato Ciclase (AbDGC) e uma Fosfodiesterase (AbPDE), proteínas envolvidas na síntese e degradação do mensageiro secundário c-di-GMP e UMP Fosfatase (UmpH) envolvida na homeostase de pirimidinas. Visando validar e caracterizar essas interações, ensaios de co-precipitação com as proteínas purificadas de *A. brasilense* e também de *Escherichia coli* foram realizados. As proteínas PII interagiram com os quatro alvos *in vitro*. A interação com Rho de *A. brasilense* (AbRho) ocorreu de forma estável com ambas as PII dessa bactéria, GlnZ e GlnB, na presença de ATP e ADP, a interação foi inibida na presença de 2-OG, também efetor de PII. Rho de *E. coli* (EcRho) também interagiu com as duas PII, GlnB e GlnK, no entanto a interação foi mais estável na presença de ADP. 2-OG também foi o fator limitante desse complexo. As proteínas AbDGC e AbPDE de *A. brasilense* já haviam sido caracterizadas como alvos de GlnZ *in vitro*, aqui as mostramos como alvos também de GlnB, a interação foi observada na presença de ATP e ADP e inibida por 2-OG. A proteína UmpH de *E. coli* interagiu somente com GlnK, a interação ocorreu na presença de ATP e ADP, e foi inibida por 2-OG. Considerando que 2-OG é uma molécula que sofre variações dentro da célula se acumulando em baixo nitrogênio e diminuindo em alto nitrogênio, é possível indicar que a interação dessas proteínas com PII deva ocorrer quando 2-OG decresce significativamente como em choque de amônio. Essas novas interações de PII sugerem função de PII na terminação da transcrição, no metabolismo do segundo mensageiro c-di-GMP e na homeostase da via de pirimidinas.

Palavras-chave: Complexo proteína-proteína, 2-oxogluturato, Terminação da transcrição, C-di-GMP, Pirimidinas.

ABSTRACT

Proteins of the PII family are widely distributed and are found in bacteria, archaea and plants. PII acts as a signal transducer and regulate central metabolism in these organisms via protein-protein interaction. Recent studies with PII from *Azospirillum brasilense* and *Escherichia coli* indicated that PII proteins may have additional targets and regulate a plethora of metabolism pathways. Four of these possible new targets are: the Rho Transcription Termination Factor, a protein known to be involved in at least half of the processes of RNA release after transcription in bacteria, a Diguanylate Cyclase (AbDGC) and a Phosphodiesterase (AbPDE), proteins involved in the synthesis and degradation of the secondary messenger c-di-GMP and UMP Phosphatase (UmpH) involved in pyrimidine homeostasis. In order to validate and characterize these novel PII interactions, co-precipitation assays were performed with purified proteins from *A. brasilense* and *E. coli*. The PII proteins interacted with the four targets *in vitro*. The interaction with *A. brasilense*'s Rho (AbRho) occurred in a stable manner with both PII paralogues present in this bacterium, GlnZ and GlnB. The AbRho-PII complex ocurred in the presence of the PII effectors ATP and ADP and was inhibited in the presence of 2-OG. *E. coli*'s Rho (EcRho) also interacted with the two PII, GlnB and GlnK, however the interaction showed a preference for ADP. 2-OG was also the limiting factor of this complex. The AbDGC and AbPDE proteins of *A. brasilense* had already been characterized as targets of GlnZ *in vitro*, here we show them as targets of GlnB, the interaction was observed in the presence of ATP and ADP and inhibited by 2-OG. The *E. coli* UmpH protein interacted only with GlnK, the interaction occurred in the presence of ATP and ADP and was inhibited by 2-OG. Considering that 2-OG is a molecule that suffers variations within the cell, accumulating in the absence of nitrogen and decreasing in high nitrogen, it is possible to indicate that the interaction of these proteins with PII must occur when 2-OG decreases significantly, in ammonium shock, for example. These new PII interactions suggest PII function in the transcription termination, metabolism of the second messenger c-di-GMP and in the pyrimidine homeostasis.

Keywords: Protein-protein complex, 2-oxogluturate, Transcription termination, C-di-GMP, Pyrimidines

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LISTA DE ABREVIATURAS

2-OG	2-Oxoglutarato
5’- pGpG	5′-fosfoguanilil-(3'-5')-guanosina
ACC	Acetil-CoA Carboxilase
ADP	5’ difosfato de adenosina
Amp^r	Resistência a ampicilina
AR	Função removedora de adenilil (ATase)
AT	Função de adenilação (ATase)
ATase	enzima adenililtransferase
ATP	5’ trifosfato de adenosina
BC	Biotina carboxilase
BCCP	Proteína carreadora de carboxi biotina
BSA	Albumina bovina
CD	Dicroísmo circular
C-di-GMP	diguanilato cíclico
DNA	Ácido desoxirribonucleico
DLS	Espalhamento dinâmico de luz (do inglês Dynamic light scattering)
DGC	Diguanilato Ciclase
DO600	Densidade óptica a 600 nm
DraG	Dinitrogenase redutase glicohidrolase
DraT	Dinitrogenase ADP-ribosil transferase
GlnZ-UMP	Proteína GlnZ uridililada
GMP	5’ monofosfato de guanosina
GOGAT	Glutamato sintase
GS	Glutamina sintetase
GTP	5’ trifosfato de guanosina
K_d	Constante de dissociação
IPTG	Isopropil β-D-1- tiogalactopiranosideo
kDa	Kilodalton

Km^r	Resistência a canamicina
LB	Luria-Bertani
MESG	2-amino-6-mercapto-7-metilpurina ribonucleosideo
NAD^+	Dinucleotideo de nicotinamida oxidado
NADH	Dinucleotideo de nicotinamida reduzido
nt	Nucleotídeo
NTP	Nucleotídeo Trifosfato
Ntr	Sistema de regulação de nitrogênio
NtrC-P	Proteína NtrC fosforilada
OB	conformação de ligação do oligonucleotídeo ou oligossacarídeo
pb	Pares de bases
PBS	Sítio primário de ligação ao RNA
pd	Polidispersividade
PDE	Fosfodiesterase
PCR	Reação em cadeia de polimerase
Pi	Fosfato inorgânico
PNP	Purina nucleosideo fosforilase
RNA	Ácido ribonucleico
SDS	Sitio secundário de ligação ao DNA
SDS	Dodecil sulfato de sódio
SDS-PAGE	Eletroforese em géis de poliacrilamida
TCA	Ciclo dos ácidos tricarboxílicos
UMP	Uridina monofosfato
UR	Removedor de uridilil
UT	Função de uridililação
UTP	Uridina trifosfato
v/v	Volume por volume

SUMÁRIO

1. INTRODUÇÃO

1.1. Proteínas PII

As proteínas PII são amplamente distribuídas nos domínios da natureza sendo encontradas, de forma conservada, em bactérias, arqueias, algas e plantas. Elas regulam vias metabólicas centrais nesses organismos através da interação com enzimas, transportadores de membrana e fatores de transcrição ou proteínas que controlam fatores de transcrição. As proteínas PII são transdutoras de sinais e sinalizam o balanço da concentração de carbono, nitrogênio e energia intracelular, via concentração de 2-oxoglutarato (2-OG), glutamina e [ATP]/[ADP], respectivamente. 2-OG pode indicar intracelularmente tanto a concentração de carbono quanto de nitrogênio (ARCONDÉGUY; JACK; MERRICK, 2001; HUERGO et al., 2012; HUERGO; CHANDRA; MERRICK, 2013).

A classificação das proteínas da família PII seguem dois critérios principais, a conservação da vizinhança gênomica e a similaridade dessas proteínas a nível da sequência primária. Essas proteínas são divididas em quatro grupos principais: 1) GlnB, predominantemente encontrado em proteobactérias e cianobactérias em que, com algumas exceções, o gene é monocistrônico, ou forma um operon com *glnA* ou *nadE*; 2) GlnK, quase invariavelmente o gene aparece no mesmo operon do transportador de amônio *amtB*; 3) NifI, genes que compõem o mesmo operon dos genes *nif*, relacionados a fixação biológica de nitrogênio, encontrado em diazotrofos metanogênicos e em algumas bactérias anaeróbias estritas; 4) PII-*New Group* (PII-NG), proteínas PII de proteobacterias no mesmo operon de genes que codificam para transportadores de metais (ARCONDÉGUY; JACK; MERRICK, 2001; SANT'ANNA et al., 2009).

Além da alta similaridade na sequência de aminoácidos as proteínas PII também apresentam alta similaridade estrutural. Proteínas PII com estruturas tridimensionais resolvidas se apresentam como proteínas homotriméricas, com uma massa molecular de aproximadamente 37 kDa, com cada subunidade contendo entre 12-13 kDa. A estrutura de barril compacto de cada monômero abriga duas α-hélices e quatro β-folhas arranjadas em motivo βαβ, conectado por uma alça longa denominada de alça T (loop T) (Fig.1). Essa alça está exposta ao solvente, é altamente flexível e conservada, sendo importante para muitas das interações realizadas por PII (CARR et al., 1996; TRUAN et al., 2010).

Além da alça T, PII apresenta mais duas alças menores, alça B e C. A alça B se encontra entre a segunda α-hélice e a quarta β-folha e a alça C está localizada na região C-

terminal da proteína. As alças T e B de uma subunidade e a alça C de outra formam uma fenda entre os monômeros importante para a ligação dos efetores (FORCHHAMMER, 2008; HUERGO; CHANDRA; MERRICK, 2013; TRUAN et al., 2010).

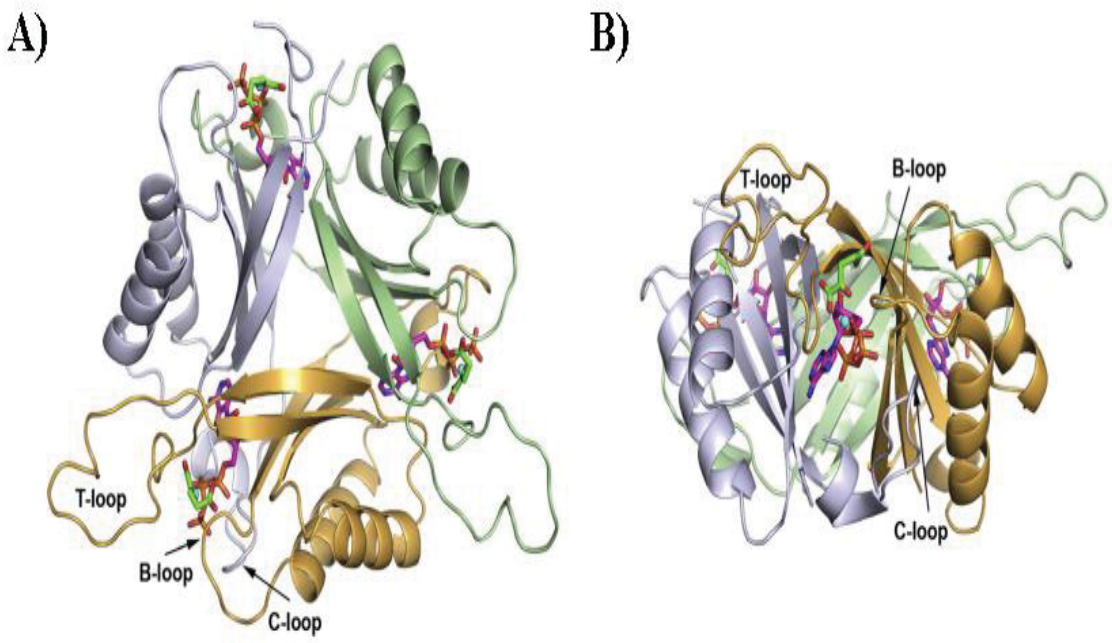

FIGURA 1. Representação do trímero de GlnZ de *A. brasilense*. O trímero está ligado à ATP (rosa), 2-OG (verde) e Mg^{2+} (azul). As alças B, C e T estão indicadas por setas. A) Vista superior. B) Vista lateral. Fonte: Truan e colaboradores, 2010.

O desempenho das PII como proteínas transdutoras de sinais depende da ligação de efetores que, ao se ligarem à proteína, mudam sua conformação permitindo a transmissão da informação para seus alvos (TRUAN et al., 2014). São efetores o ATP, ADP e 2-OG que tem sítios altamente conservados nas proteína PII e se ligam nas fendas entre os monômeros, sendo possível a ligação de ATP, ATP + Mg^{2+} (Mg.ATP), Mg.ATP + 2-OG ou ADP (HUERGO; CHANDRA; MERRICK, 2013; MERRICK, 2015; TRUAN et al., 2014). A variação [ATP]/[ADP] indica o nível energético celular (JIANG; NINFA, 2007). A variação da concentração de nitrogênio celular é sinalizado pelos níveis de glutamina e 2-OG que atuam como efetores de GlnD e PII, respectivamente (KAMBEROV; ATKINSON; NINFA, 1995). Em geral, a principal forma de assimilação de nitrogênio é através de amônio e, no caso de bactérias, geralmente ocorre pela via GS/GOGAT (glutamato sintase ou glutamina:2-oxoglutarato aminotransferase) (ARCONDÉGUY; JACK; MERRICK, 2001; MERRICK; EDWARDS, 1995).

Essa via possui dois passos, o primeiro catalisado pela proteína Glutamina Sintetase (GS):

$$NH_4^+ + \text{L-glutamato} + ATP \rightarrow \text{L-glutamina} + ADP + Pi$$

E o segundo pela Glutamato Sintase (GOGAT):

$$\text{L-glutamina} + \text{2-OG} + NADPH \rightarrow 2\ \text{L-glutamato} + NADP^+$$

Nesta via, 2-OG é o esqueleto carbônico para a síntese de glutamina e glutamato e, portanto, uma baixa concentração de 2-OG significa suficiência de nitrogênio. Ao contrário, quando sua concentração aumenta significa que os grupos nitrogenados da glutamina e glutamato estão sendo retirados para serem utilizados na biossíntese de outras moléculas, sinalizando escassez de nitrogênio (HUERGO et al., 2012; LEIGH; DODSWORTH, 2007). O 2-OG também é um intermediário do ciclo dos ácidos tricarboxílicos (TCA) e quando a célula está em limitação de carbono sua concentração cai rapidamente e de maneira significativa (YAN; LENZ; HWA, 2011; ZHANG; WEI; YE, 2013). Por isso, também é um indicador direto dos níveis de carbono.

Paralelamente à sinalização decorrente da ligação dos efetores, as proteínas PII sinalizam alterações metabólicas ao sofrerem mudança pós-traducional (CHELLAMUTHU et al., 2014). Essa modificação ocorre principalmente na alça T, e o tipo de modificação pode variar de acordo com o organismo. Em proteobactérias, usualmente ocorre uridililação da tirosina 51 (Tyr51). Em actinobactérias, a modificação é devida a adenililação também da Tyr51, enquanto que em cianobactérias ocorre fosforilação da serina 49 (Ser49) (HUERGO; CHANDRA; MERRICK, 2013; MAHESWARAN; URBANKE; FORCHHAMMER, 2004; MERRICK, 2015). Em arquéias, algas e plantas a modificação pós traducional não ocorre (CHELLAMUTHU et al., 2014). Em proteobactérias a modificação pós-traducional de PII é feita pela proteína GlnD (uridilil-transferase/removedora de uridilil), uma enzima bifuncional que liga glutamina. Em alta concentração de glutamina, esse metabólito se liga à GlnD ativando a função que remove UMP em PII, enquanto que a baixa concentração, a glutamina se desliga, prevalecendo a função que adiciona UMP em PII e essa modificação influencia as diferentes interações realizadas por PII (LEIGH; DODSWORTH, 2007).

1.2. Proteínas PII em *Azospirillum brasilense* e *Escherichia coli*

1.2.1. Proteínas PII e seus efetores

Azospirillum brasilense apresenta as proteínas GlnB e GlnZ enquanto *Escherichia coli* apresenta GlnB e GlnK (DE ZAMAROCZY, 1998; NINFA; JIANG, 2005). Nessas bactérias, a estrutura quaternária das proteínas PII é um homotrímero de aproximadamente 30 Å de altura, onde cada monômero contém 112 resíduos de aminoácidos (cerca de 12-13 kDa) e três alças funcionalmente importantes, alça T (resíduos 37-55), alça B (resíduos 82-88) e alça C (resíduos 102-105) (NINFA; JIANG, 2005; TRUAN et al., 2010). A alça T é a maior das três (contendo 18 resíduos de aminoácidos), é altamente flexível, possui o resíduo Tyr51 onde ocorre a modificação pós-traducional por uridililação e é importante para várias das interações feitas por PII (HUERGO; CHANDRA; MERRICK, 2013). As outras duas alças são importantes para ligação dos efetores ATP, ADP e 2-OG, que modificam a conformação da proteína também influenciando a interação de PII com seus alvos (NINFA; JIANG, 2005; TRUAN et al., 2010).

Os nucleotídeos de adenosina ATP e ADP se ligam nas fendas laterais entre duas subunidades de PII. A estrutura da proteína GlnZ, juntamente com ATP e ADP já foi resolvida (TRUAN et al., 2014). Assim como GlnK (CONROY et al., 2007; XU et al., 1998) e GlnB(PALANCA; RUBIO, 2017) de *E. coli*. As interações moleculares dos nucleotídeos de adenosina ligadas a GlnK e GlnB são muito similares ao reportado ao complexo visto em GlnZ de *A. brasilense* (TRUAN et al., 2014).

Na ligação de ATP em GlnB de *E. coli* os átomos N1 e N6 da adenina fazem interações de hidrogênio com a Val64 e o N3 com Thr29. A interação de N3 da adenina com Thr29 ocorre também em GlnK e GlnZ, essa interação é importante pois ela confere seletividade aos nucleotídeos de adenina contra as pirimidinas e contra a guanina, já que a guanina não tem o átomo N na posição 6 (PALANCA; RUBIO, 2017). O mutante GlnB Thr29Met em *E. coli* não liga ATP (JIANG et al., 1997).

O local de ligação do efetor 2-OG na proteína PII só foi observado em 2010, com a resolução da estrutura de GlnZ, ligada a Mg.ATP e 2-OG (TRUAN et al., 2010). Assim como os outros efetores, 2-OG também se liga nas fendas laterais entre as subunidades, no entanto, o 2-OG participa da coordenação do íon Mg^{2+} juntamente com ATP (TRUAN et al., 2010). O

íon Mg^{2+} apresenta seis pontos de coordenação em octaedro, sendo três com cada um dos grupos fosfatos do ATP, dois com 2-OG e um com o resíduo Gln39 (TRUAN et al., 2010).

A disponibilidade de nutrientes resulta em diferentes estados metabólicos da célula que afetam a concentração de ATP, ADP e 2-OG e, consequentemente, muda os ligantes de PII. Quando o nível de 2-OG é alto, por exemplo, PII está predominantemente ligada a Mg.ATP + 2-OG e a alça T projeta-se perpendicularmente ao eixo central. Quando o nível de 2-OG é baixo, PII liga-se predominantemente com ADP e a alça T assume uma conformação estendida, paralela ao eixo central (RADCHENKO; THORNTON; MERRICK, 2010; TRUAN et al., 2010).

A afinidade de PII aos efetores e a avaliação se essa ligação demonstra cooperatividade entre os três sítios de ligação, foram avaliados em *A. brasilense* e *E. coli* (JIANG; MAYO; NINFA, 2007; TRUAN et al., 2010). Foi confirmado em GlnZ e EcGlnB que 2-OG não se liga ao sítio de PII na ausência de Mg.ATP, já que Mg.ATP é necessário para estabilizar essa ligação (JIANG; MAYO; NINFA, 2007; TRUAN et al., 2010). Observou-se também que a ligação de Mg.ATP e 2-OG em GlnZ é sinérgica mas não apresenta cooperatividade (TRUAN et al., 2014). Em contraste, em EcGlnB e GlnK, na presença saturante de Mg.ATP, apenas a primeira molécula de 2-OG se liga com alta afinidade, as ligações da segunda e terceira moléculas sofrem cooperação negativa (JIANG; MAYO; NINFA, 2007; RADCHENKO; THORNTON; MERRICK, 2010).

A ligação de ATP em EcGlnB e GlnK, na presença de $MgCl_2$ (10mM), apresenta cooperatividade negativa com a afinidade diminuindo na ocupação do segundo e terceiro sítio. Na presença de 2-OG a afinidade por Mg.ATP aumenta, ocorrendo um efeito sinérgico mas a cooperatividade negativa continua (FORCHHAMMER, 2004; JIANG; NINFA, 2007). Para ADP esse efeito de cooperatividade não foi observado para EcGlnB mas sim para GlnK. É importante destacar que a ligação de ADP a GlnK é influenciada por 2-OG, ao aumentar a concentração dessa molécula a afinidade por ADP diminui (RADCHENKO; THORNTON; MERRICK, 2010). Em *A. brasilense,* o efeito de cooperatividade não foi visto em nenhuma condição para ATP ou ADP (TRUAN et al., 2014). Tanto em *E. coli* (KAMBEROV; ATKINSON; NINFA, 1995) como em *A. brasilense* (TRUAN et al., 2014) foi demonstrado que a afinidade de PII por ADP é maior que por Mg.ATP, por isso Mg.ATP não é um eficiente competidor para ADP, no entanto na presença de 2-OG a afinidade por Mg.ATP aumenta, permitindo a competição com ADP.

Os níveis intracelulares de ATP, ADP e 2-OG em *E. coli* foram medidos antes e após choque de amônio (RADCHENKO; THORNTON; MERRICK, 2010). Em células de *E. coli*

em situação de limitação de nitrogênio o nível de 2-OG é de cerca de 1,4 mM. No entanto, após a adição de 200 μM de amônio ao meio o nível de 2-OG cai rapidamente para cerca de 0,3 mM. À medida que o amônio vai sendo utilizado o nível de 2-OG retorna ao inicial (RADCHENKO; THORNTON; MERRICK, 2010).

Os níveis de ATP e ADP antes do choque de amônio alcançaram valores de aproximadamente 2 mM para ATP e 0,3 mM para ADP. Após 30 segundos da adição de amônio os níveis de ATP caíram para 1,8 mM e os de ADP subiram para 0,6 mM, no entanto em 3 minutos, as concentrações já haviam retornado ao nível pré choque de amônio, e após esse tempo ambos os efetores aumentaram gradualmente sua concentração, principalmente o ATP (RADCHENKO; THORNTON; MERRICK, 2010).

1.2.2. Regulação transcricional e pós-traducional das proteínas PII

As proteínas PII desempenham papel central no metabolismo e por isso são reguladas ao nível transcricional e pós-traducional. Em *A. brasilense* o gene *glnB* está em um operon com o gene *glnA* que codifica a proteína Glutamina Sintetase (GS) enquanto *glnZ* é monocistrônico; se diferenciando da maioria das proteínas GlnK de α-proteobactérias que formam um operon com o gene *amtB* (DE ZAMAROCZY, 1998). Em *E. coli* o gene *glnB* é monocistrônico e *glnK* é ligado a *amtB* (THOMAS; COUTTS; MERRICK, 2000; VAN HEESWIJK et al., 1996).

Em *A. brasilense* os genes *glnB* e *glnA* são co-transcritos, porém, *glnA* também pode ser transcrito como um único mRNA (DE ZAMAROCZY; DELORME; ELMERICH, 1990). Essa transcrição diferencial está relacionada com a fonte de nitrogênio. O gene *glnB* é precedido de dois promotores um promotor σ^{70} e um segundo promotor σ^{54}. O promotor σ^{70} é constitutivo e transcreve o operon *glnBA* com baixa eficiência. Na presença de amônio este operon é transcrito, no entanto, nessa condição um promotor exclusivo do gene *glnA* é regulado positivamente aumentando a transcrição desse gene. Sobre condições de fixação de nitrogênio, ou seja, baixo nitrogênio fixado, ocorre principalmente a transcrição do operon *glnBA* e esse aumento é ocasionado pelo segundo promotor σ^{54}, a frente de *glnB*. σ^{54} responde ao decréscimo de nitrogênio, via a proteína reguladora NtrC que regula positivamente o operon *glnBA*. O promotor exclusivo de *glnA* responde negativamente quando apenas nitrogênio molecular está disponível (DE ZAMAROCZY; DELORME; ELMERICH, 1990; DE ZAMAROCZY; PAQUELIN; ELMERICH, 1993; HUERGO et al., 2003). Quando há

limitação de nitrogênio o gene *glnZ* é transcrito pelo promotor σ^{54}, que é NtrC dependente (DE ZAMAROCZY, 1998).

Em *E. coli* o gene *glnB* possui quatro promotores descritos, mas nenhum deles está envolvido na regulação por nitrogênio (ARCONDÉGUY; JACK; MERRICK, 2001). Os promotores principais de *glnB* se encontram a montante do gene, mas a transcrição também pode se iniciar no promotor a montante do gene adjacente *yfhA* (LIU; MAGASANIK, 1993). Foi também descrito a inibição do gene *glnB* a partir da proteína PurF, proteína que reprime expressão de genes relacionados a síntese de purinas em condições de excesso desses nucleotídeos. Acredita-se que essa regulação pode estar envolvida em uma regulação sutil de GS, uma vez que que a glutamina está envolvida na síntese de purinas (HE; CHOI; ZALKIN, 1993).

O gene *glnK* em *E. coli* encontra-se ligado ao gene *amtB*, e amontante desse operon há um sítio de ligação de σ^{54} e um de NtrC, respectivamente. Assim, a expressão do operon é regulada de maneira dependente de NtrC (THOMAS; COUTTS; MERRICK, 2000; VAN HEESWIJK et al., 1996).

Ao nível pós-traducional a resposta ocorre de acordo à disponibilidade de nitrogênio. A regulação de PII em *A. brasilense* e *E. coli* ocorre pela ligação de um UMP no resíduo Tyr51 em cada alça T do trímero (ARAÚJO et al., 2008; FRANCIS; ENGLEMAN, 1978). Tanto o processo de uridililação quanto de desuridililação é realizado pela enzima bifuncional GlnD (Uridilil-Transferase/Removedora de Uridilil ou UTase/UR). A glutamina, possui um sítio de ligação em GlnD, que quando ligada inibe a atividade 'uridilil transferase' (UTase) e estimula a função 'removedora de uridilil' (UR). Na falta de glutamina a função UTase predomina (ZHANG et al., 2010). A presença de 2-OG e ATP são necessários para a função UTase aumentando sua atividade devido a ligação desses efetores no substrato de GlnD, a proteína PII (ARAÚJO et al., 2008; BROWN; SEGAL; STADTMAN, 1971; JIANG; PELISKA; NINFA, 1998a). A presença de um íon divalente Mg^{2+} ou Mn^{2+} também são importantes para as funções de GlnD (ZHANG et al., 2010).

A uridililação de PII ocorre à medida que o nível de amônio diminui. É possível observar 4 diferentes formas dessas proteínas em *E. coli*: PII não modificada, PII-(1)UMP, PII-(2)UMP e PII-(3)UMP. Em *A. brasilense* a formação dessas 4 formas de PII ocorre apenas em GlnZ; em GlnB é possível observar apenas as formas não modificada e a PII-(3)UMP (ARAÚJO et al., 2008; HUERGO et al., 2006b; INABA et al., 2009). Inaba e colaboradores justificaram esse fenômeno como um possível processo de cooperatividade positiva, ou seja, quando o primeiro grupo UMP é adicionado aumenta afinidade dos outros

sítios e a uridililação é tão rápida que os intermediários não são visualizados ou, ainda, que os híbridos podem não ser estáveis o suficiente e portanto não são observados.

A proteína GlnD, GlnB e GlnZ ou GlnK, juntamente com as proteínas NtrB, NtrC, GlnE e GS compõem o sistema central de regulação de nitrogênio, chamado de sistema Ntr (MERRICK; EDWARDS, 1995). Esse sistema consiste em uma cascata regulatória e as proteínas PII, como transdutoras de sinal, tem um papel importante controlando a atividade do sistema de acordo com a sinalização dos metabólitos intracelulares, glutamina, 2-OG, ATP e ADP.

1.2.3. Proteínas PII e o sistema Ntr

A enzima dodecamérica GS (Glutamina Sintetase) foi a primeira enzima descrita como regulada por proteínas PII (SHAPIRO, 1969). GS é regulada a nível transcricional e pós-traducional. A modificação pós-traducional ocorre em resposta ao aumento de amônio extracelular, através da adenililação de um resíduo de tirosina (Tyr-397) em cada uma das 12 subunidades de GS. A medida que o nível de amônio aumenta, consequentemente glutamina intracelular aumenta, grupos AMP são adicionados a GS, levando a uma progressiva inativação da enzima (REITZER, 2003). Esse processo de inativação é realizado pela proteína GlnE (Glutamina Sintetase Adenilil-Transferase ou ATase) por sua função adenilil transferase (AT). Essa enzima bifuncional é também responsável pela ação reversa em baixa glutamina a função removedora de adenilil (AR) ativando GS (AMON; TITGEMEYER; BURKOVSKI, 2010; HEESWIJK et al., 1993).

Em *A. brasilense,* testes com mutantes *glnZ*, *glnB* e duplo mutante demonstraram uma eficiente modificação de GS bem como sua reversão ao estado não modificado inferindo que PII não tem efeito sobre GlnE (DE ZAMAROCZY, 1998). Posteriormente Huergo e colaboradores (2006a) mostraram por um Western blot do padrão de modificação de GS em função do tempo após o choque de amônio, que embora GS estivesse adenililada em resposta ao aumento de amônio nos mutantes *glnB* e *glnZ*, os níveis de adenililação eram mais baixos que no selvagem, indicando uma possível atuação de PII nesse processo. Em *E. coli* a interação com GlnE ocorre tanto com GlnB como com GlnK via alça T, mas GlnB possui uma atividade mais efetiva na regulação (ATKINSON; NINFA, 1998; VAN HEESWIJK et al., 1996, 2000).

Em *E. coli,* sob limitação de nitrogênio, a concentração de glutamina intracelular é baixa e 2-OG é elevada. Nessa condição a proteína GlnB é uridililada por GlnD, se liga a

Mg.ATP + 2-OG e interage com o domínio AT de GlnE o que resulta na estimulação do domínio AR, que consequentemente ativa GS. Quando nitrogênio está em abundância, os níveis de 2-OG caem o de glutamina sobe, GlnB em sua forma não uridililada se liga preferencialmente a ADP, interage com a porção AR estimulando a função AT e GS é inativada (JIANG; NINFA, 2009; JIANG; PELISKA; NINFA, 1998a, 1998b; REITZER, 2003). Glutamina também se liga à GlnE, na porção AT, e possui a função de estabilizar a interação entre GlnB e GlnE e impedir a ligação de GlnB-UMP (JIANG; MAYO; NINFA, 2007; JIANG; NINFA, 2007).

Outro alvo de PII que ocorre em *E. coli* e *A. brasilense* é a proteína NtrB. As proteínas NtrB e NtrC fazem parte de um sistema de dois componentes, que geralmente contém uma histidina quinase (NtrB) e uma proteína regulatória (NtrC). Através da fosforilação de um resíduo conservado na histidina quinase, a mesma fosforila a proteína regulatória que por sua vez se liga às regiões regulatórias de genes alvo, induzindo ou reprimindo a transcrição (NINFA; BENNETT, 1991; NINFA et al., 1993).

Em condições limitantes de nitrogênio a concentração de glutamina intracelular é baixa e de 2-OG é alta. GlnB-UMP não consegue se ligar à NtrB, que em sua forma livre catalisa sua auto fosforilação e transfere esse grupo para o resíduo Asp54 de NtrC ativando-a. Em resposta à limitação de nitrogênio, NtrC-P, que possui um domínio C-terminal de ligação ao DNA, estimula a transcrição de genes alvos ao formar o complexo NtrC-RNA polimerase holoenzima (NORTH et al., 1996). Os produtos dos diferentes genes ativados estão envolvidos na assimilação de fontes alternativas de nitrogênio como nitrato e aminoácidos (JIANG; NINFA, 1999). Em altos níveis de nitrogênio a concentração de glutamina aumenta e de 2-OG diminui, GlnB em sua forma não modificada se liga a NtrB, inibindo sua auto fosforilação e estimula a desfosforilação de NtrC-P inativando-a (JIANG; PELISKA; NINFA, 1998c).

1.2.4. Proteínas PII e o transportador de amônio AmtB

Como mencionado em *A. brasilense* os genes *glnZ* e *amtB* não estão no mesmo operon, mas o produto proteico desses dois genes interagem intracelularmente. AmtB, é uma proteína homotrimérica transmembrana com um poro em cada subunidade que age como transportador passivo de íons amônio quando os níveis de amônio estão baixos (WINKLER, 2006).

AmtB é um dos alvos mais conhecidos de PII. Em estado de limitação de amônio, AmtB está ativa para facilitar a entrada de amônio na célula. Nessa condição a concentração de 2-OG esta alta e glutamina baixa não permitindo a interação entre AmtB e PII que estão uridililadas e ligadas a Mg.ATP + 2-OG. Com choque de amônio, a concentração de 2-OG diminui a de glutamina aumenta, PII é desuridililada e passam a interagir com AmtB inativando-a (HUERGO et al., 2006a).

Em *A. brasilense* a interação *in vitro* confirmou que a ligação de AmtB com GlnZ e GlnB só ocorre quando as mesmas não estão uridililadas e preferencialmente na presença de ADP, e é inibida na presença de Mg.ATP + 2-OG (HUERGO et al., 2007; RODRIGUES et al., 2011). Como já mencionado não é possível observar GlnB parcialmente uridililada, mas ensaios foram realizados com GlnZ parcialmente uridililadas e sua interação com AmtB foi positiva mas, à medida que mais grupos UMP foram adicionados a GlnZ a interação foi se tornando menos estável (RODRIGUES et al., 2011).

Em *E. coli,* evidências *in vivo* já mostraram a associação entre GlnK e AmtB em choque de amônio (DURAND; MERRICK, 2006) e a estrutura desse complexo também já foi resolvida (CONROY et al., 2007). A ligação de AmtB e GlnK ocorre no momento que amônio é adicionado ao meio e as concentrações de 2-OG e ATP diminui, e a de ADP aumenta, nesse momento GlnK é desuridililada, liga-se a ADP e é sequestrada por AmtB (COUTTS et al., 2002). Quando GlnK se liga à ADP a alça T adota uma conformação estendida que é crítica para a interação, pois a alça se insere profundamente nos poros da face citoplasmática de AmtB, bloqueando o transporte de amônio (CONROY et al., 2007).

A medida que a concentração de 2-OG volta a subir, Mg.ATP e 2-OG passam a ocupar os sítios de interação novamente modificando a conformação da alça T desfazendo a interação AmtB-GlnK, e GlnK volta a ser uridililada (DURAND; MERRICK, 2006). Estudos *in vitro* corroboram com o modelo em *E. coli*. A dissociação do complexo ocorre na presença de Mg.ATP + 2-OG e é estabilizado na presença de ADP (RADCHENKO; THORNTON; MERRICK, 2010).

1.2.5. Proteínas PII e a fixação biológica de nitrogênio

Os genes *nif* codificam proteínas envolvidas na fixação biológica de nitrogênio, processo realizado por apenas alguns procariotos, como *A. brasilense* mas não por *E. coli*, que converte N_2 atmosférico em NH_3 passível de ser utilizado nas funções metabólicas (DIXON; KAHN, 2004). A transcrição desses genes é altamente regulada, em *A. brasilense* a montante

desses genes um promotor σ^{54} é ativado quando a célula se encontra em situação de limitação de nitrogênio por uma proteína chamada de NifA (DRUMMOND; CONTRERAS; MITCHENALL, 1990).

NifA é um proteína conservada em proteobactérias diazotróficas. Em muitos organismos a expressão de *nifA* está sobre controle de NtrC, no entanto, em *A. brasilense* isso não ocorre e há indicativos que o gene *nifA* é constitutivamente transcrito por um promotor σ^{70} (FADEL-PICHETH et al., 1999). A regulação de sua atividade é realizada diretamente pela interação com GlnB (SOTOMAIOR et al., 2012). NifA possui domínio C-terminal de ligação ao DNA mas diferente de NtrC, que é ativada pela fosforilação, é ativada pela interação com GlnB em seu domínio GAF N-terminal (ARAÚJO et al., 2004; ARSENE; KAMINSKI; ELMERICH, 1996; SOTOMAIOR et al., 2012). GlnZ não pode substituir GlnB nessa função (DE ZAMAROCZY, 1998).

Em condições de alto nitrogênio, e consequentemente alta glutamina intracelular, os genes da fixação biológica de nitrogênio não são transcritos e a proteína NifA se encontra em sua forma livre. Nessa condição, o domínio GAF possui atividade inibitória sobre NifA inativando-a. Quando os níveis de nitrogênio diminuem a proteína GlnB é uridililada, se liga no domínio GAF e NifA se torna ativa transcrevendo os genes *nif* (ARAÚJO et al., 2004; CHEN et al., 2005; DE ZAMAROCZY; PAQUELIN; ELMERICH, 1993). A formação do complexo GlnB/domínio GAF de NifA *in vitro* ocorre na presença de Mg.ATP + 2-OG (SOTOMAIOR et al., 2012).

O processo da fixação biológica de nitrogênio é energeticamente dispendioso, por esse motivo necessita ser altamente regulado. Em *A. brasilense* essa regulação ocorre a nível transcricional através da regulação dependente de NifA e GlnB e pós-traducional chamado de *switch-off / switch-on* da Nitrogenase. O processo de fixação de nitrogênio é catalisado pelo complexo da Nitrogenase, que é composto de duas proteínas: a Dinitrogenase (NifDK) e a Dinitrogenase Redutase (NifH). A Dinitrogenase Redutase é responsável pela hidrólise do ATP e transferência de elétrons para a Dinitrogenase, a qual contém o sítio de redução de N_2 (SEEFELDT; HOFFMAN; DEAN, 2009).

As proteínas Dinitrogenase ADP-Ribosiltransferase (DraT) e Dinitrogenase Redutase Glicohidrolase (DraG) são as principais proteínas envolvidas na regulação pós-traducional da Nitrogenase. DraT é responsável por adicionar uma molécula de ADP-Ribose a partir da molécula de NAD^+ no resíduo de arginina (Arg101) de umas das subunidades da proteína homodimérica NifH, resultando em sua inativação (*switch-off*). Isso ocorre devido à adição desse grupo impedir a interação entre NifH e NifDK limitando a transferência de elétrons

essencial para a redução de N_2. DraG por sua vez possui a função de remover esse grupamento ADP-ribose ativando NifH (*switch-on*), permitindo sua interação com NifDK e consequentemente a transferência de elétrons e a fixação biológica de nitrogênio (HUERGO et al., 2006a, 2006b, 2012).

Ensaios demonstraram que a ADP-ribosilação de NifH está sincronizada com a desuridililação de PII e o sequestro principalmente de GlnZ e DraG na membrana (HUERGO et al., 2006a). Estudos posteriores mostraram que AmtB-GlnZ-DraG formavam um complexo ternário em condições de alto amônio (HUERGO et al., 2007). Ensaios de interação *in vivo* mostraram que DraT interage com GlnB desuridililada e DraG com GlnZ em ambas as formas, no entanto GlnZ-DraG é mais estável quando PII está desuridililada (HUERGO et al., 2006a, 2006b, 2012).

Estudos *in vitro* e de estrutura mostraram que a interação entre DraG-GlnZ é estabilizada por ADP. Diferentemente da maioria das interações conhecidas a interação DraG-GlnZ não ocorre via alça T, três monômeros de DraG interagem na face lateral do trímero de GlnZ na região de interface entre duas subunidades (GERHARDT et al., 2012; HUERGO et al., 2009; RAJENDRAN et al., 2011). Já DraT-GlnB, interage via alça T, a interação também é estabilizada na presença de ADP mas, nesse caso, houve interação *in vitro* em todos as condições testadas, inclusive na presença de Mg.ATP + 2-OG, no entanto essa interação foi pouco estável demonstrando que ocorre inibição nessa condição (HUERGO et al., 2009; MOURE et al., 2013).

O modelo geral da regulação de DraT e DraG discorre que em condições de alto nitrogênio como o caso de choque de amônio, o nível intracelular de glutamina aumenta, desuridililando as proteínas PII, através de GlnD (ARAÚJO et al., 2008), com a diminuição dos níveis de 2-OG, ADP se liga à PII estabilizando os complexos DraT-GlnB e DraG-GlnZ-AmtB, nessa condição DraT é ativada, inativando NifH, enquanto que DraG é sequestrada, juntamente a GlnZ pela proteína AmtB (HUERGO et al., 2009). Em baixa concentração de nitrogênio, como em fixação de nitrogênio, o nível de 2-OG aumenta, GlnD uridilila PII, que se ligam a Mg.ATP + 2-OG desfazendo seus complexos com GlnB-DraT e GlnZ-DraG-AmtB, nessa condição DraT é inativada e DraG volta ao citoplasma e retira o grupamento ADP-Ribose de NifH permitindo a transferência de elétrons e a fixação de nitrogênio pela Nitrogenase (HUERGO et al., 2012; MOURE et al., 2013).

A caracterização da interação de PII com as proteínas envolvidas no metabolismo de nitrogênio já são conhecidas e bem estabelecidas. No entanto, em 2010, Bourrellier e colaboradores publicaram o primeiro exemplo de proteína não envolvida com o metabolismo

de nitrogênio, mas cuja a atividade é influenciada por PII em *Arabdopsis thaliana* (BOURRELLIER et al., 2010). A proteína em questão era a Carreadora de Carboxil Biotina (BCCP), subunidade da proteína Acetil-CoA Carboxilase (ACC), relacionada com síntese de lipídeos.

1.2.6. Proteínas PII e seus novos alvos

A proteína ACC catalisa o passo limitante na biossíntese de lipídeos que é a produção de malonil-CoA a partir de acetil-CoA que é usado no alongamento da cadeia dos ácidos graxos. A ACC típica em bactéria é composta de 4 diferentes polipeptídios divididos em três unidades funcionais: o homodímero Biotina Carboxil (BC), a Carreadora de Carboxil Biotina (BCCP) e um heterotetrâmero a Carboxiltransferase (CT) (CRONAN; WALDROP, 2002; TONG, 2013). BC e BCCP formam um complexo estável formado de dois dímeros de BC ligados a 4 monômeros de BCCP (BROUSSARD et al., 2013). A reação ocorre em dois passos, primeiro BC catalisa a carboxilação da biotina, que está covalentemente ligada a BCCP, no segundo passo CT transfere o grupo carboxil da carboxibiotina para acetil-CoA gerando malonil-CoA (TONG, 2013).

A interação BCCP-PII que primeiramente só havia sido visualizada em planta posteriormente foi visualizada em bactéria, especificamente em *Escherichia coli* e *A. brasilense* (RODRIGUES et al., 2014). Utilizando a proteína BCCP de *E. coli* e realizando co-precipitação com PII de *A. brasilense,* GlnB e GlnZ e *E. coli*, GlnB e GlnK, foi mostrado a interação de BCCP com todas. No entanto, a interação do complexo estável e ativo, BCCP-BC, só ocorre na presença das GlnB, formando um complexo ternário. Esse complexo ternário necessita Mg.ATP para ocorrer. Mg.ATP + 2-OG inibe a formação desse complexo (GERHARDT et al., 2015).

A atividade de ACC também foi mensurada na presença e ausência de GlnB. Na reação contendo ATP (10 mM) e Mg^{2+} (5 mM) houve uma diminuição da atividade de 70% na presença de GlnB de *A. brasilense* e 40% para GlnB de *E. coli*. No entanto, na presença de 1 mM de 2-OG essa inibição era interrompida, confirmando os dados de interação. A proteína GlnB na sua forma totalmente uridililada ou com a alça T parcialmente deletada não conseguiu interagir com BC-BCCP, portanto, não teve influência na atividade de ACC (GERHARDT et al., 2015).

A regulação dessa interação seria a seguinte, quando os níveis de carbono baixam ou de nitrogênio aumentam, os níveis de 2-OG caem (YAN; LENZ; HWA, 2011; ZHANG; WEI;

YE, 2013). A redução na concentração de 2-OG permitiria a interação de GlnB com BCCP-BC inibindo ACC e consequentemente a síntese de lipídios. Quando o carbono está disponível ou nitrogênio está baixo, condição em que os níveis de 2-OG aumentam e GlnB é uridililada, impede a interação GlnB-BCCP-BC permitindo a síntese de ácidos graxos em altas taxas. Como 2-OG é o principal fator limitante nessa interação, em resumo, o nível de 2-OG só se encontra baixo quando os níveis de nitrogênio estão altos, mas os de carbono estão baixos (GERHARDT et al., 2015). Recentemente dados *in vivo* dessa interação foram observados, em células de *E. coli* super-expressando GlnB que apresentaram síntese de malonil-CoA 30% menor que as células sem a super-expressão indicando a inibição de GlnB. Enquanto que nos mutantes Δ*glnB* e Δ*glnBglnK* foi observado um acumulo de ácidos graxos de 22% e 80%, respectivamente (RODRIGUES et al., 2019).

Recentemente, Gerhardt (2015) realizou um ensaio imobilizando a proteína GlnZ de *A. brasilense* com cauda de histidina em uma coluna de afinidade e posteriormente carregou o extrato proteico dessa bactéria na coluna. Após isso as proteínas ligadas a GlnZ foram eluídas com Mg.ATP + 2-OG e identificadas. A partir desses resultados foi gerado uma lista de possíveis novos alvos de GlnZ que podem expandir o conhecimento acerca dessa proteína e dentre elas estavam o Fator de Terminação de Transcrição Rho, uma Diguanilato Ciclase, uma Fosfodiesterase. Huergo (não publicado), também realizou um ensaio semelhante, mas com as PII de *E. coli*, GlnK e GlnB, como resultado e possíveis alvos em *E. coli* foram obtidos o Fator de Terminação de Transcrição Rho e a UMP Fosfatase.

1.3. Fator de Terminação de Transcrição Rho

O processo de terminação da transcrição em procariotos pode envolver um de dois mecanismos bem conservados: a terminação intrínseca que ocorre em sequencias específicas presentes no final de operons e a terminação dependente do fator Rho que é executado pela ATPase hexamérica dependente de RNA denominada Fator de Terminação de Transcrição Rho (MITRA et al., 2017; PETERS; VANGELOFF; LANDICK, 2011). O evento de terminação programado é extremamente importante para parar a expressão gênica quando não é mais necessária e para a reciclagem da RNA polimerase (RNAP) (MITRA et al., 2017; PETERS; VANGELOFF; LANDICK, 2011; RAY-SONI; BELLECOURT; LANDICK, 2016).

O gene *rho* é encontrado em mais de 90% dos genomas bacterianos sequenciados e a proteína Rho é conservada na maioria das espécies do domínio Bacteria o que sugere que sua

origem é bastante antiga e que as funções dependentes de Rho não são facilmente substituídas (D'HEYGERE; RABHI; BOUDVILLAIN, 2013). Entre as bactérias que não possuem a proteína Rho incluem-se as do filo *Cyanobacteria*, a classe *Mollicutes* e alguns membros dos gêneros *Clostridia* e *Bacilli* (D'HEYGERE; RABHI; BOUDVILLAIN, 2013; MITRA et al., 2017). Em *E. coli* é estimado que Rho esteja envolvida em 20 a 50% dos eventos de terminação da transcrição (PETERS et al., 2009).

A forma homohexamérica de Rho é a principal forma em que essas proteínas são encontradas, mas elas podem assumir outras estruturas quaternárias dependendo da concentração de proteína, o ambiente iônico que ela se encontra ou da presença de cofatores (GEISELMANN et al., 1992). Geralmente elas possuem um formato de anel com diâmetro de 120 Å e a altura de 90 Å (YU et al., 2000). Rho na ausência de RNA pode existir em dois formatos, no de anel fechado com 6 subunidades ou no formato de anel aberto que pode ser hexamérico ou pentamérico. Ambos os formatos são funcionais e a versão aberta pode se tornar fechada na presença de uma fita simples de ácido nucléico (GAN; RICHARDSON, 1999; YU et al., 2000). As interações entre as subunidades ocorrem entre uma alça que conecta a segunda e terceira α–hélice do domínio N-terminal de uma subunidade com a região de interação da subunidade adjacente (YU et al., 2000).

A proteína Rho mais extensivamente estudada é a de *E. coli* que possui 419 aminoácidos e três sítios funcionais muito importantes por monômero (Fig. 2). O sítio de ligação primário de RNA (PBS), que se encontra no domínio N-terminal e envolve os aminoácidos 22 a 116; o sítio de ligação e hidrólise de ATP, que contém os motivos Walker A e B, está localizada dentro do domínio C-terminal e envolve os aminoácidos 179 a 183, essa região possui um alça chamada de alça P, que carrega significante homologia com proteína F1 ATPase (DOMBROSKI et al., 1988; DOMBROSKI; PLATT, 1988); e, por fim, o sítio de ligação secundário de RNA (SBS) no domínio C-terminal e que compreende duas alças, a Q (aminoácidos 278 a 290) e a R (aminoácidos 322 a 326).

A região N-terminal onde se encontra o sítio PBS, contém três α–hélices e quatro β–folhas que formam a chamada conformação de ligação à oligonucleotídeo (OB). Essa conformação é suficiente para ligação de bases pirimídicas mas é pequena para a ligação das púricas (BOGDEN et al., 1999; SKORDALAKES; BERGER, 2003; WEI; RICHARDSON, 2001). E essa é uma das razões da Rho ter mais afinidade de ligação por sequencias ricas em pirimidinas que purinas.

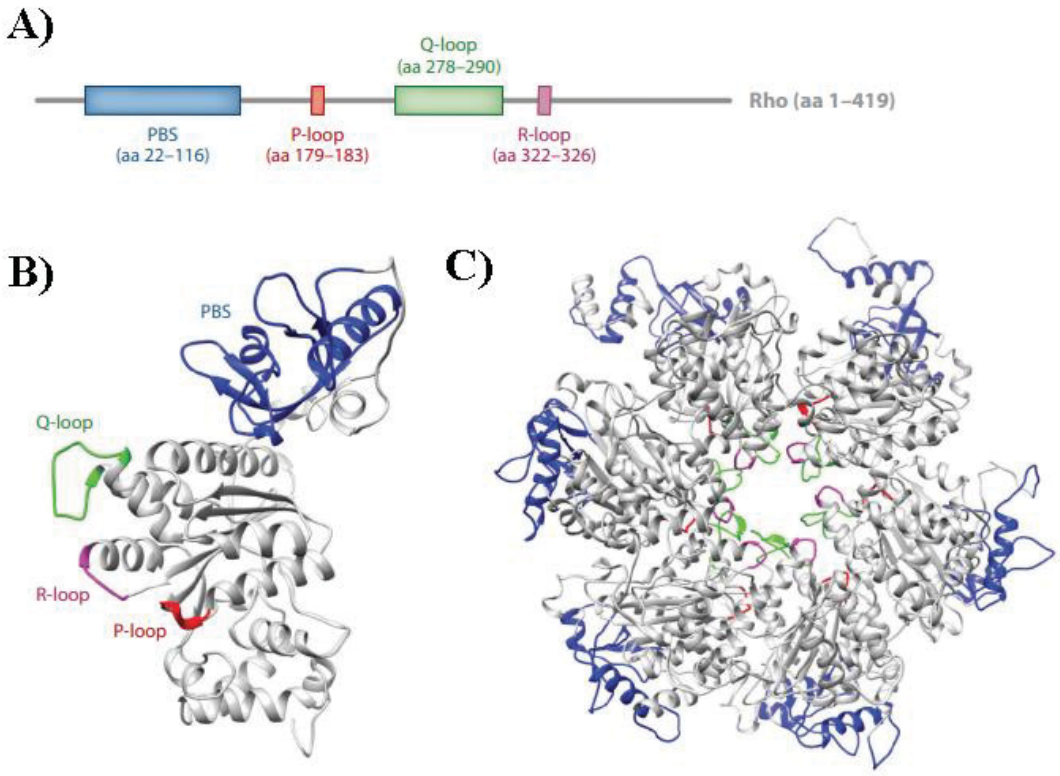

FIGURA 2. Representação do hexâmero de Rho de *E. coli*. O sítio primário de ligação do RNA (PBS) (azul), pode se ligar a fitas simples de DNA ou RNA. A alça P (vermelho) é associada a ligação de ATP. O sítio secundário de ligação ao RNA (SBS) compreende a alça Q (verde) e alça R (roxo). A) Organização dos domínios de Rho de *E. coli* B) Monômero C) Hexâmero. Fonte: Mitra e colaboradores, 2017.

O sítio de reconhecimento e ligação da proteína Rho ao RNA é chamado de *rut* (*Rho utilization*) (CHEN; RICHARDSON, 1987). Apesar da importância desse sítio, não há característica consenso que permita defini-lo ou detectá-lo precisamente. A sequência é altamente degenerada e tem por volta de 70-80 nucleotídeos que são requeridos para abranger toda periferia da porção terminal N-terminal de Rho (PBS), sendo que em cada monômero se ligam duas bases pirimídicas, citosina preferencialmente, e 9-13 bases fazem as ligações entre os monômeros. Geralmente esse sítio não apresenta estrutura secundária (ALIFANO et al., 1991; BEAR et al., 1985; MORGAN et al., 1985; NADIRAS et al., 2018).

O sítio de ligação do ATP da proteína Rho está localizado na interface da região C-terminal das subunidades. Todas as seis fendas entre as subunidades tem habilidade de ligar nucleotídeos, mas apenas três moléculas de ATP se ligam, alternadas com moléculas de RNA apresentando cooperatividade negativa (GEISELMANN; VON HIPPEL, 1992; STITT, 2001). O sítio SBS é caracterizado pelas alças Q e R. Enquanto a alça R está envolvida na ligação do

ATP e do RNA (XU; KOHN; WIDGER, 2002), a alça Q se estende ao centro do anel formado por Rho e interage unicamente com o RNA (KIM; PATEL, 2001).

O mecanismo de terminação da transcrição é complexo e envolve vários passos. Primeiramente Rho precisa se ligar ao sítio *rut* (CHEN; RICHARDSON, 1987). O RNA se envolve ao redor de PBS de cada monômero e então passa através do centro do anel fazendo contanto com SBS. A atividade de hidrólise de ATP é então ativada e Rho começa a se movimentar pelo RNA no sentido 5'→ 3'. A proximidade da alça R ao domínio de ligação a ATP ou a alça P sugerem uma explicação para a ligação de RNA e a hidrólise de ATP estarem acopladas (KIM; PATEL, 2001).

O processo de translocação ainda está em debate, mas o modelo mais aceito é o chamado *Tethered tracking model* (Fig. 3). Esse modelo propõe que após Rho estar ligada ao RNA nos dois sítios, ela se desloca ao longo do RNA mas ainda ligada ao sítio *rut,* formando uma estrutura em formato de zíper (STEINMETZ; PLATT, 1994). A zona de terminação geralmente se encontra de 60 a 90 nucleotídeos a jusante de *rut* e a RNAP faz pausas nessa zona, no entanto, nem todo sítio de pausa da RNAP é zona de terminação (KASSAVETIS; CHAMBERLIN, 1981). A terminação é induzida quando Rho entra em contato com o canal de saída de RNA da RNAP, liberando o transcrito. O mecanismo do acesso de Rho ao híbrido DNA:RNA para liberar RNA ainda está sendo investigado. Entre as hipóteses está a que Rho empurraria o complexo de alongamento para ter acesso ao híbrido ou que a interação com o canal de saída da RNAP provoca uma mudança conformacional na proteína permitindo o acesso (Fig. 3) (DUTTA; CHALISSERY; SEN, 2008; RICHARDSON, 2002).

A liberação do transcrito envolve a capacidade helicase de Rho para desprender o RNA sintetizado do DNA molde. A taxa de translocação de Rho sobre o RNA ou no híbrido DNA:RNA, é a mesma (20 nucleotídeos/segundos a 37°C). A taxa de hidrólise de ATP também não é alterada quando Rho está sobre um RNA simples fita ou sobre um híbrido (WALSTROM et al., 1997; WALSTROM; DOZONO; VON HIPPEL, 1997).

1.3.1. Funções do Fator de Terminação de Transcrição Rho

A proteína Rho desempenha uma variedade de funções na fisiologia celular, sendo a liberação do transcrito a função mais conhecida (CARDINALE et al., 2008; PETERS et al., 2009, 2012). Como em bactérias não há a presença de uma membrana envoltória para o DNA os processos de transcrição e tradução geralmente ocorrem em simultâneo com os ribossomos se ligando no mRNA nascente e bloqueando o acesso das proteínas Rho aos sítios de ligação

(HARINARAYANAN; GOWRISHANKAR, 2003; ROBERTS, 1969). Quando a tradução cessa por limitação de nutriente, por exemplo, os ribossomos se desligam desbloqueando o sítio *rut* e possibilitando acesso da proteína Rho que termina a transcrição, prevenindo o acúmulo de mRNA, uma vez que Rho libera o mRNA no citoplasma, deixando-o passível de ser degradado pelas ribonucleases (HARINARAYANAN; GOWRISHANKAR, 2003; ROBERTS, 1969).

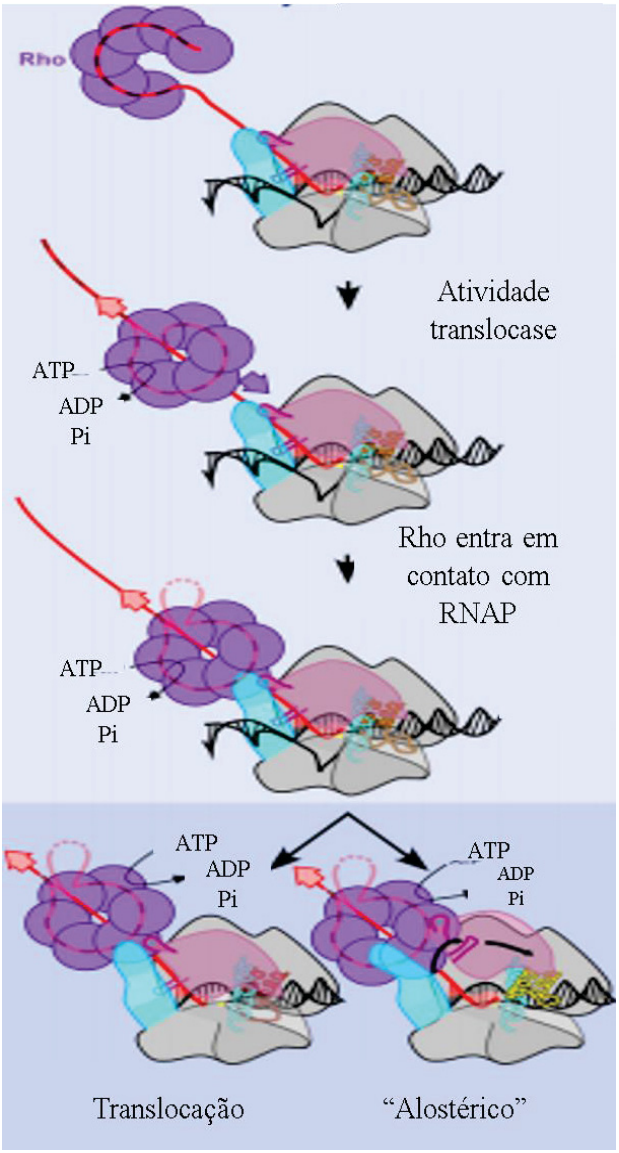

FIGURA 3. Representação do modelo da terminação mediada pelo fator Rho. A proteína Rho se liga ao sítio *rut* do RNA alvo via sitio PBS encontrado na periferia da proteína Rho, posteriormente o RNA é levado ao sítio SBS e a translocação dependente de ATP se inicia. Durante a translocação o sitio *rut* permanece ligado a PBS e o RNA e forma uma estrutura em formato de zíper até encontrar a RNAP liberando o transcrito. Fonte: Peters e colaboradores, 2011.

Além de contribuir com a terminação da transcrição, Rho também regula este evento. A transcrição do próprio gene *rho* é autorregulada e estudos sobre a região a montante do transcrito já identificaram até cinco sequencias atenuadoras que são dependentes de Rho, ou seja, que inibem a transcrição na presença da proteína (BARIK; BHATTACHARYA; DAS, 1985; MATSUMOTO et al., 1986). Recentemente foi observado o envolvimento de RNAs curtos ou sRNAs (do inglês small RNAs) nesse processo (SILVA et al., 2019). O sRNA SraL que é altamente expresso em anaerobiose e na fase estacionária tardia, mas pode ser encontrado em outros momentos metabólicos como na fase exponencial, pode se ligar na região 5' não traduzida (5'-UTR) do mRNA-rho, impedindo a ligação de Rho nessa região do transcrito, protegendo-o de uma terminação prematura (SILVA et al., 2019).

Outro exemplo envolvendo sRNA é o gene *rpoS* que codifica para o fator de transcrição σ^S. Esse fator se liga nas regiões regulatórias do DNA quando a célula está sob limitação nutricional ou alguma outra condição adversa. Geralmente *rpoS* não é transcrito durante a fase exponencial de crescimento, e essa regulação é feita pela proteína Rho que, nesta fase, se liga a um sítio *rut* presente na região 5'-UTR do mRNA-rpoS, terminando a transcrição prematuramente. Em *E. coli*, na fase estacionária ou sob condições de estresse, os sRNA DsrA, ArcZ e RprA se acumulam e se ligam na região 5'-UTR do mRNA-rpoS não permitindo o acesso de Rho e consequentemente possibilitando a transcrição (SEDLYAROVA et al., 2016).

A regulação de Rho também foi vista no transportador de Mg^{2+} CorA em *Salmonella enterica* (KRINER; GROISMAN, 2015). O mecanismo desta regulação ainda não está bem estabelecido, no entanto, já se sabe que a montante do gene *corA* há uma pequena região codificadora (ORF), chamada *corL*, que possui um sítio *rut* em sua proximidade. O RNA é transcrito como um todo, ou seja com as duas ORF, no entanto na região contendo *corL* o RNA pode assumir estrutura secundária que o deixa passível de ser traduzido ou não. Quando ele está organizado de forma a não ser traduzido o sítio *rut* fica inacessível a Rho, e o gene *corA* é livremente transcrito. Quando *corL* é eficientemente traduzido, o sítio *rut* fica disponível e ocorre a terminação da transcrição de *corA* (KRINER; GROISMAN, 2015).

A proteína Rho também está envolvida no controle por *riboswitches*, sequencias nucleotídicas, conhecidas como elementos cis de RNA, presentes geralmente na região 5'-UTR que regulam a expressão gênica em resposta a abundância de metabólicos ou íons metálicos (BÉDARD; HIEN; LAFONTAINE, 2020). O RNA assume diferentes estruturas em resposta a diferentes ligantes, permitindo ou reprimindo a transcrição (SERGANOV; NUDLER, 2013). Em *S. enterica* foi observado que em alto Mg^{2+} a região líder do gene *mgtA*,

que codifica uma proteína sensora de Mg^{2+}, apresenta uma conformação que permite a ligação de Rho e o consequente término transcrição (HOLLANDS et al., 2012). Enquanto que em baixo Mg^{2+} a conformação da região líder é sequestrada na formação de uma alça que não permite a interação com Rho. Em *E. coli*, Hollands e colaboradores (2012) reportaram a relação de Rho com *riboswitches* através da região líder do gene *ribB*, que codifica uma proteína envolvida na síntese de riboflavinas. Os autores observaram que na presença de flavina mononucleotídeo (FMN) a terminação da transcrição do gene *ribB* por Rho é estimulada.

Outra função que pode ser associada a Rho é a repressão de DNA exógenos (CARDINALE et al., 2008). Um organismo procariótico pode ser infectado por profagos que passam a fazer parte de seu genoma e como esses profagos podem expressar genes tóxicos, o hospedeiro desenvolveu algumas maneiras de reprimir a expressão destes genes. A terminação da transcrição mediada por Rho pode ser uma das maneiras, uma vez que já foi mostrado que a redução da atividade de Rho levou ao aumento da expressão dos genes de profágos (CARDINALE et al., 2008).

O avanço da transcritômica permitiu observar que ao longo do genoma de procariotos algumas sequencias antisense podem ser transcritas em larga escala a partir de sítios de início inespecíficos ou de promotores fracos. Este evento recebeu o nome de transcrição antisenso pervasiva (do inglês *antisense pervasive transcription)* que pode gerar uma situação conhecida como ruído transcricional (do inglês *transcriptional noise)* (GEORG; HESS, 2011). Quando presentes em elevada concentração estes transcritos podem ser deletérios para a célula e estudos com *E. coli* e *Bacillus subtilis* mostraram que a redução da atividade da proteína Rho leva ao aumento da transcrição de RNAs antisense (PETERS et al., 2012). Rho também auxilia na integralidade do genoma, os processos de replicação, transcrição e recombinação podem gerar estruturas contendo DNA ligado a RNA, especialmente durante a replicação a forquilha pode encontrar uma transcrição ainda não terminada, e Rho pode auxiliar na liberação do DNA através da sua atividade helicase (DUTTA et al., 2011; WASHBURN; GOTTESMAN, 2011).

1.3.2. Interações realizadas por Rho

Além de reconhecer sequencias nucleotídicas (sitio *rut*), a proteína Rho é capaz de estabelecer interação com a proteína NusG, um fator de alongamento de transcrição capaz de acelerar o processo por sua atividade anti-pausa (TOMAR; ARTSIMOVITCH, 2013). NusG

possui dois domínios conectados por uma alça flexível, o domínio N-terminal contém o sítio de interação com a RNAP enquanto que o C-terminal, apresenta o motivo KOW que pode se ligar tanto com Rho como com a proteína S10 ou NusE, uma componente do ribossomo 30S (BURMANN et al., 2010; CHALISSERY et al., 2011). Uma região hidrofóbica (aminoácidos 203-208) próxima da interface entre duas subunidades de Rho foi identificada como local de interação com NusG. Ainda que Rho possua seis sítios de interação com NusG, a interação ocorre com apenas uma NusG por vez, indicando algum tipo de cooperatividade negativa (CHALISSERY et al., 2011; PASMAN; VON HIPPEL, 2000; VALABHOJU; AGRAWAL; SEN, 2016).

NusG não possui capacidade de aumentar a taxa de translocação, ATPásica ou de ligação de Rho no RNA (NEHRKE; ZALATAN; PLATT, 1993) mas aumenta a taxa de liberação de transcritos em zonas de terminação quando está ligada a ambos Rho e RNAP. Mais de um terço das terminações dependentes de Rho é também dependente de NusG (PETERS et al., 2012; SHASHNI et al., 2014). A interação de NusG com S10 acopla transcrição e tradução criando um bloqueio físico para o acesso de Rho a NusG e, com isso, reduzindo a eficiência de terminação de transcritos NusG dependentes. No entanto, a afinidade de NusG por S10 é menor que por Rho, ocorrendo competitividade entre essas duas proteínas para se ligarem a NusG (BURMANN et al., 2010; PASMAN; VON HIPPEL, 2000).

Outro fator de alongamento que está relacionado com Rho, mas que não interage diretamente é o fator NusA. Ao ligar-se à RNAP e ao RNA NusA impede a ligação de Rho e impede sua ação de terminadora de transcrição (BURNS; RICHARDSON; RICHARDSON, 1998; QAYYUM; DEY; SEN, 2016).

Outras proteínas foram relatadas como inibidores do fator de transcrição Rho como YaeO e Hfq em *E. coli* e Psu, do bacteriófago P4. A proteína YaeO inibe a atividade de Rho diretamente ao se ligar ao sítio SBS (GUTIÉRREZ et al., 2007). A interação com Hfq permite a ligação de Rho ao RNA mas inibe a ação ATPásica de Rho e sua atividade helicase (RABHI et al., 2011). Psu é uma proteína do bacteriófago P4 que interage com a proteína Rho diminuindo sua atividade ATPásica (PANI et al., 2006).

1.4. Diguanilato Ciclases e Fosfodiesterases

O diguanilato cíclico ou bis-(3'-5')-monofosfato dimérico cíclico de guanosina (di-GMP cíclico ou c-di-GMP) é reconhecido como um mensageiro secundário universal dentro domínio Bactéria e seu nível intracelular envolve as enzimas Diguanilato ciclase (DGC) e

Fosfodiesterase (PDE), responsáveis por sua síntese e degradação respectivamente (HENGGE, 2009). As proteínas Diguanilato ciclases promovem a condensação de duas moléculas de GTP para produzir uma molécula de c-di-GMP, que atua como sinalizador, sendo efetor de muitas proteínas que promovem as alterações celulares. O c-di-GMP é degradado por Fosfodiesterases (PDE) que o transformam em 5′-fosfoguanilil-(3'-5')-guanosina (5'-pGpG) e que, por sua vez, é reciclado em dois GMP. Ambas as proteínas, DCG e PDE, podem ser encontradas no citoplasma ou ancoradas à membrana plasmática (DAHLSTROM; O'TOOLE, 2017; HENGGE, 2009; ROMLING; GALPERIN; GOMELSKY, 2013).

DGC e PDE foram identificadas na maioria das bactérias, mas estão ausentes em arqueias e eucariotos (GALPERIN; NIKOLSKAYA; KOONIN, 2001). Elas trabalham em oposição, balanceando a formação/degradação de c-di-GMP influenciando vários processos como formação de biofilme, motilidade, ativação de virulência, biossíntese de moléculas e respostas ao estresse (DAHLSTROM; O'TOOLE, 2017; KULASAKARA et al., 2006; MARTINEZ-GIL; RAMOS-GONZALEZ; ESPINOSA-URGEL, 2014; SRIVASTAVA et al., 2013). A grande parte das bactérias codificam mais de uma proteína envolvida na síntese e degradação do c-di-GMP, sendo que em algumas foram encontradas mais de 60 (GALPERIN; NIKOLSKAYA; KOONIN, 2001). Apesar do grande número dessas proteínas, muitos estudos tem demonstrado que a maioria das DGC estão envolvidas em apenas um ou dois processos de sinalização, indicando que algum tipo de mecanismo leva à ativação de uma DGC específica (HA; RICHMAN; O'TOOLE, 2014; MATSUYAMA et al., 2016; NEWELL et al., 2011; TUCKERMAN et al., 2009).

A atividade de DGC é regulada por dois motivos altamente conservados GGDEF (Gly-Gly-Asp-Glu-Phe) ou GGEEF (Gly-Gly-Glu-Glu-Phe) que formam o domínio GGDEF geralmente na região C-terminal, chamado de sítio A (CHAN et al., 2004). Na estrutura da proteína, geralmente dimérica, dois desses domínios são justapostos em um arranjo antiparalelo, e ambos podem aceitar a molécula de GTP para síntese de c-di-GMP. A presença de dois íons divalentes é requerida para formação da ligação fosfodiéster, geralmente Mg^{2+} ou Mn^{2+} (Fig. 4A e B) (CHAN et al., 2004; PAUL et al., 2004; WASSMANN et al., 2007). Os dois primeiros resíduos de Gly do motivo estão envolvidos na ligação de GTP, o quarto resíduo (Glu) na coordenação do íon metálico e o terceiro resíduo que pode ser Asp ou Glu é indispensável para a catálise participando também da coordenação do íon metálico (CHAN et al., 2004; WASSMANN et al., 2007). Muitas proteínas podem apresentar o domínio GGDEF degenerado, perdendo sua capacidade catalítica, mas que pode funcionar como regulador

alostérico. Existem algumas exceções a esses sítios que mantém sua atividade como GGEEF, AGDEF, GGDEM e SGDEF (DAHLSTROM; O'TOOLE, 2017).

A formação do homodímero de DGC cataliticamente ativo pode ser regulado de duas formas. Primeiro pela ligação de efetores alostéricos nos domínios sensores que essas proteínas possuem. Um exemplo é a DgcZ de *E. coli*, em situações específicas íons zinco se ligam à ela próximo ao sitio GGDEF inibindo a atividade de formação de c-di-GMP (RÖMLING; LIANG; DOW, 2017). O segundo mecanismo é por retroalimentação, a cinco resíduos a partir do domínio GGDEF em direção ao N-terminal pode-se encontrar outro motivo conservado chamado de RxxD ou RxxE. Esse motivo funciona como um sítio de inibição alostérica para ligação de c-di-GMP de maneira não competitiva, também pode ser chamado de sítio auto inibitório (sítio I) (Fig. 4A) (CHRISTEN et al., 2006).

As proteínas PDE podem ser divididas em dois grupos principais devido a sua estrutura, as que possuem o domínio EAL e as que possuem o domínio HD-GYP. No primeiro grupo estão as PDE que possuem o domínio EAL que contém o motivo EAL (Glu-Ala-Leu), esse domínio compreende as fosfodiesterases que transformam o c-di-GMP no produto 5′-pGpG. O domínio EAL é conservado estruturalmente, apresentando oito α-hélices e oito β-folhas alternadas com uma organização bastante flexível (BARENDS et al., 2009).

Essas enzimas geralmente possuem grande afinidade pelo substrato, ao redor de nano molar (SUNDRIYAL et al., 2014), e necessitam de um íon divalente para desempenhar sua atividade sendo, na maioria dos casos, Mg^{2+} ou Mn^{2+}, enquanto Ca^{2+} ou Zn^{2+} inibem sua atividade (Fig. 4C) (TAL et al., 1998; TCHIGVINTSEV et al., 2010). São comumente encontradas como dímeros, no entanto estudos mostram que a PDE tem atividade como monômero e pode ser encontrada como oligômeros maiores *in vitro* (BARENDS et al., 2009; SCHMIDT; RYJENKOV; GOMELSKY, 2005; TCHIGVINTSEV et al., 2010).

O segundo grupo de PDEs é o que possui o domínio HD-GYP e se caracteriza por hidrolisar c-di-GMP e 5'-pGpG para a formação de GMP, podendo influenciar diretamente os níveis de c-di-GMP, hidrolisando-o, ou indiretamente hidrolisando o produto 5'-pGpG e prevenindo inibição de domínios EAL (COHEN et al., 2015; ORR et al., 2015).

Algumas proteínas podem ter os dois sítios, de síntese e degradação de c-di-GMP (SCHIRMER, 2016). Como mencionado, alguns domínios GGDEF podem se tornar degenerados e não ser mais catalíticamente ativos, no entanto podem passar a apresentar função alostérica como receptores de c-di-GMP (regulação negativa) ou GTP (regulação positiva) de PDE por exemplo. O mesmo pode ocorrer com sítios EAL e HD-GYP (HOBLEY et al., 2012; OZAKI et al., 2014). Mas pode ocorrer de ambos os sítios serem funcionais,

nesse caso os domínios são diferencialmente regulados por fatores ambientais e/ou sinais intracelulares que faz um domínio prevalecer sobre o outro em determinadas condições (BHARATI et al., 2012). A regulação nesses casos pode incluir ligação de efetor, percepção de sinal, interação proteína-proteína, entre outras (FEIRER et al., 2015; MILLS et al., 2015; QI et al., 2009).

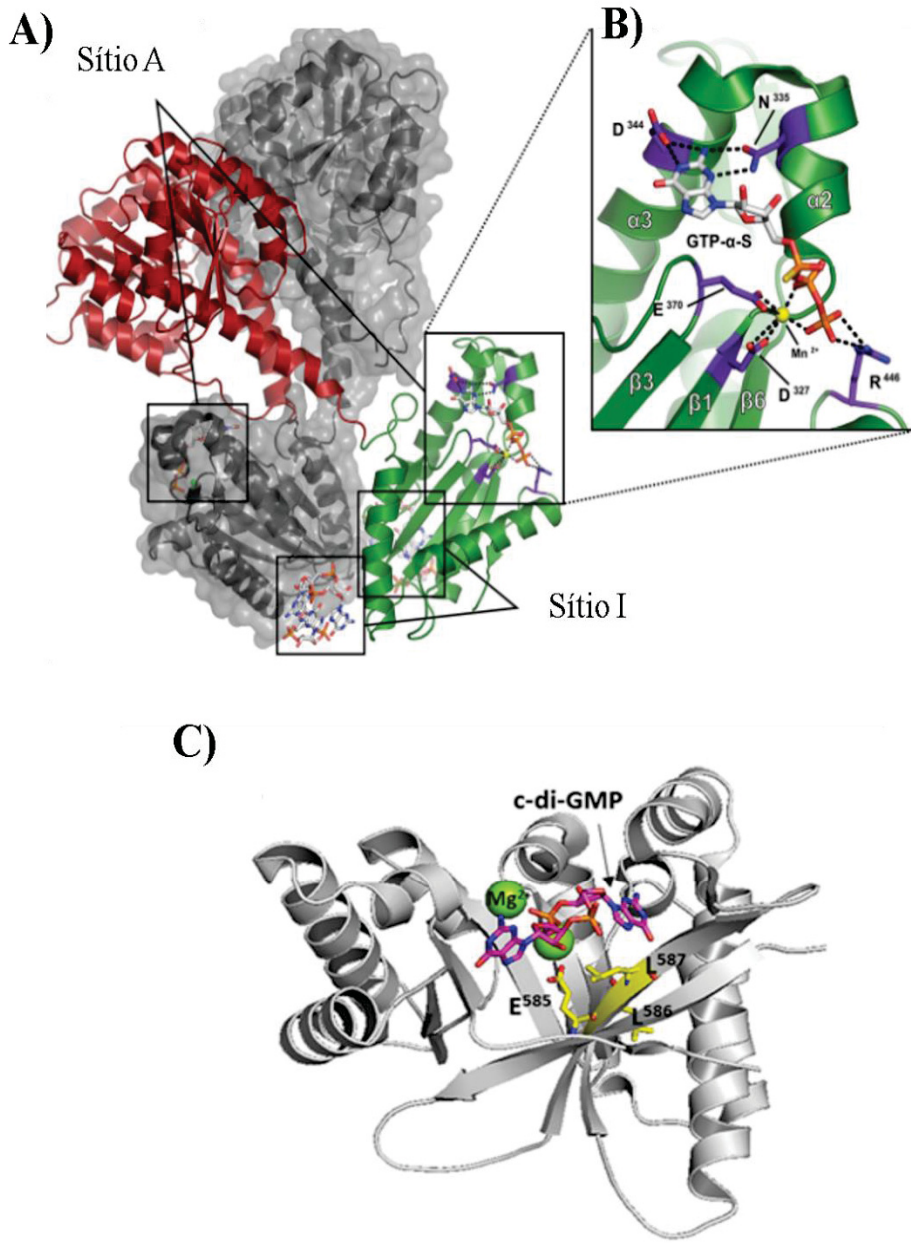

FIGURA 4. Estrutura das proteínas PleD (DGC) de *Caulobacter crescentus* e RbdA (PDE) de *Pseudomonas aeruginosa*. A) Estrutura da proteína PleD (DGC), demonstra o dímero (um monômero colorido e outro cinza), contendo domínio REC (vermelho) e GGDEF (verde), no domínio GGDEF ligado GTP-α-S no sitio A e c-di-GMP no sítio I. B) aproximação do motivo GGDEF, Mg^{2+} (amarelo). C) Proteína RbdA (PDE), com motivo ELL (amarelo) ligado a c-di-GMP e Mg^{2+} (verde). Fonte: Adaptado de Wiggers e colaboradores, 2018 e Xin e colaboradores, 2019.

Além desses dois motivos principais as proteínas DGC e PDE podem ter outras regiões, especialmente no domínio N-terminal, que podem estar associados à percepção de estímulos ambientais como nível de oxigênio, potencial redox ou luz (PAUL et al., 2004). Os demais domínios comumente encontrados em DGC e PDE constituem-se dos domínios REC, GAF, PAS e HAMP. O domínio REC, é responsável por regular os níveis de c-di-GMP em resposta a sinais intracelulares ou extracelulares recebidos por seu sensor cognato de histidina quinase (PAUL et al., 2004). O domínio GAF, pode ligar GMP cíclico (cGMP), aumentando a síntese de c-di-GMP (AN et al., 2013). O domínio PAS pode ter várias funções como promover a sinalização da transferência de grupos fosforil e interação proteína-proteína (VOROBIEV et al., 2012). HAMP encontrado em muitos receptores transmembranas, que transmitem sinais do periplasma ao citoplasma via mudança conformacional (MASCHER; HELMANN; UNDEN, 2006; PAPPALARDO et al., 2003). Outros domínios podem ser encontrados em menores proporções como MASE sensor associado a membrana, CHASE ciclase ou histidina quinase associado ao sensoriamento extracelular e CACHE canais de Ca^{2+} e receptores de quimiotaxia (ANANTHARAMAN; ARAVIND, 2001; MASCHER; HELMANN; UNDEN, 2006; ZHULIN; NIKOLSKAYA; GALPERIN, 2003).

1.4.1. Funções do c-di-GMP

A principal função desempenhada por c-di-GMP é a transição entre a fase móvel da bactéria para sua fase séssil. O aumento intracelular de c-di-GMP sinaliza para diminuição da velocidade da motilidade bacteriana, formação de biofilme e entrada em seu estado séssil, enquanto que a diminuição de c-di-GMP sinaliza motilidade e dispersão e esses processos podem envolver uma grande gama de proteínas (Fig. 5).

Foi descoberto que nas enterobactérias o movimento flagelar que envolve muitas proteínas é mediado por c-di-GMP. O rotor flagelar é um complexo proteico localizado na face citoplasmática do flagelo e é composto pelas subunidades FliG, FliM e FliN. A rotação é alimentada por um gradiente iônico realizado pelas proteínas transmembrana MotA e MotB, com FliG sendo crítica para a conversão do fluxo de prótons de MotA e MotB para o movimento que gera a rotação do corpo flagelar (ROMLING; GALPERIN; GOMELSKY, 2013). A proteína YcgR que liga c-di-GMP interfere na movimentação flagelar e dois modelos foram propostos para sua ação. O primeiro indica a interação de YcgR as proteínas FliG e FliM, que se intensifica na presença de c-di-GMP gerando mudança conformacional e consequente diminuição da motilidade. No segundo modelo YcgR ligada à c-di-GMP se

ligaria a MotA interferindo no gradiente iônico diminuindo a rotação do flagelo (PAUL et al., 2010).

Um dos processos que está intimamente relacionado com motilidade em bactérias é quimiotaxia, e uma das proteínas responsáveis por entregar sinais quimiotáticos ao flagelo é a proteína de resposta CheY em sua forma fosforilada (CheY-P). Essa proteína se liga à FliM direcionando o movimento flagelar (TOKER; MACNAB, 1997). A proteína CheY-P é inativada pela fosfatase CheX (MOTALEB et al., 2005). Sultan e colaboradores (2011) apresentaram evidencias que na bactéria patogênica *Borrelia burgdorferi* a proteína PlzA, uma receptora de c-di-GMP, se liga a fosfatase CheX, de maneira c-di-GMP dependente, desfosforilando CheY-P, e consequentemente diminuindo a motilidade.

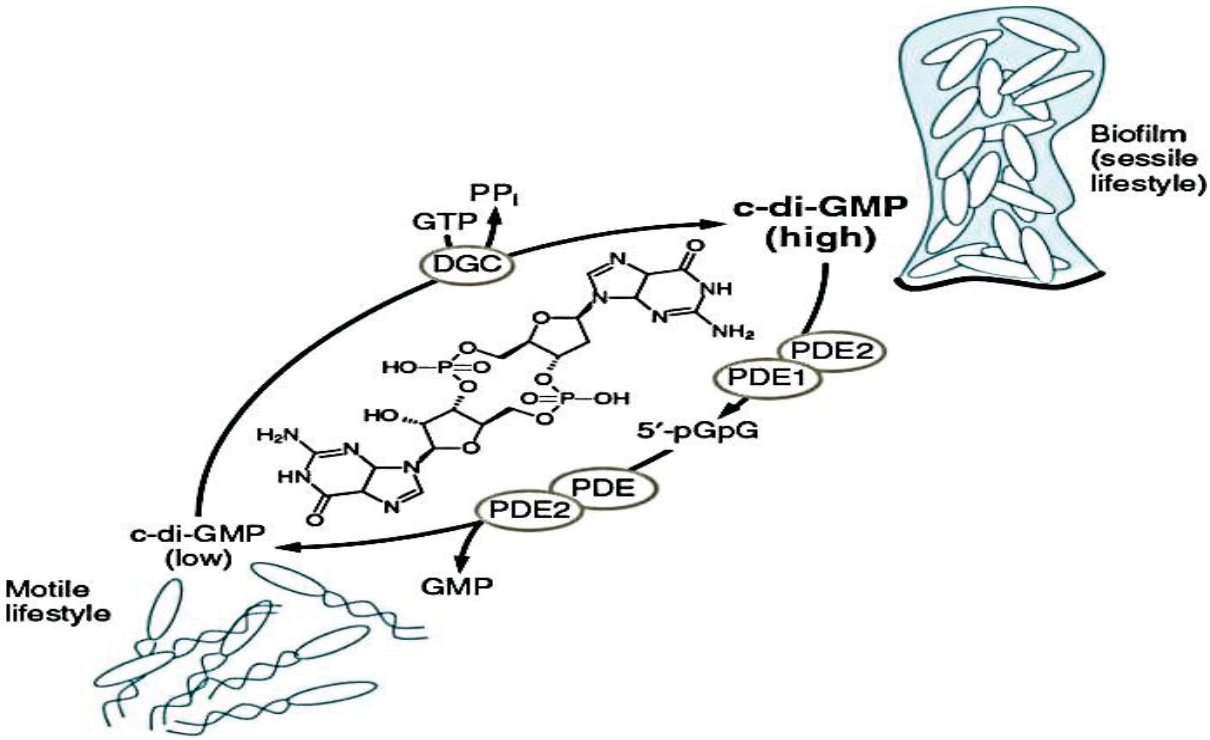

FIGURA 5. Controle da formação do c-di-GMP. A formação e degradação do segundo mensageiro c-di-GMP é controlado, respectivamente, por Diguanilato Ciclases (DGC) e Fosfodiesterases (PDE). DGC possui um domínio GGDEF responsável pela condensação de duas moléculas de GTP na formação do c-di-GMP, alto c-di-GMP sinaliza entrada no estado séssil. PDE que possuem domínio EAL degradam c-di-GMP no produto 5'-pGpG, enquanto as que possuem domínio HD-GYP podem degradar o c-di-GMP ou 5'-pGpG em GMP, baixa concentração intracelular de c-di-GMP sinaliza dispersão. Fonte: Flemming e colaboradores (2016).

Em *A. brasilense,* a proteína Tlp1 está envolvida no processo de quimiotaxia e de colonização das raízes de plantas. Tlp1 está ancorada na membrana com o domínio N-terminal voltado para espaço periplasmático com uma provável região sensorial e um domínio

C-terminal citoplasmático que interage com as proteínas envolvidas na sinalização para quimiotaxia e nesse domínio inclui o sítio de ligação a c-di-GMP (O'NEAL et al., 2017). Os níveis de c-di-GMP em *A. brasilense* variam de acordo com a concentração de oxigênio. Quando os níveis de O_2 estão baixos o nível de c-di-GMP aumenta, promovendo redução da motilidade, quando os níveis de O_2 aumentam, c-di-GMP diminui, aumentando a motilidade de *A. brasilense* (RUSSELL et al., 2013). As mudanças nos níveis de c-di-GMP permite que *A. brasilense* otimize a procura por ambientes onde maximiza seu metabolismo microaeróbico (ZHULIN et al., 1996).

Como citado acima, o aumento c-di-GMP pode favorecer o estado séssil através de sistemas que diminuem a motilidade, atingindo o flagelo diretamente, ou de sinais quimiotáticos que diminuem a velocidade da motilidade bacteriana. No entanto o c-di-GMP também pode promover o estado séssil estimulando proteínas e fatores de transcrição de genes relacionados a formação de biofilme, pili adesivos e adesinas.

Muitas bactérias possuem a capacidade de biossíntese de celulose, um componente comum dos biofilmes bacterianos (OGAWA; MAKI, 2003). Ross e colaboradores (1987) demonstraram que em *Komagataeibacter xylinus* o c-di-GMP é o ativador alostérico da síntese de celulose via Celulose Sintase. Essa proteína contém um domínio conhecido como PilZ onde se liga o c-di-GMP (AMIKAM; GALPERIN, 2006) para então favorecer o aumento da síntese de celulose (WEINHOUSE et al., 1997).

A síntese de poli-β-1,6-N-acetilglicosamina (PAG), um exopolissacarídeo muito comum dos biofilmes bacterianos, também é regulado por c-di-GMP. Em *E. coli* duas DGC (DosC e YdeH) são requeridas para a formação de biofilme dependente de PAG. Enquanto DosC afeta a transcrição regulando positivamente o operon da biossíntese de PAG, *pgaABCD*, a atividade de YdeH estabiliza a proteína PgaD a nível pós-transcricional sendo necessária para biossíntese do exopolissacarídeo (BOEHM et al., 2009; TAGLIABUE et al., 2010). A biossíntese de outros polissacarídeos como alginato, Pel e Psl presentes em diversas espécies bacterianas, também estão relacionados com os níveis de c-di-GMP (BARAQUET et al., 2012; LIAO; SAUER, 2012).

Outros reguladores transcricionais dependentes de c-di-GMP já foram relatados. O fator de transcrição VpsT em *Vibrio cholerae* se liga a c-di-GMP e sofre uma mudança na sua oligomerização permitindo a regulação positiva de genes relacionados com formação de biofilme e negativa de genes relacionados com motilidade (KRASTEVA et al., 2010). O fator de transcrição FleQ de *Pseudomonas aeruginosa* também regula a formação de biofilme e motilidade. Quando não ligado a c-di-GMP esse fator ativa a transcrição de genes flagelares e

reprime genes de formação de biofilme, quando o nível de c-di-GMP aumenta, o efetor se liga a FleQ, mudando sua estrutura quaternária, a proteína se desliga do DNA permitindo a transcrição dos genes de formação de biofilme e diminuindo a transcrição dos flagelares (MATSUYAMA et al., 2016).

Pili são longos apêndices filamentosos não flagelares compostos de proteínas que se encontram na superfície bacteriana e estão comumente associados com a formação de biofilmes em superfícies bióticas e abióticas (BARNHART; CHAPMAN, 2006). Em *Klebsiella pneumoniae* a proteína DGC YfiN estimula a expressão do pili tipo 3, enquanto que a PDE MrkJ regula negativamente sua expressão (JOHNSON et al., 2011). Em *P. aeruginosa* também foi demonstrado que a elevada concentração de c-di-GMP produzido pela DGC MorA aumenta a expressão de CupA envolvida na biossíntese do pili (MEISSNER et al., 2007).

Além do pili as adesinas também contribuem para a formação de biofilme uma vez que estão presentes na superfície celular e servem de ponto de aderência do substrato e também estabilizam a matriz extracelular. O sistema Lap em *Pseudomonas fluorescens* é um dos exemplos mais estudados do circuito do efetor c-di-GMP. As proteínas do sistema Lap contém: adesina de membrana (LapA), protease periplasmatica (LapG), proteína efetora (LapD) e o transportador ABC (LapB, C e E) responsável pela secreção de LapA (HINSA et al., 2003). A DGC GcbC interage fisicamente com LapD e isso está diretamente ligado com a habilidade de sinalização provavelmente ao passar o c-di-GMP para LapD (DAHLSTROM et al., 2015; NEWELL; MONDS; O'TOOLE, 2009). A proteína LapD, encontrada na membrana, se liga a c-di-GMP em seu domínio EAL, e sofre mudança estrutural no seu domínio HAMP (NAVARRO et al., 2011). Essa mudança estrutural promove o sequestro da protease periplasmática LapG (BOYD et al., 2012) que deixa de clivar LapA que, por sua vez, está na membrana externa e age como adesina se fixando ao substrato e influenciando na formação de biofilme (BOYD et al., 2014).

Esse sistema responde a concentração de fosfato inorgânico, em condições de baixo fosfato inorgânico, o gene *rapA* é transcrito e produz uma PDE que degrada o c-di-GMP que se desliga de LapD que, consequentemente, permite a proteólise da adesina LapA (CHATTERJEE et al., 2012, 2014).

Muitas proteínas DGC e PDE além de ter o sitio para formação ou degradação de c-di-GMP, respectivamente, possuiu outros domínios sensores. Dois exemplos são proteínas de *E. coli*, uma DGC chamada de DosC e uma PDE chamada de DosP que são sensoras de oxigênio (TUCKERMAN et al., 2009). A primeira possui um domínio com uma globina modificada e

o segundo um domínio PAS. Juntas modulam o nível de c-di-GMP produzido em resposta ao oxigênio molecular. Já foi observado que essas proteínas interagem, abrindo a possibilidade que DGC e PDE podem trabalhar juntas como um complexo para regular a concentração de c-di-GMP relacionado a um efetor específico (TUCKERMAN et al., 2009).

Muitas são as possibilidades de ação de c-di-GMP intracelularmente e sua função envolve muitos processos metabólicos importantes. Recentemente foi observada a interação de proteínas envolvidas na síntese e degradação de c-di-GMP com a proteína GlnZ de *A. brasilense*. Dentre mais de 30 possíveis DGC e PDE em *A. brasilense* somente duas delas foram indicadas como prováveis novos alvos de GlnZ (GERHARDT, 2015), o que corrobora com a ideia que as proteínas DGC e PDE estão envolvidas em poucas vias recebendo sinais específicos. GlnZ forma parte das proteínas PII sensoras dos níveis energéticos e principalmente de nitrogênio e carbono intracelular, a interação observada de GlnZ com especificas DGC e PDE ocorre em baixa concentração de 2-OG *in vitro*, ou seja, extrapolando para o fisiológico essa condição traduz a sinalização de alto nível de nitrogênio, abrindo a possibilidade de uma nova regulação da síntese de c-di-GMP via interação com a proteína GlnZ (URBANSKI, 2018).

1.5. UMP Fosfatase (UmpH)

A UmpH de *Escherichia coli* é uma proteína envolvida na homeostase da via *de novo* de pirimidinas (REAVES et al., 2013). O gene que codifica para essa proteína é chamado de *nagD* e é conhecido há muito tempo (PLUMBRIDGE, 1989), no entanto, a função fisiológica de UmpH só foi descoberta recentemente (REAVES et al., 2013). O gene *nagD* faz parte de um operon divergente em *E. coli*, o operon *nagE-nagBACD* (ALVAREZ-AÑORVE et al., 2009; PLUMBRIDGE, 1989), e seus produtos estão relacionados com o processamento de N-Acetilglicosamina (GlncNAc), um componente essencial para a síntese da parede celular bacteriana (PERI; GOLDIE; WAYGOOD, 1990).

A regulação da expressão do operon contendo *nagD* é realizada pelo regulador transcricional NagC um dos produtos do operon (FERNANDEZ; PLUMBRIDGE, 2019). NagC reprime a expressão do operon, e essa repressão é revertida quando essa proteína interage com N-Acetilglicosamina-6-Fosfato (GlcNAc6P), o produto do transporte de GlcNAc para dentro da célula, que é utilizado por NagB e NagA (ALVAREZ-AÑORVE et al., 2009). UmpH, no entanto, não é necessária para o uso eficiente de GlcNAc (PLUMBRIDGE, 1991).

UmpH pertence à superfamília das dehalogenases caracterizada por um domínio principal (*core domain*) altamente conservado (KOONIN; TATUSOV, 1994). A família compreende numerosas proteínas que se distribuem de procariontes a eucariontes superiores, incluindo seres humanos. A grande maioria das enzimas são fosforil transferases, embora a superfamília tenha o nome de dehalogenases (LU; DUNAWAY-MARIANO; ALLEN, 2005). Quando as sequências de aminoácidos de todas as suas regiões codificadoras são comparadas, as semelhanças entre os membros da familia geralmente não são muito altas (15 a 30% de idêntidade), no entanto suas regiões centrais envolvidas na atividade catalítica são altamente conservadas (KUZNETSOVA et al., 2006; LU; DUNAWAY-MARIANO; ALLEN, 2005).

A estrutura da proteína UmpH de *E. coli* já foi resolvida, a mesma possui 250 resíduos de aminoácidos e por volta de 27 kDa por monômero (TREMBLAY; DUNAWAY-MARIANO; ALLEN, 2006). Dados da estrutura e gel filtração demonstram que essa proteína se apresenta como monômero em solução (TREMBLAY; DUNAWAY-MARIANO; ALLEN, 2006). Assim como os demais membros da família das dehalogenases, UmpH possui um domínio principal (*core domain*) altamente conservado de estruturas secundárias α/β alternadas, que suportam o sítio catalítico, composto por quatro alças nomeadas de alças I, II, III e IV (Fig. 5) (ALLEN; DUNAWAY-MARIANO, 2004). A organização dos resíduos nesse sítio interagem com cofator Mg^{2+} e com o substrato. O domínio principal e o substrato entram em contato através do grupo fosforil que é transferido. Um segundo domínio variável nessas proteínas é um domínio móvel chamado CAP que também pode interagir com o substrato dessolvatando o sitio catalítico e confere especificidade (Fig. 6) (TREMBLAY; DUNAWAY-MARIANO; ALLEN, 2006).

1.5.1. Função de UmpH

As pimidinas são bases nitrogenadas (citosina, uracila e timina), produzidas a partir do anel pirimídico. Essas bases são utilizadas na formação de nucleotídeos que são moléculas obrigatórias em todos os organismos. Os nucleotídeos são substratos para síntese de RNA e DNA e são utilizados como os principais doadores de energia nos processos celulares (KILSTRUP et al., 2005). Os nucleotídeos podem ser sintetizados a partir de aminoácidos ou outras moléculas pequenas através da via *de novo*, ou através de nucleobases e nucleosídeos pré formados pela via chamada de salvamento (KILSTRUP et al., 2005).

A síntese *de novo* de pirimidinas é realizada em cinco reações principais catalisadas por cinco enzimas (HOVE-JENSEN, 1989). A regulação da homeostase da via das

pirimidinas envolve duas estratégias a primeira chamada de retroalimentação canônica e a segunda de metabolismo de transbordamento (REAVES et al., 2013). A retroalimentação canônica é conhecida e altamente estudada. Nessa regulação ocorre a retroalimentação alostérica negativa da primeira enzima da via *de novo*, e da enzima anterior. A primeira enzima da via é a Aspartato transcarbamoilase (ATCase) e ela é inibida quando ocorre aumento dos produtos finais UTP e mais fortemente pelo CTP (KANTROWITZ, 2012). A enzima anterior é a Carbamoil Fosfato Sintetase (CPSase) responsável pela síntese de carbamoil-fosfato, substrato da ATCase, e a mesma é inibida pelo intermediário da via UMP, portanto quando os níveis de UMP, UTP e CTP aumentam ocorre a inibição dessas enzimas diminuindo suas atividades (KANTROWITZ, 2012; PETERSON; COCKRELL; KANTROWITZ, 2012; WILD; LOUGHREY-CHEN; CORDER, 1989). CPSase não é parte direta da via *de novo* pois também alimenta a via de biossíntese de arginina (KANTROWITZ, 2012).

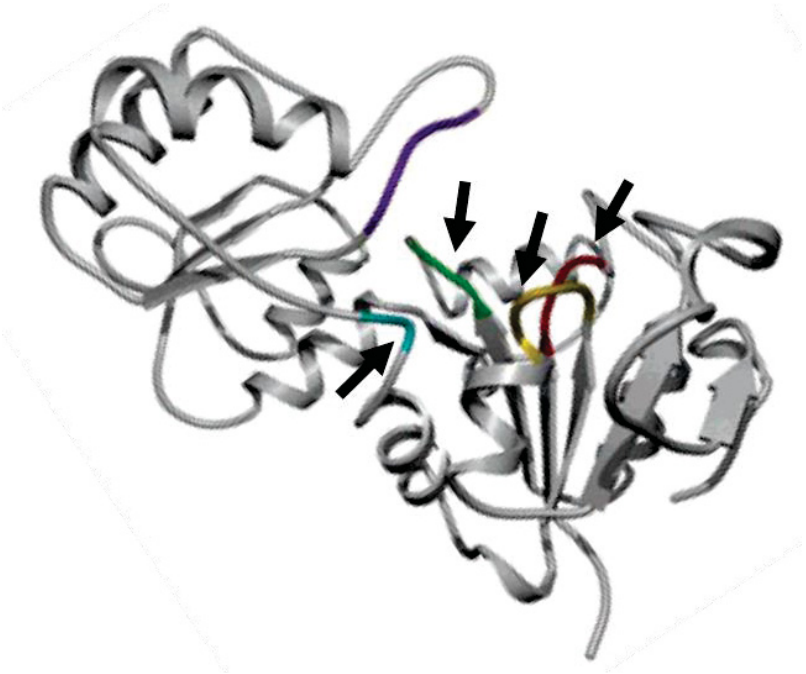

FIGURA 6. Estrutura da proteína UmpH de *Escherichia coli*. As alças estão indicadas por setas sendo a alça I (vermelho), II (verde), III (azul) e IV (dourado). A alça móvel do domínio CAP está em roxo. Fonte: Tremblay e colaboradores (2006)

A regulação por metabolismo de transbordamento foi proposta recentemente por Reaves e colaboradores (2013). No trabalho foram mutados os genes que codificam para as duas enzimas envolvidas na regulação pela retroalimentação canônica, ATCase (*pyrI*) e CPSase (*carB*), sem a regulação dessas proteínas os autores esperavam que os níveis dos nucleotídeos trifosfatados aumentassem, no entanto isso não foi observado, o que foi possível

notar foi a concentração de uracila subir.

Considerando que UMP pode originar uridina e uracila os autores deletaram 18 genes que poderiam estar relacionados com a interconversão dessas três moléculas, na tentativa de encontrar a proteína que estaria desviando a via de síntese *de novo* para uracila. A mutação de dos genes *nagD* que codifica UmpH e *surE* que codifica UmpG, diminui o acumulo de uracila por volta de 40% e 45% respectivamente. O duplo mutante desses dois genes diminuiu o acumulo de uracila em 80% indicando que esses dois genes estão relacionados com o desvio de UMP da biossíntese *de novo* de pirimidinas para a formação de uracila. Outro dado que suporta a ação conjunta de UmpH e UmpG é que apenas no mutante duplo que há acumulo do produto final UTP, indicando que UmpH pode assumir a função de UmpG e vice-versa (REAVES et al., 2013). As proteínas UmpH e UmpG transformam UMP em uridina mas não em uracila, essa etapa é realizada pela enzima Udp (REAVES et al., 2013).

Outro dado relatado pelos autores foi com a proteína UMP Quinase, responsável pela conversão de UMP em UDP. Essa enzima forma um ponto de estrangulamento quando os níveis de UTP aumentam. UTP pode se ligar de maneira cooperativa na UMP quinase inibindo a formação de UDP e aumentando os níveis de UMP, substrato de UmpH e UmpG.

Em resumo quando os níveis dos produtos finais UTP e CTP ou do intermediário UMP aumentam há inibição por retroalimentação canônica, mas o aumento de UMP pode levar ao desvio dessa molécula para a formação de uridina catalisada por UmpH ou UmpG. A concentração de UMP na célula no estado estacionário é de 52 μM, e o $K_{M\ UMP}$ para UmpH é de 120 μM indicando que o controle de UmpH ocorre pela concentração do substrato. A uridina é substrato da enzima Udp que a transforma em ribose-1-P e uracila que pode ser excretada, por isso essa via é chamada de metabolismo de transbordamento (REAVES et al., 2013).

Estudos com proteína UmpH revelaram que ela consegue utilizar uma gama de substratos como UMP, CMP, GMP, AMP, ribose 5-P, pirodoxal 5'-P, glicerol 3-P, Glicose 6-P, N-glicosamina 6-P, dTMP, dCMP, dAMP, dGMP entre outros, no entanto, a atividade mais eficiente é na presença de UMP (TREMBLAY; DUNAWAY-MARIANO; ALLEN, 2006). Entretanto, não há registro de interação da proteína UmpH com outras proteínas.

2. OBJETIVOS

Objetivo Geral

Analisar a relação funcional das proteínas PII sobre o Fator de Terminação de Transcrição Rho, uma Diguanilato Ciclase (AbDGC), uma Fosfodiesterase (AbPDE) e a UMP Fosfatase (UmpH).

Objetivos específicos

- ✓ Expressar e purificar as proteínas de interesse.
- ✓ Validar e caracterizar interação de PII com o Fator de terminação de Transcrição Rho em *A. brasilense* e em *E. coli*
- ✓ Caracterizar a interação entre a proteína GlnB com AbDGC e AbPDE em *A. brasilense*
- ✓ Validar e caracterizar a interação entre as proteínas PII com UmpH em *E. coli*
- ✓ Caracterizar as enzimas através de ensaios cinéticos.
- ✓ Determinar o efeito das proteínas PII sobre a atividade das enzimas.

3. CAPÍTULO I

THE TRANSCRIPTION TERMINATOR FACTOR RHO INTERACTS *IN VITRO* WITH THE SIGNAL TRANSDUCTION PII PROTEINS OF *Azospirillum brasilense* AND *Escherichia coli*

Abstract

Transcription factor Rho is a ring-shaped, homohexameric protein that causes transcript termination through actions on nascent RNAs. The process of transcription termination is essential to proper expression and regulation of bacterial genes. The PII proteins are widely distributed in nature and are pivotal players in control of metabolism. PII controls the activity of a diverse range of targets via protein-protein interaction, and in recent years the extend of these interactions have increased. Here, we show that the signal transduction protein PII associates with Rho from two different organisms, *Azospirillum brasilense* and *Escherichia coli.* Using the pull-down approach, we were able to characterize the interactions *in vitro* of Rho proteins with PII. The PII-Rho complexes in *A. brasilense* occurred in the presence of ATP and ADP, however, increasing 2-oxoglutarate levels in the presence Mg.ATP the interactions were inhibited. *E. coli*'s interactions occured preferably in the presence of ADP and were also inhibited increasing 2-oxoglutarate levels in the presence of Mg.ATP. We performed assays on Rho activity *in vitro* in the presence of PII proteins using ATP and poly[C], therefore the presence of PII did not change Rho activity under the tested conditions.

Introduction

The *rho* gene has been found in more than 90% of the sequenced bacterial genomes, suggesting an ancient origin. Futhermore, the functions associated to Rho are not easily substituted in bacterial cells (D'Heygere, Rabhi and Boudvillain, 2013). Typically, Rho is a ring-shaped homohexameric enzyme with multiple sites for interaction with RNA and ATP. It couples the energy of ATP hydrolysis to promote translocation in the 5'→ 3' direction on nascent single stranded RNA transcripts to terminate transcription (Boudvillain, Figueroa-Bossi and Bossi, 2013).

Rho-mediated termination requires protein binding to specific sequences called *rut* (rho utilization) sites, C-rich and secondary poorly-structure sites in the nascent RNA. Upon Rho anchoring the RNA, the nascent RNA passes through the homohexameric ring, and the ATPase activity fuels the translocation of Rho along nascent transcript removing bound obstacles until it catches up with RNA polymerase (RNAP) and triggers dissociation of the transcription elongation complex (Peters, Vangeloff and Landick, 2011; D'Heygère *et al.*, 2015).

The Rho factor also controls the expression of non-coding RNAs (Peters, Vangeloff and Landick, 2011), prevents formation of toxic RNA-DNA structures during transcription (R-loop) (Krishna Leela *et al.*, 2013), silences foreign DNA (Cardinale *et al.*, 2008), controls gene expression (Ciampi, 2006), regulates specific genes/operons in response to environmental cues (Bossi *et al.*, 2012; Hollands *et al.*, 2012), prevents spurious transcription and has involvement in maintenance of genome integrity (Cardinale *et al.*, 2008; Peters *et al.*, 2009, 2012).

The PII family comprises a very widespread group of signal transduction proteins that are present in Bacteria, Archaea and in plant chloroplasts (Forchhammer, 2008). The three dimensional structure of PII from several organisms have been determined showing highly conserved structure. PII proteins are homotrimers and form a compact barrel-like structure. Each monomer carries three protruding loops called loop B, C and - the most flexible - T; the last one emerges from each subunit and is vital for some of PII functions (Carr *et al.*, 1996; Xu *et al.*, 2003; Yutaka Mizuno *et al.*, 2007; Truan *et al.*, 2010).

The PII proteins regulate nitrogen metabolism through protein-protein interaction with transporters, key enzymes of nitrogen assimilation, and transcription factors or proteins that control transcriptional factors (Huergo, Chandra and Merrick, 2013). PII proteins bind ATP, ADP and 2-oxoglutarate (2-OG) (Ninfa and Jiang, 2005; Jiang, Mayo and Ninfa, 2007; Truan *et al.*, 2010). ATP or ADP binds each of the three lateral clefts located between the subunits formed by the two smaller loops (B and C loops) (Conroy *et al.*, 2007; Jiang and Ninfa, 2009; Truan *et al.*, 2010). 2-OG can only bind PII protein in the presence of Mg.ATP and the three binding sites for 2-OG have been identified near Mg.ATP (Truan *et al.*, 2010).

Considering that PII can bind ATP, ADP and 2-OG, this protein has the ability to sense the intracellular energy status through ATP/ADP ratio and nitrogen and carbon status binding 2-OG (Jiang and Ninfa, 2009; Fokina *et al.*, 2010; Truan *et al.*, 2010; Gerhardt *et al.*, 2012). The cellular levels of these metabolites can vary depending of the environment. The 2-OG levels vary according to the availability of nitrogen (Leigh and Dodsworth, 2007; Huergo *et al.*, 2012). When the nitrogen levels are high, 2-OG is used as carbon skeleton for the assimilation of nitrogen and the intracellular pool of 2-OG drops; while under low nitrogen availability, 2-OG accumulates (Schutt and Holzer, 1972; Yuan *et al.*, 2009; Radchenko, Thornton and Merrick, 2010). The ATP and ADP also vary in post-ammonium-shocked cells, at the first moment the ATP pool decreases whereas the ADP increases reciprocally (RADCHENKO; THORNTON; MERRICK, 2010). The fluctuations in the intracellular levels of these compounds affect the PII structure as well as its protein-protein interactions. In

Proteobacteria, PII is also regulated by reversible uridylylation of the T-loop. In low nitrogen the T-loop is covalently bond by UMP, whereas in high nitrogen this modification is reversed. This post-translational change also influences PII activities (Atkinson *et al.*, 1994; Araújo *et al.*, 2008).

Azospirillum brasilense is a diazotrophic α-proteobacterium found in association with several plants of agricultural interest (Dobereiner and Pedrosa, 1988). *A. brasilense* encodes two PII proteins GlnB and GlnZ (de Zamaroczy, 1998). Their most well-known function is regulation of nitrogen metabolism, but also PII can regulate fatty acid biosynthesis by the multi-subunit acetyl-CoA carboxylase/complex (ACC) in *A. brasilense*, *Escherichia coli*, *Arabidopsis thaliana* and *Synechocystis* sp. (Feria Bourrellier *et al.*, 2010; Rodrigues *et al.*, 2014; Gerhardt *et al.*, 2015; Hauf *et al.*, 2016).

Given the ability of PII acting as sensors of a variety of signals, the targets of proteins regulated by interaction with PII are likely to be larger. An interactome analyses with GlnZ from *A. brasilense* 2812 support that PII may regulate a large pool of proteins, and the Transcription Terminator Factor Rho was identified as a potential new target (Gerhardt, 2015). In this work, we confirm and explore, *in vitro*, the interactions between PII from *A. brasilense* FP2 (GlnZ and GlnB) and its Rho protein. In order to evaluate if this interaction would be conserved in other organisms, we also tested Rho protein from *E. coli* with both PII from this organism GlnB and GlnK. The Rho protein from both organisms interacted with PII in the presence of ATP and ADP and the interaction was inhibited in the presence of the 2-OG molecule.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *A. brasilense* FP2 was grown at 30°C in NFbHP medium (Machado *et al.*, 1991) containing 20 mM NH_4Cl as nitrogen source. *E. coli* strains were grown in LB medium (Sambrook *et al*., 1989) at 37°C.

TABLE 1. Bacterial strains and plasmids

Strains/plasmid	Genotype/phenotype	Source/reference
A. brasilese		
FP2	Wild-type	Pedrosa and Yates, 1984
E. coli		
DH10B	Sm^r, F‘ [*pro*AB^+ lacZΔM15]	Invitrogen
BL21 (λDE3)	Expresses T7 RNA polymerase	Agilent
Plasmids		
pET29a	Km^r. Expression vector T7 promoter	Novagen
pET28a	Km^r. Expression vector T7 promoter	Novagen
pMSA3	Km^r. (pET28a). Expresses the *A. brasilense* GlnZ carrying a 6xHis tag at N-terminal	Araujo *et al.*, 2004
pMSA4	Km^r. (pET-28a). Expresses *A. brasilense* untagged GlnZ	Moure *et al.*, 2012
pLMA-MLV1	Km^r. (pET28a). Expresses the *A. brasilense* GlnB carrying a 6xHis tag at N-terminal	Huergo *et al.*, 2007
pLH25PET	Km^r. (pET-28a). Expresses *A. brasilense* untagged GlnB	Araujo *et al.*, 2004
pGAHisGlnZΔloop	Km^r. (pET28a). Expresses the *A. brasilense* GlnZ carrying a T-loop deletion and a 6xHis tag at N-terminal	Araújo, 2018
pTRpetHisGlnK	Km^r.(pET28a). Expresses the *E. coli* GlnK carrying a 6xHis tag at N-terminal	Rodrigues *et al.*, 2014
pJT25	Amp^r. (pT7-7). Expresses *E. coli* untagged GlnK	Radchenko, Thornton and Merrick, 2010
pTRpetHisGlnB	Km^r. (pET28a). Expresses the *E. coli* GlnB carrying a 6xHis tag at N-terminal	Gerhardt *et al.*, 2015
pTRPETGlnB	Km^r. (pET-29a). Expresses *E. coli* untagged GlnB	Gerhardt *et al.*, 2015
pLHpet29RHO.3	Km^r. (pET-29a). Expresses *A. brasilense* untagged Rho	This Work
pEt28b-Rho	Km^r. (pET-28a). Expresses *E. coli* untagged Rho	Boudvillain *et al.*, 2009

Cloning and molecular biology methods

Isolation of plasmid DNA, gel electrophoresis, bacterial transformation and cloning were performed as described (Sambrook *et al*., 1989). Enzymes were obtained from commercial sources and used according to the manufacturers’ instructions. DNA sequencing was performed using dye-labeled terminators in an automated DNA sequencer ABI 3500 from Applied Biosystems.

The *A. brasilense* FP2 *rho* gene was amplified by PCR using the *A. brasiliense* FP2 genomic DNA as template, the primers: 5-CCCCCCATATGCATCTCCAAGAGCTGAAG–3' in combination with 5'-GCGGTAAGCTTCTATTGGTTCATGGACTCGAAGAA–'3 (Restriction sites for *NdeI* and *HindIII* are underlined), and high-fidelity *pfu* DNA polymerase. The PCR products were digested with *Nde*I and *Hind*III and ligated into pET29a, previously digested with the same enzymes. The resulting pLHpet29RHO.3 plasmid was sequenced to check its integrity.

Protein methods

Electrophoresis of proteins was carried out by SDS-PAGE (Laemmli, 1970) and gels were Coomassie blue stained. Protein concentrations were determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as standard.

Protein sequence analysis of AbRho and EcRho were performed on ClustalW program (Thompson, Higgins and Gibson, 1994). Domains prediction was performed on Pfam (Finn *et al.*, 2016)

Protein expression and purification
Rho from Azospirillum brasilense and Escherichia coli

BL21 (λDE3) cells carrying the pLHpet29RHO.3 or pEt28b-Rho plasmid were cultivated on 400 ml LB at 37°C to a 0.5 OD_{600}. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) was added and after 3 hours cells were harvested by centrifugation (5.000 × *g* for 10 minutes at 4°C). The pellet was resuspended in 20 ml of buffer 1 (50 mM Tris-HCl pH 8.0, 0.1 M NaCl). Cells were disrupted by sonication (10 minutes with cycles of 15 seconds) and cellular extracts were clarified by centrifugation (20.000 × *g* for 20 minutes at 4°C). The soluble fraction was first loaded onto an 1 ml heparin column (GE-healthcare) at room temperature, which was pre-equilibrated with buffer 1. The bound proteins were washed by 10 ml of buffer 1. After, bound proteins were eluted with a NaCl gradient (0.1 to 0.75 M of NaCl in buffer 1). Second, fractions containing protein of interest were loaded onto a 320 ml size exclusion column Sephacryl S-200 (GE HealthCare), which was pre-equilibrated with buffer 1, the isocratic elution occurred at a flow rate of 0.5 ml per minute at room temperature. Fractions containing AbRho or EcRho were pooled and dialyzed in buffer 2 (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 10% glycerol (v/v)).

Purification of PII

The expression and purification of His-tagged proteins (His-AbGlnB, His-AbGlnZ, His-AbGlnZΔLoop, His-EcGlnB and His-EcGlnK) were performed according to Rodrigues *et al*. (2014) and the native PII proteins (AbGlnB, AbGlnZ, EcGlnB e EcGlnK) were expressed and purified according to Gerhardt *et al*. (2015).

Complex co-purification

PII-His proteins and the untagged Rho proteins overexpressed pellets were resuspended in 10 ml of buffer 3 (50 mM Tris-HCl pH 8.0, 0.1 M KCl). Cells were disrupted by sonication (10 minutes with cycles of 15 seconds), and cellular extracts were clarified by centrifugation (20.000 × *g* for 20 minutes at 4°C). The soluble extracts containing one PII-His protein and one untagged Rho protein were combined, after adding ADP (1 mM) and $MgCl_2$ (5 mM), and loaded on the 1 ml HiTrap chelating column. Elution was performed increasing the imidazole concentration (0.1 – 1 M) at room temperature. The elution pattern was analyzed in 15% SDS-PAGE stained with Coomassie blue.

In vitro uridylylation of PII

Purified His-PII (Rodrigues *et al.*, 2014), was subjected to *in vitro* uridylylation in a 500 µl reaction mixtures containing 100 µM His-PII (considered as monomer), 1 mM ATP, 2 mM UTP, 5 mM 2-OG, 1 µM of purified *E. coli* GlnD (kindly provided by Dr. Thiago E. Rodrigues). The reaction was performed using 100 mM Tris–HCl pH 7.5, 100 mM KCl and 25 mM $MgCl_2$ as buffer. The reactions were incubated at 37ºC three hours and terminated by heating at 70ºC for 15 min to allow GlnD denaturation, after the reaction was put on ice for 10 minutes, and GlnD is further removed by centrifugation (20.000 × *g* for 15 minutes at 4°C). The His-PII-UMP was dialyzed against buffer 2 (50 mM Tris HCl pH 8.0, 100 mM KCl, glycerol 10% (v/v)). The His-PII uridylylation was determined by Native-PAGE as described previously (Inaba *et al.*, 2009).

Gel filtration chromatography

Analytical gel filtration chromatography was performed on a 25 ml Superose 6 10/30 column (GE Healthcare) at a flow rate of 0.5 ml per minute using buffer 1 at room temperature. The column was calibrated with the following molecular mass markers from Bio-Rad: Thyroglobulin (670 kDa); gama-globulin (158 kDa); ovalbulmin (44 kDa), myoglobin (17 kDa) and B12 Vitamin (1.35 kDa).

Circular dichroism (CD)

Circular dichroism (CD) was performed on a JASCO J-815 (JAPAN Spectroscopy & Chromatography Technology) spectropolarimeter coupled to a temperature controller. Proteins were diluted in 20 mM TrisHCl pH 8.0 to final hexamer concentration of 0.5 µM. The CD spectra were recorded at 25°C in the measure range of 200–300 nm. The baseline was corrected subtracting the buffer spectrum. The secondary structure content was calculated using K2D3 software (Louis-Jeune, Andrade-Navarro and Perez-Iratxeta, 2012).

Dynamic Light Scattering (DLS)

DLS analysis were carried out on a Dynapro NanoStar (Wyatt Technologies) in 10 µl volume disposable cuvette. Protein concentration of 35 µM (considering hexamer) was found to be sufficient for the assay. We performed the test with and without poly[C] (2.5 µg). Data were analysed using the software Dynamics 7.1.9, associated with the DLS machine.

NTP hydrolysis assay

Rho NTP hydrolysis activities were determined with the EnzCheck Phosphate Assay kit (Molecular Probes). In the presence of inorganic phosphate (Pi), the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is converted enzymatically by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. Enzymatic conversion of MESG results in a shift in absorbance at 360 nm. The final reactions were prepared in 200 µl and placed in a 96-well transparent plate. The reactions contained Hepes buffer pH 7.9 (20 mM), KCl (50 mM), $MgCl_2$ (1mM), MESG (0.2 mM), PNP (1 U/mL) in NTPase buffer (1 mM $MgCl_2$, 50mM Tris-HCl pH7.5, 0.1 mM

sodium azide), poly[rC] (2.5 μg) and Rho (60 nM considering hexamer). The reaction components changing will be indicated when necessary. After equilibration at 30°C for 1 min, NTP were added to the reaction, and the activity was monitored for 2 minutes. Absorbance measurements were performed in TECAN plate reader at 360 nm every 10 sec. For the determination of catalytic constants, the data were fitted to Michaelis–Menten equation using GraphPad Prism software.

Data were analyzed using One-way ANOVA followed by Tuckey test (GraphPad Prism 6.0). Probabilities of < 0.05 were accepted as significant.

Protein co-precipitation using magnetic beads (Pull-down)

In vitro complex formation was performed at room temperature using His-Magnetic beads (Promega) as described (Huergo *et al.*, 2007), with modifications. All reactions were conducted in buffer containing 50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 5 mM de $MgCl_2$, 0.05% Triton X-100 (v/v), 10% glycerol (v/v), 20 mM imidazole in the presence or absence of effectors as indicated in each experiment. Five microliters of beads were equilibrated washing with 200 μl of buffer. Binding reactions were performed in 400 μl of buffer by adding 20 μg His-PII and then 40 μg untagged Rho. To assess the specificity of the interaction between Rho and PII we used 20 μg of two different His-tag proteins, PyrE from *E. coli* (kindly provided by MSc. Ana C. Goedert) and Lonely Guy from *A. brasilense* (kindly provided MSc. Isabela C. Rodrigues). The proteins were mixed at room temperature. The beads were washed three times with 200 μl of buffer. Then, the samples were mixed with sample buffer and analyzed in 15% SDS-PAGE stained with Coomassie blue.

Gel band densitometry analyses were performed using Gel Analyzer 19.1 program.

Results

Rho protein from Azospirillum brasilense FP2 is hexameric in solution

Protein sequence analysis of AbRho and EcRho were performed and showed an identity of 68% and a similarity of 85% (Fig. S1). The organization of the domains in these proteins are also similar predicting three domains for both proteins: N-terminal domain, RNA-binding domain and nucleotide-binding domain (Fig. S1).

The Rho proteins from both organisms were similarly expressed and purified (Fig S2). The proteins showed different elution patterns in the heparin column (Fig. S2A and C), AbRho protein began to elute in the first fractions (≈ 150 mM NaCl), with the elution peak at 200 mM (Fig. S2A). EcRho protein required a higher salt concentration for its elution, the peak was at 500 mM, but the total elution extended from 350 to 550 mM (Fig. S2C). The second step of purification occurred by size exclusion column. In this step, both proteins had a similar elution pattern (Fig. S2B and D).

Typically, Rho factor, in a variety of organisms, is a ring-shaped homohexameric enzyme (Boudvillain, Nollmann and Margeat, 2010) and EcRho has previously presented this pattern (Skordalakes and Berger, 2003). To evaluate the pattern of AbRho we used Superose 6 analytical gel filtration column. AbRho monomer is 46.9 kDa and EcRho 47 kDa. Considering that both proteins are hexameric, the expected elution pattern would be around 282 kDa. The assay was performed with both proteins, AbRho and EcRho, and their elutions patterns were similar (Fig. 1A and B). EcRho protein eluted by 13.15 ml reaching a molecular weight of 270.97 kDa compared to the calibration curve, which was close to expected (~ 282 kDA) (Fig. 1A and C). AbRho protein eluted at 12.95 ml resulting in a molecular weight of 307.91 kDa also similar to that expected for a hexameric protein (Fig. 1B and C). The results showed that the proteins are in their quaternary structure in solution. Dynamic light scattering (DLS) analysis showed that the majority of the proteins in solution were in quaternary structure (Fig. S3B). The molecular weight obtained from DLS was 209 kDa for AbRho and 200 kDa for EcRho with a polydispersity (pd) of 13.2% and 15.9% respectively. In the presence of the cofactor poly[C] the molecular weight increased to 258 kDa (14% pd) for AbRho and 260 (20.9% pd) for EcRho, it is known that poly[C] stabilizes the Rho protein hexamer (Geiselmann *et al.*, 1992).

Comparing secondary structure of these proteins by circular dichroism (CD) (Fig. S3A), we found for EcRho 36.01% α-helix and 18.82% β-strand, similar to results found by Thomsen et al. (2016). For AbRho we observed a 25.6% α-helix and 24.17% β-strand.

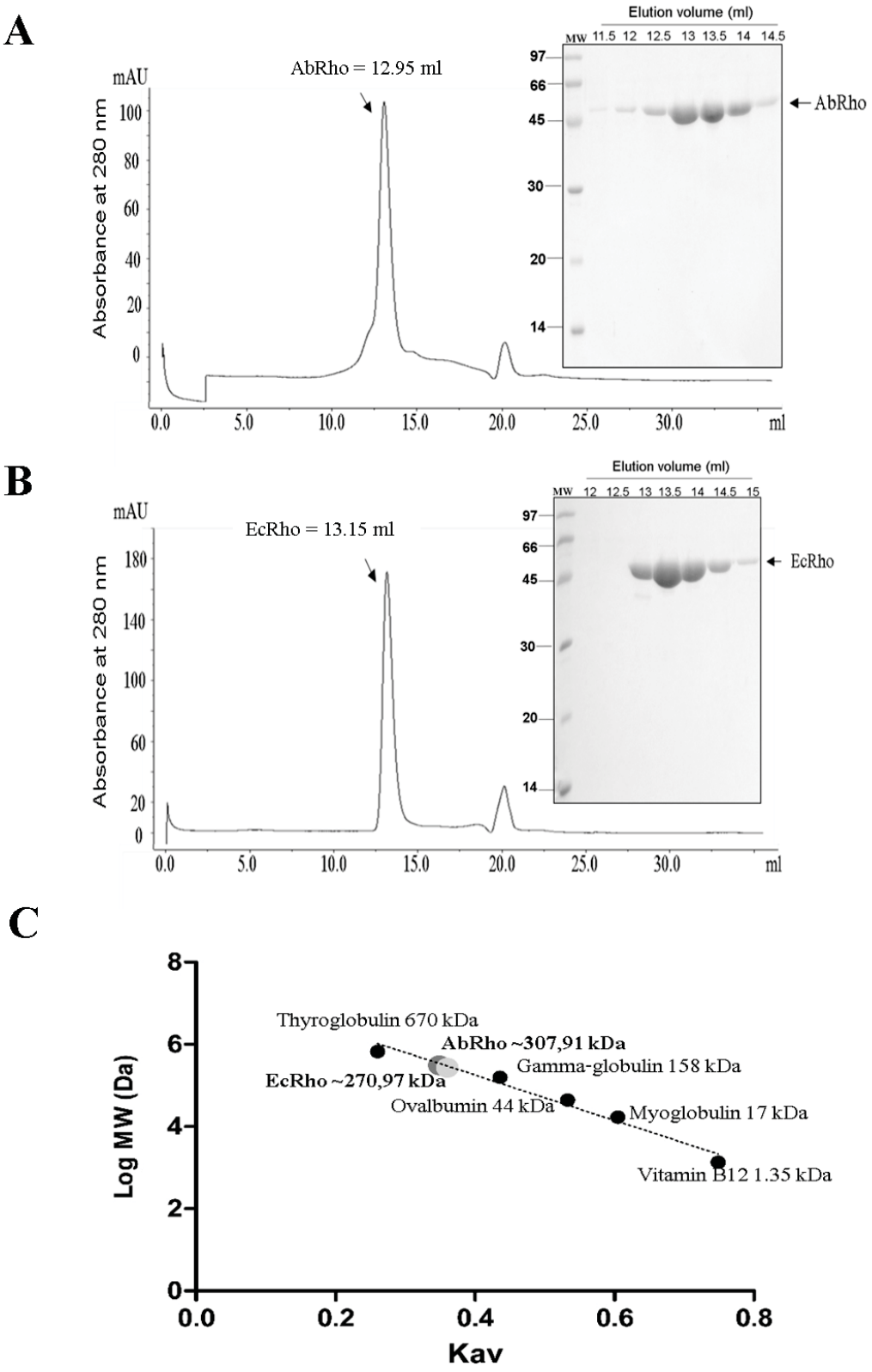

FIGURE 1. Gel filtration analysis of purified Rho proteins from *A. brasilense* and *E. coli*. A) Elution profile of purified AbRho on a Superose 6 column (GE Healthcare). B) Elution profile of purified EcRho on a Superose 6 column (GE Healthcare). C) Log MW vs Kav ($(V_e-V_0)/(V_t-V_0)$) plots of molecular mass standards (BioRad) and Rho proteins.

AbRho is an NTPase inhibited by ADP

Characterization assays were performed with *Azospirillum brasilense* Rho protein. We first tested Rho activity with its usual substrate, adenosine triphosphate (ATP). AbRho was able to use ATP as substrate. Plotting the initial velocities against increasing concentrations of ATP we got a typical Michaelis-Menten hyperbolic response (Fig. 2A). The $K_{M\text{-}ATP}$ for this enzyme was 30μM ± 2.6, similar to the range for other Rho proteins like *E.coli*'s Rho (11 μM ± 0.5) (Park *et al.,* 1995) and *Mycobacterium tuberculosis*' Rho (70 μM ± 14) (Kalarickal *et al.*, 2010). In *E. coli* all six pockets have the ability to bind nucleotides, but studies indicated that Rho hexamer binds only three ATP or RNA molecules at the alternate sites owing to negative cooperativity (Stitt, 1988; Geiselmann and Von Hippel, 1992). The calculated K_{CAT} Of *E. coli* Rho protein is 10 s^{-1} and takes into account three active sites per hexamer (Stitt and Xu, 1998). Assuming that in *A. brasilense* this phenomenon can also occur, the K_{CAT} calculated for three active sites was 5.5 s^{-1} (± 0.17), a result similar to that seen in *E. coli*.

All assays were performed in the presence of commercial poly[C], a RNA cofactor which is an important factor for AbRho activity (Fig. 2B).

We investigated whether AbRho had substrate preference within all triphosphate nucleotides. Just like EcRho (Nowatzke and Richardson, 1996), AbRho protein showed the ability of utilize all triphosphate nucleotides (Fig. 2). Statistical analysis showed a preference of AbRho for ATP and UTP.

The diphosphate nucleotide ADP is the reaction product and is a competitive inhibitor of EcRho (Galluppi and Richardson, 1980). For AbRho a dose dependent inhibition was observed when we tested different ADP concentrations at fixed ATP (1mM) (Fig. 2D).

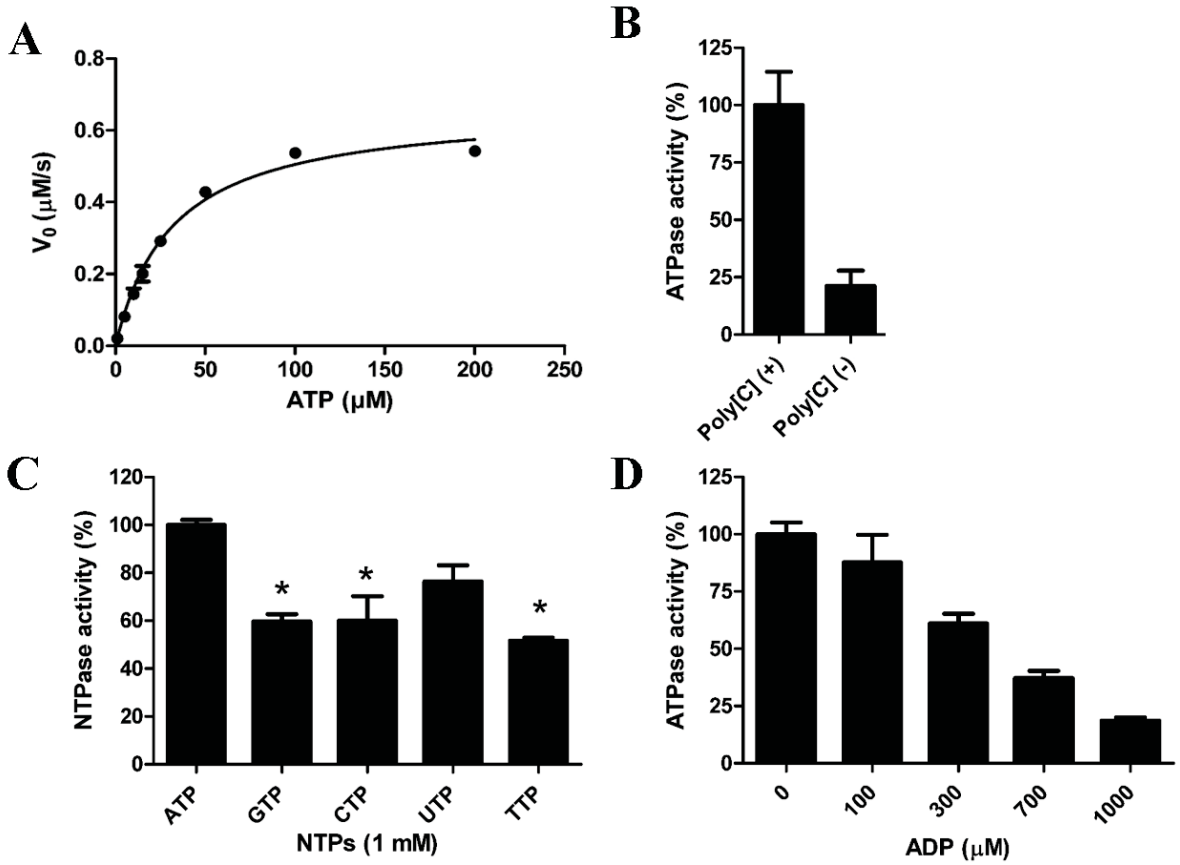

FIGURE 2. RNA-dependent NTP hydrolysis activities of the AbRho protein. AbRho activity was determined with the EnzCheck Phosphate Assay kit. Data are reported as the average of duplicate experiments showing the standard deviation. A) AbRho activity in the presence of increasing concentration of ATP (1-200 µM). B) Stimulation of the ATPase activity of AbRho by polyribonucleotide poly[rC]. The reaction was conduct with and without poly [rC] (2.5 µg) C) Comparison of the NTPase activities under the same standard conditions. All nucleotides used were at 1 mM. Statistical Analysis was performed with One-way ANOVA followed by Tukey test. * p≤ 0,05 compared to ATP D) Effect of the presence of different concentrations of ADP on AbRho activity, the concentrations of ATP in the assays was 1 mM. The different ADP concentrations were added to reaction mixtures in the presence of AbRho, ATP was the last component added and was used to start the catalysis.

AbRho and EcRho Interact with PII Signal Transducer Proteins

Gerhardt (2015) developed an interactome assay showing possible new GlnZ targets in *A. brasilense* 2812 and one of them was the transcription termination factor Rho. *A. brasilense* has two PII proteins, GlnZ and GlnB. In order to validate the AbRho-GlnZ interaction and test if this interaction could also occur with GlnB, *A. brasilense* PII proteins were purified in a N-terminal 6xHis-tag form and were used for *in vitro* co-precipitation (pull-down) assays with native AbRho. To evaluate if this interaction could be conserved in other

organisms we also tested Rho protein from *E. coli* with both PII from this organism EcGlnB and EcGlnK.

AbRho protein showed a low affinity for the bead (Fig. 3 lanes 2, 5 and 8), however, it is clear the enrichment that occurs in the presence of PII. AbRho interacts with both PII proteins (GlnB and GlnZ), in the presence of ATP and ADP, but in the presence of Mg.ATP and 2-OG, the interaction does not occur (Fig. 3). EcRho also interacts with the two PII proteins; however, EcRho in the performed assay shows greater complex with GlnB than GlnK, the EcRho-GlnK complex was observed only in the presence of ADP and in the EcRho-GlnB complex, ADP showed a positive effect in complex formation (Fig.3). Mg.ATP + 2-OG inhibit the EcRho-PII complexes.

We performed a co-purification assay to confirm the complexes between PII and Rho *in vitro* (Fig. S4). We used soluble extracts containing His-PII protein and untagged Rho overexpressed. Both extracts were combined, 1 mM ADP were added and loaded on HiTrap chelating column. 6xHis-PII has affinity and binds to HiTrap column while the native AbRho and EcRho do not (Fig.S4C and F). After imidazole elution all the complexes, AbRho-GlnB, AbRho-GlnZ, EcRho-GlnB and EcRho-GlnK, remained stable (Fig.S4A, B D and E) confirming complexes formation.

To verify whether the interaction between AbRho and EcRho with PII proteins was specific, we used two random 6xHis proteins and performed a pull-down assay together with AbRho and EcRho. The *E. coli* random protein was PyrE, involved in pyrimidine synthesis and *A.brasilense* random protein was the Lonely Guy (LOG), which participated on the cytokinin metabolism. AbRho and EcRho did not interact with random 6x-His proteins, showing that the interaction with PII is specific (Fig. S5). We also found that AbRho protein interacts with PII in the absence of effectors and in the presence of only 2-OG (1 mM), reinforcing the need for Mg.ATP to respond to 2-OG. While for EcRho, it does not interact with PII without effectors or when in the presence of only 2-OG (Fig. S6).

Rho and PII proteins can bind the Mg^{2+} ion as cofactor. We investigated whether the absence of this ion would interfere with the formation of the complexes. The Mg^{2+} binding on PII protein requires ATP, for that reason the reaction were performed at fixed concentration of ATP (1 mM). For AbRho-GlnB and AbRho-GlnZ we found that the absence of Mg^{2+} decreases the formation of the complexes but they still occurring (Fig. 4). The EcRho-GlnK did not occur in any tested condition when ATP was the effector. EcRho-GlnB complex decreases in the absence of Mg^{2+} (Fig. 4).

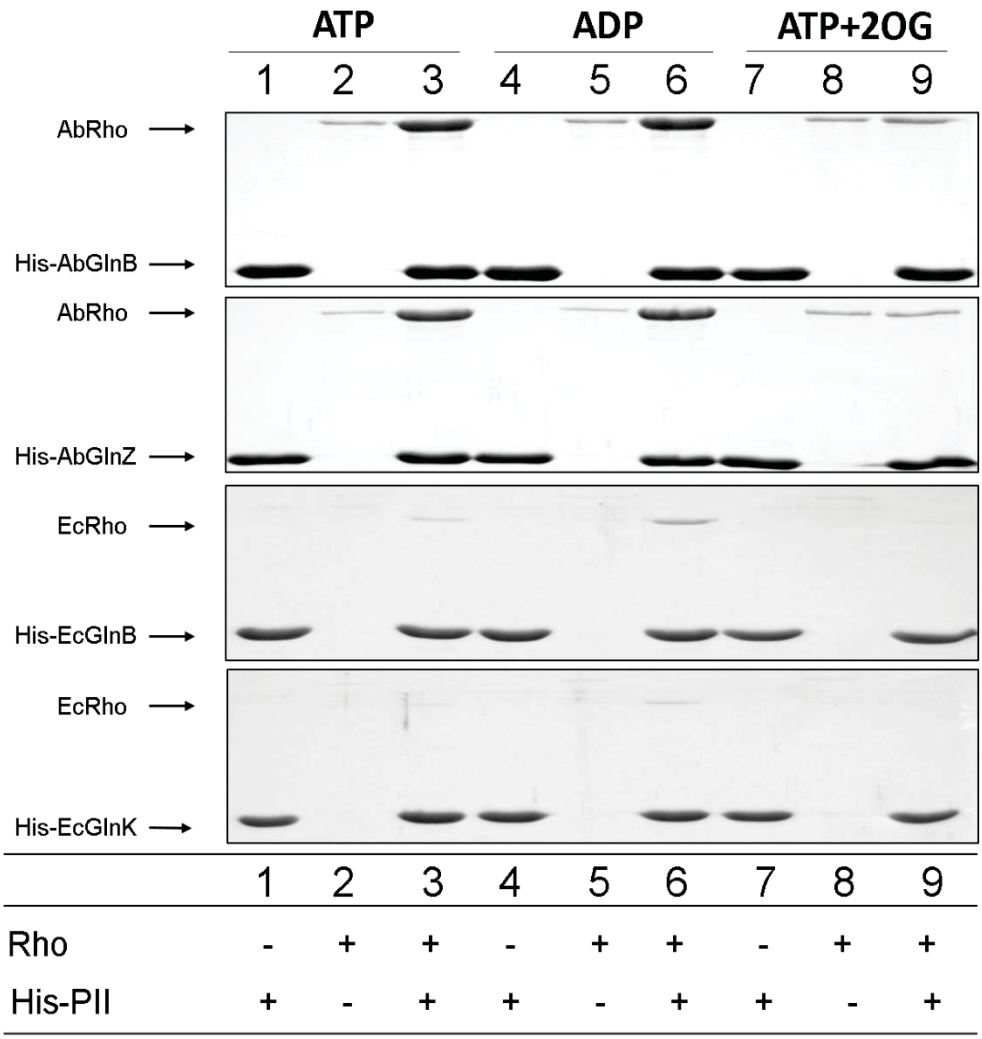

FIGURE 3. *In vitro* formation of the AbRho-PII and EcRho-PII complexes. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of $MgCl_2$ (5mM) and the effectors ATP, ADP and/or 2-OG at 1mM as indicated. Binding reactions were conducted in 400 µl of buffer adding purified His-PII (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

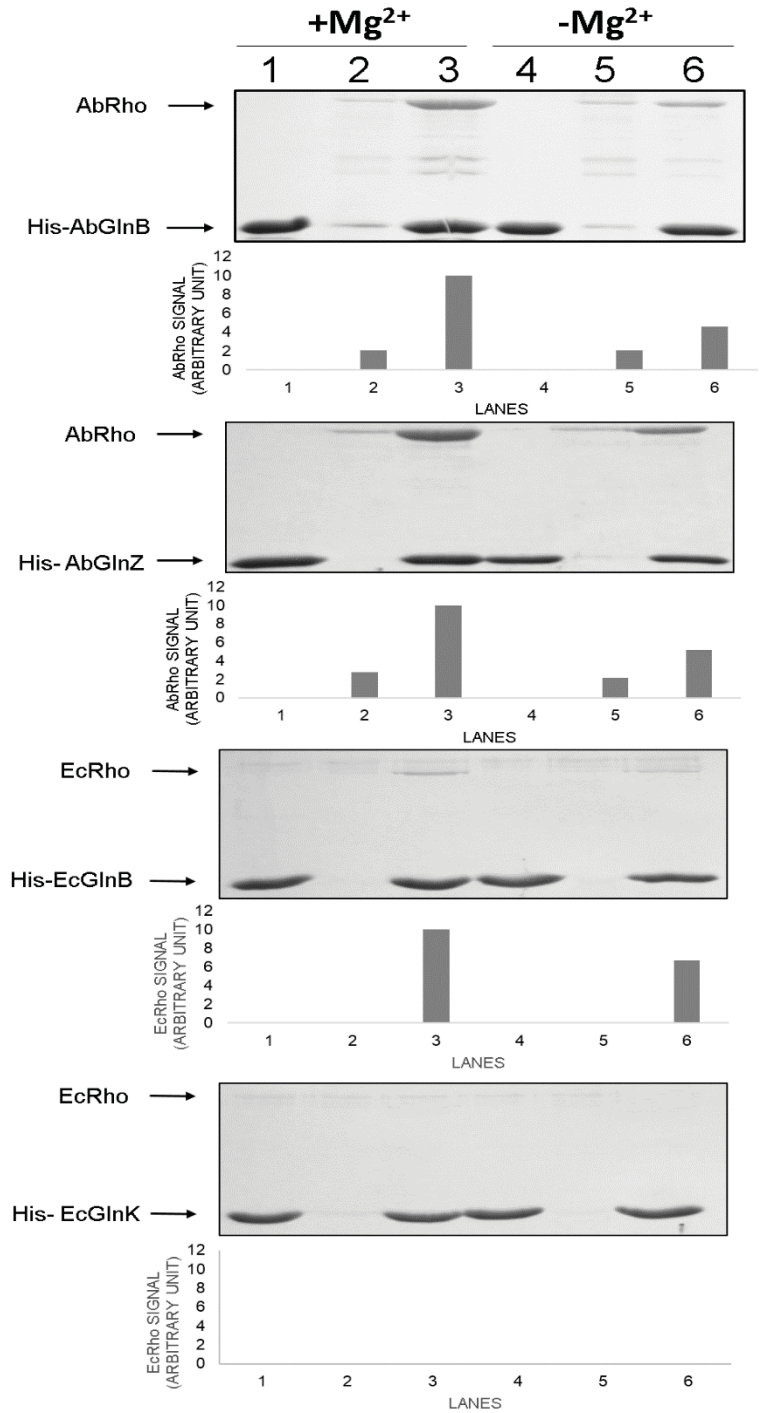

FIGURE 4. ***In vitro*** **formation of the AbRho-PII and EcRho-PII complexes are stabilized by Mg^{2+}.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed ATP concentration (1 mM) in the presence or absence of $MgCl_2$ (5mM) as indicated. Reactions were conducted in 400 µl of buffer adding purified His-PII (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

2-OG prevents formation of the Rho-PII complex of A. brasilense and E. coli

Since high 2-OG inhibits Rho-PII complex formation (Fig. 3), we investigated the influence of 2-OG on the Rho-PII interaction. A new pull-down assay was performed increasing the 2-OG concentration in the presence of ATP (1 mM) and Mg^{2+} (5 mM). We found that increasing 2-OG concentration inhibits the complex formation in a dose dependent manner for AbRho-GlnZ, AbRho-GlnB and EcRho-GlnB complexes (Fig. 5).

For AbRho-GlnZ and AbRho-GlnB interactions, 0.01 mM 2-OG concentration decreased by half the amount of complexes formed when compared to the interaction in the absence of 2-OG (Fig. 5 and S7, lanes 3 and 4). For the highest concentrations tested almost no complexes were detected.

EcRho-GlnB complex occurs in the presence of 2-OG at concentrations 0.01 and 0.1 mM (Fig. 5 and S7, lanes 3, 4 and 5). The interaction in the absence of 2-OG and at a concentration of 0.01 mM of 2-OG was similar, dropping by half at a concentration of 0.1 mM (Fig. 5, lane 5 and Fig. S7), for 1 mM and 2 mM no interaction was detected. EcRho-GlnK interaction was not detected in any tested condition (Fig. 5 and S7).

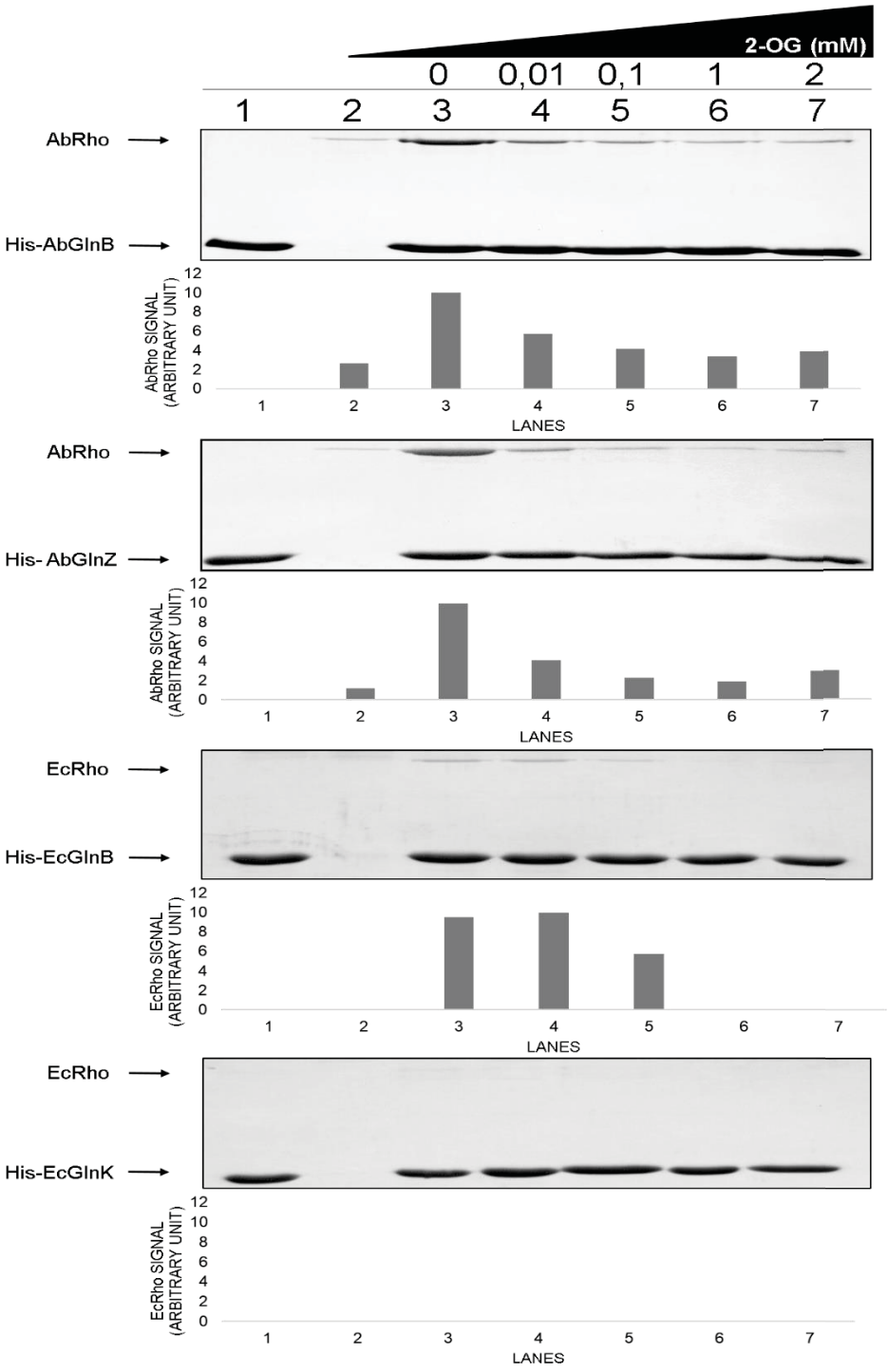

FIGURE 5. ***In vitro*** **formation of the AbRho-PII and EcRho-PII complexes are dependent on the 2-OG concentrations.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of ATP (1 mM) and $MgCl_2$ (5mM), increasing concentrations of 2-OG as indicated. Reactions were conducted in 400 µl of buffer adding purified His-PII (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

Rho-PII complexes formation are positively influenced by ADP concentration for Escherichia coli, but not for Azospirillum brasilense

We evaluated the influence of ATP and ADP in Rho-PII complexes. Assays were performed by pull-down with different concentrations of these two effectors (Fig.6). The AbRho-PII interactions were not influenced by ATP or ADP, and complexes formation occurred with GlnB and GlnZ in all tested effector's concentrations (Fig.6). In *E. coli* ADP positively influences *in vitro* interaction with GlnB and GlnK (Fig. 6). The higher is ADP concentration, the more stable the interaction becomes, with more complexes being detected when the ADP concentration became higher than ATP (Fig. 6, lane 7).

We performed pull-down increasing the concentration of ATP and ADP effectors independently. As expected, AbRho-PII complexes formation were not influenced by these effectors under any tested conditions (Fig. S8 and S9). For EcRho, the results were different for each PII. EcRho-GlnB interaction occurred in an ADP-dependent manner. It started at 0.01 mM and stabilized in 0.75 mM (Fig. S8). At different ATP concentrations, the interaction occurred in a similar way under all tested conditions, showing that lower ATP concentrations stabilize the complex, but the amount of complexes formed is low (Fig. S9). The EcRho-GlnK interaction started in the presence of a higher concentration of ADP (0.1 mM) compared with GlnB (Fig. S8), and no interactions were visualized in the presence of ATP (0.01 – 1 mM) (Fig. S9).

One assay containing the three effectors was also performed. In general, AbRho interacted likewise with GlnB and GlnZ, and the interaction occurred in the presence of 2-OG (0.1 mM), but was inhibited as the concentration of 2-OG increased, except in the absence of ATP (Fig. S10). It is noted in interactions with EcRho that the interaction increases as the concentration of ADP increased, indicating that ADP is binding GlnB preventing the inhibitory action of 2-OG. In high 2-OG and in the presence of Mg.ATP the EcRho-PII interactions are totally inhibited (Fig. S10).

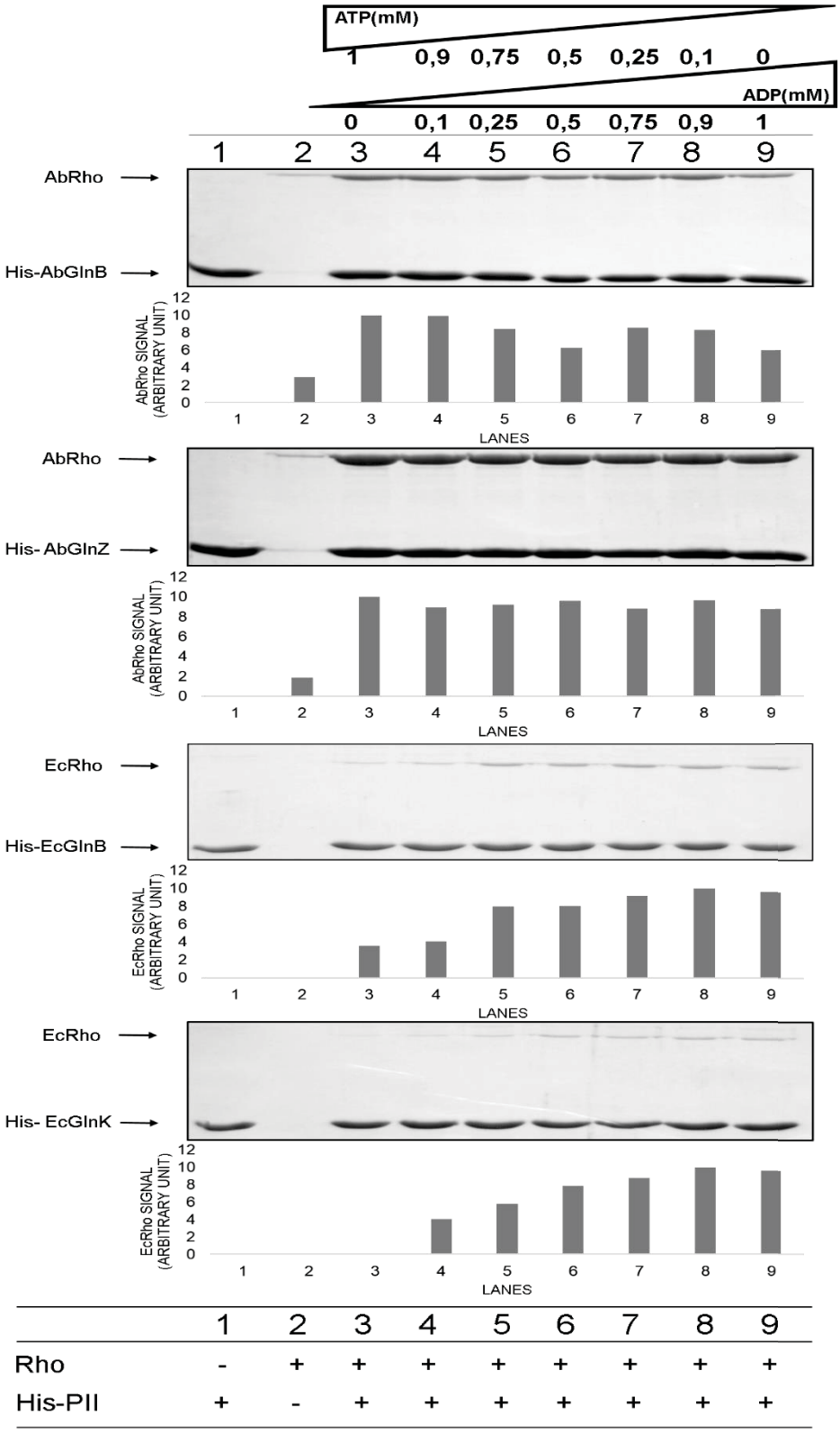

FIGURE 6. ***In vitro*** **formation of the EcRho-PII complexes increase as the [ATP]/[ADP] ratio decreases, but [ATP]/[ADP] ratio does not affect the AbRho-PII complexes.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under different concentration of ATP and ADP as indicated and fixed concentration of $MgCl_2$ (5mM). Reactions were conducted in 400 µl of buffer adding purified His-PII (20 µg) mixed native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

AbRho interacts with PII in the absence of T loop and when it is post-translational modified

In order to investigate whether the interaction between AbRho and PII occurs via T loop, we used the T loop partially truncated *glnZ* gene, expressing the GlnZΔLoop protein to perform the interaction assays. AbRho-GlnZΔLoop interaction occurred even in the presence of Mg.ATP + 2-OG, indicating that this interaction does not occur via T Loop (Fig. 7).

During nitrogen increase, PII suffers a post-translational modification, called uridylylation. Hence, we investigated if this modification could interfere in the interaction with AbRho. The assay was performed with GlnB and GlnZ partially uridylylated (Fig. S11) and we observed that this post-translational modification does not prevents the interaction (Fig. 8).

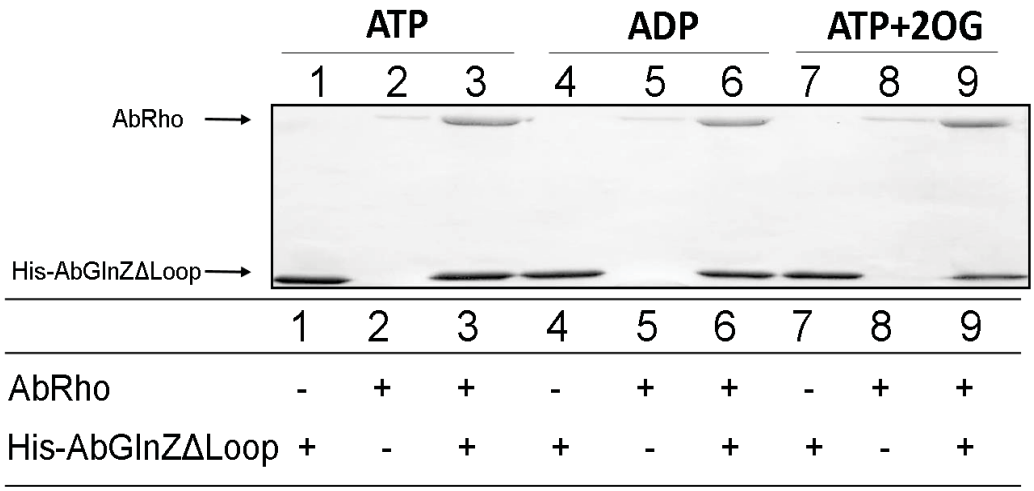

FIGURE 7. *In vitro* formation of the AbRho-GlnZ complex does not require the T loop. Complex formation was assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of the effectors ATP, ADP and/or 2-OG at 1mM, as indicated and fixed concentration of $MgCl_2$ (5 mM). Binding reactions were conducted in 400 µl of buffer adding purified His-GlnZΔLoop (20 µg), mixed with AbRho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

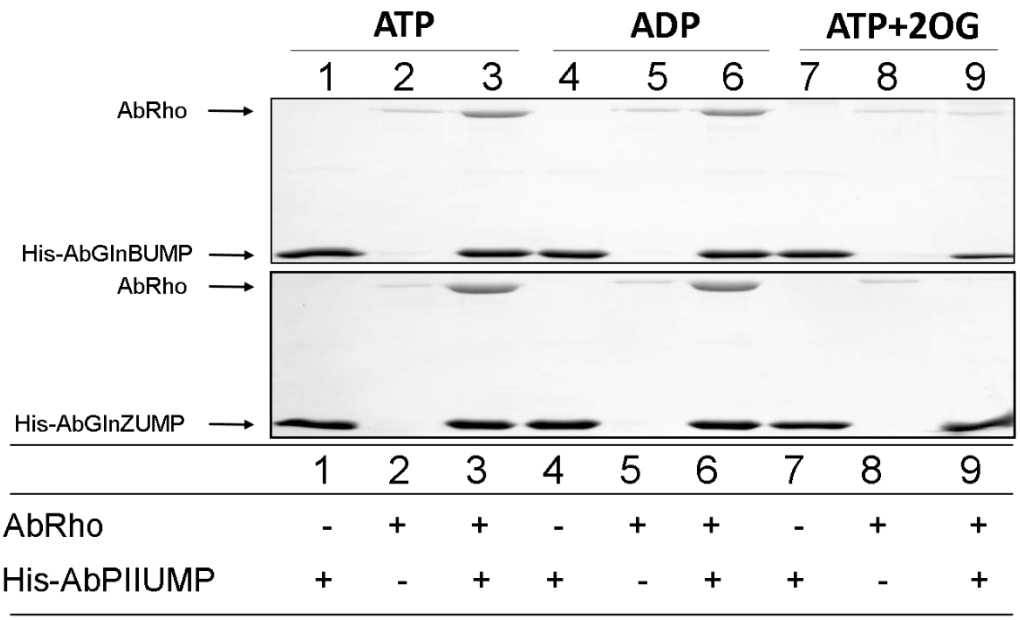

FIGURE 8. *in vitro* formation of the AbRho-PII complexes occur independently of the uridylylated form of PII. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of the effectors ATP, ADP and/or 2-OG at 1mM, as indicated and fixed concentration of $MgCl_2$ (5 mM). Binding reactions were conducted in 400 µl of buffer adding purified His-PII (20 µg) uridylylated, mixed with AbRho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

AbRho and EcRho activity in the presence of PII

In order to examine whether the presence of PII proteins influence Rho activity, the ATPase activity was measured. Both AbRho and EcRho showed the same ATPase activity in the presence or absence of PII proteins (Fig. 9A). ATP concentration was varied for AbRho from 1 to 200 µM in the presence and absence of GlnB, but the results showed the same K_M, $V_{Max,}$ and K_{CAT} (Fig. 9B and C) with or without PII in the reaction. What may have occurred is that the Rho-PII interaction do not influence ATPasic activity, affecting another Rho's activity such as helicase, or that this complex needs a specific condition for the effect to be visualized that has not been tested in the reactions or that in a specific condition Rho is sequestered by the membrane by PII as observed for DraG (Huergo *et al.*, 2007, 2009). Something recurring in Rho interactions is that the influence of other proteins on its activity can be masked by the cofactor poly[C], since it has a great affinity for Rho, and it can mask the influence of other proteins on Rho's activity (Pani *et al.*, 2006; Rabhi *et al.*, 2011).

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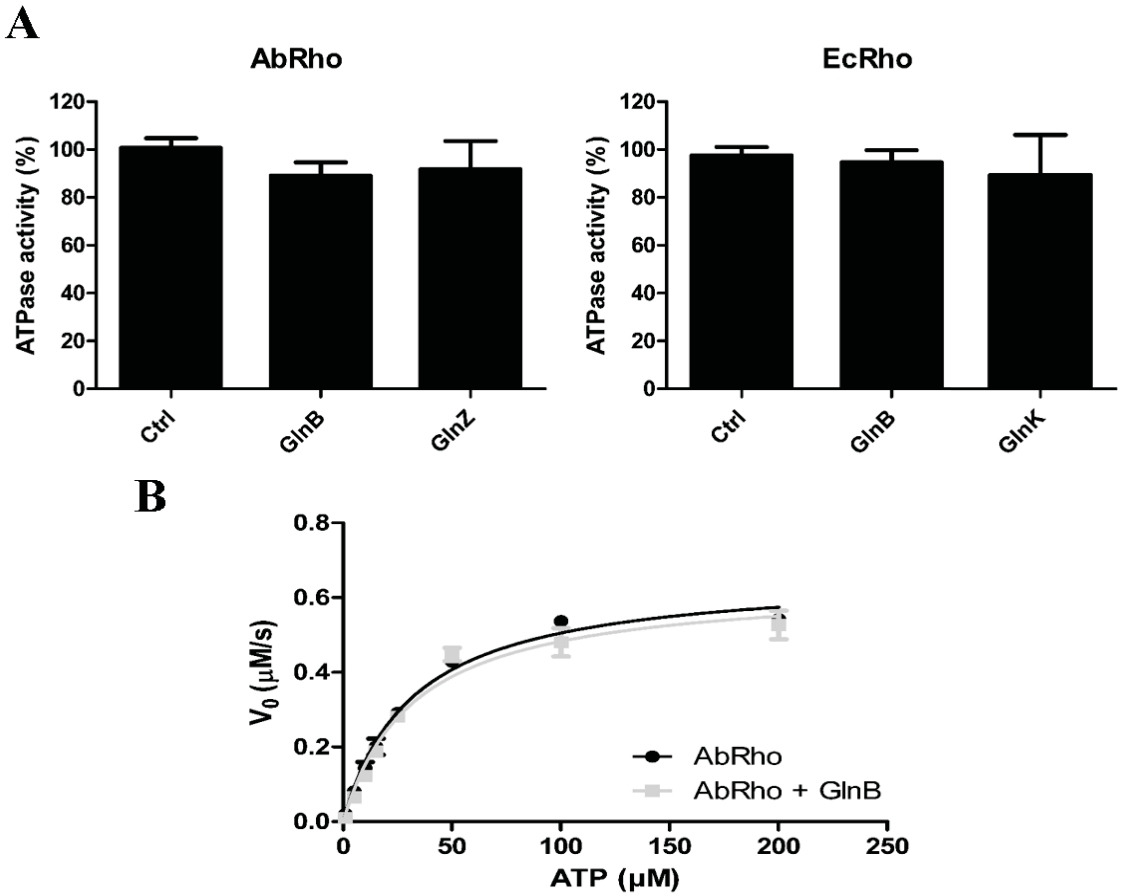


C

	$K_{M\text{-}ATP}$ (µM)	V_{Max} (µM/s)	K_{CAT}* (s^{-1})
AbRho	31.72 ± 2.86	0.66 ± 0.02	5.5 ± 0.35
AbRho + GlnB	32.16 ± 4.35	0.64 ± 0.03	5.3 ± 0.51

FIGURE 9. The effects of PII protein on Rho activity. AbRho activity was determined with the EnzCheck Phosphate Assay kit. Data are reported as the average of duplicate experiments showing the standard deviation (±). In all conditions tested, the concentration of Rho was 60 nM, PII was 600 nM and poly[C] was 2,5 µg. A) AbRho and EcRho activity in the presence of PII protein. B) AbRho activity in the presence of increasing concentration of ATP in the presence of GlnB. C) The $K_{M\text{-}ATP}$, V_{Max} and the K_{CAT} determined for AbRho and AbRho + GlnB by fitting the data into Michaelis–Menten equation using the GraphPad Prism software. *K_{CAT} was calculated considering three active sites.

Discussion

In our study we characterized Rho from *A. brasilense*, and compared it to the well-studied *E. coli*'s Rho protein. The primary structure of these two protein are similar (Fig. S1). The oligomeric propensities of EcRho is widely known, the homohexameric form was found

to be the major state of EcRho in the presence of ATP and RNA (Geiselmann *et al.*, 1992). In this work we observed AbRho assuming an hexameric structure in solution.

The $K_{M\text{-}ATP}$ obtained for the AbRho protein is in the same range compared to other Rho proteins already analyzed. Considering that in *E. coli* the level of ATP is stable (2 mM), generally two orders of magnitude greater than the $K_{M\text{-}ATP}$ of EcRho (Radchenko, Thornton and Merrick, 2010) and assuming that a similar pattern occur in *A. brasilense*, AbRho and EcRho can operate at V_{Max} in the cell. EcRho (Nowatzke and Richardson, 1996) and AbRho can also hydrolyze other nucleotides than ATP, showing a broad nucleotide substrate specificity (Fig. 2C). The diphosphate nucleotide ADP is the reaction product and is a competitive inhibitor of EcRho ATPase activity (Galluppi and Richardson, 1980). A similar effect was observed here with AbRho (Fig. 2D). High ADP concentrations regulate the Rho activity, indicating that ADP and ATP compete for the same site in AbRho.

Rho protein has many functions and among them this protein acts in the regulation of gene transcription, a function shared with other PII targets like NtrB and NifA (Huergo, Chandra and Merrick, 2013). In all assays to investigate the interaction of AbRho-PII and EcRho-PII we observed the complex formation was inhibited in the presence of Mg.ATP + 2-OG (Fig. 3 and Fig. 5). The binding of Mg.ATP + 2-OG to PII causes a conformational changes in PII, especially in T-Loop, which causes inhibition of many interactions of PII with their targets (Jiang and Ninfa, 2007; Truan *et al.*, 2010).

The interaction between Rho and PII can only happen when the level of 2-OG is low. This state usually occurs when the nitrogen available is high, which increases the concentration of intracellular glutamine and decreases the concentration of 2-OG (Leigh and Dodsworth, 2007). 2-OG in *E. coli* has already been quantified, the lower limit of detection of this metabolite was determined minutes after an abrupt increase of ammonium in nitrogen-limiting cells, this made 2-OG levels reach 0.3 mM (Radchenko, Thornton and Merrick, 2010), just above the minimum for interaction shown in *in vitro* assays (Fig.5). Other *in vitro* assays have also shown total limitation of interaction at low levels of 2-OG, with 0.01 mM for AmtB-GlnZ (Huergo *et al.*, 2007) and 0.1 mM for DraG-GlnZ (Huergo *et al.*, 2009), and both proteins are regulated by PII *in vivo*. We also have to take into account that 2-OG quantification is challenging due to difficulties involved in this determination, such as 2-OG leakage or degradation by cell metabolism, or fluctuations in cell volume under the sampling conditions (Yan, Lenz and Hwa, 2011).

The dissociation constant (K_d) of 2-OG in saturated ATP was evaluated in *E. coli* (Jiang and Ninfa, 2007) and *A. brasilense* (Truan *et al.*, 2014). In *E. coli*, the binding of 2-OG

shows negative cooperativity and therefore has three K_d, they are 0.012 mM, 0.12 mM and 5 mM. Relating to the data obtained for *E. coli*'s Rho-PII complexes (Fig. 5), when 2-OG binds the first site, it already causes inhibition of the interaction, the binding in the second increases the inhibition and in the above concentrations, the interaction is completely inhibited. In *A. brasilense* there is no cooperativity, and the K_d for 2-OG is 0.048 mM. Therefore, in 0.1 mM of 2-OG all PII sites would be occupied. The data obtained from the AbRho-PII interaction showed this relation, since from 0.1 mM band densitometry in the AbRho-PII interaction was not significantly altered (Fig. 5 and Fig. S7).

Differently of 2-OG, ATP and ADP binding cause only a slight change in the conformation of PII. Almost all know PII interactions were detected in ADP or ATP presence. Unlike mostly know PII interactions, AbRho-PII complexes are formed in presence of ADP and ATP, without dose dependence and they also occur in effector absence. EcRho-PII complexes, however, requires ADP and/or ATP. EcRho-GlnB is stable in the presence of ATP, without dose dependence, however for ADP, the formation of this complex increase as the level of ADP increases. EcRho-GlnK was only formed in the presence of ADP (Fig. 3 and Fig. 6). The requirement of ATP or ADP for PII complexes formation have already been reported. ADP is generally the required effector, as example for AmtB-GlnK interaction (Radchenko, Thornton and Merrick, 2010), AmtB-GlnZ (Rodrigues *et al.*, 2011), and the proteins involved in the regulation of Nitrogenase DraT with GlnB and DraG with GlnZ (Huergo *et al.*, 2009).

Many of PII interactions occur via the T loop, a flexible and expose region in PII surface. For AbRho-GlnZ, however, T loop was not required for complex formation, indicating that interaction did not occur via T loop (Fig. 7). Although not common *A. brasilense* DraG, also does not interact with GlnZ by T loop, interacting on the lateral face of GlnZ (Rajendran *et al.*, 2011). Despite the fact of T loop was not required for AbRho-GlnZ complex, the interaction of AbRho-GlnZΔloop in the presence of Mg.ATP + 2-OG (Fig. 7) suggests that the T-loop structure assumed when its these effectores is important for the inhibition of the complex. The fact that T loop is not required for AbRho-GlnZ complex formation in reinforced by detection of interaction with uridilylated PII. Since this post-translational modification occurs in Tyr51 in T loop, the presence of a charged group can impair interaction with same PII targets, especially when this interaction is made via T loop.

All PII, AbRho and EcRho binds Mg^{2+} as cofactor. The interaction in the presence and absence of magnesium revealed that the lack of this ion interferes with the AbRho-GlnZ, AbRho-GlnB and EcRho-GlnB interactions (Fig. 4). It has already been reported that Rho

protein binds to Mg^{2+} and that the lack of this ion can decrease the activity of this protein (Stitt, 1988), while PII can bind both ATP and Mg.ATP. Considering that ATP was the effector used in this interaction, probably the conformation achieved with Rho and PII bound to Mg.ATP favored the interaction.

PII can influence the activity of its targets in many ways. We tried to assess whether PII interfered in Rho's ATPase activity. However, under the conditions tested we were not successful to detect any effect of PII on the activity of Rho proteins (Fig. 9). This absence of effect could be related with RNA cofactor (poly[C]) used in our essay. Previous assays with EcRho showed an interaction with Hfq protein, a post-transcriptional regulator. When it binds to Rho, Hfq prevents Rho from ending transcription. This regulation were only detected when the authors used a RNA similar to natural *rut* site (Rabhi *et al.*, 2011), in the presence of poly[C], an artificial RNA cofactor, any regulation was observed (Rabhi *et al.*, 2011).

Another similar case occurred with the Psu protein of bacteriophage P4. This protein also suppresses Rho termination activity. However, in the presence of poly[C] it is not possible to observe this effect. The authors also tried to assess whether the K_M changed in the presence of Psu and poly[C] but were unsuccessful, they only observed the inhibition of Psu when they used a natural RNA (Pani *et al.*, 2006). The explanation for these cases is that the presence of a cofactor as strong as poly[C], which has a high affinity for binding sites in Rho, can mask possible effects in Rho leaving its activity at maximum.

In a study carried out with different Rho mutants in *E. coli*, while many had their activity decreased in the presence of a natural mRNA, in the presence of poly[C] the activity of mutant Rho remained, indicating that poly[C] caused an optimal ATPase conformation even in Rho mutants (Chen and Stitt, 2004). Because poly[C] is a very strong cofactor, this may have masked the effect of PII on Rho activity (Fig.9).

Considering all the *in vitro* data obtained, we can formulate a possible cellular condition for Rho-PII interaction (Fig. 10). Under low glutamine the intracellular levels of and 2-OG accumulate. The PII proteins are fully uridylylated and presumably bound to Mg.ATP + 2-OG. Which do not result in PII-Rho complexes. When nitrogen starved cells receive an ammonium shock, the intracellular level of glutamine increases, PII is deuridylylated and the intracellular 2-OG concentration drops and there are a transitory fluctuation on ATP/ADP ratio, increasing the concentration of ADP and decreasing ATP. In this condition, Rho could interacts with PII, being able to inhibit or stimulate Rho activity, until the levels of 2-OG increase again. Furthermore, the interaction of PII with Rho can have a variety of functions such as preventing Rho from binding with other proteins at low 2-OG

levels, or for being sequestered from the cytoplasm to the membrane as occurs with DraG which is sequestered by the AmtB-GlnZ complex in ammonium shock conditions in *A. brasilense* (Huergo *et al.*, 2007, 2009).

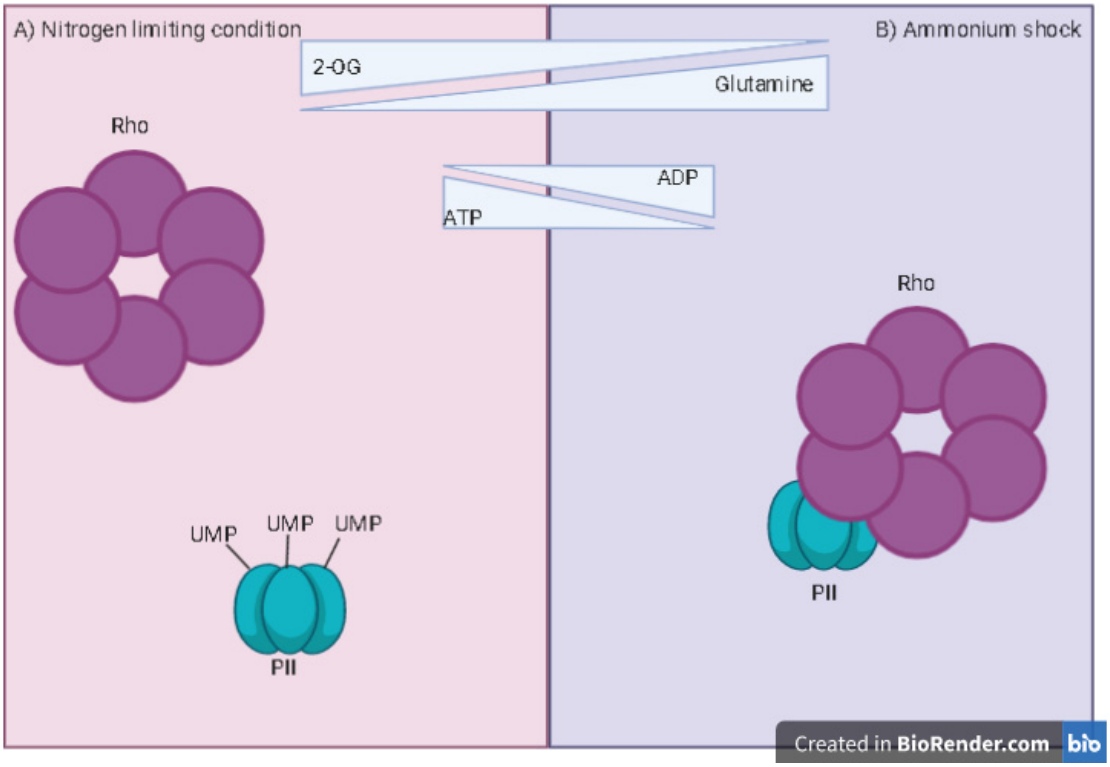


FIGURE 10. Interaction model of Rho-PII complex. *In vitro* assays showed that, the Rho-PII interaction occur when the intracellular 2-OG level is low. The ammonium shock occurs when a nitrogen limited bacterial cell receive a high ammonium concentration, in this particular state the 2-OG level drops, probably allowing Rho-PII interaction. ADP also increases in this metabolic state, positively influencing the interaction. When ammonium is consumed 2-OG level increases and it binds PII, no longer allowing PII to interact with Rho.

Conclusion

New targets for PII proteins are being discovered and this increases the importance of this protein within microbial metabolism. PII are signal transducer proteins, which the effectors binding or post-translational modification, cause a conformational change influencing its interactions with their targets, modulating different cellular behaviors. Several PII targets are transcriptional regulators and enzymes, in this work we described the interaction of PII proteins in *A. brasilense* and *E. coli* with their Rho proteins, whose main functions is to terminate bacterial transcription using its ATPase activity and transcription regulation. The main inhibitory effector of PII-Rho interactions in *A. brasilense* and *E. coli*, is the PII's effector 2-OG, probably this molecule binds PII protein causing a conformational

changing, preventing the PII-Rho complexes formation. Therefore, this interaction probably occurs when the 2-OG is low, one example of a metabolic state when 2-OG drops is when nitrogen-limiting cell encounters high available nitrogen concentration. This condition causes a decrease in the intracellular levels of 2-OG, allowing the interaction between PII and Rho.

References

Araújo, L. M. *et al.* (2008) 'Different responses of the GlnB and GlnZ proteins upon in vitro uridylylation by the Azospirillum brasilense GlnD protein.', *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas*, 41(4), pp. 289–94. doi: 10.1590/s0100-879x2008000400006.

Araujo, M. S. *et al.* (2004) 'In vitro uridylylation of the Azospirillum brasilense N-signal transducing GlnZ protein', *Protein Expression and Purification*, 33(1), pp. 19–24. doi: 10.1016/j.pep.2003.08.024.

Araujo, G. A. T. (2018). Caracterização *in vitro* das enzimas málica MaeB e N-acetil glutamato quinase NAGK e interação com proteínas PII em *Azospirillum brasilense. Dissertação (Mestrado em Ciências - Bioquímica) - Set. Ciências Biológicas, Univ. Fed. do Paraná.*, 1–97.

Atkinson, M. R. *et al.* (1994) 'Reversible uridylylation of the Escherichia coli PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC).', *The Journal of biological chemistry*, 269(45), pp. 28288–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7961766 (Accessed: 7 July 2018).

Bossi, L. *et al.* (2012) 'A role for Rho-dependent polarity in gene regulation by a noncoding small RNA.', *Genes & development*. Cold Spring Harbor Laboratory Press, 26(16), pp. 1864–73. doi: 10.1101/gad.195412.112.

Boudvillain, M. *et al.* (2009) 'Simple Enzymatic Assays for the In Vitro Motor Activity of Transcription Termination Factor Rho from Escherichia coli', in *Methods in molecular biology (Clifton, N.J.)*, pp. 137–154. doi: 10.1007/978-1-60327-355-8_10.

Boudvillain, M., Figueroa-Bossi, N. and Bossi, L. (2013) 'Terminator still moving forward: expanding roles for Rho factor', *Current Opinion in Microbiology*, 16(2), pp. 118–124. doi: 10.1016/j.mib.2012.12.003.

Boudvillain, M., Nollmann, M. and Margeat, E. (2010) 'Keeping up to speed with the transcription termination factor Rho motor', *Transcription*, 1(2), pp. 70–75. doi: 10.4161/trns.1.2.12232.

Bradford, M. (1976) 'A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding', *Analytical Biochemistry*, 72(1–2), pp. 248–254. doi: 10.1006/abio.1976.9999.

Cardinale, C. J. *et al.* (2008) 'Termination Factor Rho and Its Cofactors NusA and NusG Silence Foreign DNA in E. coli', *Science*, 320(5878), pp. 935–938. doi: 10.1126/science.1152763.

Carr, P. D. *et al.* (1996) 'X-ray structure of the signal transduction protein from Escherichia

coli at 1.9 Å', *Acta Crystallographica Section D Biological Crystallography*, 52(1), pp. 93–104. doi: 10.1107/S0907444995007293.

Chen, X. and Stitt, B. L. (2004) 'The Binding of C $_{10}$ Oligomers to *Escherichia coli* Transcription Termination Factor Rho', *Journal of Biological Chemistry*, 279(16), pp. 16301–16310. doi: 10.1074/jbc.M313640200.

Ciampi, M. S. (2006) 'Rho-dependent terminators and transcription termination', *Microbiology*, 152(9), pp. 2515–2528. doi: 10.1099/mic.0.28982-0.

Conroy, M. J. *et al.* (2007) 'The crystal structure of the Escherichia coli AmtB-GlnK complex reveals how GlnK regulates the ammonia channel', *Proceedings of the National Academy of Sciences*, 104(4), pp. 1213–1218. doi: 10.1073/pnas.0610348104.

D'Heygère, F. *et al.* (2015) 'ATP-dependent motor activity of the transcription termination factor Rho from *Mycobacterium tuberculosis*', *Nucleic Acids Research*, 43(12), pp. 6099–6111. doi: 10.1093/nar/gkv505.

D'Heygere, F., Rabhi, M. and Boudvillain, M. (2013) 'Phyletic distribution and conservation of the bacterial transcription termination factor Rho', *Microbiology*, 159(Pt_7), pp. 1423–1436. doi: 10.1099/mic.0.067462-0.

Dobereiner, J. and Pedrosa, F. O. (1988) 'Nitrogen-Fixing Bacteria in Nonleguminous Crop Plants. Johanna Dobereiner , Fabio O. Pedrosa , Thomas D. Brock', *The Quarterly Review of Biology*. Stony Brook Foundation, Inc., 63(3), pp. 338–339. doi: 10.1086/415968.

Feria Bourrellier, A. B. *et al.* (2010) 'Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit', *Proceedings of the National Academy of Sciences*, 107(1), pp. 502–507. doi: 10.1073/pnas.0910097107.

Finn, R. D. *et al.* (2016) 'The Pfam protein families database: towards a more sustainable future', *Nucleic Acids Research*, 44(D1), pp. D279–D285. doi: 10.1093/nar/gkv1344.

Fokina, O. *et al.* (2010) 'Mechanism of 2-oxoglutarate signaling by the Synechococcus elongatus PII signal transduction protein.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(46), pp. 19760–5. doi: 10.1073/pnas.1007653107.

Forchhammer, K. (2008) 'PII signal transducers: novel functional and structural insights', *Trends in Microbiology*, 16(2), pp. 65–72. doi: 10.1016/j.tim.2007.11.004.

Galluppi, G. R. and Richardson, J. P. (1980) 'ATP-induced changes in the binding of RNA synthesis termination protein Rho to RNA.', *Journal of molecular biology*, 138(3), pp. 513–39. doi: 10.1016/s0022-2836(80)80016-7.

Geiselmann, J. *et al.* (1992) 'Physical properties of the Escherichia coli transcription termination factor rho. 1. Association states and geometry of the rho hexamer', *Biochemistry*. American Chemical Society, 31(1), pp. 111–121. doi: 10.1021/bi00116a017.

Geiselmann, J. and Von Hippel, P. H. (1992) 'Functional interactions of ligand cofactors with *Escherichia coli* transcription termination factor rho. I. Binding of ATP', *Protein Science*, 1(7), pp. 850–860. doi: 10.1002/pro.5560010703.

Gerhardt, E. C. M. *et al.* (2012) 'Influence of the ADP/ATP ratio, 2-oxoglutarate and divalent ions on Azospirillum brasilense PII protein signalling', *Microbiology*, 158(Pt_6), pp. 1656–

1663. doi: 10.1099/mic.0.058446-0.

Gerhardt, E. C. M. (2015). Identificação e estudo *in vitro* da interação entre proteínas PII e proteínas alvo. *Dissertação (Mestrado em Ciências - Bioquímica) - Set. Ciências Biológicas, Univ. Fed. do Paraná.*, 1–158.

Gerhardt, E. C. M. *et al.* (2015) 'The Bacterial signal transduction protein GlnB regulates the committed step in fatty acid biosynthesis by acting as a dissociable regulatory subunit of acetyl-CoA carboxylase', *Molecular Microbiology*. John Wiley & Sons, Ltd (10.1111), 95(6), pp. 1025–1035. doi: 10.1111/mmi.12912.

Hauf, W. *et al.* (2016) 'Interaction of the Nitrogen Regulatory Protein GlnB (PII) with Biotin Carboxyl Carrier Protein (BCCP) Controls Acetyl-CoA Levels in the Cyanobacterium Synechocystis sp. PCC 6803', *Frontiers in Microbiology*, 7, p. 1700. doi: 10.3389/fmicb.2016.01700.

Hollands, K. *et al.* (2012) 'Riboswitch control of Rho-dependent transcription termination', *Proceedings of the National Academy of Sciences*, 109(14), pp. 5376–5381. doi: 10.1073/pnas.1112211109.

Huergo, L. F. *et al.* (2007) 'Ternary complex formation between AmtB, GlnZ and the nitrogenase regulatory enzyme DraG reveals a novel facet of nitrogen regulation in bacteria', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2007.06016.x.

Huergo, L. F. *et al.* (2009) '*In Vitro* Interactions between the P_{II} Proteins and the Nitrogenase Regulatory Enzymes Dinitrogenase Reductase ADP-ribosyltransferase (DraT) and Dinitrogenase Reductase-activating Glycohydrolase (DraG) in *Azospirillum brasilense*', *Journal of Biological Chemistry*, 284(11), pp. 6674–6682. doi: 10.1074/jbc.M807378200.

Huergo, L. F. *et al.* (2012) 'PII signal transduction proteins: pivotal players in post-translational control of nitrogenase activity', *Microbiology*, 158(1), pp. 176–190. doi: 10.1099/mic.0.049783-0.

Huergo, L. F., Chandra, G. and Merrick, M. (2013) 'PII signal transduction proteins: nitrogen regulation and beyond', *FEMS Microbiology Reviews*, 37(2), pp. 251–283. doi: 10.1111/j.1574-6976.2012.00351.x.

Inaba, J. *et al.* (2009) 'Azospirillum brasilense PII proteins GlnB and GlnZ do not form heterotrimers and GlnB shows a unique trimeric uridylylation pattern', *European Journal of Soil Biology*. Elsevier Masson, 45(1), pp. 94–99. doi: 10.1016/J.EJSOBI.2008.08.006.

Jiang, P., Mayo, A. E. and Ninfa, A. J. (2007) 'Escherichia coli Glutamine Synthetase Adenylyltransferase (ATase, EC 2.7.7.49): Kinetic Characterization of Regulation by PII, PII-UMP, Glutamine, and α-Ketoglutarate', *Biochemistry*, 46(13), pp. 4133–4146. doi: 10.1021/bi0620510.

Jiang, P. and Ninfa, A. J. (2007) '*Escherichia coli* PII Signal Transduction Protein Controlling Nitrogen Assimilation Acts As a Sensor of Adenylate Energy Charge *in Vitro* †', *Biochemistry*, 46(45), pp. 12979–12996. doi: 10.1021/bi701062t.

Jiang, P. and Ninfa, A. J. (2009) 'Sensation and Signaling of α-Ketoglutarate and Adenylylate Energy Charge by the *Escherichia coli* PII Signal Transduction Protein Require Cooperation of the Three Ligand-Binding Sites within the PII Trimer', *Biochemistry*, 48(48), pp. 11522–11531. doi: 10.1021/bi9011594.

Kalarickal, N. C. *et al.* (2010) 'A Bacterial Transcription Terminator with Inefficient

Molecular Motor Action but with a Robust Transcription Termination Function', *Journal of Molecular Biology*, 395(5), pp. 966–982. doi: 10.1016/j.jmb.2009.12.022.

Krishna Leela, J. *et al.* (2013) 'Rho-dependent transcription termination is essential to prevent excessive genome-wide R-loops in Escherichia coli', *Proceedings of the National Academy of Sciences*, 110(1), pp. 258–263. doi: 10.1073/pnas.1213123110.

Laemmli, U. K. (1970) 'Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4', *Nature*, 227(5259), pp. 680–685. doi: 10.1038/227680a0.

Leigh, J. A. and Dodsworth, J. A. (2007) 'Nitrogen Regulation in Bacteria and Archaea', *Annual Review of Microbiology*, 61(1), pp. 349–377. doi: 10.1146/annurev.micro.61.080706.093409.

Louis-Jeune, C., Andrade-Navarro, M. A. and Perez-Iratxeta, C. (2012) 'Prediction of protein secondary structure from circular dichroism using theoretically derived spectra', *Proteins: Structure, Function, and Bioinformatics*, 80(2), pp. 374–381. doi: 10.1002/prot.23188.

Machado, H. B. *et al.* (1991) 'Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine', *Canadian Journal of Microbiology*. NRC Research Press Ottawa, Canada , 37(7), pp. 549–553. doi: 10.1139/m91-092.

Moure, V. R. *et al.* (2012) 'Heat stability of Proteobacterial P II protein facilitate purification using a single chromatography step', *Protein Expression and Purification*, 81(1), pp. 83–88. doi: 10.1016/j.pep.2011.09.008.

Ninfa, A. J. and Jiang, P. (2005) 'PII signal transduction proteins: sensors of α-ketoglutarate that regulate nitrogen metabolism', *Current Opinion in Microbiology*. (Cell Regulation), 8(2), pp. 168–173. doi: 10.1016/j.mib.2005.02.011.

Nowatzke, W. L. and Richardson, J. P. (1996) 'Characterization of an Unusual Rho Factor from the High G + C Gram-positive Bacterium *Micrococcus luteus*', *Journal of Biological Chemistry*, 271(2), pp. 742–747. doi: 10.1074/jbc.271.2.742.

Pani, B. *et al.* (2006) 'Mechanism of Inhibition of Rho-dependent Transcription Termination by Bacteriophage P4 Protein Psu', *Journal of Biological Chemistry*, 281(36), pp. 26491–26500. doi: 10.1074/jbc.M603982200.

Park, H. *et al.* (1995) 'Bicyclomycin and Dihydrobicyclomycin Inhibition Kinetics ofEscherichia colirho-Dependent Transcription Termination Factor ATPase Activity', *Archives of Biochemistry and Biophysics*, 323(2), pp. 447–454. doi: 10.1006/abbi.1995.0066.

Pedrosa, F. O. and Yates, M. G. (1984) 'Regulation of nitrogen fixation *(nif)* genes of *Azospirillum brasilense* by *nifA* and *ntr (gln)* type gene products', *FEMS Microbiology Letters*. Wiley/Blackwell (10.1111), 23(1), pp. 95–101. doi: 10.1111/j.1574-6968.1984.tb01042.x.

Peters, J. M. *et al.* (2009) 'Rho directs widespread termination of intragenic and stable RNA transcription', *Proceedings of the National Academy of Sciences*, 106(36), pp. 15406–15411. doi: 10.1073/pnas.0903846106.

Peters, J. M. *et al.* (2012) 'Rho and NusG suppress pervasive antisense transcription in Escherichia coli', *Genes & Development*, 26(23), pp. 2621–2633. doi: 10.1101/gad.196741.112.

Peters, J. M., Vangeloff, A. D. and Landick, R. (2011) 'Bacterial Transcription Terminators:

The RNA 3′-End Chronicles', *Journal of Molecular Biology*, 412(5), pp. 793–813. doi: 10.1016/j.jmb.2011.03.036.

Rabhi, M. *et al.* (2011) 'The Sm-like RNA chaperone Hfq mediates transcription antitermination at Rho-dependent terminators.', *The EMBO journal*, 30(14), pp. 2805–16. doi: 10.1038/emboj.2011.192.

Radchenko, M. V., Thornton, J. and Merrick, M. (2010) 'Control of AmtB-GlnK Complex Formation by Intracellular Levels of ATP, ADP, and 2-Oxoglutarate', *Journal of Biological Chemistry*, 285(40), pp. 31037–31045. doi: 10.1074/jbc.M110.153908.

Rajendran, C. *et al.* (2011) 'Crystal structure of the GlnZ-DraG complex reveals a different form of PII-target interaction', *Proceedings of the National Academy of Sciences*, 108(47), pp. 18972–18976. doi: 10.1073/pnas.1108038108.

Rodrigues, T. E. *et al.* (2011) 'In vitro interaction between the ammonium transport protein AmtB and partially uridylylated forms of the PII protein GlnZ', *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*. Elsevier, 1814(9), pp. 1203–1209. doi: 10.1016/J.BBAPAP.2011.05.012.

Rodrigues, T. E. *et al.* (2014) 'Search for novel targets of the P_{II} signal transduction protein in Bacteria identifies the BCCP component of acetyl-CoA carboxylase as a P_{II} binding partner', *Molecular Microbiology*. John Wiley & Sons, Ltd (10.1111), 91(4), pp. 751–761. doi: 10.1111/mmi.12493.

Rodrigues, T. E. (2018). Regulação da acetil-CoA carboxilase pelas proteínas PII e cristalização da enzima GlnD de *Escherichia coli. Dissertação (Mestrado em Ciências - Bioquímica) - Set. Ciências Biológicas, Univ. Fed. do Paraná.*, 1–158.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, n. 2.

Schutt, H. and Holzer, H. (1972) 'Biological Function of the Ammonia-Induced Inactivation of Glutamine Synthetase in Escherichia coli', *European Journal of Biochemistry*, 26(1), pp. 68–72. doi: 10.1111/j.1432-1033.1972.tb01740.x.

Skordalakes, E. and Berger, J. M. (2003) 'Structure of the Rho transcription terminator: mechanism of mRNA recognition and helicase loading.', *Cell*, 114(1), pp. 135–46. doi: 10.1016/s0092-8674(03)00512-9.

Stitt, B. L. (1988) 'Escherichia coli transcription termination protein rho has three hydrolytic sites for ATP.', *The Journal of biological chemistry*, 263(23), pp. 11130–7.

Stitt, B. L. and Xu, Y. (1998) 'Sequential hydrolysis of ATP molecules bound in interacting catalytic sites of Escherichia coli transcription termination protein Rho.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 273(41), pp. 26477–86. doi: 10.1074/jbc.273.41.26477.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) 'CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice', *Nucleic Acids Research*, 22(22), pp. 4673–4680. doi: 10.1093/nar/22.22.4673.

Thomsen, N. D. and Berger, J. M. (2009) 'Running in Reverse: The Structural Basis for Translocation Polarity in Hexameric Helicases', *Cell*, 139(3), pp. 523–534. doi: 10.1016/j.cell.2009.08.043.

Thomsen, N. D. et al. (2016) 'Molecular mechanisms of substrate-controlled ring dynamics and substepping in a nucleic acid-dependent hexameric motor.', *Proceedings of the National Academy of Sciences of the United States of America.* National Academy of Sciences, 113(48), pp. E7691–E7700. doi: 10.1073/pnas.1616745113.

Truan, D. *et al.* (2010) 'A New PII Protein Structure Identifies the 2-Oxoglutarate Binding Site', *Journal of Molecular Biology*, 400(3), pp. 531–539. doi: 10.1016/j.jmb.2010.05.036.

Truan, D. *et al.* (2014) 'Structure and Thermodynamics of Effector Molecule Binding to the Nitrogen Signal Transduction PII Protein GlnZ from Azospirillum brasilense', *Journal of Molecular Biology*, 426(15), pp. 2783–2799. doi: 10.1016/j.jmb.2014.05.008.

Xu, Y. *et al.* (2003) 'The structures of the PII proteins from the cyanobacteria *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803', *Acta Crystallographica Section D Biological Crystallography*. International Union of Crystallography, 59(12), pp. 2183–2190. doi: 10.1107/S0907444903019589.

Yan, D., Lenz, P. and Hwa, T. (2011) 'Overcoming Fluctuation and Leakage Problems in the Quantification of Intracellular 2-Oxoglutarate Levels in Escherichia coli', *Applied and Environmental Microbiology*, 77(19), pp. 6763–6771. doi: 10.1128/AEM.05257-11.

Yuan, J. *et al.* (2009) 'Metabolomics-driven quantitative analysis of ammonia assimilation in E. coli', *Molecular Systems Biology*, 5, p. 302. doi: 10.1038/msb.2009.60.

Yutaka Mizuno *et al.* (2007) 'Crystal Structure of Arabidopsis PII Reveals Novel Structural Elements Unique to Plants†,‡'. American Chemical Society . doi: 10.1021/BI062149E.

de Zamaroczy, M. (1998) 'Structural homologues P(II) and P(Z) of Azospirillum brasilense provide intracellular signalling for selective regulation of various nitrogen-dependent functions.', *Molecular microbiology*, 29(2), pp. 449–63.

Supplementary material

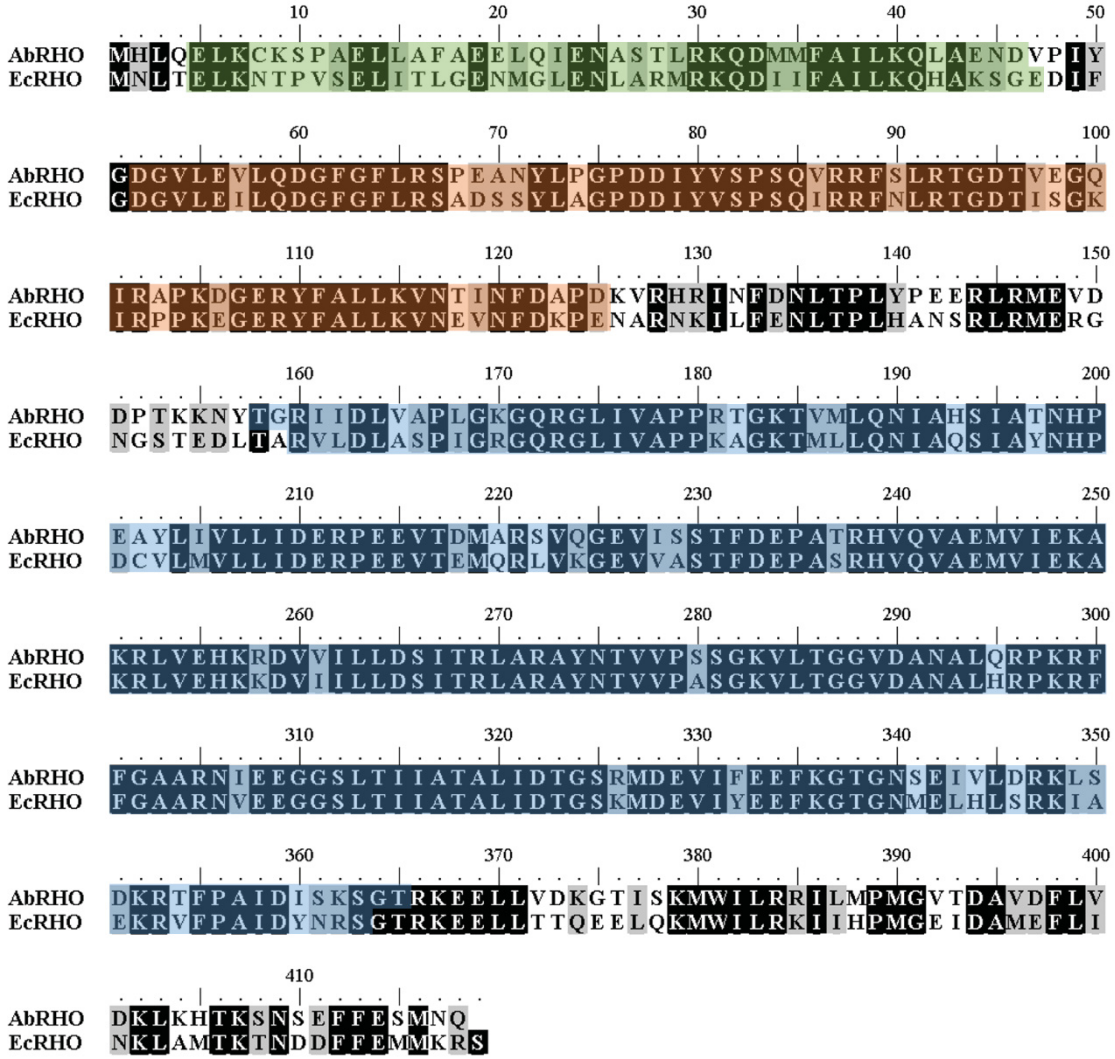
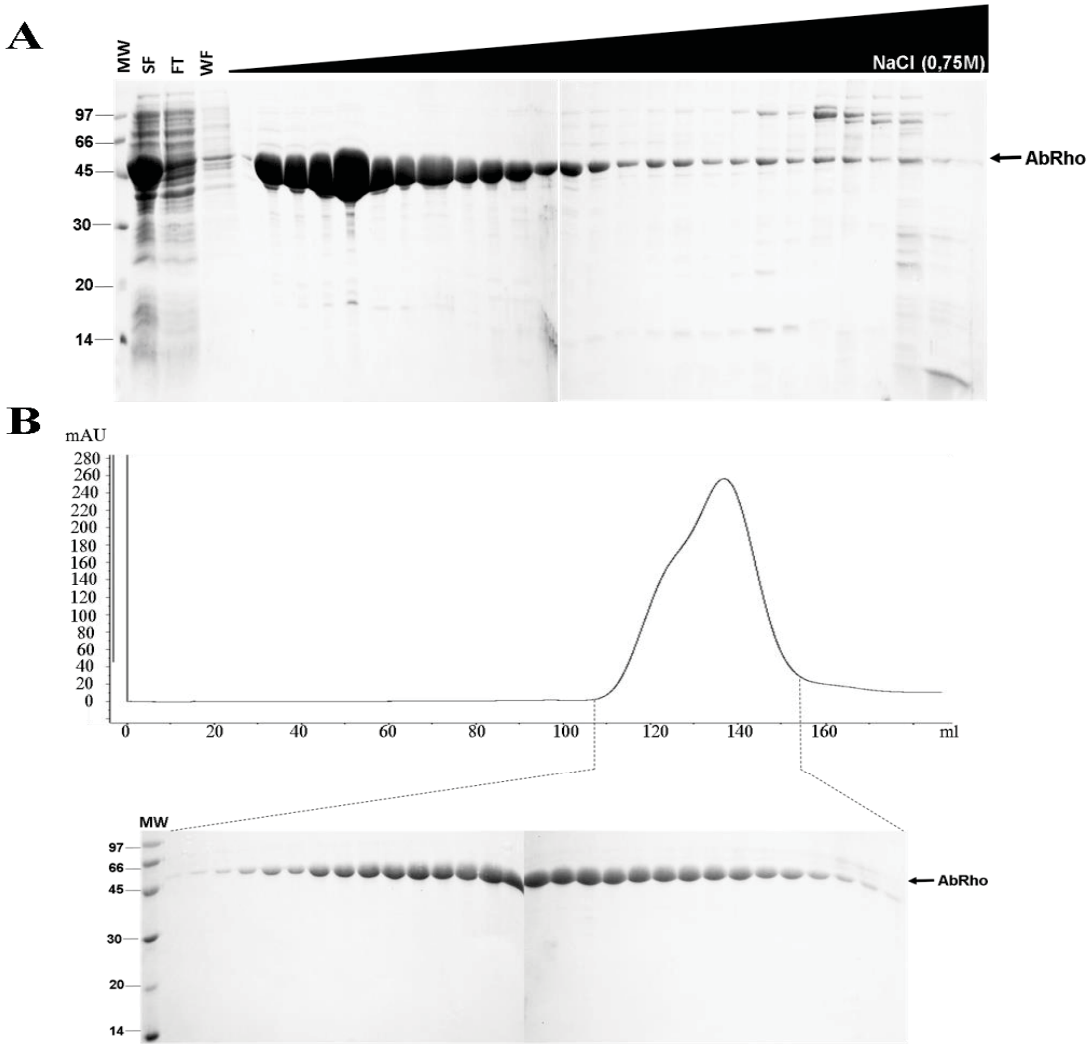


FIGURE S1. Alignment of Rho proteins from *Azospirillum brasilense* (AbRho) and *Escherichia coli* (EcRho). Analyzes using Pfam predicted three domains in both proteins. In green is the N-terminal domain; in red is the RNA-binding domain and in blue is the ATP synthase alpha/beta family, nucleotide-binding domain.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AbRho
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AbRho

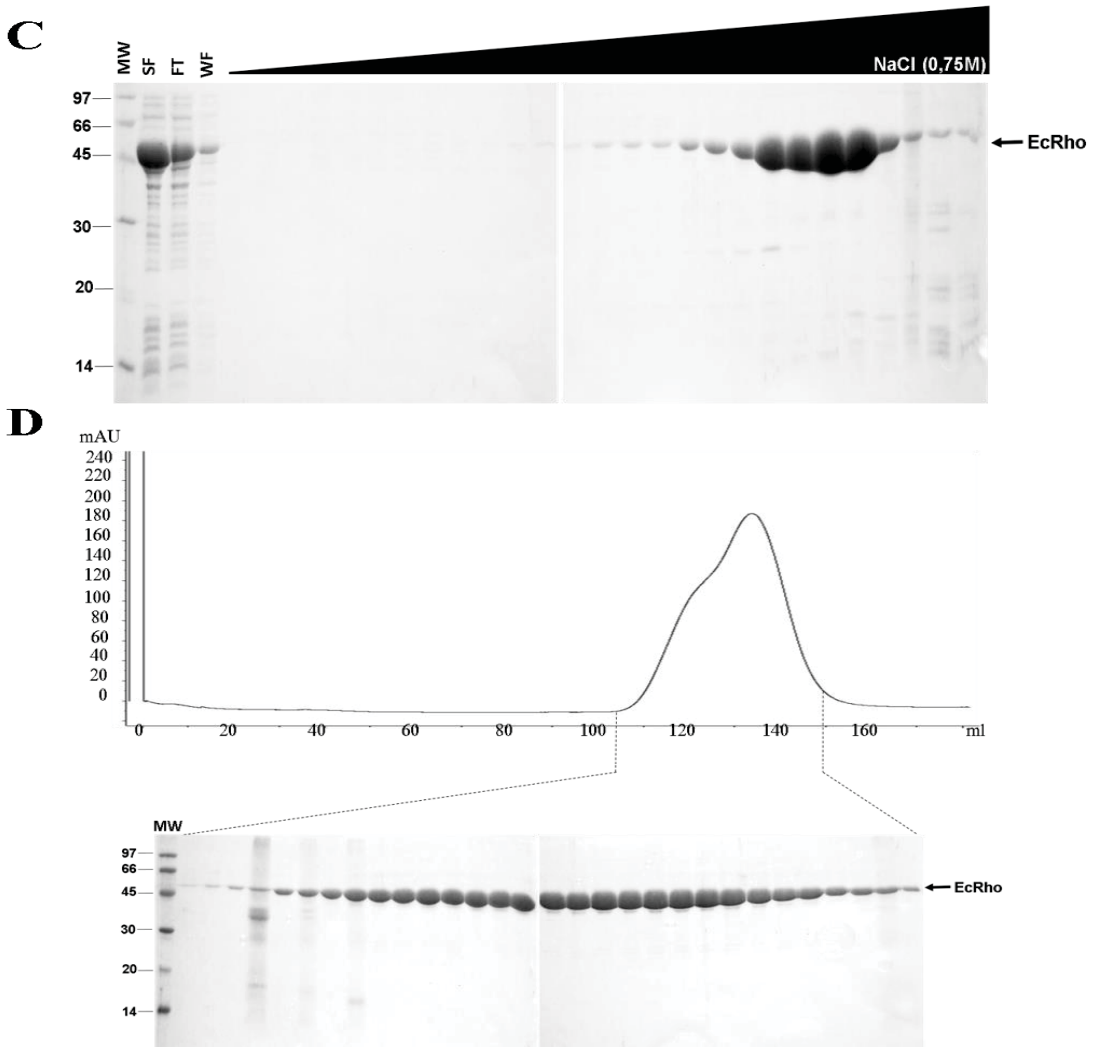


FIGURE S2. Purification of AbRho and EcRho proteins. Sonication buffer = Tris HCl pH 8.0 (0.05 M) and NaCl (0.1 M). The elution gradient range was 0.1 to 0.75 M of NaCl on Heparin column and isocratic in Gel Filtration (NaCl – 0.1 M). MW = Molecular Weight; SF = Soluble Fraction; FT = Flow Through; WF = Washing Fraction. A) Elution profile of the AbRho protein after purification with the Heparin column. Samples were collected every 1 ml and the 10 µl were analysed in 12.5% SDS-PAGE gels, the gel was stained with Coomassie blue. B) Elution profile of the AbRho protein after passing through the Gel Filtration. Samples were collected every 500 µl and the 10 µl were analysed in 12.5% SDS-PAGE gels, the gel was stained with Coomassie blue. C) Elution profile of the EcRho protein after purification with the Heparin column. Samples were collected every 1 ml and the 10 µl were analysed in 12.5% SDS-PAGE gels, the gel was stained with Coomassie blue. D) Elution profile of the EbRho protein after passing through the Gel Filtration. Samples were collected every 500 µl and the 10 µl were analysed in 12.5% SDS-PAGE gels, the gel was stained with Coomassie blue.

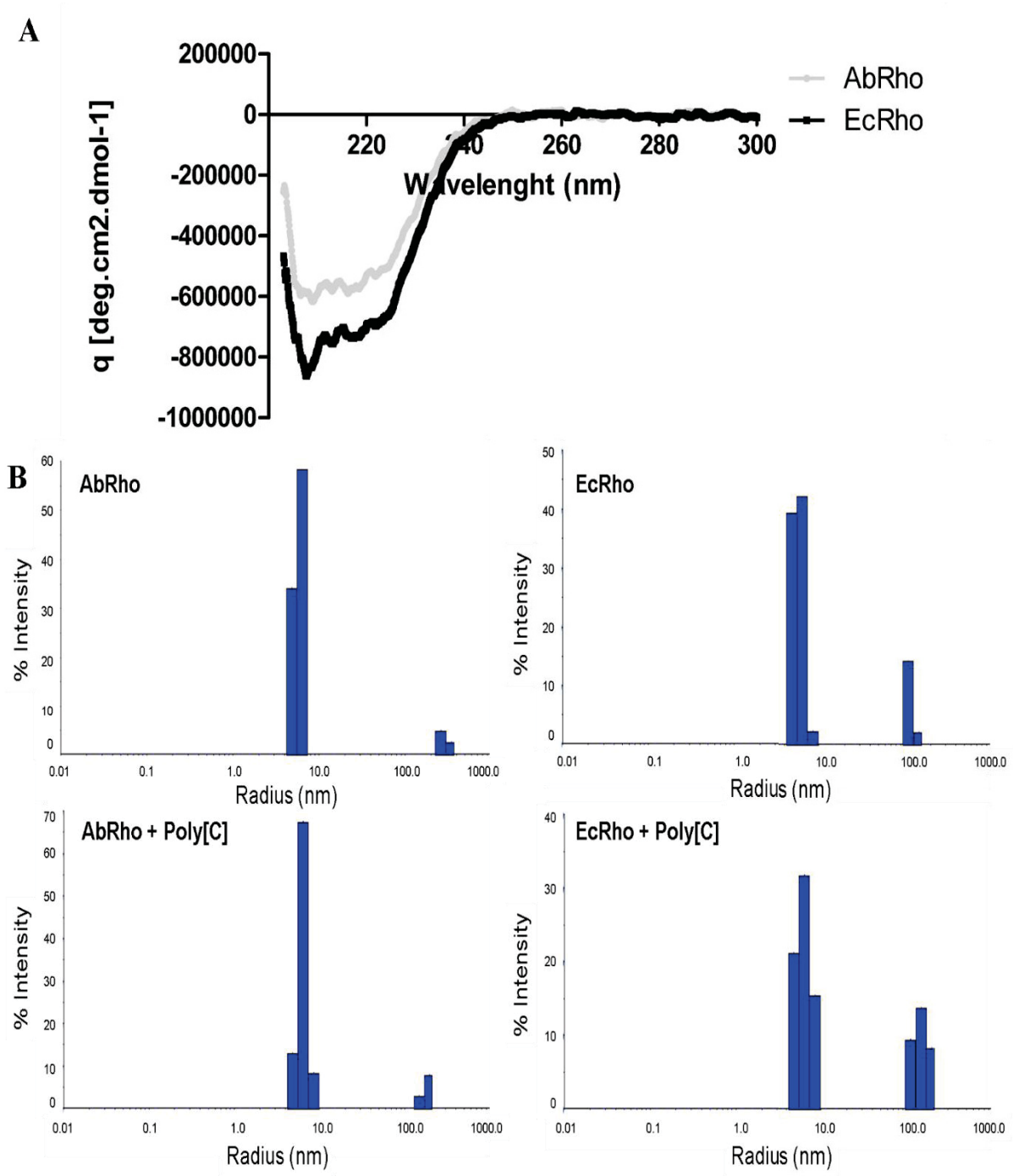
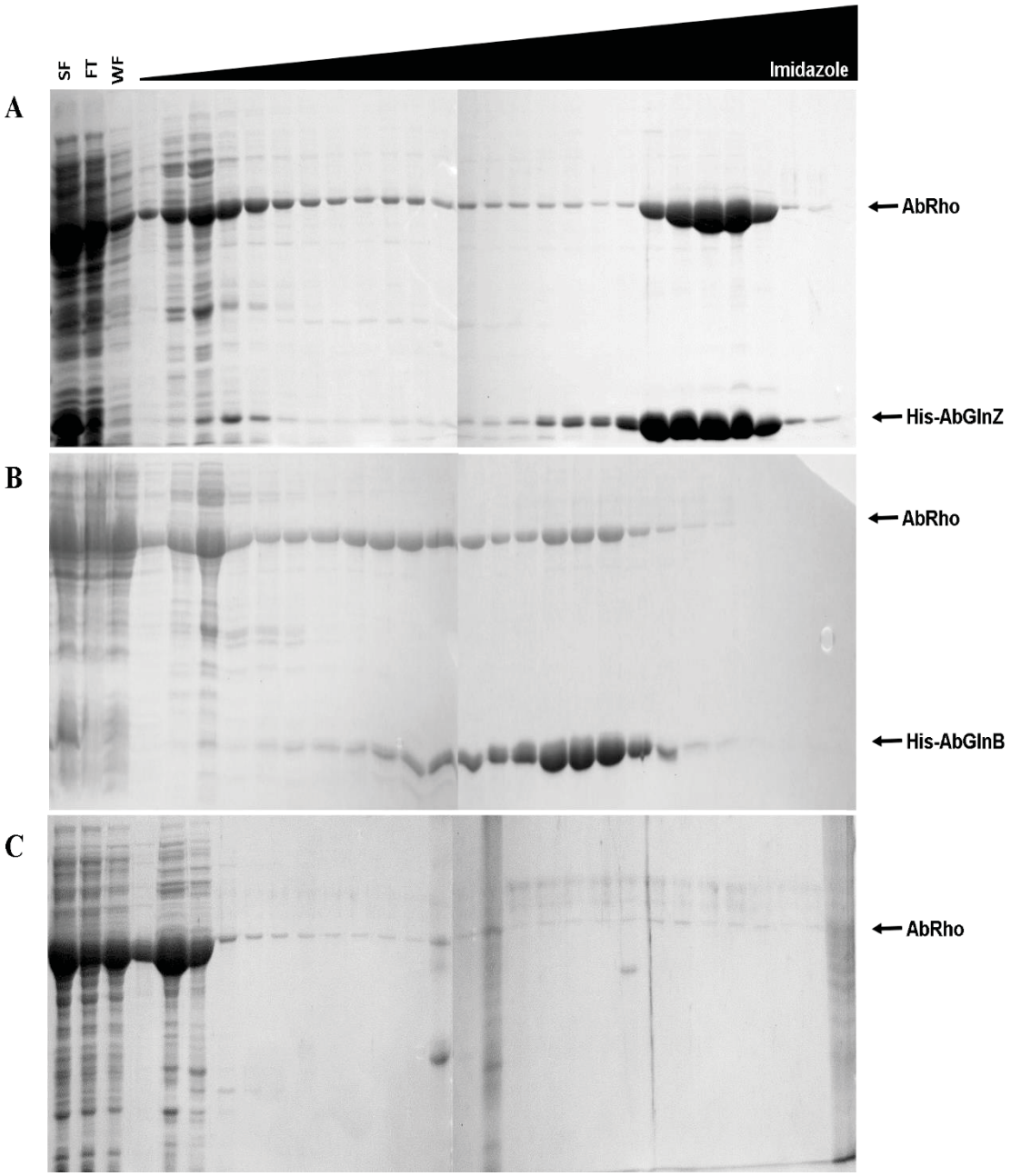


FIGURE S3. Structural analysis of Rho proteins by Circular dichroism (CD) and Dynamic light scattering (DLS). A) CD spectrum of AbRho and EcRho (~ 0,5 µM considering the hexamer). CD was recorded at 30 °C. The secondary structure content was calculated using K2D3 software. B) Dynamic Light Scattering profile of AbRho and EcRho alone (~35 µM considering the hexamer) and with poly[C] cofactor.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B
AbRho
His-AbGlnB
C
AbRho

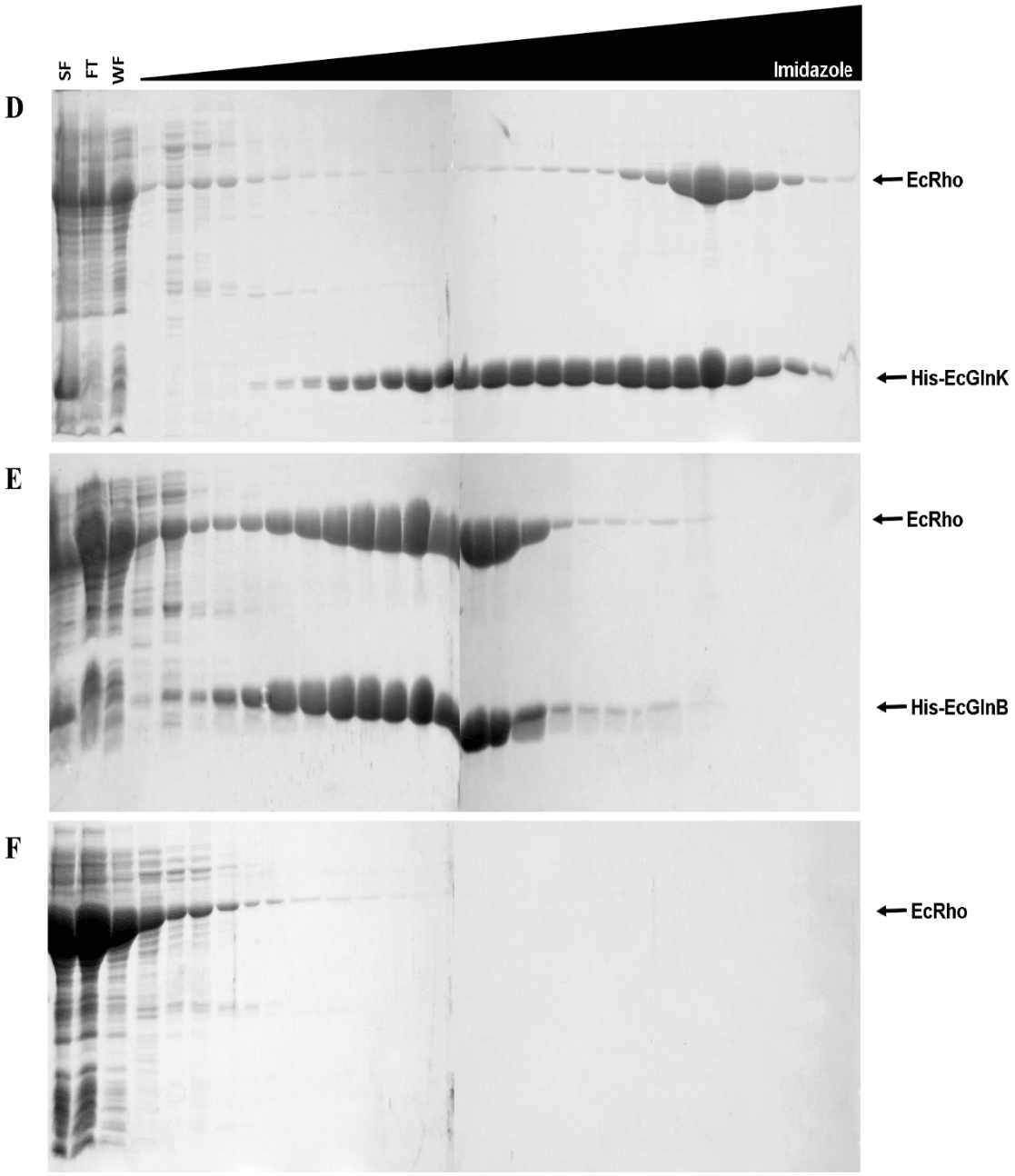


FIGURE S4. Co-purification of AbRho and EcRho proteins with PII. Sonication buffer = Tris HCl pH 8.0 (0.05 M); KCl (0.1 M); ADP (1 mM) and $MgCl_2$ (5mM). The elution gradient range was 0.1 to 1 M of imidazole on HiTrap chelating column. Samples were collected every 1 ml and the 10 µl were analysed in 12.5% SDS-PAGE gels, the gel was stained with Coomassie blue. MW = Molecular Weight; SF = Soluble Fraction; FT = Flow Through; WF = Washing Fraction. A) Elution profile of the AbRho-GlnZ complex B) Elution profile of the AbRho-GlnB complex C) Elution profile of the AbRho only (control) D) Elution profile of the EcRho-GlnK complex E) Elution profile of the EcRho-GlnB complex F) Elution profile of the EcRho only (control).

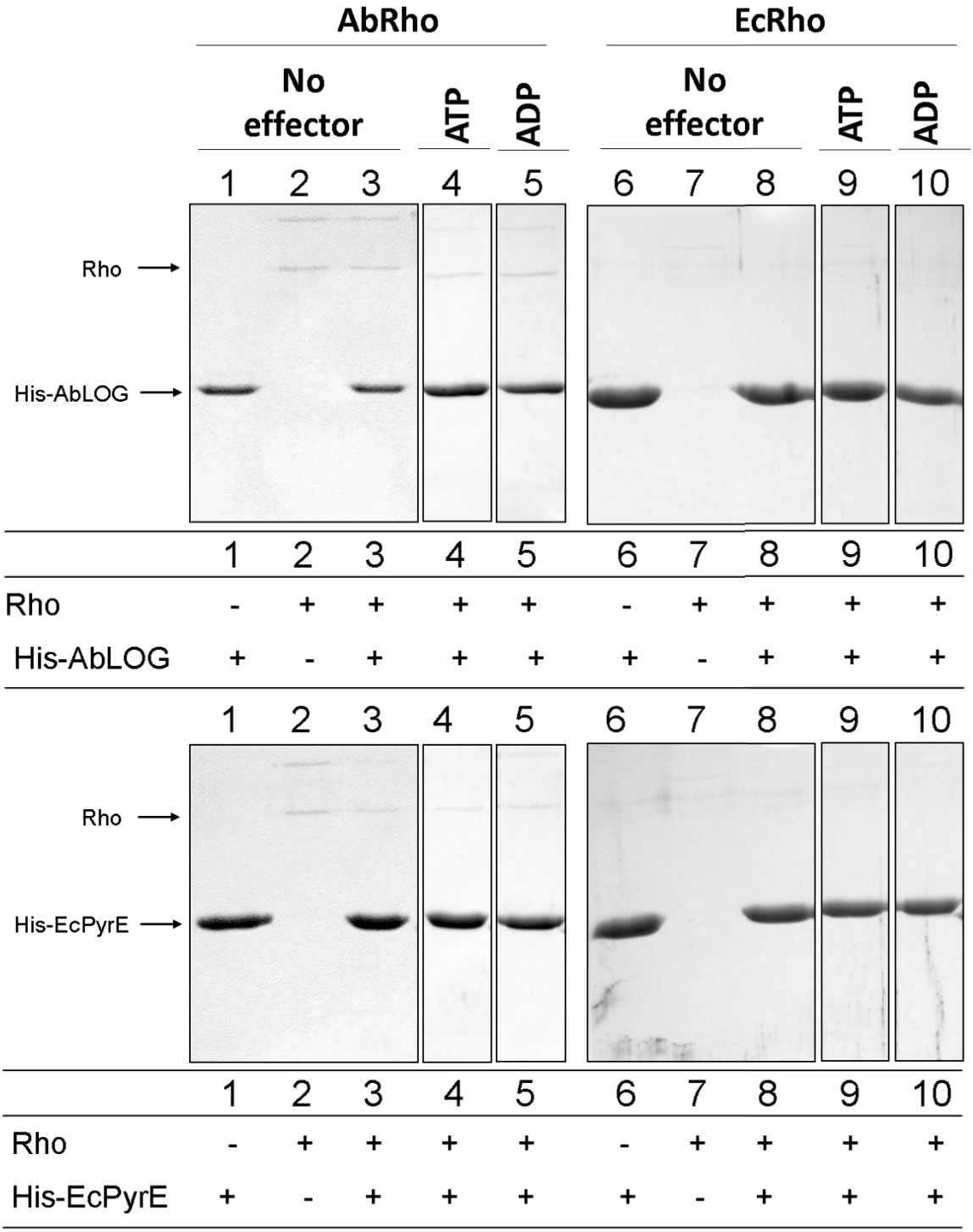


FIGURE S5. AbRho and EcRho does not pull-down unspecifically. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in absence of effectors and under fixed ATP or ADP concentration (1 mM) in presence of $MgCl_2$ (5mM). Reactions were conducted in 400 µl of buffer adding random purified His proteins (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue. Lanes 1 and 6: Control with His-PII only; lanes 2 and 7: Control with native Rho only; lanes 3 and 8: Pull down without effectors; lanes: 4 and 9: Pull down in the presence of ATP; lanes: 5 and 10: Pull down in the presence of ADP.

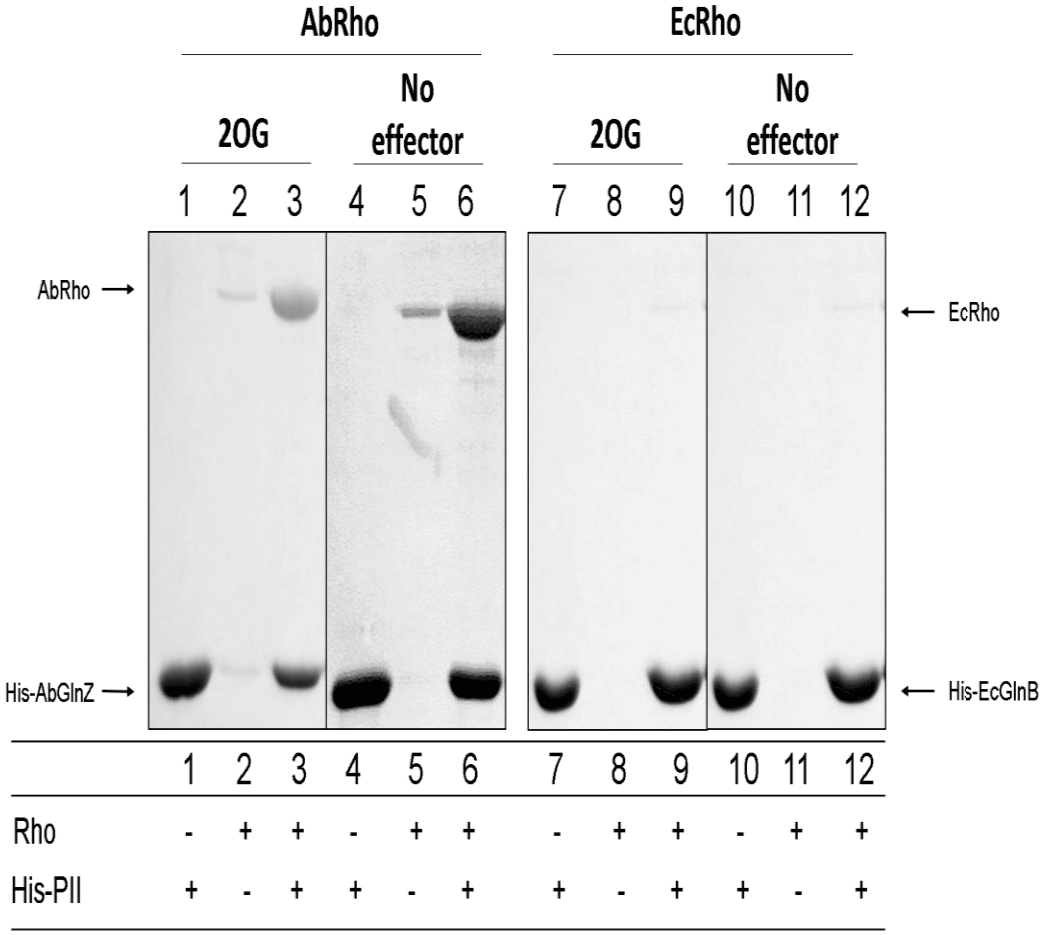


FIGURE S6. AbRho interacts *in vitro* with PII in the absence of effector, whereas EcRho does not. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in absence of effectors and under fixed 2-OG concentration (1 mM) as indicated. All assays were in presence of $MgCl_2$ (5mM). Reactions were conducted in 400 µl of buffer adding random purified His proteins (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

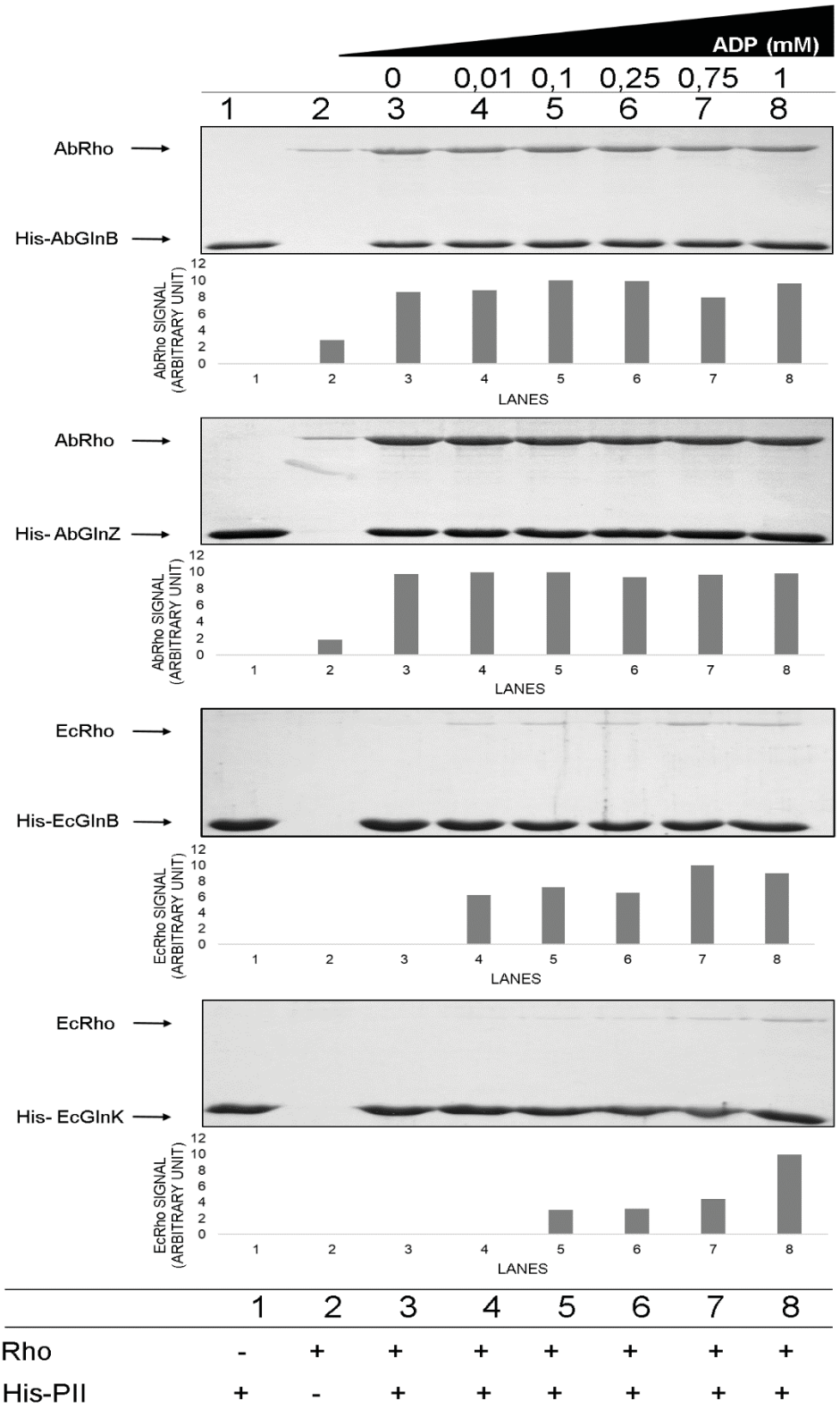


FIGURE S7. *In vitro* formation of the EcRho-PII complexes occur in an ADP-dependent manner whereas AbRho-PII complexes occur independly of the ADP concentration. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of $MgCl_2$ (5mM), increasing concentrations of ADP as indicated. Reactions were conducted in 400 µl of buffer adding purified His-PII (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

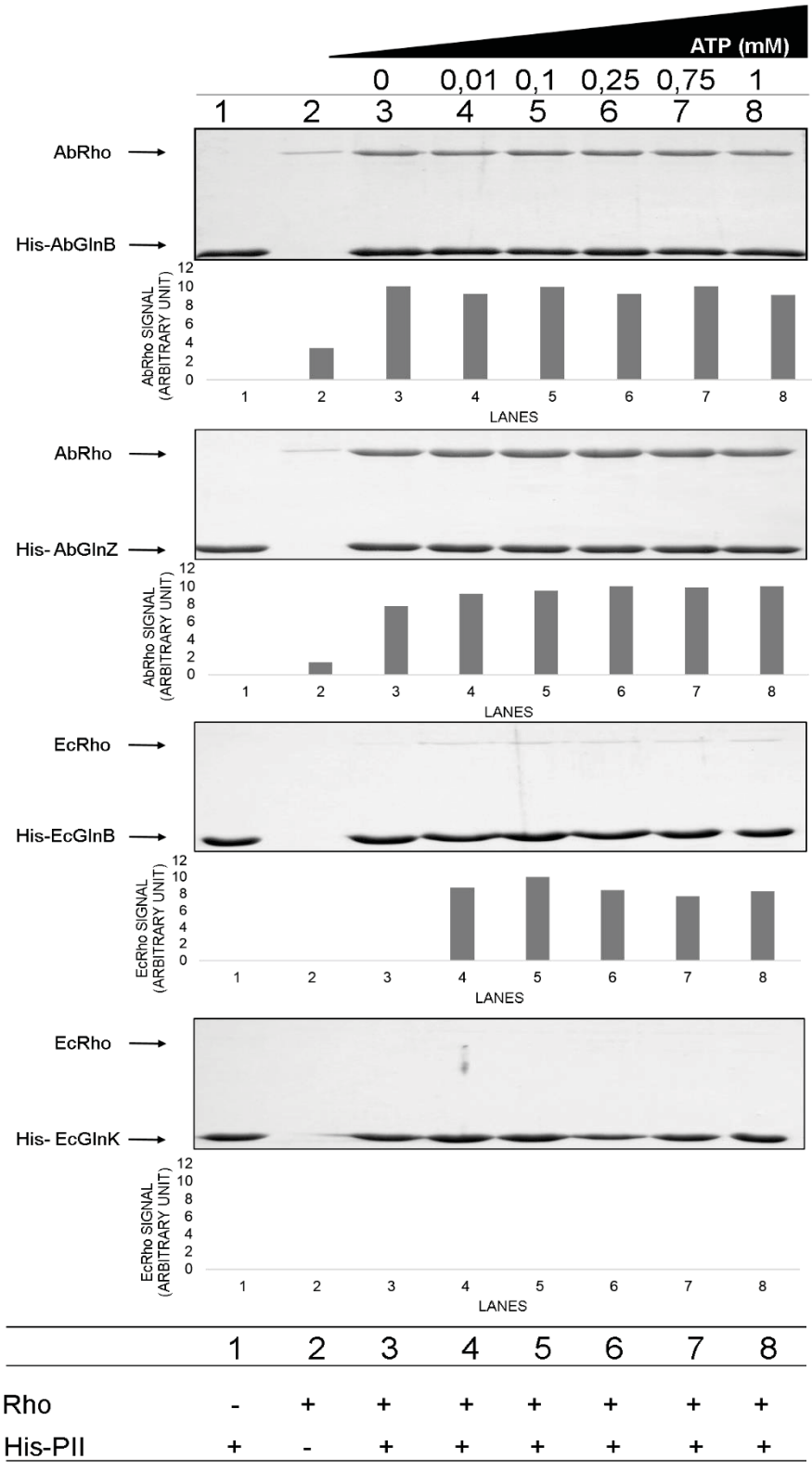
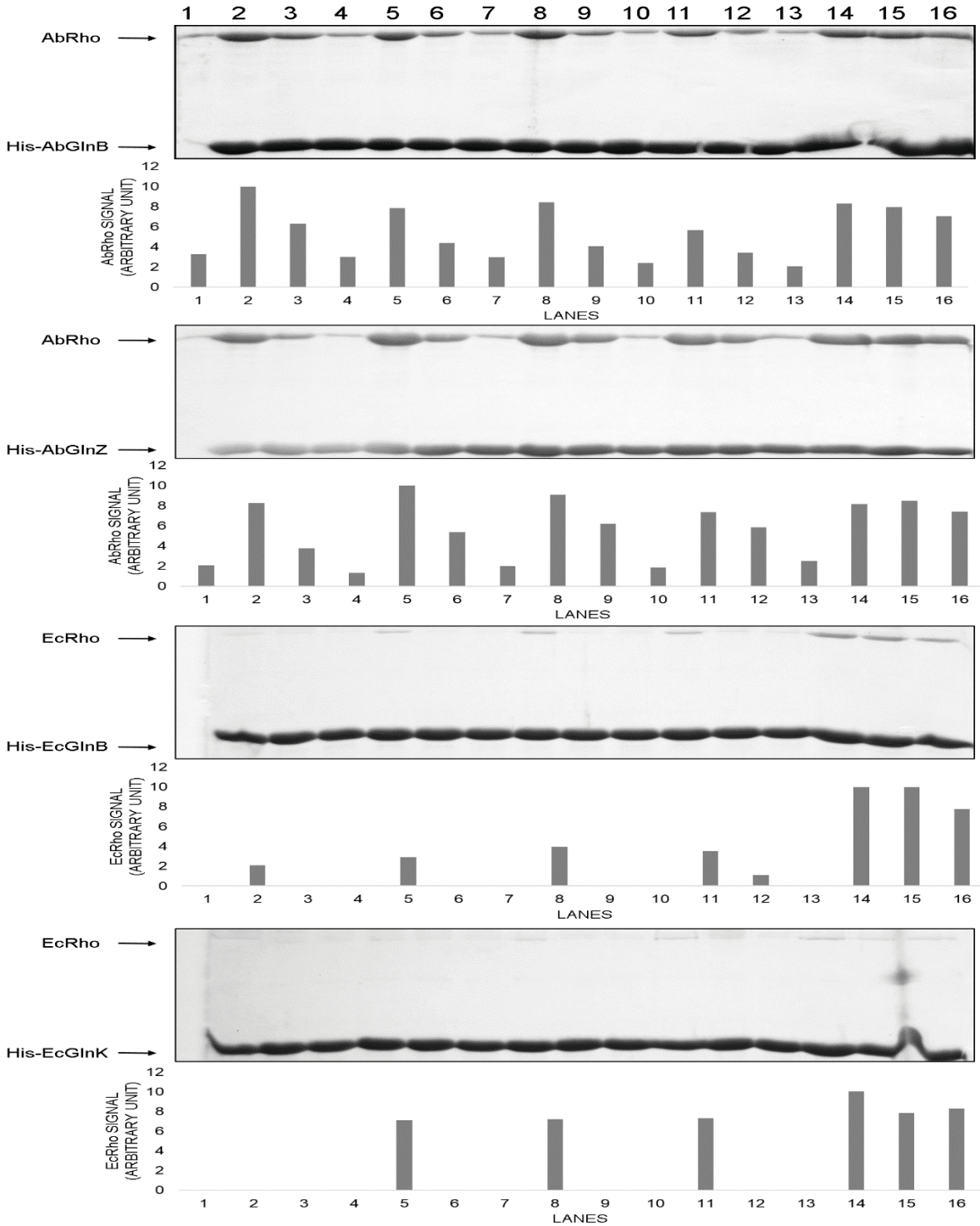


FIGURE S8. ***In vitro*** **formation of the AbRho-PII and EcRho-GlnB complexes occur similarly in different concentrations of the ATP concentration. EcRho-GlnK do not occur in the presence of only ATP.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of $MgCl_2$ (5mM), increasing concentrations of ATP as indicated. Reactions were conducted in 400 µl of buffer adding purified His-PII (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
ATP (mM)	0	1	1	1	0,9	0,9	0,9	0,75	0,75	0,75	0,5	0,5	0,5	0	0	0
ADP (mM)	0	0	0	0	0,1	0,1	0,1	0,25	0,25	0,25	0,5	0,5	0,5	1	1	1
2-OG (mM)	0	0	0,1	1	0	0,1	1	0	0,1	1	0	0,1	1	0	0,1	1

FIGURE S9. Effect of different concentrations of the PII effectors on *in vitro* formation of AbRho-PII and EcRho-PII complexes. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in different concentrations of the effectors ATP, ADP and 2-OG, as indicated and fixed concentration of $MgCl_2$ (5 mM). Binding reactions were conducted in 400 µl of buffer adding purified His-PII, mixed with native Rho. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

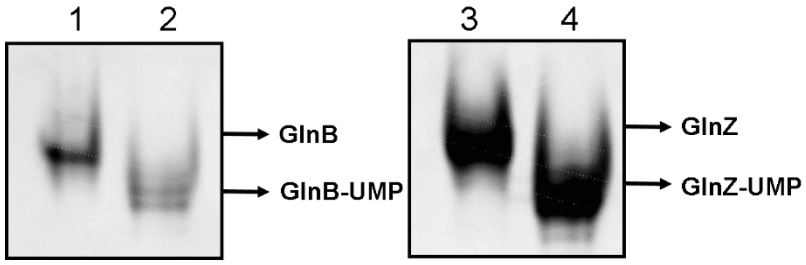


FIGURE S10. PII of *A. brasilense* partially uridylylated by GlnD of *E. coli*. The reaction was performed in 500 µl reaction mixtures containing 100 µM His-PII (considered as monomer), 1 mM ATP, 2 mM UTP, 5 mM 2-OG, 1 µM of purified *E. coli* GlnD, 100 mM Tris–HCl pH 7.5, 100 mM KCl and 25 mM $MgCl_2$. The uridylylation His-PII was determined by Native-PAGE.

4. CAPÍTULO II

A DIGUANYLATE CYCLASE AND A PHOSPHODIESTERASE FROM *Azospirillum brasilense* SP245 INTERACT WITH GlnB *IN VITRO*

Abstract

Cyclic diguanylate (c-di-GMP) is a near universal signaling molecule produced by Diguanylate Cyclases (DGC) and is degraded by Phosphodiesterases (PDE) and the balance of this molecule influences in bacterial behavior. These proteins are associated with motility, biofilm formation, virulence and other processes. Many DGC and PDE studied to date have been found to signal for a specific c-di-GMP related process. Physical interaction of DGC and PDE with its target proteins have been suggested as a mechanism to provide signaling fidelity. Recently, the physical interaction between the GlnZ protein with specific DGC and PDE in *A. brasilense* SP245 was identified *in vitro*. GlnZ is part of the PII protein family, whose main function is to transduce signals via physical interaction with their targets. The interaction was characterized *in vitro* and demonstrated to occur in the presence of ATP and ADP, two GlnZ effectors, and was inhibited in the presence of the third GlnZ effector, 2-OG, in the presence of ATP and Mg^{2+}. However, *A. brasilense* has a second PII called GlnB, whose interaction with DGC and PDE was identified and characterized, using pull-down assays. As with GlnZ, the interaction with GlnB occurred in the presence of ATP and ADP, but was inhibited by 2-OG, in the presence of ATP and Mg^{2+}. Different ATP and ADP concentrations did not affect the interaction, but the interaction responded 2-OG in a dependent manner. The interaction with PDE was Mg^{2+} dependent, an important effector of these proteins, whereas DGC-GlnB did not have this dependence. Knowing that in general DGC and PDE proteins respond to specific signals to have their actions stimulated and inhibited, the interaction with PII indicates a new way of controlling these proteins and, consequently, cellular behavior.

Introduction

Bis-(3',5')-cyclic dimeric GMP (c-di-GMP) is a seconder messenger involved in the regulation of several bacterial processes, such as motility, virulence and biofilm formation (Hengge, 2009; Romling, Galperin and Gomelsky, 2013). The cellular concentration of c-di-GMP is controlled by two enzymatic domains with opposing activities: GGDEF domain with synthesis activity and EAL or HD-GYP domains with degradation activity (Chan *et al.*, 2004; Römling, Liang and Dow, 2017). Diguanylate Ciclases (DGC) enzymes catalyze the synthesis of c-di-GMP using two GTP molecules, which binds two GGDEF domains, in two different monomers, usually arranged in a dimer conformation forming the site for the condensation of the molecules, also called A site. GGDEF domain presents a GGDEF or a GGEEF motif. Some of the DGC proteins possess a second binding site called inhibitory site or I site. This is a regulatory site containing an RxxD motif. When c-di-GMP binds to the RxxD motif, this

domais act to inhibit DGC activity thereby providing a negative feedback inhibition loop (Chan *et al.*, 2004; Wassmann *et al.*, 2007).

The hydrolysis of the c-di-GMP is catalyzed by EAL or HD-GYP domains, which is harbored by the Phosphodiesterase enzymes (PDE). The EAL domain transforms c-di-GMP into the product 5′-phosphoguanylyl-(3'-5 ')-guanosine (5'-pGpG) (Tal *et al.*, 1998; Tchigvintsev *et al.*, 2010) and HD-GYP domain is characterized by hydrolyzing c-di-GMP and 5'-pGpG into GMP. In some bacteria, PDE HD-GYP can directly influence levels of c-di-GMP, hydrolyzing it, or indirectly hydrolyzing the 5'-pGpG product preventing inhibition of EAL domains (Cohen *et al.*, 2015; Orr *et al.*, 2015).

Many proteins can have both c-di-GMP synthesis and degradation sites. When the both sites are functional, the domains are differentially regulated by environmental factors and/or intracellular signals that make one domain prevail over the other under certain conditions (Bharati *et al.*, 2012). Regulation in these cases may include effector binding, signal perception, protein-protein interaction, among others (Qi *et al.*, 2009; Feirer *et al.*, 2015; Mills *et al.*, 2015). On the other hand, some GGDEF domains may become degenerate and no longer be catalytically active. However, they may have an allosteric function such as c-di-GMP (negative regulation) or GTP (positive regulation) PDE receptors, for example. The same can occur with EAL and HD-GYP domains (Hobley *et al.*, 2012; Ozaki *et al.*, 2014).

PII signal transduction proteins control metabolic status in organisms ranging from bacteria to higher plants. *Azospirillum brasilense* has two PII proteins named GlnZ and GlnB. The binding of effectors in PII is essential for the performance of their functions as a signal transducer protein. The binding of ATP, ADP and 2-OG effectors in PII is highly conserved, being possible the binding of ATP, ATP + Mg^{2+} (Mg.ATP), Mg.ATP + 2-OG or ADP. The metabolic balance of these effectors dictates the ability of PII to interact with its target proteins. The conformation change caused by effector binding allows PII to transmit information to other proteins (Huergo, Chandra and Merrick, 2013; Truan *et al.*, 2014; Merrick, 2015) via protein-protein interaction (Arcondéguy, Jack and Merrick, 2001; Huergo *et al.*, 2012; Huergo, Chandra and Merrick, 2013).

In addition to the signaling performed by PII through the binding of effectors, the perception of signals in this protein can also occur due to post-translational modification. This modification occurs in the most flexible protein structure named T loop. The type of modification can vary according to the organism, in proteobacteria usually is a uridylylation of a tyrosine residue (Tyr51) (Forchhammer, 2004; Maheswaran, Urbanke and Forchhammer, 2004; Merrick, 2015).

Recently, Urbanski (2018) characterized *in vitro* interaction of a Diguanylate Cyclase and a Phosphodiesterase from *A. brasilense* SP245, here named AbDGC and AbPDE respectively, with GlnZ protein, one of the PII of this bacterium. The complexes AbDGC-GlnZ and AbPDE-GlnZ were formed in the presence of ATP and ADP, and was inhibited by the addition of 2-OG and Mg.ATP (Urbanski, 2018).

A. brasilense is found in the soil and is a metabolically versatile bacterium that uses chemotaxis to find microaerophilic environments which is, an ideal condition for its growth. The levels of c-di-GMP is involved in this process (Russell *et al.*, 2013; O'Neal *et al.*, 2017). *A. brasilense* needs to sense and respond quickly to changes in the environment, to decide between its sessile or mobile state, and the integration of the functions of c-di-GMP with the signals performed by PII would be helpful to this bacterium mechanism.

In this work we characterize *in vitro* the interaction of the AbDGC and AbPDE proteins of *A.brasilense* SP245 (Urbanski, 2018), with the GlnB, a second protein of the PII family found in this bacterium. As seen for GlnZ (Urbanski, 2018), GlnB also interacts with AbDGC and AbPDE in the presence of ATP and ADP and the interaction was inhibited by 2-OG.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1.

Molecular biology methods

Gel electrophoresis, isolation of plasmids, bacterial transformation were performed as described by Sambrook *et al.*, (1989). The enzymes used were obtained from commercial sources and used according to the instructions provided by the manufacturers.

Protein methods

Proteins Electrophoresis was carried out by SDS-PAGE (Laemmli, 1970) and gels were Coomassie blue stained. Protein concentrations were determined by the Bradford assay using bovine serum albumin as standard (Bradford, 1976).

TABLE 1. Bacterial strains and plasmids

Strains/plasmid	Genotype/phenotype	Source/reference
E. coli		
DH10B	Sm^r, F‘ [*pro*AB^+ lacZΔM15]	Invitrogen
BL21 (λDE3)	Expresses T7 RNA polymerase	Agilent
Plasmids		
pET29a	Km^r. Expression vector T7 promoter	Novagen
pET28a	Km^r. Expression vector T7 promoter	Novagen
pMSA3	Km^r. (pET28a). Expresses the *A. brasilense* GlnZ carrying a 6xHis tag at N-terminal	Araujo *et al.*, 2004
pLMA-MLV1	Km^r. (pET28a). Expresses the *A. brasilense* GlnB carrying a 6xHis tag at N-terminal	Huergo *et al.*, 2007
pGAHisGlnZΔloop	Km^r. (pET28a). Expresses the *A. brasilense* GlnZ carrying a T-loop deletion and a 6xHis tag at N-terminal (GlnZΔLoop)	Araújo, 2018
pTRpetHisGlnK	Km^r.(pET28a). Expresses the *E. coli* GlnK carrying a 6xHis tag at N-terminal	Rodrigues *et al.*, 2014
pTRpetHisGlnB	Km^r. (pET28a). Expresses the *E. coli* GlnB carrying a 6xHis tag at N-terminal	Gerhardt *et al.*, 2015
pAHUDGC	Km^r (pET-29a). Expresses *A. brasilense* untagged AbDGC	Urbanski, 2018
pAHUPDE	Km^r (pET-29a). Expresses *A. brasilense* untagged AbPDE	Urbanski, 2018

Protein expression and purification

Expression of AbDGC and AbPDE

Escherichia coli BL21 (λDE3) cells carrying the pAHUDGC or pAHUPDE plasmid were cultivated on 400 ml LB at 37°C to an OD_{600} of 0.5. After reached the expected OD, cells were transferred to 16°C for 30 minutes and protein expression was induced by adding 0.25 mM Isopropyl-β-D-thiogalactopyranoside (IPTG). After 16 hours, cells were harvested by centrifugation (5.000 × *g* for 10 minutes at 4°C). The pellet was resuspended in 20 ml of buffer 1 (50 mM Tris-HCl pH 8.0, 0.1 M KCl). Cells were disrupted by sonication (10 minutes with cycles of 15 seconds); cell extracts were clarified by centrifugation (20.000 × *g* for 20 minutes at 4°C).

Ammonium sulphate precipitation of AbDGC and AbPDE

Ammonium sulphate precipitation was carried out as described by Urbanski (2018) with modifications. Ammonium sulphate was added slowly to the soluble fractions containing AbDGC and AbPDE expression up to 20% saturation. The samples were incubated for one hour at 0°C. After this period the supernatant was separated from the precipitate by centrifugation for 10 minutes, (10.000 × *g*) at 4°C. The precipitate was resuspended in 5 ml of buffer 1. The resulting resuspended protein was dialyzed in buffer 2 (Tris HCl pH 8.0 (50 mM), KCl (100 mM) and Glycerol (10% v/v)), for 16 hours at 4°C.

Purification of PII

The His-tagged proteins (His-AbGlnB, His-AbGlnZ, His-AbGlnZΔLoop, His-EcGlnB and His-EcGlnK) were expressed in *E. coli* BL21 (λDE3), OD_{600} of 0.5 added IPTG for 3 hours at 37°C. Cells were harvested by centrifugation and resuspended in 20 ml of buffer 1. Cells were disrupted by sonication (10 minutes with cycles of 15 seconds), and the extracts were clarified by centrifugation (20.000 × *g* for 20 minutes at 4°C). The next purification step was performed using a heating step as described (Moure *et al.*, 2012). After, the cells were centrifuged (20.000 × *g* for 20 minutes at 4°C) and the supernatant was loaded onto a 1 ml HiTrap chelating column (GE Healthcare) containing Ni^{2+} (100mM) previously equilibrated with buffer 1. The bound proteins were washed with 10 ml of buffer 1. The elution was performed with increasing imidazole gradient (0.1 to 1M in buffer 1).

Protein fractions were analyzed by SDS-PAGE, and the fractions containing the protein of interest were dialyzed in buffer 2.

Purification of AbDGC and AbPDE using His-GlnZ protein

His-GlnZ was expressed (see purification of PII), and the extract containing the His-GlnZ expression was mixed with the extract containing the overexpression of the AbDGC or AbPDE protein on buffer 1 (20 ml). The extracts were sonicated together for 10 minutes with cycles of 15 seconds. The soluble fraction was obtained by centrifugation for 20 minutes (20.000 *x g* at 4 ° C). After that ATP (1 mM) was added, and the resulting fraction was loaded on the 1 ml HiTrap chelating column equilibrated with buffer 1. His-GlnZ binded to the column held AbDGC or AbPDE and a washed step were performed with 50 mM of imidazole

in buffer 1. The elution of AbDGC or AbPDE was made adding 2-OG (2 mM), ATP (1 mM) and $MgCl_2$ (5 mM) in buffer 1. The proteins were observed in SDS-PAGE gels, stained with Coomassie blue.

In vitro uridylylation of PII

In vitro uridylylation of His-PII was carried out according to a modified version of the protocol previously described (Rodrigues *et al.*, 2014). Purified His-PII was subjected to *in vitro* uridylylation in a 500 µl reaction mixtures containing 100 µM His-PII (considered as monomer), ATP (1 mM), UTP (2 mM), 2-OG (5 mM), 1 µM of purified *E. coli* GlnD (kindly provided by Dr. Thiago E. Rodrigues), Tris–HCl pH 7.5 (100 mM), KCl (100 mM) and $MgCl_2$ (25 mM). The reactions were incubated at 37ºC for three hours and terminated by heating at 60ºC for 15 min then we put the solution on ice for 15 minutes, GlnD was removed by centrifugation (20.000 × *g* for 20 minutes at 4°C). The His-PII-UMP was dialyzed against buffer 2 (50 mM Tris HCl pH 8.0, 100 mM KCl, glycerol 10%). The His-PII uridylylation was determined by Native-PAGE as described previously (Inaba *et al.*, 2009).

Gel filtration chromatography

Analytical gel filtration chromatography of AbDGC and AbPDE were performed on a 25 ml Superose 6 10/30 column (GE Healthcare) at room temperature and a flow rate of 0.5 ml per minute using buffer 1. The column was calibrated with the following molecular mass markers from Bio-Rad: Thyroglobulin (670 kDa); gama-globulin (158 kDa); ovalbulmin (44 kDa), myoglobin (17 kDa) and Vitamin B12 (1.35 kDa).

Protein co-precipitation using magnetic beads (Pull-down)

Five microliters of HisMagnetic beads (Promega) were equilibrated by wash with 200 µl of buffer containing 50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 0.1% Triton X-100 (v/v), 10% glycerol (v/v), 20 mM imidazole in the presence or absence of effectors as indicated in each experiment. The interactions were conducted in 400 µl of buffer adding 20 µg His-PII and then 40 µg untagged AbDGC or AbPDE. The proteins were mixed at room temperature; The beads were washed three times with 200 µl of buffer. Then, the samples were boiled and analyzed in 15% SDS-PAGE stained with Coomassie blue.

Gel band densitometry analyses were performed using Gel Analyzer 19.1 program.

Results

AbDGC and AbPDE proteins from A. brasilense SP245 assume quarternary structure in vitro

The AbDGC protein monomer is about 38 kDa while the AbPDE is 93 kDa. Generally, these types of proteins are found in quaternary structure. In order to know the organization of these proteins we used an analytical gel filtration chromatography column. However, before the analytical step, we tried to purify the AbDGC and AbPDE proteins with different approaches.

First, proteins were precipitate with ammonium sulphate. The resulting proteins presented many contaminants to analytical gel filtration step. Following we tried to purify AbDGC and AbPDE using different ionic exchange columns, but the proteins did not showed affinity to any column tested (Heparin, Q, DEAE,CM and SP). Then, as we knew AbDGC and AbPDE interacted with the GlnZ and that interactions were abolished in the presence of Mg.ATP + 2-OG (Urbanski, 2018), we purified the proteins using an Mg.ATP + 2-OG complex inhibition approach.

We mixed either HisGlnZ-AbDGC or HisGlnZ-AbPDE extracts in the presence of ATP (1 mM), sonicated them together loaded the soluble fraction into the HiTrap chelating column. Then we eluted the content bound to GlnZ with Mg.ATP + 2-OG, as the AbDGC and AbPDE proteins were overexpressed they were bound in greater amounts to His-GlnZ and at elution it was possible to observe them relatively purified, while His-GlnZ remains bound to the column (Fig. S1A and B). The resulting purified proteins were loaded in the analytical column Superose 6.

The AbDCG protein eluted in the volume 14.64 ml, it corresponds to 96 kDa, this size is close to the expected for a dimer or trimer (Fig. 1A and C). Usually DGC are observed as dimers for binding two GTP in each monomer, to form the c-di-GMP (Chan *et al.*, 2004; Wassmann *et al.*, 2007). The AbPDE protein was eluted at 12.36 ml, which corresponds to a molecular weight of 565 kDa, size expected for a hexamer (fig. 1B and C). Studies showed that PDE proteins are generally found as dimers, however *in vitro* oligomerization can reach larger sizes (Barends *et al.*, 2009).

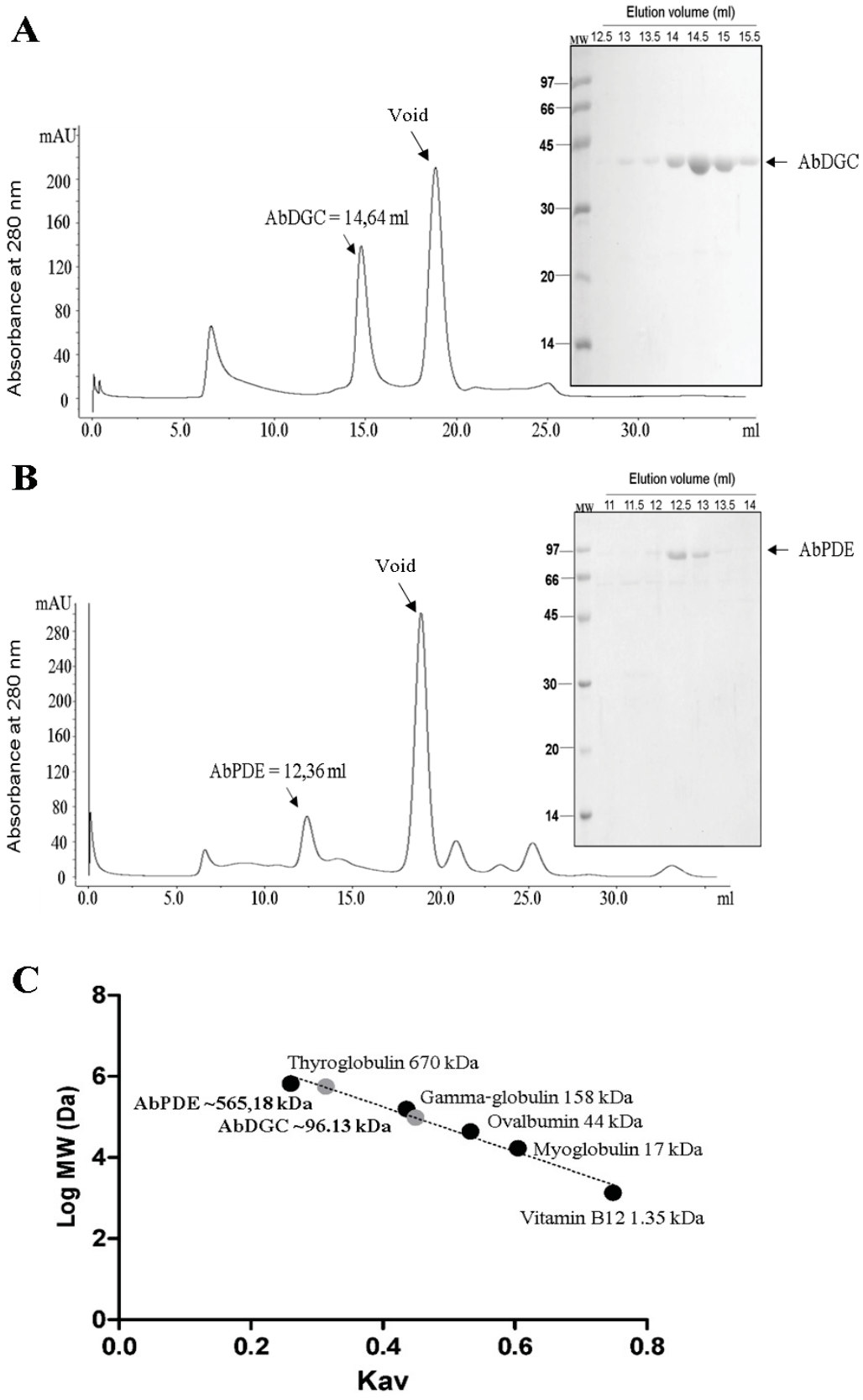


FIGURE 1. Analysis of purified *A. brasilense* AbDGC e AbPDE. Superose 6 column has a total volume of 25 ml. A) Elution profile of purified AbDGC and B) AbPDE on a Superose 6 column (GE Healthcare). C) Log MW vs Kav plots of molecular mass standards (BioRad), AbDGC and AbPDE.

AbDGC and AbPDE proteins also interact with GlnB of A. brasilense SP245

Urbanski (2018) characterized the interaction between AbDGC and AbPDE proteins with the GlnZ from *A. brasilense* SP245. However, this bacterium has a second PII protein called GlnB. Here we evaluated whether AbDGC and AbPDE also interac with this protein. To evaluate the interaction we used the pull-down technique with AbDGC and AbPDE partially purified using ammonium sulphate precipitation.

As seen for GlnZ (Urbanski, 2018), the interaction showed to occurs in the presence of ATP and ADP and is inhibited in the presence of Mg.ATP + 2-OG (Fig. 2). We evaluated whether the interaction occurs in the absence of effectors and the result was also positive for both proteins, as well as in the presence of only 2-OG (Fig. S3).

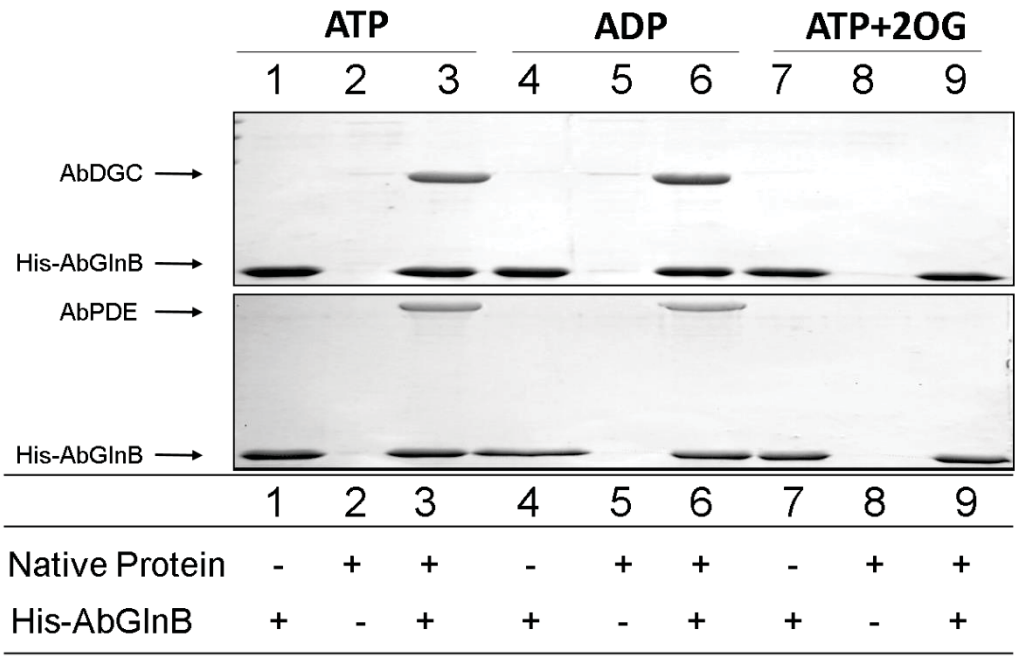


FIGURE 2. *In vitro* formation of the complexes AbDGC-GlnB and AbPDE-GlnB. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of $MgCl_2$ (5mM) and the effectors ATP, ADP and/or 2-OG at 1mM. Binding reactions were conducted in 400 µl of buffer adding purified His-GlnB mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

2-OG is the main effector in the GlnB-AbDGC and GlnB-AbPDE complexes formation

Effector binding causes conformational changes in PII that can alter the interaction with their targets. We performed tests with the PII effectors (ATP, ADP and 2-OG) to verify if they affect AbDGC-GlnB or AbPDE-GlnB complexes. First, we evaluated different

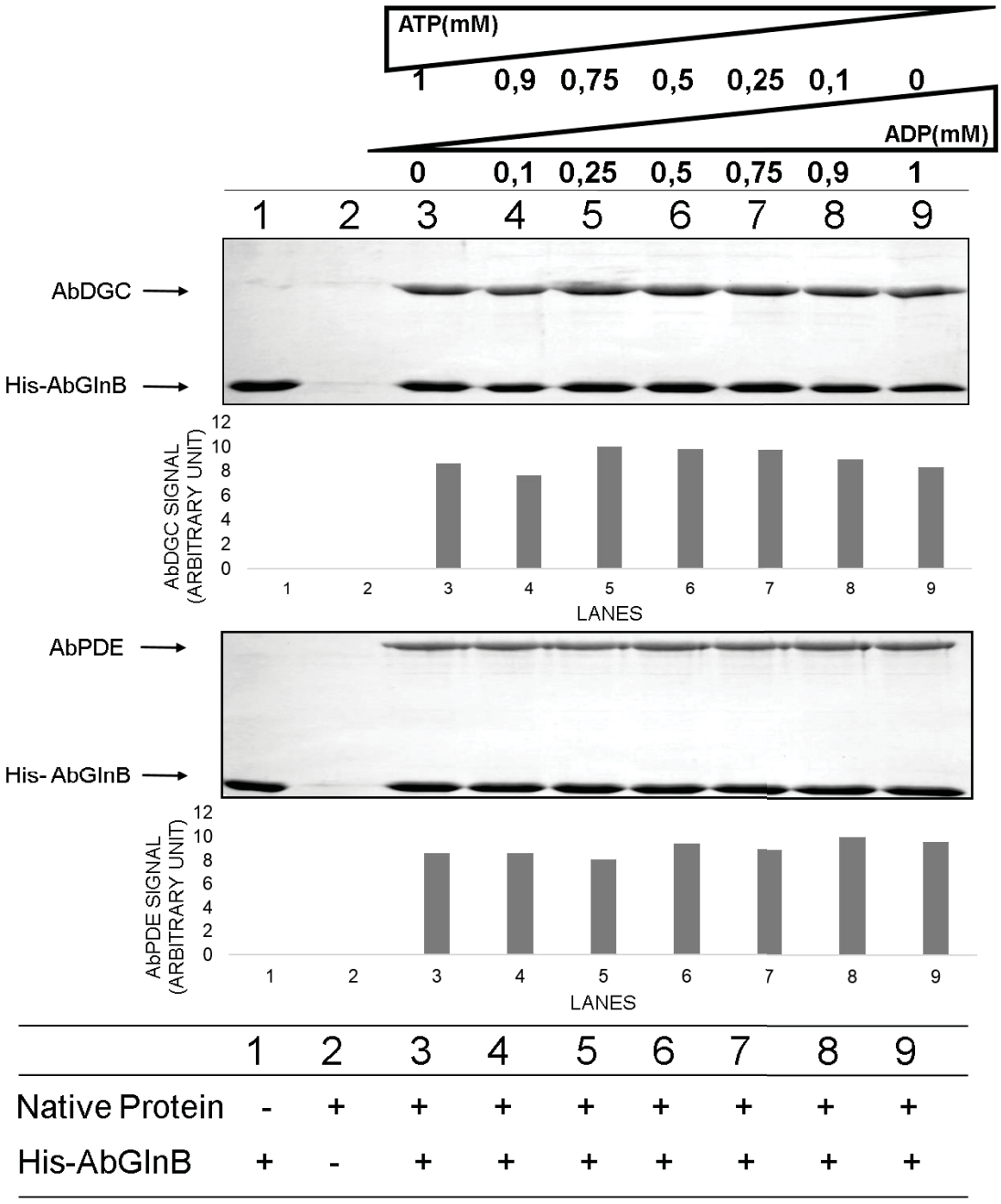
concentrations of ATP and ADP together (Fig. 3) and independently (Fig. S4 and S5). In all tested concentrations and combinations, the interactions of GlnB with both proteins occured and remained stable.

As observed, the interaction of AbDGC-GlnB and AbPDE-GlnB in the presence of Mg.ATP + 2-OG (1 mM) do not occur (Fig. 1). We tested different concentrations of 2-OG in the complex formation process and we noticed that the complex formation is inhibited as 2-OG concentration increases (Fig. 4). It is still possible to visualize AbDGC-GlnB complex at 0.1 mM of 2-OG, but above concentrations the interaction was totally inhibited. For AbPDE-GlnB it is not possible to view the interaction from 0.1 mM of 2-OG (Fig. 4).

Tests were also performed in the presence of the three PII effectors. The results of both proteins, AbDGC and AbPDE, were similar. As expected, in the absence of 2-OG and at different concentrations of ATP and ADP, the interaction occurs in a stable manner. In the presence of low 2-OG (0.1 mM) the interaction is inhibited, mainly for AbPDE, however, as the concentration of ADP increases the interaction of GlnB with both proteins also increases, indicating that ADP is binding GlnB preventing the inhibitory activity of 2-OG. In high 2-OG (1 mM) the interaction with GlnB is almost completely prevented with both proteins, unless if there is an absence of ATP (Fig. 5).

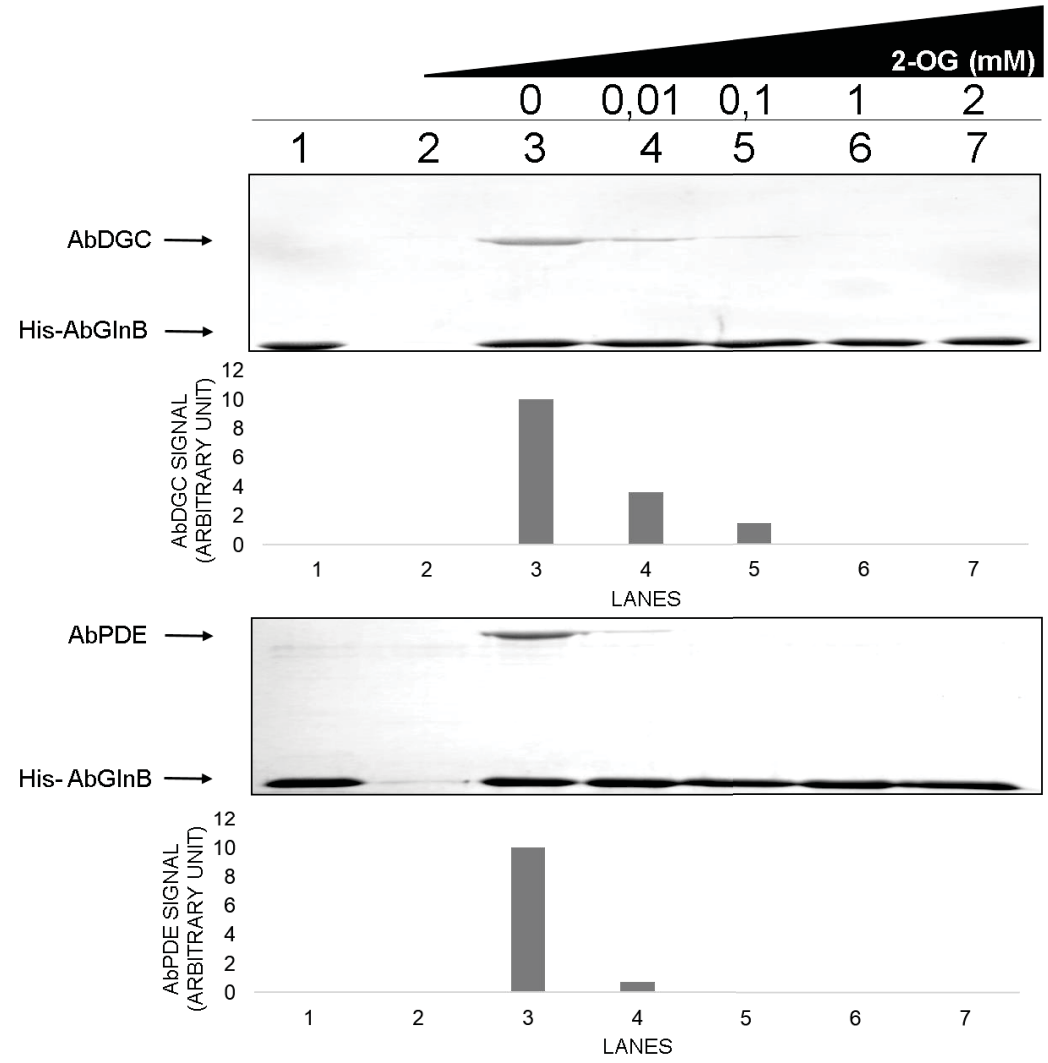
Interaction between GlnB and AbPDE is Mg^{2+} dependent, whereas with AbDGC is not

We tested AbDGC-GlnB and AbPDE-GlnB interactions in the presence or absence of $MgCl_2$ (5 mM) at fixed ATP concentration (1 mM). The interaction with AbDGC continued to occur regardless of the absence of Mg^{2+}, unlike what happened with AbPDE. The AbPDE-GlnB interaction was totally inhibited in the absence of the divalent ion (Fig. 6).



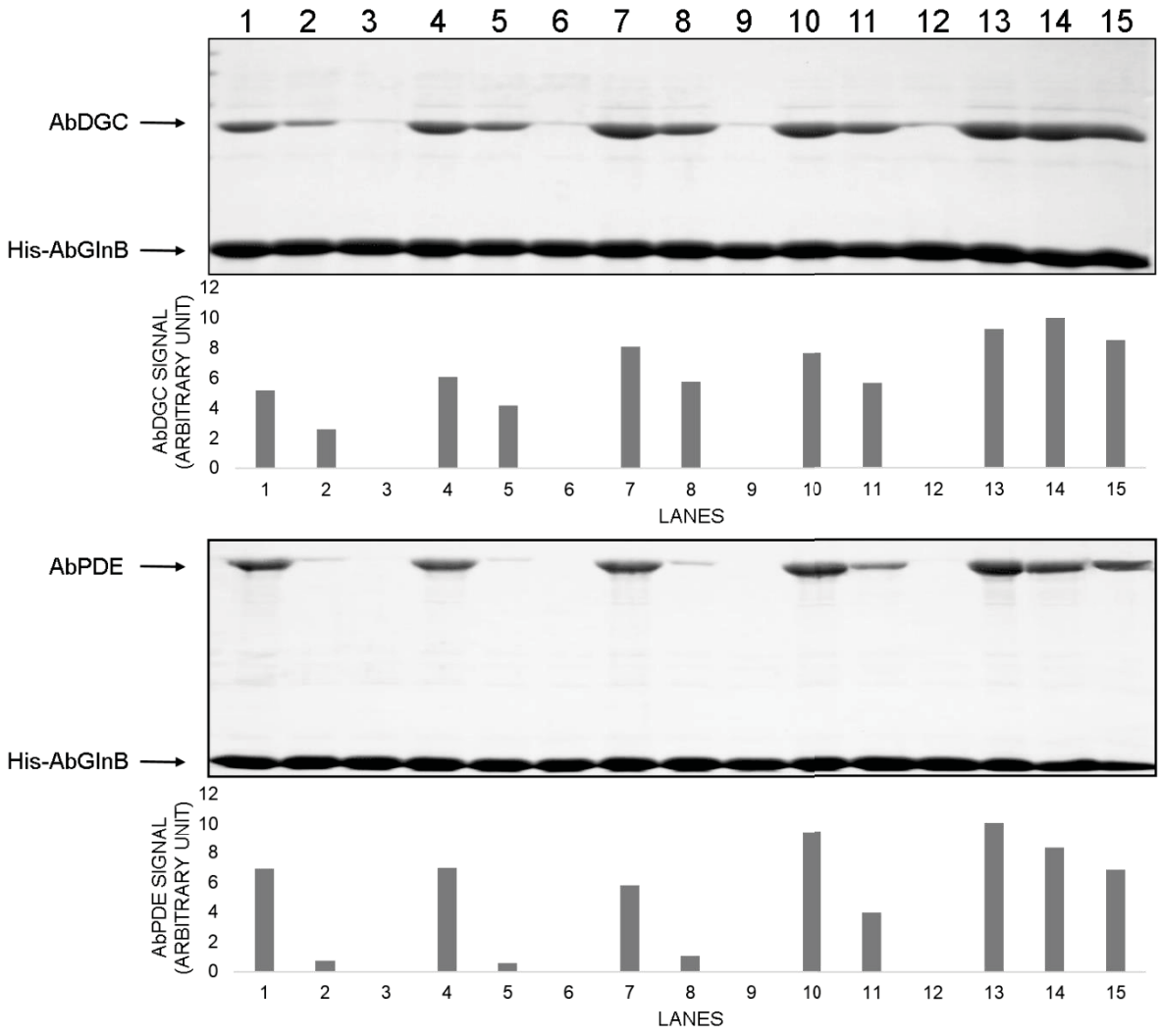
	1	2	3	4	5	6	7	8	9
Native Protein	-	+	+	+	+	+	+	+	+
His-AbGlnB	+	-	+	+	+	+	+	+	+

FIGURE 3. ***In vitro*** **formation of the complexes AbDGC-GlnB and AbPDE-GlnB are independent on the [ATP]/[ADP] ratio.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under different concentrations of ATP and ADP as indicated and fixed concentration of $MgCl_2$ (5mM). Reactions were conducted in 400 µl of buffer adding purified His-GlnB mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.



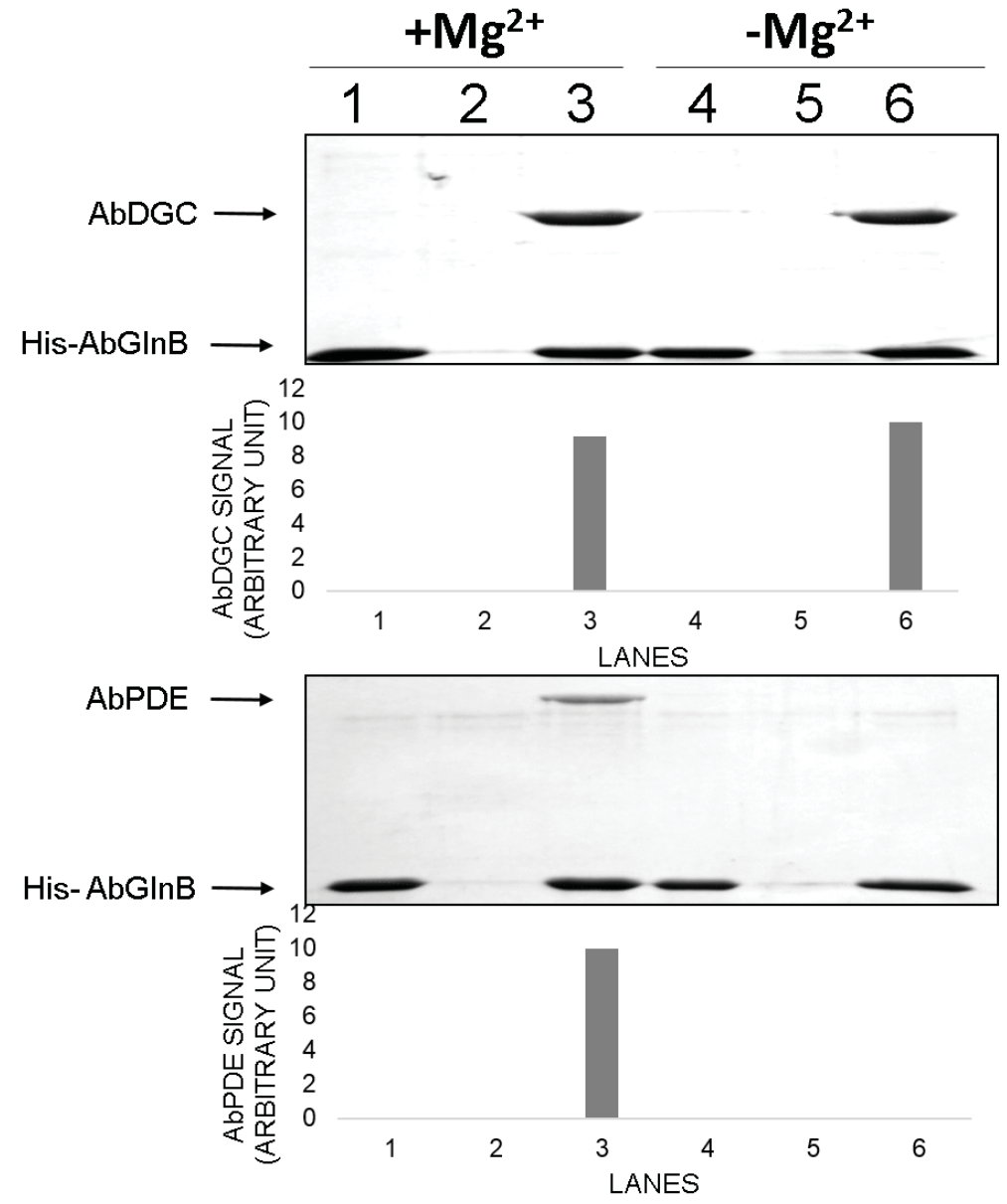
	1	2	3	4	5	6	7
Native Protein	-	+	+	+	+	+	+
His-AbGlnB	+	-	+	+	+	+	+

FIGURE 4. ***In vitro*** **formation of the complexes AbDGC-GlnB and AbPDE-GlnB are dependent on the 2-OG concentrations.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of ATP (1 mM) and $MgCl_2$ (5mM), increasing concentrations of 2-OG as indicated. Reactions were conducted in 400 µl of buffer adding purified His-GlnB mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ATP (mM)	1	1	1	0,9	0,9	0,9	0,75	0,75	0,75	0,5	0,5	0,5	0	0	0
ADP (mM)	0	0	0	0,1	0,1	0,1	0,25	0,25	0,25	0,5	0,5	0,5	1	1	1
2-OG (mM)	0	0,1	1	0	0,1	1	0	0,1	1	0	0,1	1	0	0,1	1

FIGURE 5. Effect of different concentrations of the PII effectors on *in vitro* formation of AbDGC-GlnB and AbPDE-GlnB complexes. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in different concentrations of the effectors ATP, ADP and 2-OG, as indicated and fixed $MgCl_2$ concentration (5 mM). Binding reactions were conducted in 400 µl of buffer adding purified His-GlnB, mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.



	1	2	3	4	5	6
Native Protein	-	+	+	-	+	+
His-AbGlnB	+	-	+	+	-	+

FIGURE 6. Mg^{2+} is required for *in vitro* formation of the AbPDE-GlnB complex but not for AbDGC-GlnB. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed ATP concentration (1 mM) in presence or absence of $MgCl_2$ (5mM) as indicated. Reactions were conducted in 400 µl of buffer adding purified His-GlnB mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

AbDGC and AbPDE do not need T-Loop to interact with PII, but the covalent binding of the UMP to T-Loop interferes with the AbPDE-GlnB interaction

Using the GlnZ protein with the partially truncated T-Loop, called GlnZΔLoop, we performed the interaction with AbDGC and AbPDE, the interaction occurred as usual indicating that these interactions do not need T-Loop to occur (Fig. 7). We partially uridylylated the GlnB protein and tested the interaction with AbPDE and AbDGC (Fig. S6). Surprisingly, the interaction with AbPDE was inhibited in the presence of the UMP group (Fig. 8).

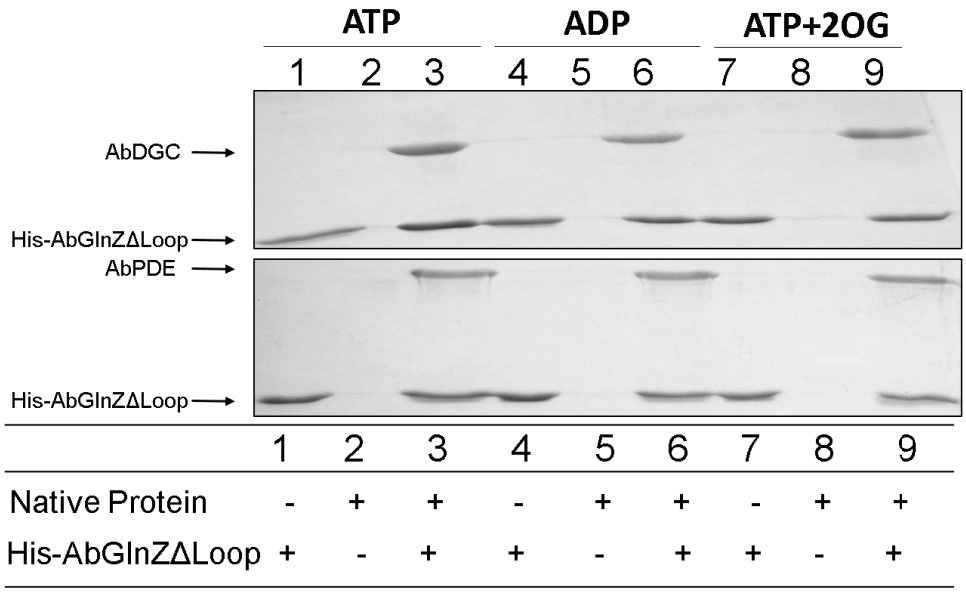


FIGURE 7. ***In vitro*** **formation of the complexes AbDGC-GlnZ and AbPDE-GlnZ does not require the T loop**. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of the effectors ATP, ADP and/or 2-OG at 1mM and $MgCl_2$ (5 mM). Binding reactions were conducted in 400 µl of buffer adding purified His-GlnZΔLoop, mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

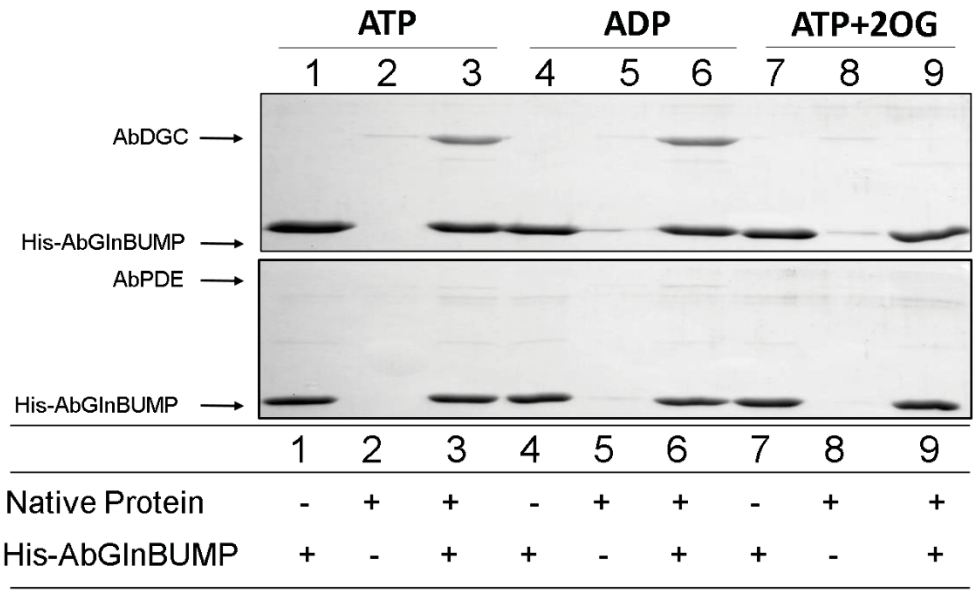


FIGURE 8. Partially uridylylated form of PII interferes in the *in vitro* formation of the AbPDE-GlnB complex, but does not affect AbDGC-GlnB complex. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of the effectors ATP, ADP and/or 2-OG at 1mM and $MgCl_2$ at 5 mM. Binding reactions were conducted in 400 µl of buffer adding purified His-GlnB uridylylated, mixed with AbDGC or AbPDE, as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

AbDGC and AbPDE interact with E. coli's PII proteins

We also performed a heterologous assay using proteins from different organisms *(A. brasilense* and *E. coli*) to verify if there is a conserved structure in the interaction. We performed the interaction in the presence of the *E. coli* GlnB with the *A. brasilense* SP245's AbDGC and AbPDE proteins. The interaction with AbDGC occurred as usual in the presence of ATP and ADP, and was inhibited in the presence of Mg.ATP + 2-OG. The interaction with AbPDE also occurred but only in the presence of ATP, not being observed in the presence of ADP and Mg.ATP + 2-OG (Fig. 9). We also performed interaction with the second *E. coli* PII protein, GlnK, and the interaction occurred with both proteins in the presence of ATP and ADP and was inhibited in the presence of Mg.ATP + 2-OG (Fig. S7).

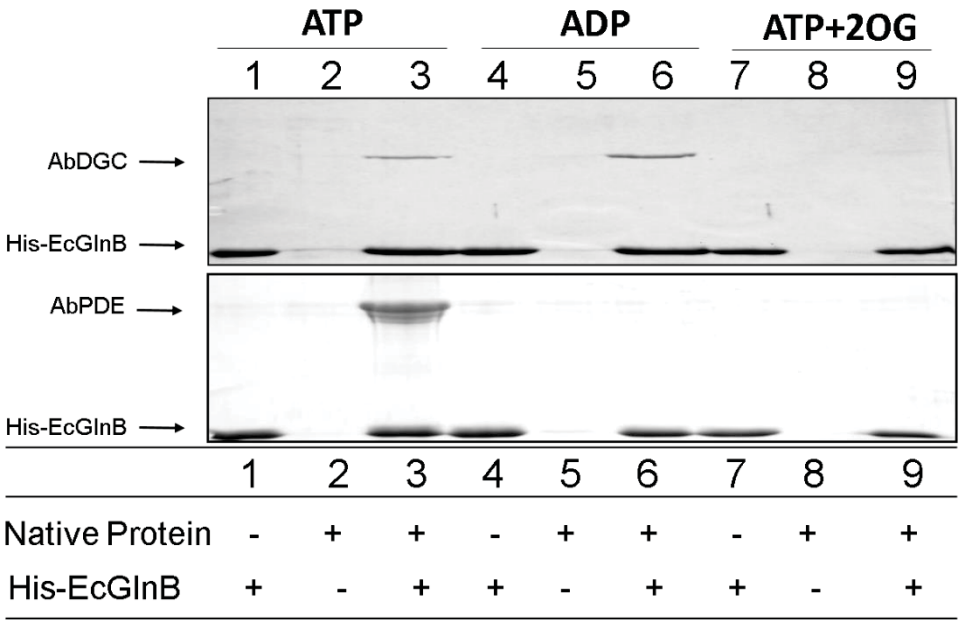


FIGURE 9. *In vitro* formation of the complexes AbDGC-EcGlnB and AbPDE-EcGlnB. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of $MgCl_2$ (5mM) and the effectors ATP, ADP and/or 2-OG at 1mM. Binding reactions were conducted in 400 µl of buffer adding purified His-EcGlnB mixed with AbPDE or AbDGC. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

Discussion

Analysis of the *A. brasilense* genome performed by Urbanski (2018), revealed to have 21 sequences of probable DGC proteins containing only the GGDEF domain and another 10 proteins sequences containing EAL and GGDEF domains. However, only one of each (AbDGC and AbPDE) was predicted to interact with GlnZ according to Gerhardt (2015).

The AbDGC protein has one predict GGDEF domain, with the GGEEF motif (A site). The AbPDE protein has five domains: an EAL domain containing the EAL motif, a GGDEF domain with a degenerate motif SADEF and three PAS domains (Urbanski, 2018). PAS domains have a variety of functions, usually act as a molecular sensor, whereby small molecules and/or other proteins associate via physical interaction (Vorobiev *et al.*, 2012).

Complex formation of AbDGC and AbPDE with GlnB occurred in the presence of ATP and ADP, but with high 2-OG in the presence of ATP and Mg^{2+} (Mg.ATP) the complexes were not formed (Fig. 2). The same pattern occurred with GlnZ (Urbanski, 2018). The binding of 2-OG requires previous binding of Mg.ATP (Truan *et al.*, 2010). This explain why the interaction occurs in the presence of high 2-OG without Mg.ATP (Fig. S3).

This effect of Mg.ATP + 2-OG promoting inhibition of interactions is quite common among proteins that interact with PII. The binding of 2-OG causes a conformational change in PII that leads to this effect (Truan *et al.*, 2010). Some examples are the BCCP-GlnB interaction in *A. brasilense* and *E. coli* (Gerhardt *et al.*, 2015), in *Synechococcus elongatus*, the interaction between GlnB and NAGK (Fokina *et al.*, 2010) and both PII GlnZ and GlnB from *A. brasilense* do not form an *in vitro* complex with AmtB in the presence of 0.1 mM of 2-OG (Huergo *et al.*, 2007). The levels of 2-OG have already been measured in *E. coli* and the minimum reached was around 0.3 mM under ammonium-shock conditions (Radchenko, Thornton and Merrick, 2010), probably the interaction of PII with AbDGC and AbPDE should occur in this metabolic condition.

Low levels of 2-OG are important to many interactions of PII, the interaction of AmtB with PII in *A. brasilense* is an example of an interaction that follows this pattern. The AmtB protein is an ammonium transporter that is inhibited by PII when ammonium levels rise *in vivo*. The ammonium-shock rises the levels of intracellular glutamine and decrease the levels of 2-OG reciprocally. In this condition PII interacts with AmtB in the membrane until the levels of 2-OG increase (Huergo *et al.*, 2006).

The PII T-loop region is the most flexible and changes in response to the binding of effectors leading to a conformational state that promotes or inhibits the interaction with its targets (Xu *et al.*, 1998; Truan *et al.*, 2010). Most of the interactions described for PII and its targets occur in the T-loop region (Huergo, Chandra and Merrick, 2013). However, the deletion of the T-loop did not prevent the interaction between AbDGC and AbPDE with GlnZΔLoop, and 2-OG failed to negatively modulate the complex formation (Fig. 7). This suggests that the T-loop structure is important for the conformational change in PII induced by 2-OG binding, and consequently inhibition of the complexes with AbDGC and AbPDE, but it is not the site of interaction.

The T-loop can be also post-translational changed, UMP is added in response to increased nitrogen (Atkinson and Ninfa, 1998; Araújo *et al.*, 2008). The complex formation of the partially uridylylated GlnB (His-GlnBUMP) and AbDGC (Fig.8) occurs as without modification. For AbPDE, different from that observed by Urbanski (2018) with GlnZ, was not possible to observe the interaction in the presence of partially uridylylated GlnB. Other interactions of PII have already been inhibited in the presence of UMP groups in *A. brasilense*, for example with DraG, DraT, AmtB and NtrB (Jiang and Ninfa, 1999; Huergo *et al.*, 2006, 2007; Radchenko, Thornton and Merrick, 2010). Probably the conformation

assumed after adding UMP or the presence of the charged UMP group in the Tyr51 region interfered with the interaction with AbPDE.

With the results *in vitro* is possible to propose a cellular condition for the complex formation in *A. brasilense* (Fig. 10). Under conditions of nitrogen fixation, the cellular glutamine level is low and the levels of 2-OG rises, preventing the interaction of PII with AbDGC and AbPDE. In ammonium shock conditions, the level of 2-OG drops, while glutamine increases, low 2-OG levels allow to GlnB-AbPDE and GlnB-AbDGC complexes formation.

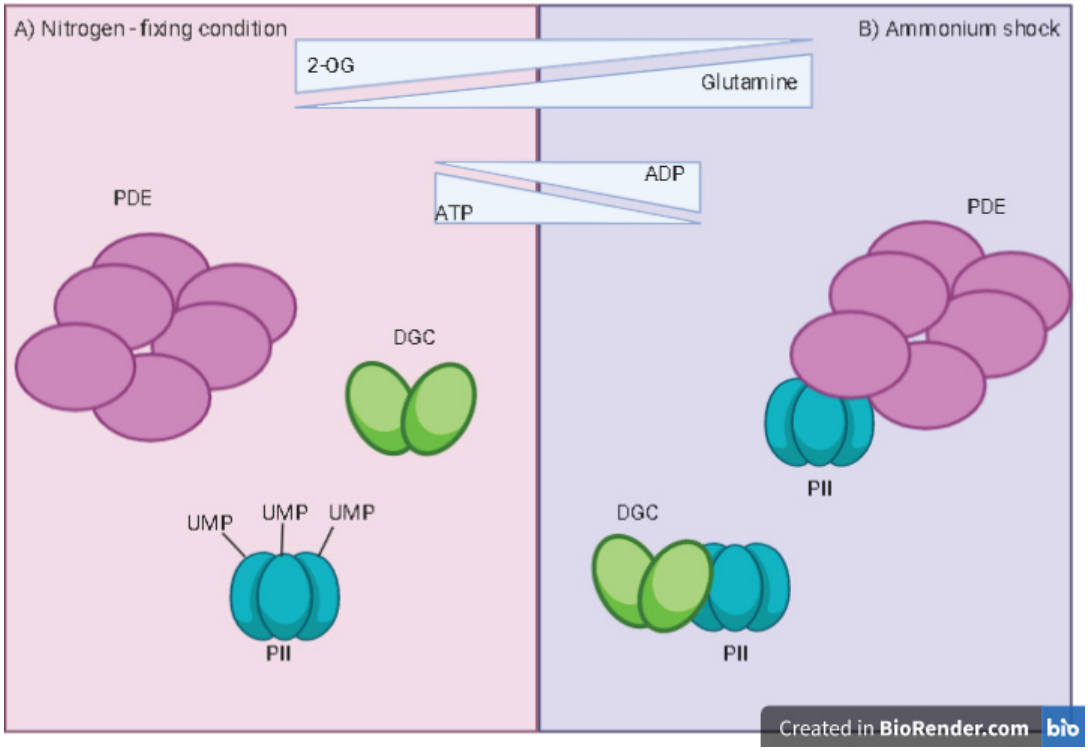


FIGURE 10. Interaction model of AbDGC-PII and AbPDE-PII complexes. Previously *in vitro* data showed that the AbDGC-PII and AbPDE-PII interactions are inhibited by the PII's effector 2-OG. The level of 2-OG drops when the level of available nitrogen increases, once 2-OG is used on the formation of glutamine, a metabolite which rises in high nitrogen. A particular metabolic state which these interactions would occur could be in the ammonium shock. When a nitrogen limited bacterial cell receive a high ammonium concentration the 2-OG level in the bacterial cell drops, probably allowing AbDGC-PII and AbPDE-PII complexes formation. When ammonium is consumed 2-OG level increases and it binds PII, no longer allowing PII to interact with AbDGC or AbPDE.

Conclusion

DGC and PDE proteins seem to respond specific signals for their activation or inhibition, these signals can be via effector binding or physical interaction with other proteins. Recently

possible new targets for the PII signal transducer protein of *A. brasilense* were discovered, and two of them are proteins AbDGC and AbPDE. PII proteins receive signals by binding effectors ATP, ADP and 2-OG, which signals energy status and availability of intracellular nitrogen and carbon. Considering that, AbDGC and AbPDE synthesize the compound c-di-GMP, an important component in the transition of mobile to the sessile state in bacteria, interaction with a protein that senses different states of the cell would be advantageous. The interaction of AbDGC and AbPDE occurs with the two PII proteins of *A. brasilense*, GlnB and GlnZ, and that this interaction probably occurs in ammonium shock, a cellular moment that 2-OG levels decrease, the main inhibitory effector of the interaction with both proteins. Further *in vitro* and *in vivo* studies are needed to observe the functions of these complexes.

References

Araújo, L. M. *et al.* (2008) 'Different responses of the GlnB and GlnZ proteins upon in vitro uridylylation by the Azospirillum brasilense GlnD protein.', *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas*, 41(4), pp. 289–94. doi: 10.1590/s0100-879x2008000400006.

Araujo, M. S. *et al.* (2004) 'In vitro uridylylation of the Azospirillum brasilense N-signal transducing GlnZ protein', *Protein Expression and Purification*, 33(1), pp. 19–24. doi: 10.1016/j.pep.2003.08.024.

Araujo, G. A. T. (2018). Caracterização *in vitro* das enzimas málica MaeB e N-acetil glutamato quinase NAGK e interação com proteínas PII em *Azospirillum brasilense. Dissertação (Mestrado em Ciências - Bioquímica) - Set. Ciências Biológicas, Univ. Fed. do Paraná.*, 1–97.

Arcondéguy, T., Jack, R. and Merrick, M. (2001) 'PII Signal Transduction Proteins, Pivotal Players in Microbial Nitrogen Control', *Microbiology and Molecular Biology Reviews*, 65(1), pp. 80–105. doi: 10.1128/MMBR.65.1.80-105.2001.

Atkinson, M. R. and Ninfa, A. J. (1998) 'Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*', *Molecular Microbiology*, 29(2), pp. 431–447. doi: 10.1046/j.1365-2958.1998.00932.x.

Barends, T. R. M. *et al.* (2009) 'Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase', *Nature*, 459(7249), pp. 1015–1018. doi: 10.1038/nature07966.

Bharati, B. K. *et al.* (2012) 'A full-length bifunctional protein involved in c-di-GMP turnover is required for long-term survival under nutrient starvation in Mycobacterium smegmatis', *Microbiology*, 158(6), pp. 1415–1427. doi: 10.1099/mic.0.053892-0.

Bradford, M. (1976) 'A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding', *Analytical Biochemistry*, 72(1–2), pp. 248–254. doi: 10.1006/abio.1976.9999.

Chan, C. *et al.* (2004) 'Structural basis of activity and allosteric control of diguanylate cyclase', *Proceedings of the National Academy of Sciences*, 101(49), pp. 17084–17089. doi:

10.1073/pnas.0406134101.

Cohen, D. *et al.* (2015) 'Oligoribonuclease is a central feature of cyclic diguanylate signaling in *Pseudomonas aeruginosa*', *Proceedings of the National Academy of Sciences*, 112(36), pp. 11359–11364. doi: 10.1073/pnas.1421450112.

Feirer, N. *et al.* (2015) 'A Pterin-Dependent Signaling Pathway Regulates a Dual-Function Diguanylate Cyclase-Phosphodiesterase Controlling Surface Attachment in Agrobacterium tumefaciens', *mBio*. Edited by M. Parsek and E. P. Greenberg, 6(4), p. e00156. doi: 10.1128/mBio.00156-15.

Fokina, O. *et al.* (2010) 'Mechanism of 2-oxoglutarate signaling by the Synechococcus elongatus PII signal transduction protein.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(46), pp. 19760–5. doi: 10.1073/pnas.1007653107.

Forchhammer, K. (2004) 'Global carbon/nitrogen control by PII signal transduction in cyanobacteria: from signals to targets', *FEMS Microbiology Reviews*, 28(3), pp. 319–333. doi: 10.1016/j.femsre.2003.11.001.

Gerhardt, E. C. M. *et al.* (2015) 'The Bacterial signal transduction protein GlnB regulates the committed step in fatty acid biosynthesis by acting as a dissociable regulatory subunit of acetyl-CoA carboxylase', *Molecular Microbiology*. John Wiley & Sons, Ltd (10.1111), 95(6), pp. 1025–1035. doi: 10.1111/mmi.12912.

Hengge, R. (2009) 'Principles of c-di-GMP signalling in bacteria', *Nature Reviews Microbiology*, 7(4), pp. 263–273. doi: 10.1038/nrmicro2109.

Hobley, L. *et al.* (2012) 'Discrete Cyclic di-GMP-Dependent Control of Bacterial Predation versus Axenic Growth in Bdellovibrio bacteriovorus', *PLoS Pathogens*. Edited by A. Camilli, 8(2), p. e1002493. doi: 10.1371/journal.ppat.1002493.

Huergo, L. F. *et al.* (2006) 'Interactions between PII proteins and the nitrogenase regulatory enzymes DraT and DraG in Azospirillum brasilense', *FEBS Letters*, 580(22), pp. 5232–5236. doi: 10.1016/j.febslet.2006.08.054.

Huergo, L. F. *et al.* (2007) 'Ternary complex formation between AmtB, GlnZ and the nitrogenase regulatory enzyme DraG reveals a novel facet of nitrogen regulation in bacteria', *Molecular Microbiology*. doi: 10.1111/j.1365-2958.2007.06016.x.

Huergo, L. F. *et al.* (2012) 'PII signal transduction proteins: pivotal players in post-translational control of nitrogenase activity', *Microbiology*, 158(1), pp. 176–190. doi: 10.1099/mic.0.049783-0.

Huergo, L. F., Chandra, G. and Merrick, M. (2013) 'PII signal transduction proteins: nitrogen regulation and beyond', *FEMS Microbiology Reviews*, 37(2), pp. 251–283. doi: 10.1111/j.1574-6976.2012.00351.x.

Inaba, J. *et al.* (2009) 'Azospirillum brasilense PII proteins GlnB and GlnZ do not form heterotrimers and GlnB shows a unique trimeric uridylylation pattern', *European Journal of Soil Biology*. Elsevier Masson, 45(1), pp. 94–99. doi: 10.1016/J.EJSOBI.2008.08.006.

Jiang, P. and Ninfa, A. J. (1999) 'Regulation of autophosphorylation of Escherichia coli nitrogen regulator II by the PII signal transduction protein.', *Journal of bacteriology*, 181(6), pp. 1906–11.

Laemmli, U. K. (1970) ‘Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4’, *Nature*, 227(5259), pp. 680–685. doi: 10.1038/227680a0.

Maheswaran, M., Urbanke, C. and Forchhammer, K. (2004) ‘Complex formation and catalytic activation by the PII signaling protein of N-acetyl-L-glutamate kinase from Synechococcus elongatus strain PCC 7942.’, *The Journal of biological chemistry*, 279(53), pp. 55202–10. doi: 10.1074/jbc.M410971200.

Merrick, M. (2015) ‘Post-translational modification of PII signal transduction proteins’, *Frontiers in Microbiology*, 5, p. 763. doi: 10.3389/fmicb.2014.00763.

Mills, E. *et al.* (2015) ‘A direct screen for c-di-GMP modulators reveals a *Salmonella* Typhimurium periplasmic L-arginine–sensing pathway’, *Science Signaling*, 8(380), pp. ra57–ra57. doi: 10.1126/scisignal.aaa1796.

Moure, V. R. *et al.* (2012) ‘Heat stability of Proteobacterial P II protein facilitate purification using a single chromatography step’, *Protein Expression and Purification*, 81(1), pp. 83–88. doi: 10.1016/j.pep.2011.09.008.

O’Neal, L. *et al.* (2017) ‘Optogenetic Manipulation of Cyclic Di-GMP (c-di-GMP) Levels Reveals the Role of c-di-GMP in Regulating Aerotaxis Receptor Activity in Azospirillum brasilense’, *Journal of Bacteriology*. Edited by A. M. Stock, 199(18). doi: 10.1128/JB.00020-17.

Orr, M. W. *et al.* (2015) ‘Oligoribonuclease is the primary degradative enzyme for pGpG in *Pseudomonas aeruginosa* that is required for cyclic-di-GMP turnover’, *Proceedings of the National Academy of Sciences*, 112(36), pp. E5048–E5057. doi: 10.1073/pnas.1507245112.

Ozaki, S. *et al.* (2014) ‘Activation and polar sequestration of PopA, a c-di-GMP effector protein involved in *C aulobacter crescentus* cell cycle control’, *Molecular Microbiology*, 94(3), pp. 580–594. doi: 10.1111/mmi.12777.

Qi, Y. *et al.* (2009) ‘A Flavin Cofactor-Binding PAS Domain Regulates c-di-GMP Synthesis in *Ax* DGC2 from *Acetobacter xylinum*’, *Biochemistry*, 48(43), pp. 10275–10285. doi: 10.1021/bi901121w.

Radchenko, M. V., Thornton, J. and Merrick, M. (2010) ‘Control of AmtB-GlnK Complex Formation by Intracellular Levels of ATP, ADP, and 2-Oxoglutarate’, *Journal of Biological Chemistry*, 285(40), pp. 31037–31045. doi: 10.1074/jbc.M110.153908.

Rodrigues, T. E. *et al.* (2014) ‘Search for novel targets of the P II signal transduction protein in Bacteria identifies the BCCP component of acetyl-CoA carboxylase as a P II binding partner’, *Molecular Microbiology*. John Wiley & Sons, Ltd (10.1111), 91(4), pp. 751–761. doi: 10.1111/mmi.12493.

Romling, U., Galperin, M. Y. and Gomelsky, M. (2013) ‘Cyclic di-GMP: the First 25 Years of a Universal Bacterial Second Messenger’, *Microbiology and Molecular Biology Reviews*, 77(1), pp. 1–52. doi: 10.1128/MMBR.00043-12.

Römling, U., Liang, Z.-X. and Dow, J. M. (2017) ‘Progress in Understanding the Molecular Basis Underlying Functional Diversification of Cyclic Dinucleotide Turnover Proteins.’, *Journal of bacteriology*. American Society for Microbiology, 199(5), pp. e00790-16. doi: 10.1128/JB.00790-16.

Russell, M. H. *et al.* (2013) ‘Integration of the second messenger c-di-GMP into the chemotactic signaling pathway.’, *mBio*. American Society for Microbiology, 4(2), pp.

e00001-13. doi: 10.1128/mBio.00001-13.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, n. 2.

Tal, R. *et al.* (1998) 'Three cdg operons control cellular turnover of cyclic di-GMP in Acetobacter xylinum: genetic organization and occurrence of conserved domains in isoenzymes.', *Journal of bacteriology*, 180(17), pp. 4416–25.

Tchigvintsev, A. *et al.* (2010) 'Structural Insight into the Mechanism of c-di-GMP Hydrolysis by EAL Domain Phosphodiesterases', *Journal of Molecular Biology*, 402(3), pp. 524–538. doi: 10.1016/j.jmb.2010.07.050.

Truan, D. *et al.* (2010) 'A New PII Protein Structure Identifies the 2-Oxoglutarate Binding Site', *Journal of Molecular Biology*, 400(3), pp. 531–539. doi: 10.1016/j.jmb.2010.05.036.

Truan, D. *et al.* (2014) 'Structure and Thermodynamics of Effector Molecule Binding to the Nitrogen Signal Transduction PII Protein GlnZ from Azospirillum brasilense', *Journal of Molecular Biology*, 426(15), pp. 2783–2799. doi: 10.1016/j.jmb.2014.05.008.

Urbanski, A. H. (2018). Investigação e caracterização da interação i*n vitro* entre as proteínas PII, GlnZ e GlnB com uma diguanilato ciclase (AZOBR_140132) e uma fosfodiesterase (AZOBR_p1130052) de *Azospirillum brasilense* SP245. *Dissertação (Mestrado em Ciências - Bioquímica) - Set. Ciências Biológicas, Univ. Fed. do Paraná.*, 1–97.

Vorobiev, S. M. *et al.* (2012) 'Crystal structure of a catalytically active GG(D/E)EF diguanylate cyclase domain from Marinobacter aquaeolei with bound c-di-GMP product', *Journal of Structural and Functional Genomics*. Springer Netherlands, 13(3), pp. 177–183. doi: 10.1007/s10969-012-9136-4.

Wassmann, P. *et al.* (2007) 'Structure of BeF3−-Modified Response Regulator PleD: Implications for Diguanylate Cyclase Activation, Catalysis, and Feedback Inhibition', *Structure*. Cell Press, 15(8), pp. 915–927. doi: 10.1016/J.STR.2007.06.016.

Xu, Y. *et al.* (1998) 'GlnK, a PII-homologue: structure reveals ATP binding site and indicates how the T-loops may be involved in molecular recognition', *Journal of Molecular Biology*, 282(1), pp. 149–165. doi: 10.1006/jmbi.1998.1979.

Supplementary Material

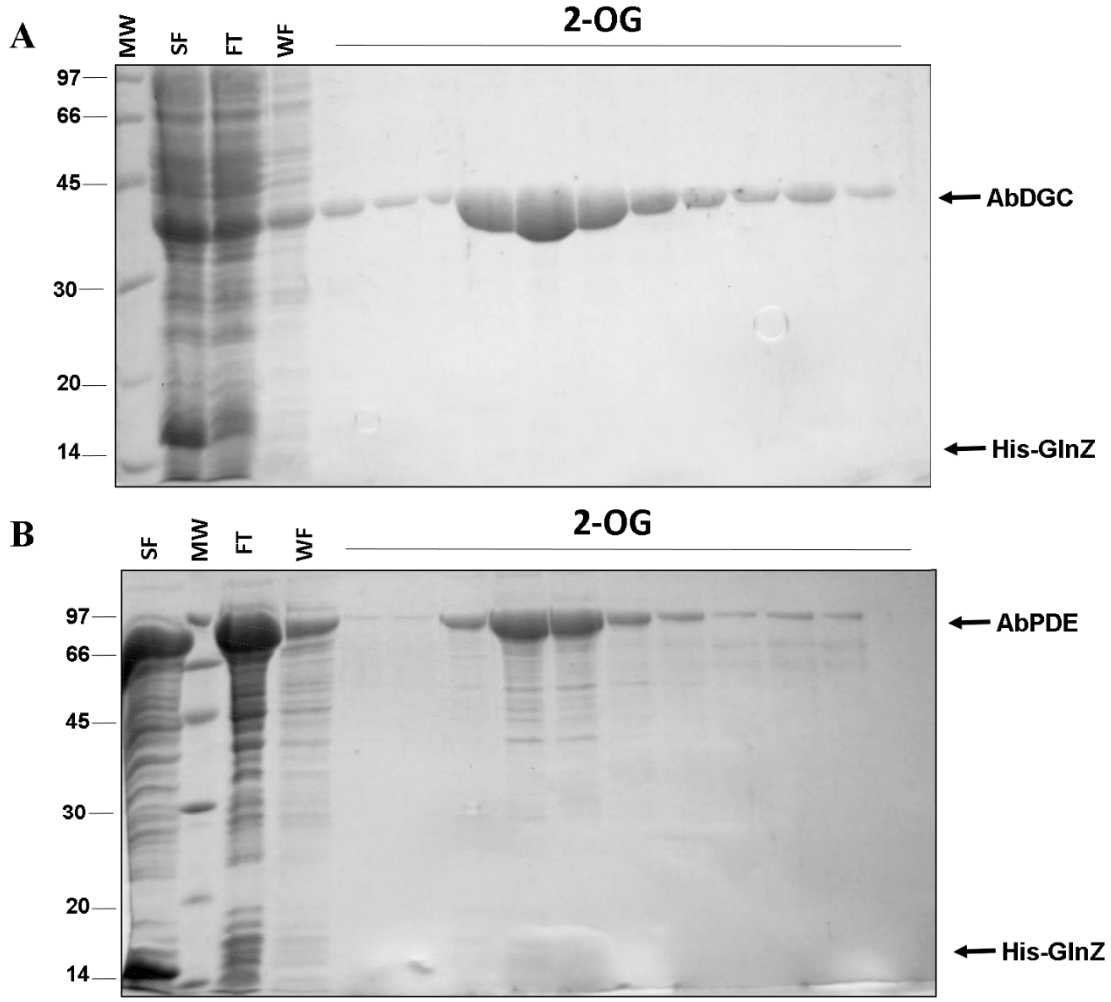


FIGURE S1. Purification of AbDGC and AbPDE eluting them from their complex with His-GlnZ. The His-GlnZ protein was bound to the HiTrap chelating column associated with native A) DGC or B) PDE, only the native proteins associated, were eluted with 2-OG (2 mM), ATP (1 mM) and MgCl2 (5 mM). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

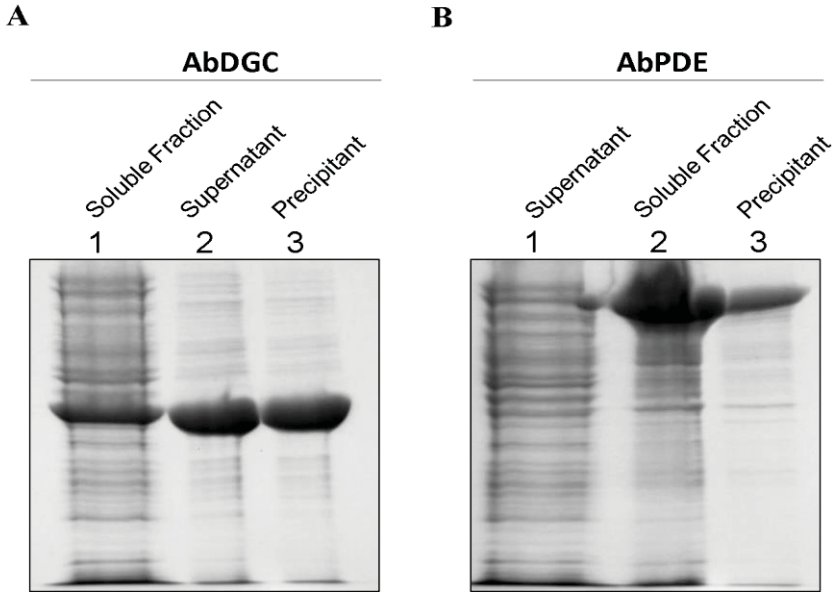


FIGURE S2. Ammonium sulphate precipitation of AbDGC and AbPDE proteins. Sonication buffer = Tris HCl pH 8.0 (0.05 M) and KCl (0.1 M). A) AbDGC precipitation using ammonium sulfate (20% saturation). Lane 1: soluble fraction containing AbDGC expressed. Lane 2: Supernatant after ammonium sulfate precipitation. Lane 3: Resuspended precipitate in buffer. B) AbPDE precipitation using ammonium sulfate (20% saturation). Lane 1: Supernatant after ammonium sulfate precipitation. Lane 2: soluble fraction containing AbPDE expressed. Lane 3: Resuspended precipitate in buffer.

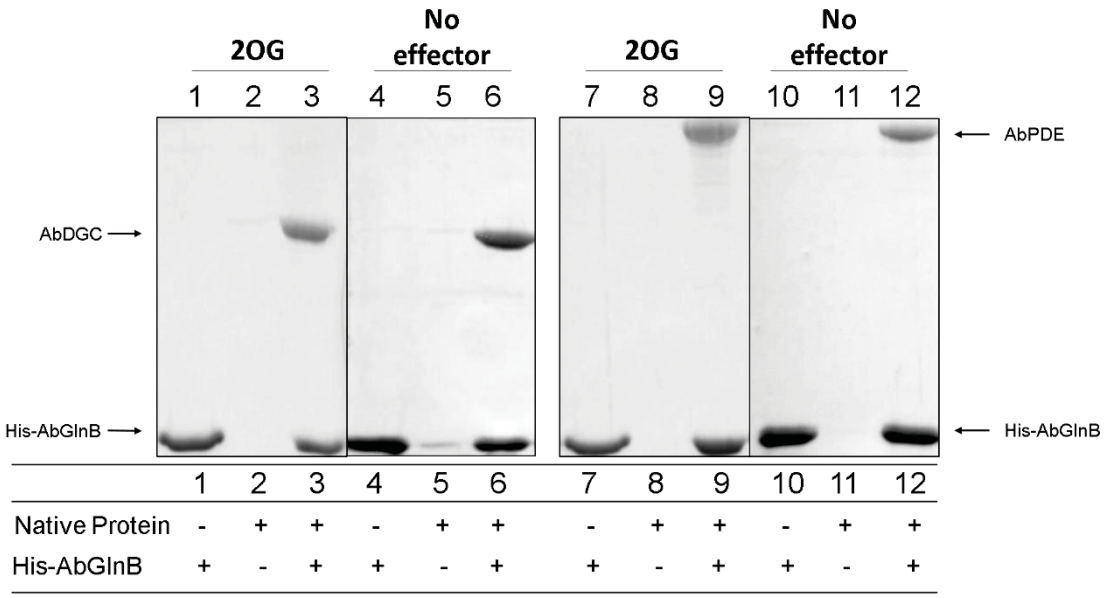


FIGURE S3. AbDGC and AbPDE interacts *in vitro* with PII in the absence of effector and in the presence of only 2-OG. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in absence of effectors and under fixed 2-OG concentration (1 mM) as indicated. All assays were in presence of $MgCl_2$ (5mM). Reactions were conducted in 400 µl of buffer adding random purified His proteins (20 µg) mixed with native Rho (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

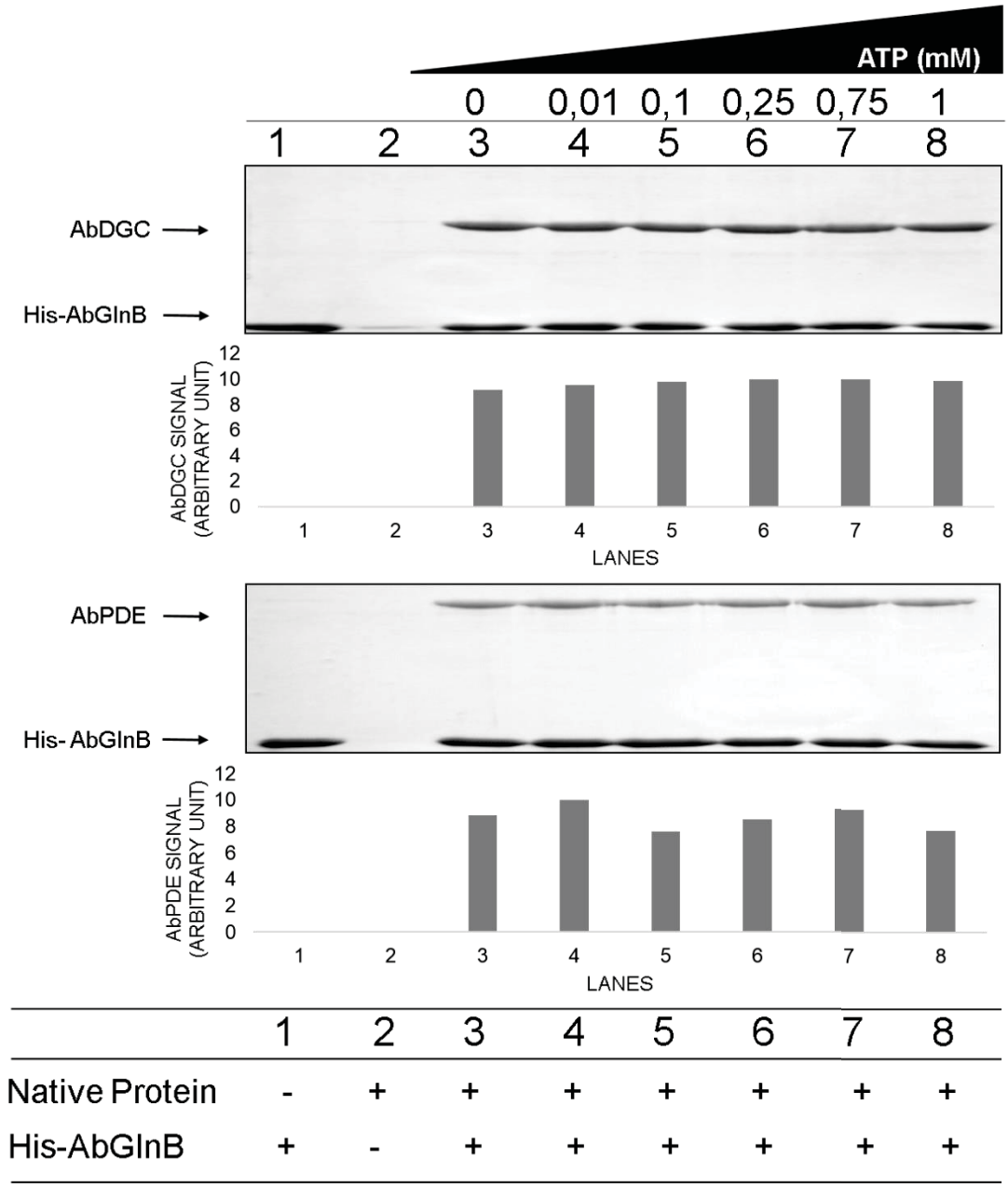


FIGURE S4. ***In vitro*** **formation of the complexes AbDGC-GlnB and AbPDE-GlnB occur independly of the ATP concentration.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of $MgCl_2$ (5mM), increasing concentrations of ATP as indicated. Reactions were conducted in 400 µl of buffer adding purified His-GlnB mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

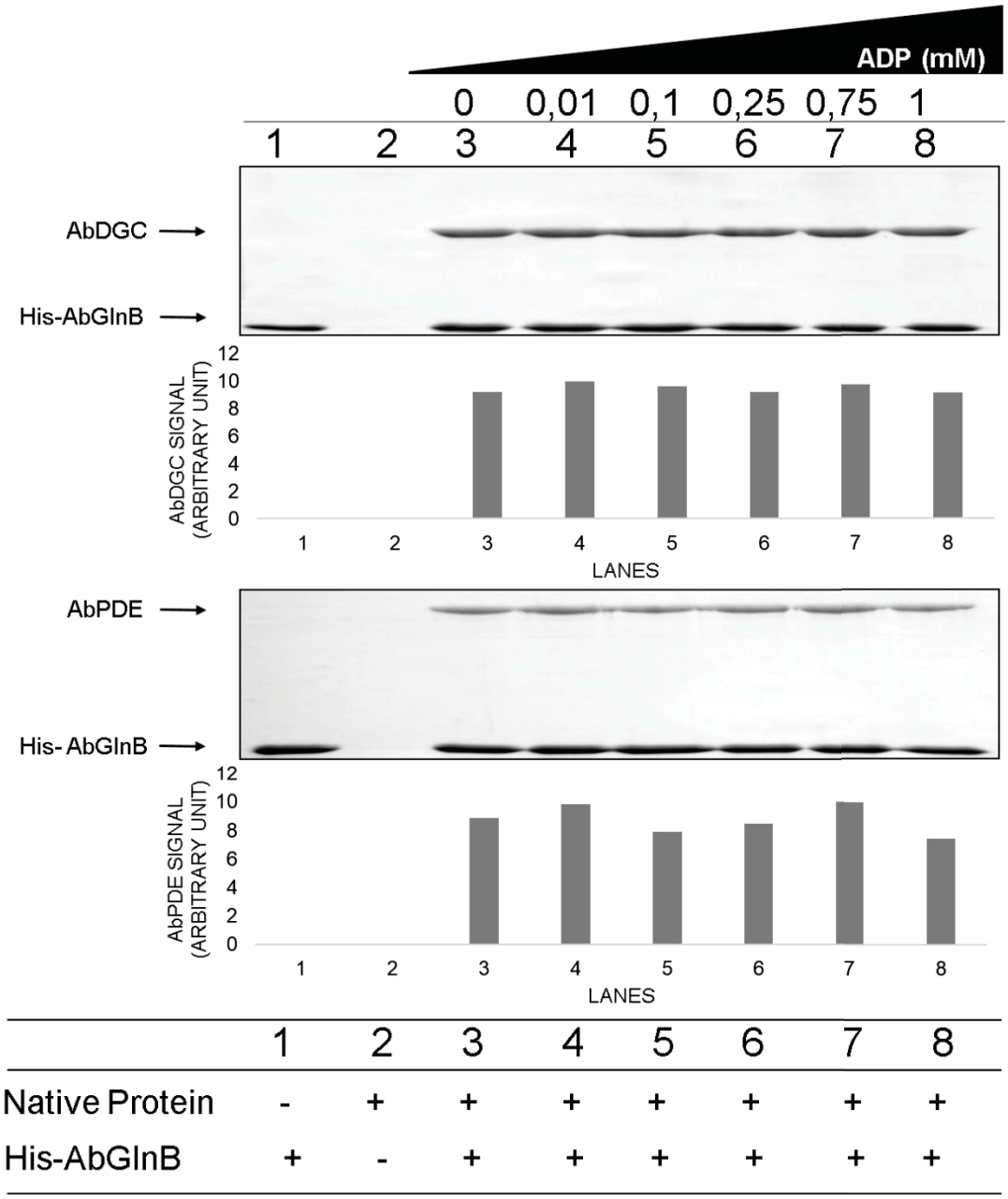


FIGURE S5. ***In vitro*** **formation of the complexes AbDGC-GlnB and AbPDE-GlnB occur independly of the ADP concentration.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of $MgCl_2$ (5mM), increasing concentrations of ADP as indicated. Reactions were conducted in 400 µl of buffer adding purified His-GlnB mixed with AbDGC or AbPDE as indicated. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

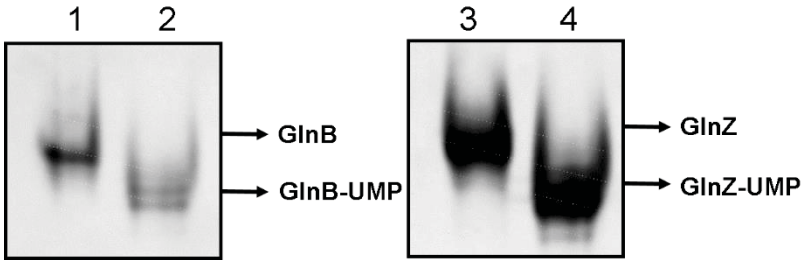


FIGURE S6. PII of *A. brasilense* partially uridylylated by GlnD of *E. coli*. The reaction was performed in 500 µl reaction mixtures containing 100 µM His-PII (considered as monomer), 1 mM ATP, 2 mM UTP, 5 mM 2-OG, 1 µM of purified *E. coli* GlnD, 100 mM Tris–HCl pH 7.5, 100 mM KCl and 25 mM $MgCl_2$. The uridylylation His-PII was determined by Native-PAGE.

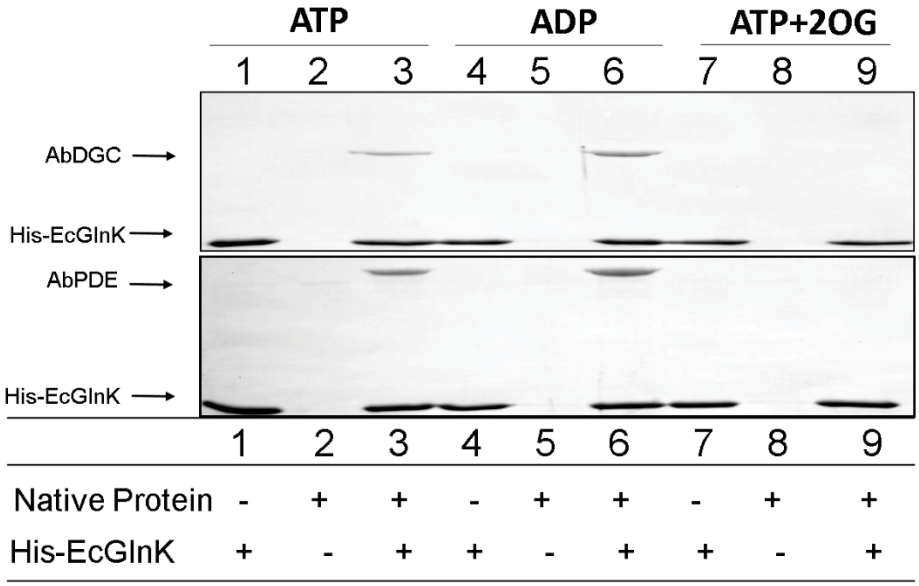


FIGURE S7. *In vitro* formation of the complexes AbDGC-EcGlnK and AbPDE-EcGlnK. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of $MgCl_2$ (5mM) and the effectors ATP, ADP and/or 2-OG at 1mM. Binding reactions were conducted in 400 µl of buffer adding purified His-GlnK mixed with AbPDE or AbDGC. The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

5. CAPÍTULO III

THE UmpH PROTEIN, INVOLVED IN THE HOMEOSTASIS OF THE PYRIMIDINE PATHWAY, INTERACTS *IN VITRO* WITH THE SIGNAL TRANSDUCER PROTEIN GlnK OF *Escherichia coli*

Abstract

Through an interactome approach it was indicated that the enzyme involved in pyrimidine homeostasis by overflow metabolism, UmpH, interact with the signal transducer protein GlnK in *E. coli*. In order to validate this interaction *in vitro*, we used pull-down assays with the His tail GlnK protein and the native UmpH protein. We observed interaction occurring in the presence of the GlnK effectors ATP and ADP, but not in the presence of Mg^{2+} + ATP and 2-OG, another effector of GlnK. These results justify a more detailed investigation of the physiological function of this interaction *in vivo*.

Introduction

Escherichia coli UMP Phosphatase (UmpH) is a protein involved in pyrimidine pathway homeostasis (Reaves *et al.*, 2013). Regulation of this pathway involves two strategies; the first is called canonical feedback and the second is named the overflow metabolism, which UmpH is involved (Reaves *et al.*, 2013). When the levels of UTP or CTP (products of the pathway) increase, the UMP intermediate accumulates and is diverted to the formation of uridine by UmpH. Uridine is subsequently transformed into uracil, which is excreted. This deviation helps to keep pyrimidine levels constant (Reaves *et al.*, 2013).

Recently, Huergo (unpublished) using an interactome approach found a clue of a possible interaction of UmpH with a PII signal transducer protein. The PII family are responsible for regulating metabolism by binding effectors that signal the state of carbon, nitrogen and intracellular energy (Huergo, Chandra and Merrick, 2013). *Escherichia coli* has two PII proteins, named GlnB and GlnK, which binds three effectors ATP, ADP and 2-OG. The PII proteins are homotrimeric and the effectors bind in the lateral clefts between the monomers. ATP and ADP bind competitively and 2-OG binds synergistically in the presence of Mg^{2+} + ATP (Mg.ATP) (Jiang and Ninfa, 2007; Truan *et al.*, 2010).

The binding of these effectors promotes conformational change in PII that influences the different interactions performed by it and, consequently, their different physiological actions (Huergo, Chandra and Merrick, 2013). Many PII interactions have already been described in *E. coli* as well as their physiological functions (Atkinson and Ninfa, 1998; Radchenko, Thornton and Merrick, 2010; Gerhardt *et al.*, 2015). Here we characterize the *in vitro* interaction that occurs between GlnK and UmpH, indicating UmpH as a possible new target for PII regulation.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *E. coli* strains were grown in LB medium at 37°C (Sambrook *et al*., 1989).

TABLE 1. Bacterial strains and plasmids

Strains/plasmid	Genotype/phenotype	Source/reference
E. coli		
DH10B	Sm^r, F‘ [*pro*AB^+ lacZΔM15]	Invitrogen
BL21 (λDE3)	Expresses T7 RNA polymerase	Agilent
Plasmids		
pET29a	Km^r. Expression vector T7 promoter	Novagen
pET28a	Km^r. Expression vector T7 promoter	Novagen
pTRpetHisGlnK	Km^r.(pET28a). Expresses the *E. coli* GlnK carrying a 6xHis tag at N-terminal	Rodrigues *et al.*, 2014
pTRpetHisGlnB	Km^r. (pET28a). Expresses the *E. coli* GlnB carrying a 6xHis tag at N-terminal	Gerhardt *et al.*, 2015
pEtUmpH	Km^r. (pET29a). Expresses the untagged *E. coli* UmpH	General Biosystens

Molecular biology methods

Isolation of plasmid DNA, gel electrophoresis, bacterial transformation were performed using standard procedures (Sambrook *et al*., 1989). Enzymes were obtained from commercial sources and used according to the manufacturers’ instructions.

Protein methods

Electrophoresis of proteins was carried out by SDS-PAGE (Laemmli, 1970). Protein concentrations were determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as standard.

Protein expression and purification

E. coli BL21 (λDE3) cells carrying the plasmids pETUmpH, pTRpetHisGlnK and pTRHisGlnB were cultivated on 400 ml LB at 37°C to a 0.5 OD_{600}. Isopropyl-β-D-

thiogalactopyranoside (IPTG) (0.5 mM) was added and after 3 hours cells were harvested by centrifugation (5.000 × *g* for 10 minutes at 4°C). The pellet was resuspended in 20 ml of buffer 1 (50 mM Tris-HCl pH 8.0, 0.1 M KCl). Cells were disrupted by sonication (10 min, cycles of 15s on and 15s off); cell extracts were clarified by centrifugation (20.000 × *g* for 20 minutes at 4°C).

The soluble fraction containing the UmpH overexpressed was precipitated with ammonium sulphate added slowly up to 30% saturation. The samples were incubated for one hour at 0°C. After this period, the supernatant was separated from the precipitate by centrifugation for 10 minutes (10.000 × *g* at 4°C). The precipitate was resuspended in 5 ml of buffer 1. The resulting resuspended proteins were dialyzed in buffer 2 (Tris HCl pH 8.0 (50 mM), KCl (100 mM) and Glycerol (10% v/v)), for 16 hours at 4°C.

His-EcGlnB and His-EcGlnK was purified using a heating step as described (Moure *et al.*, 2012). After, the cells were centrifuged (20.000 × *g* for 20 minutes at 4°C) and the supernatant was loaded onto a 1 ml HiTrap chelating column (GE Healthcare) containing Ni^{2+} (100mM) previously equilibrated with buffer 1 (50 mM Tris-HCl pH 8.0, 0.1 M KCl). The bound proteins were washed with 10 ml of buffer 1. The elution was performed with imidazole gradient (0.1 to 1M in buffer 1). Protein fractions were dialyzed in buffer 2.

Protein co-precipitation using magnetic beads

In vitro complex formation was performed using His-Magnetic beads (Promega). All reactions were conducted in buffer containing 50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 5 mM de $MgCl_2$, 0.05% Tween 20 (v/v), 10% glycerol (v/v), 20 mM imidazole in the presence or absence of effectors as indicated in each experiment. Four microliters of beads were equilibrated by wash with 200 µl of buffer. Binding reactions were performed in 400 µl of buffer by adding 20 µg His-PII and then 40 µg untagged UmpH. The proteins were mixed at room temperature; the beads were washed three times with 200 µl of buffer. Then, the samples were mixed with sample buffer and analyzed in 15% SDS-PAGE stained with Coomassie blue.

Gel band densitometry analyses were performed using Gel Analyzer 19.1 program.

Results

UmpH protein interacts with GlnK, but not with GlnB

Huergo (unpublished), using an interactome approach, pointed out that the UmpH protein could interact with the GlnK protein in *E. coli*. In this work, using pull-down assay, we confirmed and characterized this interaction *in vitro*. *E. coli* has two PII proteins, GlnK and GlnB, and we analyzed the ability of UmpH to interact with both proteins. UmpH only co-precipitated with GlnK (Fig. 1) in the presence of ATP and ADP and the interaction was inhibited in the presence of Mg.ATP + 2-OG.

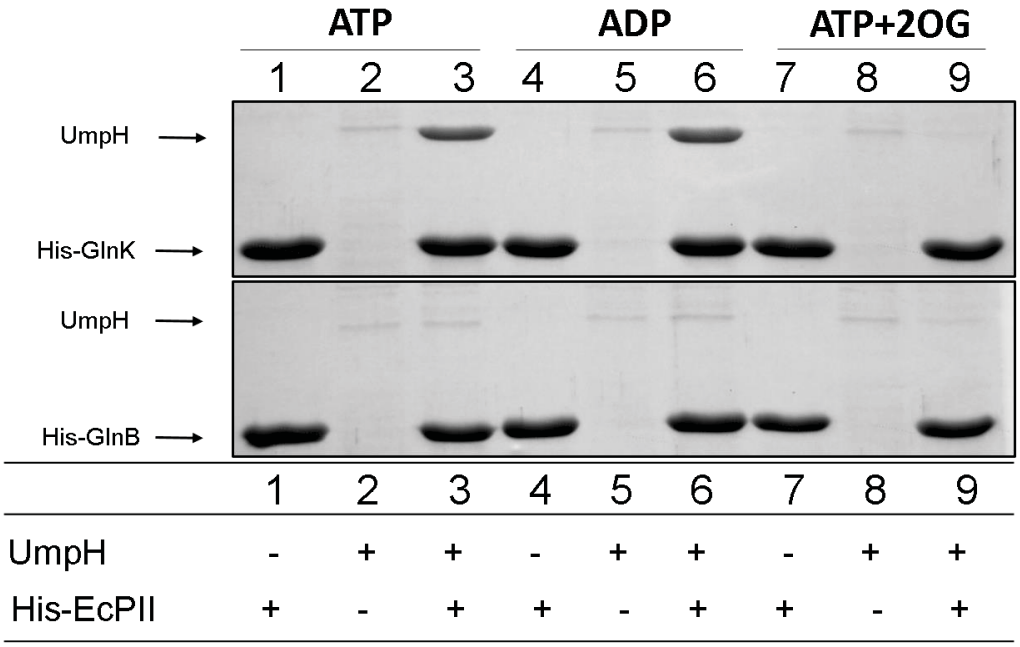


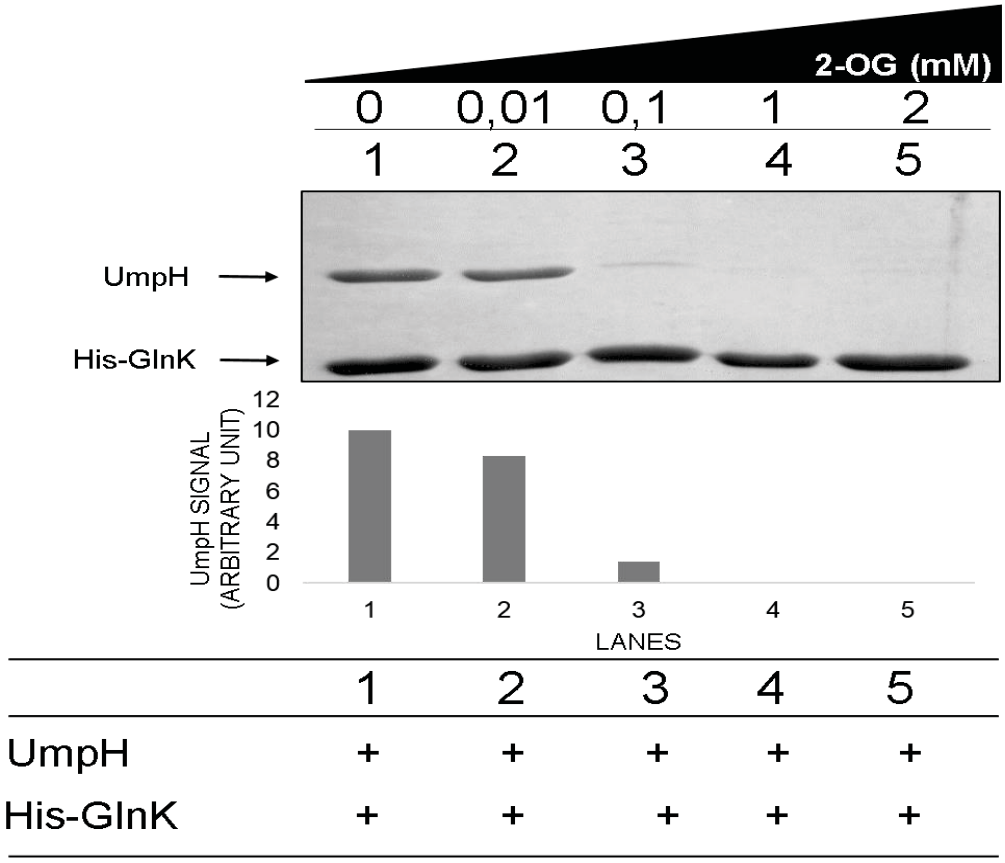
FIGURE 1. The *E. coli* UmpH protein forms a complex *in vitro* with the GlnK protein, but not with GlnB. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in the presence of $MgCl_2$ (5mM) and the effectors ATP, ADP and/or 2-OG at 1mM as indicated. Binding reactions were conducted in 400 µl of buffer adding purified His-EcPII (20 µg) mixed with UmpH (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

2-OG inhibits the UmpH-GlnK interaction

PII proteins can bind to three effectors ATP, ADP and 2-OG, and the binding of these molecules modify the proteins conformation, helping or inhibiting the interaction with other proteins. It is known in *E. coli* that the binding of 2-OG to PII requires the Mg.ATP prior binding (Jiang and Ninfa, 2007). To evaluate the effect of 2-OG on UmpH-GlnK interaction

we perform pull-down assay with different concentrations of 2-OG, but with fixed ATP (1 mM) and Mg^{2+} (5 mM) concentrations.

We observe that the inhibition of the complex UmpH-GlnK, occurs in a 2-OG dependent manner (Fig.2). Quantifying the density of bands, we showed that in 0.01 mM of 2-OG the interaction occurred as the control, decreased significantly with increase 2-OG concentration (0.1 mM) and was totally inhibited at 1 mM.



	1	2	3	4	5
UmpH	+	+	+	+	+
His-GlnK	+	+	+	+	+

FIGURE 2. *In vitro* formation of the UmpH-GlnK complex is dependent on the 2-OG concentrations. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of ATP (1 mM), ADP (1 mM) and $MgCl_2$ (5 mM), increasing concentrations of 2-OG as indicated. Reactions were conducted in 400 µl of buffer adding purified His-GlnK (20 µg) mixed with UmpH (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

Different concentrations of ATP and ADP, together or separately, were tested for their influence in the complex formation. When assessing ATP and ADP separately, we notice that interaction increases as the effector concentration increases (Fig. 3). However, there is no difference in the complex formation when one effector is changed by another (Fig. 4). This was confirmed when we used the same ATP and ADP concentrations (Fig. 5 – line 10)

When the interaction was evaluated in the presence of the three effectors, we notice

that in the presence of 0.1 mM 2-OG the interaction visibly decreases and is highly inhibited in presence of 1 mM 2-OG. It is possible to notice that as the level of ADP increases the formation of the complex also increases (Fig. 5).

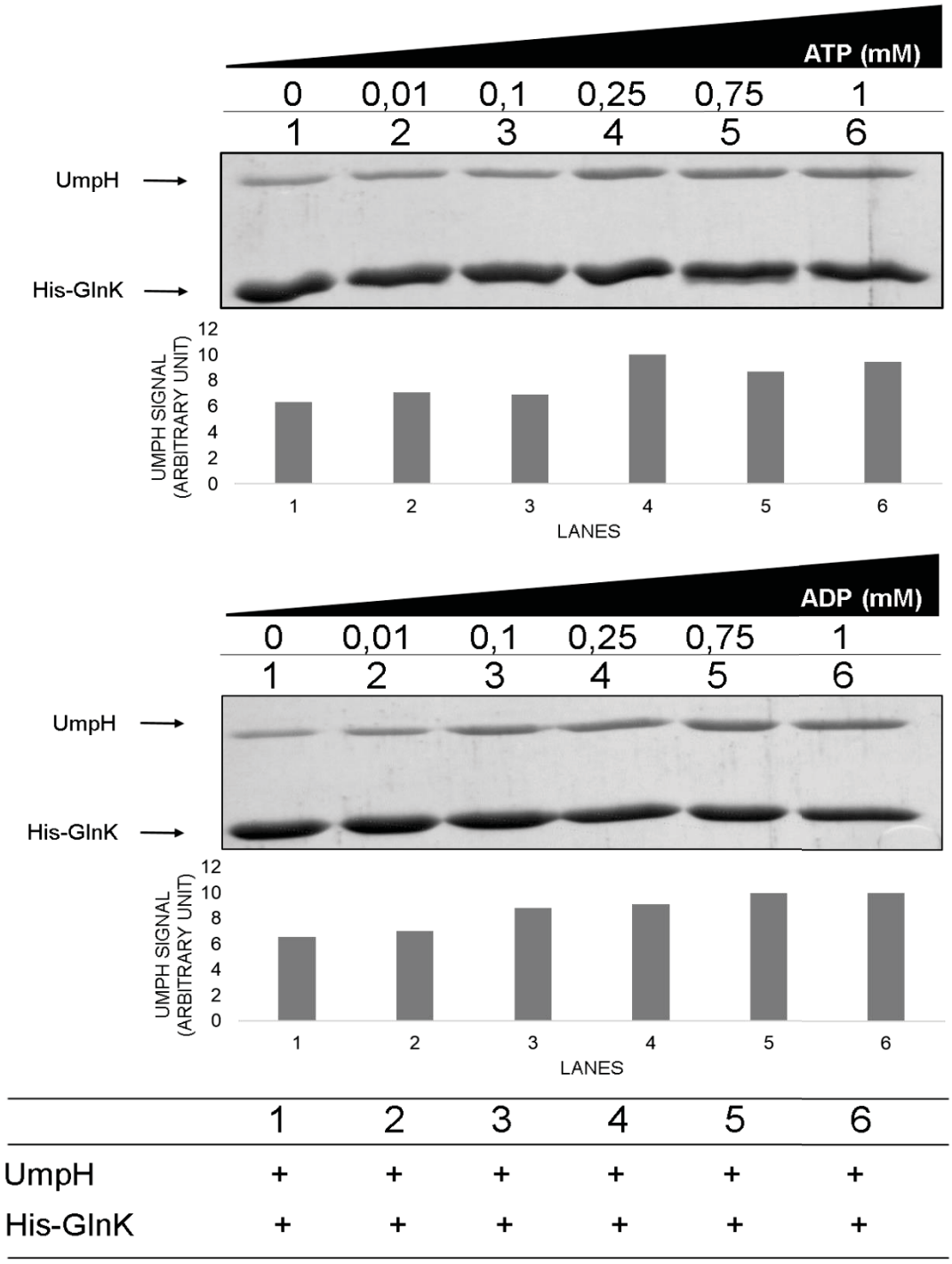


FIGURE 3. *In vitro* formation of the UmpH-GlnK is slightly stabilized by ADP and ATP. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed concentration of $MgCl_2$ (5mM), increasing concentrations of ATP or ADP as indicated. Reactions were conducted in 400 µl of buffer adding purified His-GlnK (20 µg) mixed with UmpH (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

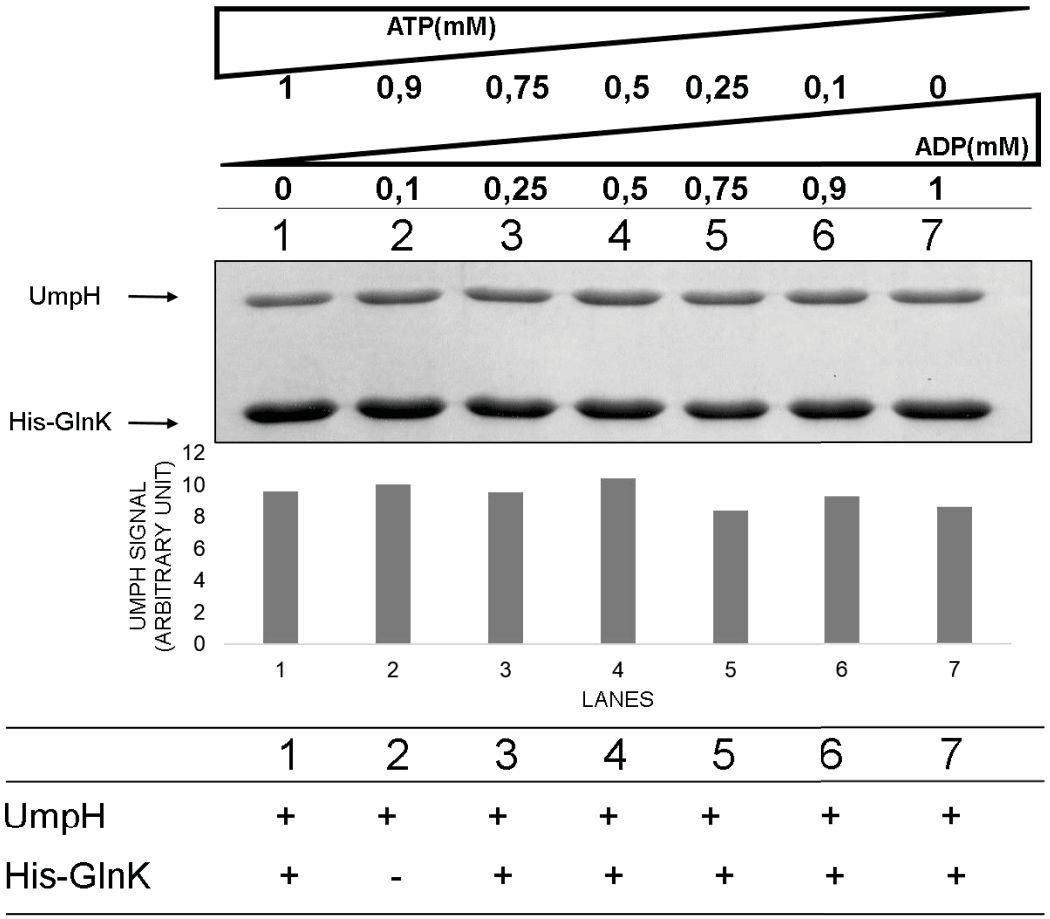
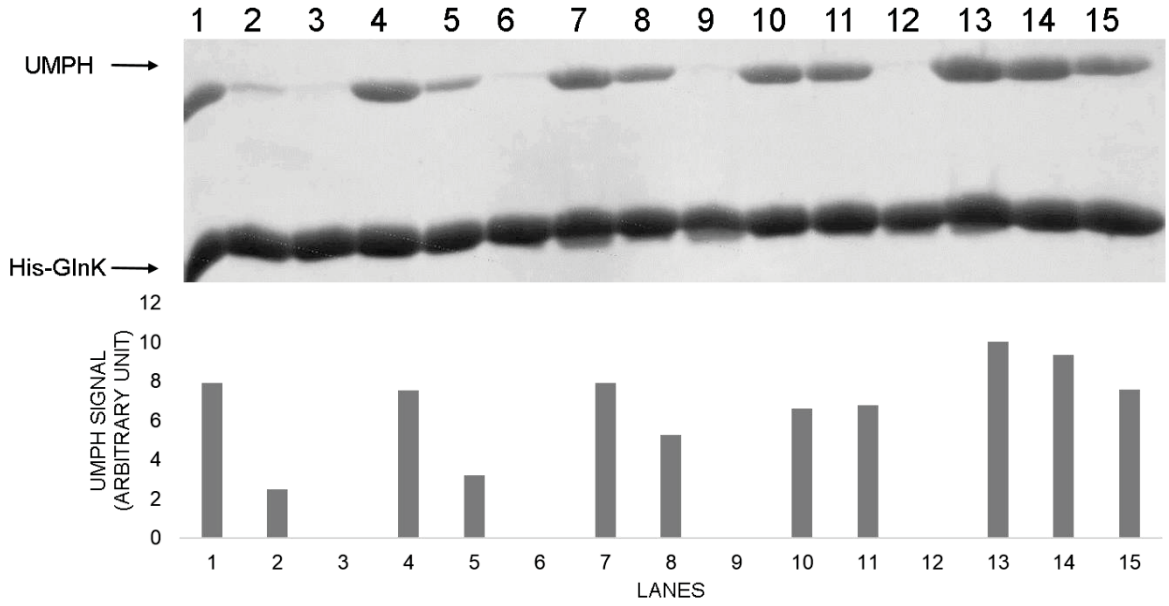


FIGURE 4. ***In vitro*** **formation of the UmpH-GlnK complex does not change in variable [ATP]/[ADP] ratios.** Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under different concentrations of ATP and ADP as indicated and fixed concentration of $MgCl_2$ (5mM). Reactions were conducted in 400 µl of buffer adding purified His-GlnK (20 µg) mixed with UmpH (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

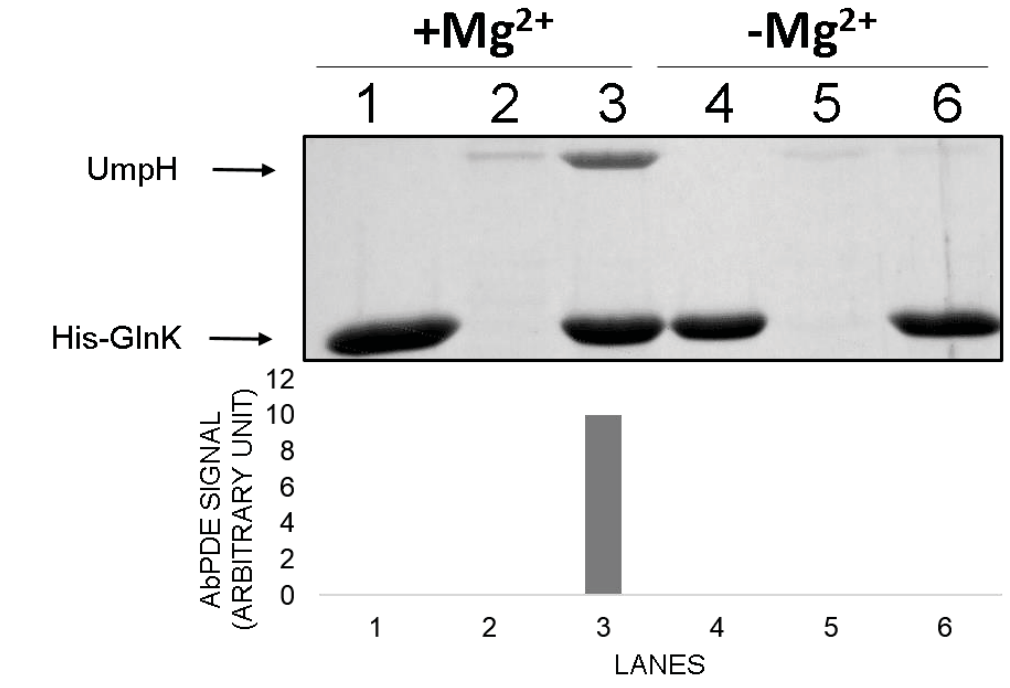


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ATP (mM)	1	1	1	0,9	0,9	0,9	0,75	0,75	0,75	0,5	0,5	0,5	0	0	0
ADP (mM)	0	0	0	0,1	0,1	0,1	0,25	0,25	0,25	0,5	0,5	0,5	1	1	1
2-OG (mM)	0	0,1	1	0	0,1	1	0	0,1	1	0	0,1	1	0	0,1	1

FIGURE 5. Effect of different concentrations of the PII effectors on *In vitro* formation of UmpH-GlnK complex. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed in different concentrations of the effectors ATP, ADP and 2-OG, as indicated and $MgCl_2$ at fixed concentration (5 mM). Binding reactions were conducted in 400 µl of buffer adding purified His-GlnK (20 µg) mixed with UmpH (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

The formation of the UmpH-GlnK complex is Mg^{2+} dependent

PII protein can bind to the divalent ion Mg^{2+}, and it is known that UmpH uses this ion as a cofactor. We investigated whether the absence of Mg^{2+} would interfere in the binding of UmpH to GlnK and we observed that the interaction did not occur in absence of this cofactor (Fig. 6).



	1	2	3	4	5	6
UmpH	-	+	+	-	+	+
His-GlnK	+	-	+	+	-	+

FIGURE 6. *In vitro* formation of the UmpH-GlnK complex is stabilized by Mg^{2+}. Complexes formation were assessed by pull-down using Ni^{2+} beads. Reactions were performed under fixed ATP concentration (1 mM) in presence or absence of $MgCl_2$ (5mM) as indicated. Reactions were conducted in 400 µl of buffer adding purified His-GlnK (20 µg) mixed with UmpH (40 µg). The eluted fractions from the Ni^{2+} beads were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue.

Discussion

Nucleotides are among the most important nitrogen compounds in all living organisms. Besides, pyrimidine and purine nucleotides are essential precursors for nucleic acids, they are also important metabolites in bio-energetic processes and in the synthesis of macromolecules (Stasolla *et al.*, 2003). Here we describe the interaction *in vitro* of the UmpH, a protein involved in pyridine homeostasis, with the GlnK, a PII – family protein, in *E. coli*. GlnK are widely known for regulating nitrogen metabolism by binding 2-OG, which is used as a carbon skeleton in the biosynthesis of nitrogen compounds (Leigh and Dodsworth, 2007). The direct sensing performed by GlnK of nitrogen levels could suggest the participation of PII proteins in the regulation of nitrogenous compounds synthesis such as pyrimidine.

The interaction with GlnK occurred in the presence of ATP and ADP and was inhibited in the presence of Mg.ATP + 2-OG (Fig. 1) in a 2-OG dependent manner (Fig.2). Many of the known interactions of PII in *E. coli* are inhibited in the presence of high 2-OG as in the case of the histidine kinase protein NtrB (Jiang, Peliska and Ninfa, 1998), the ammonium transporter AmtB (Radchenko, Thornton and Merrick, 2010) and the Acetyl-CoA Carboxylase subunit, BCCP (Biotin Carboxyl Carrier Protein) (Gerhardt *et al.*, 2015). Binding of Mg.ATP + 2-OG causes a conformation change in PII protein, inhibiting a variety of interactions (Truan *et al.*, 2010).

When the pull-down assays were carried out in the presence of different combinations of the ATP, ADP and 2-OG, we visualized that a 2-OG high concentration (1 mM) completely inhibited the UmpH-GlnK interaction (Fig. 5). The 0.1 mM 2-OG concentration impaired the formation of the UmpH-GlnK complex at ADP low levels (0 – 0.25 mM), however in higher concentrations the complex was normally formed. At 1 mM ADP and no ATP, even at 1 mM 2-OG did not affect the UmpH-GlnK complex. One explanation for these patterns could be that ATP and ADP bind to PII competitively (Jiang and Ninfa, 2007), and as the level of ADP increases it displaces Mg.ATP, preventing the 2-OG binding and allowing interaction (Fig. 5, lanes 11 to 15). The dissociation constant (K_d) presented by ATP and ADP for GlnK also supports this model. In 0.1 mM of 2-OG the affinity for ADP is greater than for ATP and in 1 mM of 2-OG the opposite occurs (Radchenko, Thornton and Merrick, 2010).

It is known that when 2-OG binds PII, 2-OG, ATP and side chain Q39 coordinate the Mg^{2+} ion at the PII interaction site. In addition 2-OG also forms a salt bridge with the K58 side chain (Truan *et al.*, 2010). However, when ADP binds PII, the side chain Q39 interacts with K58, not allowing the 2-OG binding, also explaining, in a structural view, the ADP effect on 2-OG binding limitation (Jiang and Ninfa, 2007; Truan *et al.*, 2010).

The structure of the UmpH protein was already been resolved and it was found as monomer in solution that requires Mg^{2+} as a cofactor (Tremblay, Dunaway-Mariano and Allen, 2006). As mentioned, the GlnK protein also binds to Mg^{2+} and when evaluating whether Mg^{2+} would interfere in the formation of the complex, the result was positive (Fig. 6). Considering that ATP is present in both assays performed, with and without Mg^{2+}, probably was binding of Mg^{2+} that caused a conformational change in UmpH or GlnK to stabilize the complex.

The quantification of intracellular levels of ATP, ADP and 2-OG in *E. coli* (Radchenko, Thornton and Merrick, 2010) and the data obtained *in vitro* allowed us to suggest a cellular condition for UmpH-GlnK interaction. When *E. coli* is nitrogen supplied, in

ammonium shock for example, the level of intracellular glutamine increases, decreasing the concentration of 2-OG, which is used on glutamine biosynthesis (Merrick and Edwards, 1995). With low 2-OG levels, the interaction of UmpH and GlnK occurs. After two minutes post ammonium shock, glutamine drops and 2-OG increases progressively. This condition would inhibit the UmpH-GlnK interaction, once under high 2-OG and low glutamine levels GlnK is uridilylated in a post-translational event (Francis and Engleman, 1978). In summary, the interaction between UmpH-GlnK should probably occur in situations of high available nitrogen and low 2-OG (Fig. 7).

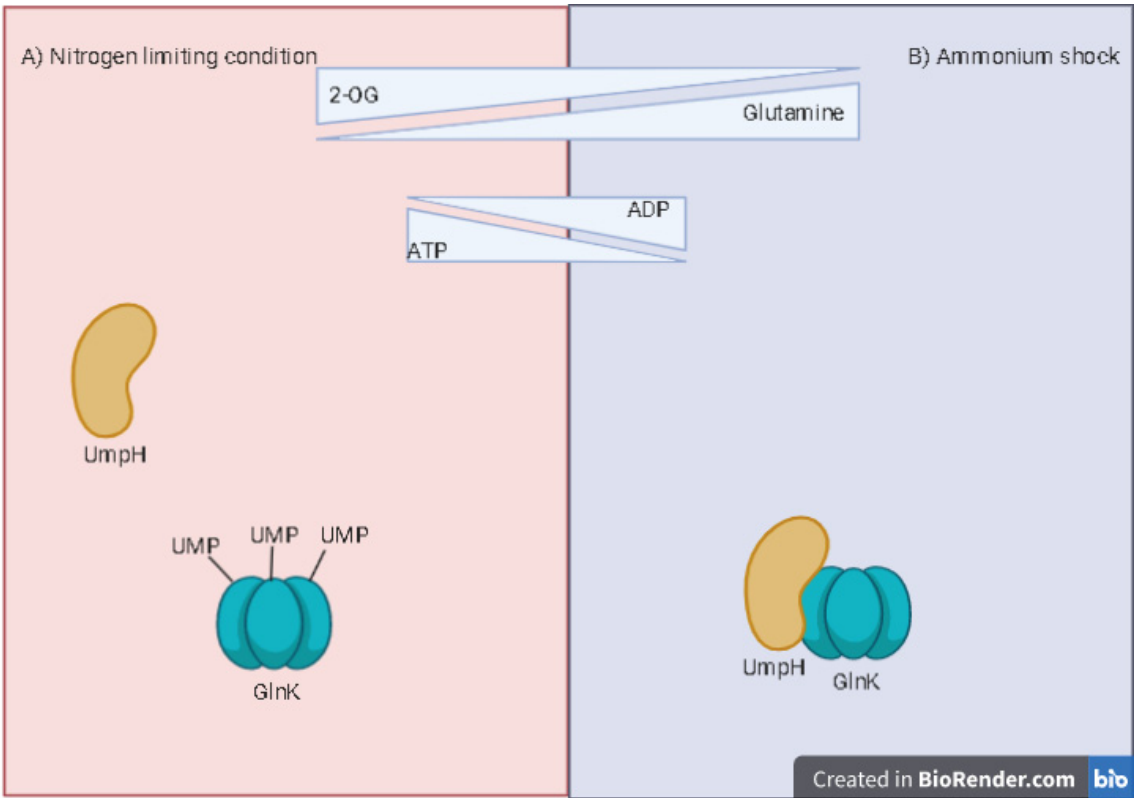


FIGURE 7. Interaction model of UmpH-GlnK complex. *In vitro* data showed that the UmpH-GlnK interaction is inhibited by the PII's effector 2-OG. A particular metabolic state which this interaction would occur could be in the ammonium shock. When a nitrogen limited bacterial cell receive a high ammonium concentration the 2-OG drops, allowing UmpH-GlnK complex formation. When ammonium is consumed 2-OG level increases and it binds GlnK, no longer allowing the complex formation with UmpH.

Conclusion

We validated and characterized the interaction between UmpH and GlnK *in vitro*. The interaction occurs in the presence of ATP and ADP and is inhibited in the presence of MgATP + 2-OG, indicating that the physiological interaction occurs only when the levels of intracellular 2-OG are low.

References

Atkinson, M. R. and Ninfa, A. J. (1998) 'Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*', *Molecular Microbiology*, 29(2), pp. 431–447. doi: 10.1046/j.1365-2958.1998.00932.x.

Bradford, M. (1976) 'A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding', *Analytical Biochemistry*, 72(1–2), pp. 248–254. doi: 10.1006/abio.1976.9999.

Francis, S. H. and Engleman, E. G. (1978) 'Cascade control of E. coli glutamine synthetase', *Archives of Biochemistry and Biophysics*, 191(2), pp. 590–601. doi: 10.1016/0003-9861(78)90397-1.

Gerhardt, E. C. M. *et al.* (2015) 'The Bacterial signal transduction protein GlnB regulates the committed step in fatty acid biosynthesis by acting as a dissociable regulatory subunit of acetyl-CoA carboxylase', *Molecular Microbiology*. John Wiley & Sons, Ltd (10.1111), 95(6), pp. 1025–1035. doi: 10.1111/mmi.12912.

Huergo, L. F. *et al.* (2007) 'Ternary complex formation between AmtB, GlnZ and the nitrogenase regulatory enzyme DraG reveals a novel facet of nitrogen regulation in bacteria', *Molecular Microbiology*, 0(0), pp. 071119190133008-??? doi: 10.1111/j.1365-2958.2007.06016.x.

Huergo, L. F., Chandra, G. and Merrick, M. (2013) 'PII signal transduction proteins: nitrogen regulation and beyond', *FEMS Microbiology Reviews*, 37(2), pp. 251–283. doi: 10.1111/j.1574-6976.2012.00351.x.

Jiang, P. and Ninfa, A. J. (2007) '*Escherichia coli* PII Signal Transduction Protein Controlling Nitrogen Assimilation Acts As a Sensor of Adenylate Energy Charge *in Vitro* †', *Biochemistry*, 46(45), pp. 12979–12996. doi: 10.1021/bi701062t.

Jiang, P., Peliska, J. A. and Ninfa, A. J. (1998) 'Reconstitution of the Signal-Transduction Bicyclic Cascade Responsible for the Regulation of Ntr Gene Transcription in *Escherichia coli* †', *Biochemistry*, 37(37), pp. 12795–12801. doi: 10.1021/bi9802420.

Laemmli, U. K. (1970) 'Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4', *Nature*, 227(5259), pp. 680–685. doi: 10.1038/227680a0.

Leigh, J. A. and Dodsworth, J. A. (2007) 'Nitrogen Regulation in Bacteria and Archaea', *Annual Review of Microbiology*, 61(1), pp. 349–377. doi: 10.1146/annurev.micro.61.080706.093409.

Merrick, M. J. and Edwards, R. A. (1995) 'Nitrogen control in bacteria.', *Microbiological Reviews*, 59(4), pp. 604–622.

Moure, V. R. *et al.* (2012) 'Heat stability of Proteobacterial P II protein facilitate purification using a single chromatography step', *Protein Expression and Purification*, 81(1), pp. 83–88. doi: 10.1016/j.pep.2011.09.008.

Radchenko, M. V., Thornton, J. and Merrick, M. (2010) 'Control of AmtB-GlnK Complex Formation by Intracellular Levels of ATP, ADP, and 2-Oxoglutarate', *Journal of Biological Chemistry*, 285(40), pp. 31037–31045. doi: 10.1074/jbc.M110.153908.

Reaves, M. L. *et al.* (2013) 'Pyrimidine homeostasis is accomplished by directed overflow

metabolism', *Nature*, 500(7461), pp. 237–241. doi: 10.1038/nature12445.

Rodrigues, T. E. *et al.* (2014) 'Search for novel targets of the P $_{II}$ signal transduction protein in Bacteria identifies the BCCP component of acetyl-CoA carboxylase as a P $_{II}$ binding partner', *Molecular Microbiology*. John Wiley & Sons, Ltd (10.1111), 91(4), pp. 751–761. doi: 10.1111/mmi.12493.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, n. 2.

Stasolla, C. *et al.* (2003) 'Purine and pyrimidine nucleotide metabolism in higher plants', *Journal of Plant Physiology*, 160(11), pp. 1271–1295. doi: 10.1078/0176-1617-01169.

Tremblay, L., Dunaway-Mariano, D. and Allen, K. (2006) 'Structure and Activity Analyses of Escherichia coli K-12 NagD Provide Insight into the Evolution of Biochemical Function in the Haloalkanoic Acid Dehalogenase Superfamily†,‡'. American Chemical Society. doi: 10.1021/BI051842J.

Truan, D. *et al.* (2010) 'A New PII Protein Structure Identifies the 2-Oxoglutarate Binding Site', *Journal of Molecular Biology*, 400(3), pp. 531–539. doi: 10.1016/j.jmb.2010.05.036.

6. CONCLUSÕES

Este trabalho teve como objetivo analisar a relação funcional das proteínas PII com possíveis novos alvos, e entre eles estavam o Fator de terminação de transcrição Rho, uma diguanilato ciclase, uma Fosfodiesterase e a UMP Fosfatase (UmpH). Observamos que:

1. As proteínas AbRho, AbDGC, AbPDE e EcUmpH foram expressas e purificadas.
2. Foi estabelecido o protocolo de purificação das proteínas Rho de *A. brasilense* (AbRho) e *Escherichia coli* (EcRho).
3. A proteína AbRho é hexamérica em solução.
4. AbRho é uma NTPase.
5. A atividade de AbRho é inibida por ADP.
6. AbRho interage *in vitro* com GlnB e GlnZ na presença de ATP e ADP.
7. EcRho interage *in vitro* com GlnB na presença de ATP, mas preferencialmente na presença de ADP e com GlnK somente na presença de ADP.
8. ADP influencia positivamente a interação EcRho com PII.
9. Presença de Mg^{2+} estabiliza a interação de AbRho e EcRho com PII.
10. Na presença de Mg.ATP, 2-OG inibe a interação de AbRho e EcRho com PII.
11. AbRho não interage com GlnZ via alça T, por mais que ela seja importante para a resposta de inibição por 2-OG.
12. AbRho interage com PII mesmo estando parcialmente uridililadas.

13. Na presença de Poly[C], como cofator, PII não influencia na atividade de AbRho e EcRho.
14. *In vitro*, AbDGC e AbPDE de *A. brasilense* SP245 apresentam-se como dímero e hexâmero, respectivamente.
15. *In vitro,* AbDGC e AbPDE interagem com AbGlnB de *A. brasilense* na presença de ATP e ADP.
16. O principal fator limitante na interação de AbDGC e AbPDE com GlnB é o 2-OG, na presença de Mg.ATP.
17. A interação AbPDE-GlnB é dependente de Mg^{2+} enquanto que a interação AbDGC-GlnB independe desse íon divalente.
18. A alça T não é essencial para a formação de ambos os complexos, AbDGC-AbGlnB e AbPDE-AbGlnB, mas a uridililação inibiu a formação do complexo AbPDE-AbGlnB.
19. AbDGC e AbPDE também podem interagir com PII de *E. coli in vitro.*
20. EcUmpH interage com EcGlnK na presença de ATP e ADP. Entretanto, EcUmpH não interage com EcGlnB.
21. Na presença de Mg.ATP, 2-OG inibe a formação do complexo EcUmpH-EcGlnK.
22. A interação EcUmpH-EcGlnK é dependente de Mg^{2+}.

7. REFERÊNCIAS

ALIFANO, P. et al. A consensus motif common to all Rho-dependent prokaryotic transcription terminators. **Cell**, v. 64, n. 3, p. 553–63, 8 fev. 1991.

ALLEN, K. N.; DUNAWAY-MARIANO, D. Phosphoryl group transfer: evolution of a catalytic scaffold. **Trends in Biochemical Sciences**, v. 29, n. 9, p. 495–503, set. 2004.

ALVAREZ-AÑORVE, L. I. et al. Allosteric regulation of glucosamine-6-phosphate deaminase (NagB) and growth of Escherichia coli on glucosamine. **Journal of bacteriology**, v. 191, n. 20, p. 6401–7, out. 2009.

AMIKAM, D.; GALPERIN, M. Y. PilZ domain is part of the bacterial c-di-GMP binding protein. **Bioinformatics**, v. 22, n. 1, p. 3–6, 1 jan. 2006.

AMON, J.; TITGEMEYER, F.; BURKOVSKI, A. Common patterns – unique features: nitrogen metabolism and regulation in Gram-positive bacteria. **FEMS Microbiology Reviews**, v. 34, n. 4, p. 588–605, jul. 2010.

AN, S.-Q. et al. A cyclic GMP-dependent signalling pathway regulates bacterial phytopathogenesis. **The EMBO Journal**, v. 32, n. 18, p. 2430–2438, 23 jul. 2013.

ANANTHARAMAN, V.; ARAVIND, L. The CHASE domain: a predicted ligand-binding module in plant cytokinin receptors and other eukaryotic and bacterial receptors. **Trends in Biochemical Sciences**, v. 26, n. 10, p. 579–582, out. 2001.

ARAUJO, G. T. **Caracterização *in vitro* das enzimas málica MaeB e N-acetil glutamato**

quinase NAGK e interação com proteínas PII em *Azospirillum brasilense.* Dissertação. (Mestrado em Ciências - Bioquímica) – Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba. 2018.

ARAÚJO, L. M. et al. GlnB is specifically required for Azospirillum brasilense NifA activity in Escherichia coli. **Research in Microbiology**, v. 155, n. 6, p. 491–495, jul. 2004.

ARAÚJO, L. M. et al. Different responses of the GlnB and GlnZ proteins upon in vitro uridylylation by the Azospirillum brasilense GlnD protein. **Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas**, v. 41, n. 4, p. 289–94, abr. 2008.

ARAUJO, M. S. et al. In vitro uridylylation of the Azospirillum brasilense N-signal transducing GlnZ protein. **Protein Expression and Purification**, v. 33, n. 1, p. 19–24, jan. 2004.

ARCONDÉGUY, T.; JACK, R.; MERRICK, M. PII Signal Transduction Proteins, Pivotal Players in Microbial Nitrogen Control. **Microbiology and Molecular Biology Reviews**, v. 65, n. 1, p. 80–105, mar. 2001.

ARSENE, F.; KAMINSKI, P. A.; ELMERICH, C. Modulation of NifA activity by PII in Azospirillum brasilense: evidence for a regulatory role of the NifA N-terminal domain. **Journal of bacteriology**, v. 178, n. 16, p. 4830–8, ago. 1996.

ATKINSON, M. R. et al. Reversible uridylylation of the Escherichia coli PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC). **The Journal of biological chemistry**, v. 269, n. 45, p. 28288–93, 11 nov. 1994.

ATKINSON, M. R.; NINFA, A. J. Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*. **Molecular Microbiology**, v. 29, n. 2, p. 431–447, jul. 1998.

BARAQUET, C. et al. The FleQ protein from Pseudomonas aeruginosa functions as both a repressor and an activator to control gene expression from the pel operon promoter in response to c-di-GMP. **Nucleic acids research**, v. 40, n. 15, p. 7207–18, ago. 2012.

BARENDS, T. R. M. et al. Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase. **Nature**, v. 459, n. 7249, p. 1015–1018, 18 jun. 2009.

BARIK, S.; BHATTACHARYA, P.; DAS, A. Autogenous regulation of transcription termination factor Rho. **Journal of Molecular Biology**, v. 182, n. 4, p. 495–508, 20 abr. 1985.

BARNHART, M. M.; CHAPMAN, M. R. Curli Biogenesis and Function. **Annual Review of Microbiology**, v. 60, n. 1, p. 131–147, out. 2006.

BEAR, D. G. et al. Escherichia coli transcription termination factor rho has a two-domain structure in its activated form. **Proceedings of the National Academy of Sciences**, v. 82, n. 7, p. 1911–1915, 1 abr. 1985.

BÉDARD, A.-S. V.; HIEN, E. D. M.; LAFONTAINE, D. A. Riboswitch regulation mechanisms: RNA, metabolites and regulatory proteins. **Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms**, p. 194501, 6 fev. 2020.

BHARATI, B. K. et al. A full-length bifunctional protein involved in c-di-GMP turnover is

required for long-term survival under nutrient starvation in Mycobacterium smegmatis. **Microbiology**, v. 158, n. 6, p. 1415–1427, 1 jun. 2012.

BOEHM, A. et al. Second messenger signalling governs *Escherichia coli* biofilm induction upon ribosomal stress. **Molecular Microbiology**, v. 72, n. 6, p. 1500–1516, jun. 2009.

BOGDEN, C. E. et al. The structural basis for terminator recognition by the Rho transcription termination factor. **Molecular cell**, v. 3, n. 4, p. 487–93, abr. 1999.

BOSSI, L. et al. A role for Rho-dependent polarity in gene regulation by a noncoding small RNA. **Genes & development**, v. 26, n. 16, p. 1864–73, 15 ago. 2012.

BOUDVILLAIN, M. et al. Simple Enzymatic Assays for the In Vitro Motor Activity of Transcription Termination Factor Rho from Escherichia coli. In: **Methods in molecular biology (Clifton, N.J.)**.. v. 587p. 137–154. 2010.

BOUDVILLAIN, M.; FIGUEROA-BOSSI, N.; BOSSI, L. Terminator still moving forward: expanding roles for Rho factor. **Current Opinion in Microbiology**, v. 16, n. 2, p. 118–124, abr. 2013.

BOUDVILLAIN, M.; NOLLMANN, M.; MARGEAT, E. Keeping up to speed with the transcription termination factor Rho motor. **Transcription**, v. 1, n. 2, p. 70–75, 18 set. 2010.

BOURRELLIER, A. B. F. et al. Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate–regulated by interaction of PII with the biotin carboxyl carrier subunit. **Proceedings of the National Academy of Sciences**, v. 107, n. 1, p. 502–507, jan. 2010.

BOYD, C. D. et al. LapG, required for modulating biofilm formation by Pseudomonas fluorescens Pf0-1, is a calcium-dependent protease. **Journal of bacteriology**, v. 194, n. 16, p. 4406–14, 15 ago. 2012.

BOYD, C. D. et al. Structural Features of the Pseudomonas fluorescens Biofilm Adhesin LapA Required for LapG-Dependent Cleavage, Biofilm Formation, and Cell Surface Localization. **Journal of Bacteriology**, v. 196, n. 15, p. 2775–2788, 1 ago. 2014.

BRADFORD, M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. **Analytical Biochemistry**, v. 72, n. 1–2, p. 248–254, 7 maio 1976.

BROUSSARD, T. C. et al. The Three-Dimensional Structure of the Biotin Carboxylase-Biotin Carboxyl Carrier Protein Complex of E. coli Acetyl-CoA Carboxylase. **Structure**, v. 21, n. 4, p. 650–657, 2 abr. 2013.

BROWN, M. S.; SEGAL, A.; STADTMAN, E. R. Modulation of glutamine synthetase adenylylation and deadenylylation is mediated by metabolic transformation of the P II - regulatory protein. **Proceedings of the National Academy of Sciences of the United States of America**, v. 68, n. 12, p. 2949–53, dez. 1971.

BURMANN, B. M. et al. A NusE:NusG Complex Links Transcription and Translation. **Science**, v. 328, n. 5977, p. 501–504, 23 abr. 2010.

BURNS, C. M.; RICHARDSON, L. V; RICHARDSON, J. P. Combinatorial effects of NusA and NusG on transcription elongation and rho-dependent termination in Escherichia coli 1 1Edited by M. Gottesman. **Journal of Molecular Biology**, v. 278, n. 2, p. 307–316, 1 maio 1998.

CARDINALE, C. J. et al. Termination Factor Rho and Its Cofactors NusA and NusG Silence

Foreign DNA in E. coli. **Science**, v. 320, n. 5878, p. 935–938, 16 maio 2008.

CARR, P. D. et al. X-ray structure of the signal transduction protein from Escherichia coli at 1.9 Å. **Acta Crystallographica Section D Biological Crystallography**, v. 52, n. 1, p. 93–104, 1 jan. 1996.

CHALISSERY, J. et al. Interaction Surface of the Transcription Terminator Rho Required to Form a Complex with the C-Terminal Domain of the Antiterminator NusG. **Journal of Molecular Biology**, v. 405, n. 1, p. 49–64, 7 jan. 2011.

CHAN, C. et al. Structural basis of activity and allosteric control of diguanylate cyclase. **Proceedings of the National Academy of Sciences**, v. 101, n. 49, p. 17084–17089, 7 dez. 2004.

CHATTERJEE, D. et al. Structural characterization of a conserved, calcium-dependent periplasmic protease from Legionella pneumophila. **Journal of bacteriology**, v. 194, n. 16, p. 4415–25, 15 ago. 2012.

CHATTERJEE, D. et al. Mechanistic insight into the conserved allosteric regulation of periplasmic proteolysis by the signaling molecule cyclic-di-GMP. **eLife**, v. 3, p. e03650, 2 set. 2014.

CHELLAMUTHU, V.-R. et al. A Widespread Glutamine-Sensing Mechanism in the Plant Kingdom. **Cell**, v. 159, n. 5, p. 1188–1199, nov. 2014.

CHEN, C. Y.; RICHARDSON, J. P. Sequence elements essential for rho-dependent transcription termination at lambda tR1. **The Journal of biological chemistry**, v. 262, n. 23, p. 11292–9, 15 ago. 1987.

CHEN, S. et al. Functional analysis of the GAF domain of NifA in Azospirillum brasilense: effects of Tyr→Phe mutations on NifA and its interaction with GlnB. **Molecular Genetics and Genomics**, v. 273, n. 5, p. 415–422, 11 jun. 2005.

CHEN, X.; STITT, B. L. The Binding of C_{10} Oligomers to *Escherichia coli* Transcription Termination Factor Rho. **Journal of Biological Chemistry**, v. 279, n. 16, p. 16301–16310, 16 abr. 2004.

CHRISTEN, B. et al. Allosteric Control of Cyclic di-GMP Signaling. **Journal of Biological Chemistry**, v. 281, n. 42, p. 32015–32024, 20 out. 2006.

CIAMPI, M. S. Rho-dependent terminators and transcription termination. **Microbiology**, v. 152, n. 9, p. 2515–2528, 1 set. 2006.

COHEN, D. et al. Oligoribonuclease is a central feature of cyclic diguanylate signaling in *Pseudomonas aeruginosa*. **Proceedings of the National Academy of Sciences**, v. 112, n. 36, p. 11359–11364, 8 set. 2015.

COMMICHAU, F. M.; FORCHHAMMER, K.; STÜLKE, J. Regulatory links between carbon and nitrogen metabolism. **Current Opinion in Microbiology**, v. 9, n. 2, p. 167–172, abr. 2006.

CONROY, M. J. et al. The crystal structure of the Escherichia coli AmtB-GlnK complex reveals how GlnK regulates the ammonia channel. **Proceedings of the National Academy of Sciences**, v. 104, n. 4, p. 1213–1218, 23 jan. 2007.

COUTTS, G. et al. Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB. **The EMBO Journal**, v. 21, n. 4, p. 536–545, 15 fev. 2002.

CRONAN, J. E.; WALDROP, G. L. Multi-subunit acetyl-CoA carboxylases. **Progress in lipid research**, v. 41, n. 5, p. 407–35, set. 2002.

D'HEYGÈRE, F. et al. ATP-dependent motor activity of the transcription termination factor Rho from *Mycobacterium tuberculosis*. **Nucleic Acids Research**, v. 43, n. 12, p. 6099–6111, 13 jul. 2015.

D'HEYGERE, F.; RABHI, M.; BOUDVILLAIN, M. Phyletic distribution and conservation of the bacterial transcription termination factor Rho. **Microbiology**, v. 159, n. Pt_7, p. 1423–1436, 1 jul. 2013.

DAHLSTROM, K. M. et al. Contribution of Physical Interactions to Signaling Specificity between a Diguanylate Cyclase and Its Effector. **mBio**, v. 6, n. 6, p. e01978-15, 15 dez. 2015.

DAHLSTROM, K. M.; O'TOOLE, G. A. A Symphony of Cyclases: Specificity in Diguanylate Cyclase Signaling. **Annual Review of Microbiology**, v. 71, n. 1, p. 179–195, 8 set. 2017.

DE ZAMAROCZY, M. Structural homologues P(II) and P(Z) of Azospirillum brasilense provide intracellular signalling for selective regulation of various nitrogen-dependent functions. **Molecular microbiology**, v. 29, n. 2, p. 449–63, jul. 1998.

DE ZAMAROCZY, M.; DELORME, F.; ELMERICH, C. Characterization of three different nitrogen-regulated promoter regions for the expression of glnB and glnA in Azospirillum brasilense. **Molecular & general genetics : MGG**, v. 224, n. 3, p. 421–30, dez. 1990.

DE ZAMAROCZY, M.; PAQUELIN, A.; ELMERICH, C. Functional organization of the glnB-glnA cluster of Azospirillum brasilense. **Journal of bacteriology**, v. 175, n. 9, p. 2507–15, maio 1993.

DIXON, R.; KAHN, D. Genetic regulation of biological nitrogen fixation. **Nature Reviews Microbiology**, v. 2, n. 8, p. 621–631, ago. 2004.

DOBEREINER, J.; PEDROSA, F. O. Nitrogen-Fixing Bacteria in Nonleguminous Crop Plants. Johanna Dobereiner , Fabio O. Pedrosa , Thomas D. Brock. **The Quarterly Review of Biology**, v. 63, n. 3, p. 338–339, 22 set. 1988.

DOMBROSKI, A. J. et al. Site-directed alterations in the ATP-binding domain of rho protein affect its activities as a termination factor. **The Journal of biological chemistry**, v. 263, n. 35, p. 18802–9, 15 dez. 1988.

DOMBROSKI, A. J.; PLATT, T. Structure of rho factor: an RNA-binding domain and a separate region with strong similarity to proven ATP-binding domains. **Proceedings of the National Academy of Sciences**, v. 85, n. 8, p. 2538–2542, 1 abr. 1988.

DRUMMOND, M. H.; CONTRERAS, A.; MITCHENALL, L. A. The function of isolated domains and chimaeric proteins constructed from the transcriptional activators NifA and NtrC of Klebsiella pneumoniae. **Molecular Microbiology**, v. 4, n. 1, p. 29–37, 1 jan. 1990.

DURAND, A.; MERRICK, M. *In Vitro* Analysis of the *Escherichia coli* AmtB-GlnK Complex Reveals a Stoichiometric Interaction and Sensitivity to ATP and 2-Oxoglutarate. **Journal of Biological Chemistry**, v. 281, n. 40, p. 29558–29567, 6 out. 2006.

DUTTA, D. et al. Linking RNA Polymerase Backtracking to Genome Instability in E. coli. **Cell**, v. 146, n. 4, p. 533–543, 19 ago. 2011.

DUTTA, D.; CHALISSERY, J.; SEN, R. Transcription Termination Factor Rho Prefers

Catalytically Active Elongation Complexes for Releasing RNA. **Journal of Biological Chemistry**, v. 283, n. 29, p. 20243–20251, 18 jul. 2008.

FADEL-PICHETH, C. M. T. et al. Regulation of *Azospirillum brasilense nifA* gene expression by ammonium and oxygen. **FEMS Microbiology Letters**, v. 179, n. 2, p. 281–288, 1 out. 1999.

FEIRER, N. et al. A Pterin-Dependent Signaling Pathway Regulates a Dual-Function Diguanylate Cyclase-Phosphodiesterase Controlling Surface Attachment in Agrobacterium tumefaciens. **mBio**, v. 6, n. 4, p. e00156, 30 jun. 2015.

FERIA BOURRELLIER, A. B. et al. Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. **Proceedings of the National Academy of Sciences**, v. 107, n. 1, p. 502–507, 5 jan. 2010.

FERNANDEZ, M.; PLUMBRIDGE, J. Complex synergistic amino acid–nucleotide interactions contribute to the specificity of NagC operator recognition and induction. **Microbiology**, v. 165, n. 7, p. 792–803, 1 jul. 2019.

FLEMMING, H.-C. EPS—Then and Now. **Microorganisms**, v. 4, n. 4, p. 41, 18 nov. 2016.

FOKINA, O. et al. Mechanism of 2-oxoglutarate signaling by the Synechococcus elongatus PII signal transduction protein. **Proceedings of the National Academy of Sciences of the United States of America**, v. 107, n. 46, p. 19760–5, 16 nov. 2010.

FORCHHAMMER, K. Global carbon/nitrogen control by PII signal transduction in cyanobacteria: from signals to targets. **FEMS Microbiology Reviews**, v. 28, n. 3, p. 319–333, jun. 2004.

FORCHHAMMER, K. PII signal transducers: novel functional and structural insights. **Trends in Microbiology**, v. 16, n. 2, p. 65–72, fev. 2008.

FRANCIS, S. H.; ENGLEMAN, E. G. Cascade control of E. coli glutamine synthetase. **Archives of Biochemistry and Biophysics**, v. 191, n. 2, p. 590–601, dez. 1978.

GALLUPPI, G. R.; RICHARDSON, J. P. ATP-induced changes in the binding of RNA synthesis termination protein Rho to RNA. **Journal of molecular biology**, v. 138, n. 3, p. 513–39, 15 abr. 1980.

GALPERIN, M. Y.; NIKOLSKAYA, A. N.; KOONIN, E. V. Novel domains of the prokaryotic two-component signal transduction systems. **FEMS microbiology letters**, v. 203, n. 1, p. 11–21, 11 set. 2001.

GAN, E.; RICHARDSON, J. P. ATP and Other Nucleotides Stabilize the Rho−mRNA Complex †. **Biochemistry**, v. 38, n. 51, p. 16882–16888, 21 dez. 1999.

GEISELMANN, J. et al. Physical properties of the Escherichia coli transcription termination factor rho. 1. Association states and geometry of the rho hexamer. **Biochemistry**, v. 31, n. 1, p. 111–121, 14 jan. 1992.

GEISELMANN, J.; VON HIPPEL, P. H. Functional interactions of ligand cofactors with *Escherichia coli* transcription termination factor rho. I. Binding of ATP. **Protein Science**, v. 1, n. 7, p. 850–860, jul. 1992.

GEORG, J.; HESS, W. R. cis-Antisense RNA, Another Level of Gene Regulation in Bacteria. **Microbiology and Molecular Biology Reviews**, v. 75, n. 2, p. 286–300, 1 jun. 2011.

GERHARDT, E. C. M. et al. Influence of the ADP/ATP ratio, 2-oxoglutarate and divalent ions on Azospirillum brasilense PII protein signalling. **Microbiology**, v. 158, n. Pt_6, p. 1656–1663, 1 jun. 2012.

GERHARDT, E. C. M. et al. The Bacterial signal transduction protein GlnB regulates the committed step in fatty acid biosynthesis by acting as a dissociable regulatory subunit of acetyl-CoA carboxylase. **Molecular Microbiology**, v. 95, n. 6, p. 1025–1035, 1 mar. 2015.

GERHARDT, E. C. M. **Identificação e estudo *in vitro* da interação entre proteínas PII e proteínas alvo**. 154 f. Tese (Doutorado em Ciências - Bioquímica) - Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 2015.

GUTIÉRREZ, P. et al. Solution Structure of YaeO, a Rho-specific Inhibitor of Transcription Termination. **Journal of Biological Chemistry**, v. 282, n. 32, p. 23348–23353, 10 ago. 2007.

HA, D.-G.; RICHMAN, M. E.; O'TOOLE, G. A. Deletion Mutant Library for Investigation of Functional Outputs of Cyclic Diguanylate Metabolism in Pseudomonas aeruginosa PA14. **Applied and Environmental Microbiology**, v. 80, n. 11, p. 3384–3393, 1 jun. 2014.

HABAZETTL, J. et al. Solution Structure of the PilZ Domain Protein PA4608 Complex with Cyclic di-GMP Identifies Charge Clustering as Molecular Readout. **Journal of Biological Chemistry**, v. 286, n. 16, p. 14304–14314, 22 abr. 2011.

HARINARAYANAN, R.; GOWRISHANKAR, J. Host factor titration by chromosomal R-loops as a mechanism for runaway plasmid replication in transcription termination-defective mutants of Escherichia coli. **Journal of molecular biology**, v. 332, n. 1, p. 31–46, 5 set. 2003.

HAUF, W. et al. Interaction of the Nitrogen Regulatory Protein GlnB (PII) with Biotin Carboxyl Carrier Protein (BCCP) Controls Acetyl-CoA Levels in the Cyanobacterium Synechocystis sp. PCC 6803. **Frontiers in Microbiology**, v. 7, p. 1700, 26 out. 2016.

HE, B.; CHOI, K. Y.; ZALKIN, H. Regulation of Escherichia coli glnB, prsA, and speA by the purine repressor. **Journal of bacteriology**, v. 175, n. 11, p. 3598–606, jun. 1993.

HEESWIJK, W. C. et al. The genes of the glutamine synthetase adenylylation cascade are not regulated by nitrogen in Escherichia coli. **Molecular Microbiology**, v. 9, n. 3, p. 443–457, ago. 1993.

HENGGE, R. Principles of c-di-GMP signalling in bacteria. **Nature Reviews Microbiology**, v. 7, n. 4, p. 263–273, abr. 2009.

HINSA, S. M. et al. Transition from reversible to irreversible attachment during biofilm formation by Pseudomonas fluorescens WCS365 requires an ABC transporter and a large secreted protein. **Molecular Microbiology**, v. 49, n. 4, p. 905–918, 8 jul. 2003.

HOBLEY, L. et al. Discrete Cyclic di-GMP-Dependent Control of Bacterial Predation versus Axenic Growth in Bdellovibrio bacteriovorus. **PLoS Pathogens**, v. 8, n. 2, p. e1002493, 2 fev. 2012.

HOLLANDS, K. et al. Riboswitch control of Rho-dependent transcription termination. **Proceedings of the National Academy of Sciences**, v. 109, n. 14, p. 5376–5381, 3 abr. 2012.

HOVE-JENSEN, B. Phosphoribosylpyrophosphate (PRPP)-less mutants of Escherichia coli. **Molecular Microbiology**, v. 3, n. 11, p. 1487–1492, nov. 1989.

HUERGO, L. F. et al. Regulation of glnB gene promoter expression in Azospirillum

brasilense by the NtrC protein. **FEMS microbiology letters**, v. 223, n. 1, p. 33–40, 6 jun. 2003.

HUERGO, L. F. et al. ADP-ribosylation of dinitrogenase reductase in Azospirillum brasilense is regulated by AmtB-dependent membrane sequestration of DraG. **Molecular Microbiology**, v. 59, n. 1, p. 326–337, jan. 2006a.

HUERGO, L. F. et al. Interactions between PII proteins and the nitrogenase regulatory enzymes DraT and DraG in Azospirillum brasilense. **FEBS Letters**, v. 580, n. 22, p. 5232–5236, out. 2006b.

HUERGO, L. F. et al. Ternary complex formation between AmtB, GlnZ and the nitrogenase regulatory enzyme DraG reveals a novel facet of nitrogen regulation in bacteria. **Molecular Microbiology**, 19 nov. 2007.

HUERGO, L. F. et al. *In Vitro* Interactions between the P_{II} Proteins and the Nitrogenase Regulatory Enzymes Dinitrogenase Reductase ADP-ribosyltransferase (DraT) and Dinitrogenase Reductase-activating Glycohydrolase (DraG) in *Azospirillum brasilense*. **Journal of Biological Chemistry**, v. 284, n. 11, p. 6674–6682, 13 mar. 2009.

HUERGO, L. F. et al. PII signal transduction proteins: pivotal players in post-translational control of nitrogenase activity. **Microbiology**, v. 158, n. 1, p. 176–190, 2012.

HUERGO, L. F.; CHANDRA, G.; MERRICK, M. PII signal transduction proteins: nitrogen regulation and beyond. **FEMS Microbiology Reviews**, v. 37, n. 2, p. 251–283, mar. 2013.

INABA, J. et al. Azospirillum brasilense PII proteins GlnB and GlnZ do not form heterotrimers and GlnB shows a unique trimeric uridylylation pattern. **European Journal of Soil Biology**, v. 45, n. 1, p. 94–99, 1 jan. 2009.

JIANG, P. et al. Structure/function analysis of the PII signal transduction protein of Escherichia coli: genetic separation of interactions with protein receptors. **Journal of bacteriology**, v. 179, n. 13, p. 4342–53, jul. 1997.

JIANG, P.; MAYO, A. E.; NINFA, A. J. Escherichia coli Glutamine Synthetase Adenylyltransferase (ATase, EC 2.7.7.49): Kinetic Characterization of Regulation by PII, PII-UMP, Glutamine, and α-Ketoglutarate. **Biochemistry**, v. 46, n. 13, p. 4133–4146, abr. 2007.

JIANG, P.; NINFA, A. J. Regulation of autophosphorylation of Escherichia coli nitrogen regulator II by the PII signal transduction protein. **Journal of bacteriology**, v. 181, n. 6, p. 1906–11, mar. 1999.

JIANG, P.; NINFA, A. J. *Escherichia coli* PII Signal Transduction Protein Controlling Nitrogen Assimilation Acts As a Sensor of Adenylate Energy Charge *in Vitro* †. **Biochemistry**, v. 46, n. 45, p. 12979–12996, 13 nov. 2007.

JIANG, P.; NINFA, A. J. Sensation and Signaling of α-Ketoglutarate and Adenylylate Energy Charge by the *Escherichia coli* PII Signal Transduction Protein Require Cooperation of the Three Ligand-Binding Sites within the PII Trimer. **Biochemistry**, v. 48, n. 48, p. 11522–11531, 8 dez. 2009.

JIANG, P.; PELISKA, J. A.; NINFA, A. J. Enzymological Characterization of the Signal-Transducing Uridylyltransferase/Uridylyl-Removing Enzyme (EC 2.7.7.59) of Escherichia coli and Its Interaction with the PII Protein. **Biochemistry**, v. 37, n. 37, p. 12782–12794, set. 1998a.

JIANG, P.; PELISKA, J. A.; NINFA, A. J. The Regulation of *Escherichia coli* Glutamine Synthetase Revisited: Role of 2-Ketoglutarate in the Regulation of Glutamine Synthetase Adenylylation State †. **Biochemistry**, v. 37, n. 37, p. 12802–12810, 15 set. 1998b.

JIANG, P.; PELISKA, J. A.; NINFA, A. J. Reconstitution of the Signal-Transduction Bicyclic Cascade Responsible for the Regulation of Ntr Gene Transcription in *Escherichia coli* †. **Biochemistry**, v. 37, n. 37, p. 12795–12801, 15 set. 1998c.

JOHNSON, J. G. et al. Type 3 Fimbriae and Biofilm Formation Are Regulated by the Transcriptional Regulators MrkHI in Klebsiella pneumoniae. **Journal of Bacteriology**, v. 193, n. 14, p. 3453–3460, 15 jul. 2011.

KALARICKAL, N. C. et al. A Bacterial Transcription Terminator with Inefficient Molecular Motor Action but with a Robust Transcription Termination Function. **Journal of Molecular Biology**, v. 395, n. 5, p. 966–982, 5 fev. 2010.

KAMBEROV, E. S.; ATKINSON, M. R.; NINFA, A. J. The *Escherichia coli* PII Signal Transduction Protein Is Activated upon Binding 2-Ketoglutarate and ATP. **Journal of Biological Chemistry**, v. 270, n. 30, p. 17797–17807, 28 jul. 1995.

KANTROWITZ, E. R. Allostery and cooperativity in Escherichia coli aspartate transcarbamoylase. **Archives of Biochemistry and Biophysics**, v. 519, n. 2, p. 81–90, 15 mar. 2012.

KASSAVETIS, G. A.; CHAMBERLIN, M. J. Pausing and termination of transcription within the early region of bacteriophage T7 DNA in vitro. **The Journal of biological chemistry**, v. 256, n. 6, p. 2777–86, 25 mar. 1981.

KILSTRUP, M. et al. Nucleotide metabolism and its control in lactic acid bacteria. **FEMS Microbiology Reviews**, v. 29, n. 3, p. 555–590, ago. 2005.

KIM, D.-E.; PATEL, S. S. The Kinetic Pathway of RNA Binding to the *Escherichia coli* Transcription Termination Factor Rho. **Journal of Biological Chemistry**, v. 276, n. 17, p. 13902–13910, 27 abr. 2001.

KOONIN, E. V.; TATUSOV, R. L. Computer Analysis of Bacterial Haloacid Dehalogenases Defines a Large Superfamily of Hydrolases with Diverse Specificity. **Journal of Molecular Biology**, v. 244, n. 1, p. 125–132, 18 nov. 1994.

KRASTEVA, P. V. et al. Vibrio cholerae VpsT Regulates Matrix Production and Motility by Directly Sensing Cyclic di-GMP. **Science**, v. 327, n. 5967, p. 866–868, 12 fev. 2010.

KRINER, M. A.; GROISMAN, E. A. The Bacterial Transcription Termination Factor Rho Coordinates Mg2+ Homeostasis with Translational Signals. **Journal of Molecular Biology**, v. 427, n. 24, p. 3834–3849, 4 dez. 2015.

KRISHNA LEELA, J. et al. Rho-dependent transcription termination is essential to prevent excessive genome-wide R-loops in Escherichia coli. **Proceedings of the National Academy of Sciences**, v. 110, n. 1, p. 258–263, 2 jan. 2013.

KULASAKARA, H. et al. Analysis of Pseudomonas aeruginosa diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. **Proceedings of the National Academy of Sciences**, v. 103, n. 8, p. 2839–2844, 21 fev. 2006.

KUZNETSOVA, E. et al. Genome-wide Analysis of Substrate Specificities of the *Escherichia coli* Haloacid Dehalogenase-like Phosphatase Family. **Journal of Biological Chemistry**, v.

281, n. 47, p. 36149–36161, 24 nov. 2006.

LAEMMLI, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. **Nature**, v. 227, n. 5259, p. 680–685, 15 ago. 1970.

LEIGH, J. A.; DODSWORTH, J. A. Nitrogen Regulation in Bacteria and Archaea. **Annual Review of Microbiology**, v. 61, n. 1, p. 349–377, out. 2007.

LIAO, J.; SAUER, K. The MerR-Like Transcriptional Regulator BrlR Contributes to Pseudomonas aeruginosa Biofilm Tolerance. **Journal of Bacteriology**, v. 194, n. 18, p. 4823–4836, 15 set. 2012.

LIU, J.; MAGASANIK, B. The glnB region of the Escherichia coli chromosome. **Journal of bacteriology**, v. 175, n. 22, p. 7441–9, nov. 1993.

LOUIS-JEUNE, C.; ANDRADE-NAVARRO, M. A.; PEREZ-IRATXETA, C. Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. **Proteins: Structure, Function, and Bioinformatics**, v. 80, n. 2, p. 374–381, fev. 2012.

LU, Z.; DUNAWAY-MARIANO, D.; ALLEN, K. N. HAD Superfamily Phosphotransferase Substrate Diversification: Structure and Function Analysis of HAD Subclass IIB Sugar Phosphatase BT4131 † , ‡. **Biochemistry**, v. 44, n. 24, p. 8684–8696, 21 jun. 2005.

MACHADO, H. B. et al. Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine. **Canadian Journal of Microbiology**, v. 37, n. 7, p. 549–553, jul. 1991.

MAHESWARAN, M.; URBANKE, C.; FORCHHAMMER, K. Complex formation and catalytic activation by the PII signaling protein of N-acetyl-L-glutamate kinase from Synechococcus elongatus strain PCC 7942. **The Journal of biological chemistry**, v. 279, n. 53, p. 55202–10, 31 dez. 2004.

MARTINEZ-GIL, M.; RAMOS-GONZALEZ, M. I.; ESPINOSA-URGEL, M. Roles of Cyclic Di-GMP and the Gac System in Transcriptional Control of the Genes Coding for the Pseudomonas putida Adhesins LapA and LapF. **Journal of Bacteriology**, v. 196, n. 8, p. 1484–1495, 15 abr. 2014.

MASCHER, T.; HELMANN, J. D.; UNDEN, G. Stimulus perception in bacterial signal-transducing histidine kinases. **Microbiology and molecular biology reviews : MMBR**, v. 70, n. 4, p. 910–38, dez. 2006.

MATSUMOTO, Y. et al. Autogenous regulation of the gene for transcription termination factor rho in Escherichia coli: localization and function of its attenuators. **Journal of bacteriology**, v. 166, n. 3, p. 945–58, jun. 1986.

MATSUYAMA, B. Y. et al. Mechanistic insights into c-di-GMP–dependent control of the biofilm regulator FleQ from *Pseudomonas aeruginosa*. **Proceedings of the National Academy of Sciences**, v. 113, n. 2, p. E209–E218, 12 jan. 2016.

MEISSNER, A. et al. Pseudomonas aeruginosa cupA-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate. **Environmental Microbiology**, v. 9, n. 10, p. 2475–2485, out. 2007.

MERRICK, M. Post-translational modification of PII signal transduction proteins. **Frontiers in Microbiology**, v. 5, p. 763, 6 jan. 2015.

MERRICK, M. J.; EDWARDS, R. A. Nitrogen control in bacteria. **Microbiological Reviews**,

v. 59, n. 4, p. 604–622, dez. 1995.

MILLS, E. et al. A direct screen for c-di-GMP modulators reveals a *Salmonella* Typhimurium periplasmic L-arginine–sensing pathway. **Science Signaling**, v. 8, n. 380, p. ra57–ra57, 9 jun. 2015.

MITRA, P. et al. Rho Protein: Roles and Mechanisms. **Annual Review of Microbiology**, v. 71, n. 1, p. 687–709, 8 set. 2017.

MORGAN, W. et al. RNA sequence and secondary structure requiresments for rho-dependent transcription termination. **Nucleic Acids Research**, v. 13, n. 10, p. 3739–3754, 24 maio 1985.

MOTALEB, M. A. et al. CheX is a phosphorylated CheY phosphatase essential for Borrelia burgdorferi chemotaxis. **Journal of bacteriology**, v. 187, n. 23, p. 7963–9, dez. 2005.

MOURE, V. R. et al. Heat stability of Proteobacterial P II protein facilitate purification using a single chromatography step. **Protein Expression and Purification**, v. 81, n. 1, p. 83–88, jan. 2012.

MOURE, V. R. et al. The nitrogenase regulatory enzyme dinitrogenase reductase ADP-ribosyltransferase (DraT) is activated by direct interaction with the signal transduction protein GlnB. **Journal of bacteriology**, v. 195, n. 2, p. 279–86, jan. 2013.

NADIRAS, C. et al. A multivariate prediction model for Rho-dependent termination of transcription. **Nucleic acids research**, v. 46, n. 16, p. 8245–8260, 2018.

NAVARRO, M. V. A. S. et al. Structural Basis for c-di-GMP-Mediated Inside-Out Signaling Controlling Periplasmic Proteolysis. **PLoS Biology**, v. 9, n. 2, p. e1000588, 1 fev. 2011.

NEHRKE, K. W.; ZALATAN, F.; PLATT, T. NusG alters rho-dependent termination of transcription in vitro independent of kinetic coupling. **Gene expression**, v. 3, n. 2, p. 119–33, 1993.

NEWELL, P. D. et al. Systematic Analysis of Diguanylate Cyclases That Promote Biofilm Formation by Pseudomonas fluorescens Pf0-1. **Journal of Bacteriology**, v. 193, n. 18, p. 4685–4698, 15 set. 2011.

NEWELL, P. D.; MONDS, R. D.; O'TOOLE, G. A. LapD is a bis-(3',5')-cyclic dimeric GMP-binding protein that regulates surface attachment by Pseudomonas fluorescens Pf0-1. **Proceedings of the National Academy of Sciences**, v. 106, n. 9, p. 3461–3466, 3 mar. 2009.

NINFA, A. J.; BENNETT, R. L. Identification of the site of autophosphorylation of the bacterial protein kinase/phosphatase NRII. **The Journal of biological chemistry**, v. 266, n. 11, p. 6888–93, 15 abr. 1991.

NINFA, A. J.; JIANG, P. PII signal transduction proteins: sensors of α-ketoglutarate that regulate nitrogen metabolism. **Current Opinion in Microbiology**, Cell Regulation. v. 8, n. 2, p. 168–173, abr. 2005.

NINFA, E. G. et al. Mechanism of autophosphorylation of Escherichia coli nitrogen regulator II (NRII or NtrB): trans-phosphorylation between subunits. **Journal of bacteriology**, v. 175, n. 21, p. 7024–32, nov. 1993.

NORTH, A. K. et al. Repressor Forms of the Enhancer-binding Protein NtrC: Some Fail in Coupling ATP Hydrolysis to Open Complex Formation by σ54-Holoenzyme. **Journal of Molecular Biology**, v. 260, n. 3, p. 317–331, 19 jul. 1996.

NOWATZKE, W. L.; RICHARDSON, J. P. Characterization of an Unusual Rho Factor from the High G + C Gram-positive Bacterium *Micrococcus luteus*. **Journal of Biological Chemistry**, v. 271, n. 2, p. 742–747, 12 jan. 1996.

O'NEAL, L. et al. Optogenetic Manipulation of Cyclic Di-GMP (c-di-GMP) Levels Reveals the Role of c-di-GMP in Regulating Aerotaxis Receptor Activity in Azospirillum brasilense. **Journal of Bacteriology**, v. 199, n. 18, 15 set. 2017.

OGAWA, K.; MAKI, Y. Cellulose as Extracellular Polysaccharide of Hot Spring Sulfur-turf Bacterial Mat. **Bioscience, Biotechnology, and Biochemistry**, v. 67, n. 12, p. 2652–2654, 22 jan. 2003.

ORR, M. W. et al. Oligoribonuclease is the primary degradative enzyme for pGpG in *Pseudomonas aeruginosa* that is required for cyclic-di-GMP turnover. **Proceedings of the National Academy of Sciences**, v. 112, n. 36, p. E5048–E5057, 8 set. 2015.

OZAKI, S. et al. Activation and polar sequestration of PopA, a c-di-GMP effector protein involved in *C aulobacter crescentus* cell cycle control. **Molecular Microbiology**, v. 94, n. 3, p. 580–594, nov. 2014.

PALANCA, C.; RUBIO, V. Effects of T-loop modification on the PII-signalling protein: structure of uridylylated *Escherichia coli* GlnB bound to ATP. **Environmental Microbiology Reports**, v. 9, n. 3, p. 290–299, jun. 2017.

PANI, B. et al. Mechanism of Inhibition of Rho-dependent Transcription Termination by Bacteriophage P4 Protein Psu. **Journal of Biological Chemistry**, v. 281, n. 36, p. 26491–26500, 8 set. 2006.

PAPPALARDO, L. et al. The NMR Structure of the Sensory Domain of the Membranous Two-component Fumarate Sensor (Histidine Protein Kinase) DcuS of *Escherichia coli*. **Journal of Biological Chemistry**, v. 278, n. 40, p. 39185–39188, 3 out. 2003.

PARK, H. et al. Bicyclomycin and Dihydrobicyclomycin Inhibition Kinetics ofEscherichia colirho-Dependent Transcription Termination Factor ATPase Activity. **Archives of Biochemistry and Biophysics**, v. 323, n. 2, p. 447–454, 10 nov. 1995.

PASMAN, Z.; VON HIPPEL, P. H. Regulation of rho-dependent transcription termination by NusG is specific to the Escherichia coli elongation complex. **Biochemistry**, v. 39, n. 18, p. 5573–85, 9 maio 2000.

PAUL, K. et al. The c-di-GMP Binding Protein YcgR Controls Flagellar Motor Direction and Speed to Affect Chemotaxis by a "Backstop Brake" Mechanism. **Molecular Cell**, v. 38, n. 1, p. 128–139, 9 abr. 2010.

PAUL, R. et al. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. **Genes & development**, v. 18, n. 6, p. 715–27, 15 mar. 2004.

PEDROSA, F. O.; YATES, M. G. Regulation of nitrogen fixation *(nif)* genes of *Azospirillum brasilense* by *nifA* and *ntr (gln)* type gene products. **FEMS Microbiology Letters**, v. 23, n. 1, p. 95–101, jun. 1984.

PERI, K. G.; GOLDIE, H.; WAYGOOD, E. B. Cloning and characterization of the *N* -acetylglucosamine operon of *Escherichia coli*. **Biochemistry and Cell Biology**, v. 68, n. 1, p. 123–137, 1 jan. 1990.

PETERS, J. M. et al. Rho directs widespread termination of intragenic and stable RNA transcription. **Proceedings of the National Academy of Sciences**, v. 106, n. 36, p. 15406–15411, 8 set. 2009.

PETERS, J. M. et al. Rho and NusG suppress pervasive antisense transcription in Escherichia coli. **Genes & Development**, v. 26, n. 23, p. 2621–2633, 1 dez. 2012.

PETERS, J. M.; VANGELOFF, A. D.; LANDICK, R. Bacterial Transcription Terminators: The RNA 3′-End Chronicles. **Journal of Molecular Biology**, v. 412, n. 5, p. 793–813, 7 out. 2011.

PETERSON, A. W.; COCKRELL, G. M.; KANTROWITZ, E. R. A second allosteric site in Escherichia coli aspartate transcarbamoylase. **Biochemistry**, v. 51, n. 24, p. 4776–8, 19 jun. 2012.

PLUMBRIDGE, J. A. Sequence of the nagBACD operon in Escherichia coli K12 and pattern of transcription within the nag regulon. **Molecular Microbiology**, v. 3, n. 4, p. 505–515, abr. 1989.

PLUMBRIDGE, J. A. Repression and induction of the nag regulon of Escherichia coll K-12: the roles of nagC and nagA in maintenance of the uninduced state. **Molecular Microbiology**, v. 5, n. 8, p. 2053–2062, ago. 1991.

QAYYUM, M. Z.; DEY, D.; SEN, R. Transcription Elongation Factor NusA Is a General Antagonist of Rho-dependent Termination in Escherichia coli. **The Journal of biological chemistry**, v. 291, n. 15, p. 8090–108, 8 abr. 2016.

QI, Y. et al. A Flavin Cofactor-Binding PAS Domain Regulates c-di-GMP Synthesis in *Ax* DGC2 from *Acetobacter xylinum*. **Biochemistry**, v. 48, n. 43, p. 10275–10285, 3 nov. 2009.

RABHI, M. et al. The Sm-like RNA chaperone Hfq mediates transcription antitermination at Rho-dependent terminators. **The EMBO journal**, v. 30, n. 14, p. 2805–16, 14 jun. 2011.

RADCHENKO, M. V.; THORNTON, J.; MERRICK, M. Control of AmtB-GlnK Complex Formation by Intracellular Levels of ATP, ADP, and 2-Oxoglutarate. **Journal of Biological Chemistry**, v. 285, n. 40, p. 31037–31045, 1 out. 2010.

RAJENDRAN, C. et al. Crystal structure of the GlnZ-DraG complex reveals a different form of PII-target interaction. **Proceedings of the National Academy of Sciences**, v. 108, n. 47, p. 18972–18976, 22 nov. 2011.

RAY-SONI, A.; BELLECOURT, M. J.; LANDICK, R. Mechanisms of Bacterial Transcription Termination: All Good Things Must End. **Annual Review of Biochemistry**, v. 85, n. 1, p. 319–347, 2 jun. 2016.

REAVES, M. L. et al. Pyrimidine homeostasis is accomplished by directed overflow metabolism. **Nature**, v. 500, n. 7461, p. 237–241, 31 ago. 2013.

REITZER, L. Nitrogen Assimilation and Global Regulation in *Escherichia coli*. **Annual Review of Microbiology**, v. 57, n. 1, p. 155–176, out. 2003.

RICHARDSON, J. P. Rho-dependent termination and ATPases in transcript termination. **Biochimica et biophysica acta**, v. 1577, n. 2, p. 251–260, 13 set. 2002.

ROBERTS, J. W. Termination Factor for RNA Synthesis. **Nature**, v. 224, n. 5225, p. 1168–1174, dez. 1969.

RODRIGUES, T. E. et al. In vitro interaction between the ammonium transport protein AmtB and partially uridylylated forms of the PII protein GlnZ. **Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics**, v. 1814, n. 9, p. 1203–1209, 1 set. 2011.

RODRIGUES, T. E. et al. Search for novel targets of the P_{II} signal transduction protein in Bacteria identifies the BCCP component of acetyl-CoA carboxylase as a P_{II} binding partner. **Molecular Microbiology**, v. 91, n. 4, p. 751–761, 1 fev. 2014.

RODRIGUES, T. E. **Regulação da Acetil-CoA Carboxilase pelas proteínas PII e cristalização da enzima GlnD de *Escherichia coli***. 132 f. Tese (Doutorado em Ciências - Bioquímica) - Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 2017.

RODRIGUES, T. E. et al. Fatty acid biosynthesis is enhanced in Escherichia coli strains with deletion in genes encoding the PII signaling proteins. **Archives of Microbiology**, v. 201, n. 2, p. 209–214, 30 mar. 2019.

ROMLING, U.; GALPERIN, M. Y.; GOMELSKY, M. Cyclic di-GMP: the First 25 Years of a Universal Bacterial Second Messenger. **Microbiology and Molecular Biology Reviews**, v. 77, n. 1, p. 1–52, 1 mar. 2013.

RÖMLING, U.; LIANG, Z.-X.; DOW, J. M. Progress in Understanding the Molecular Basis Underlying Functional Diversification of Cyclic Dinucleotide Turnover Proteins. **Journal of bacteriology**, v. 199, n. 5, p. e00790-16, 1 mar. 2017.

RUSSELL, M. H. et al. Integration of the second messenger c-di-GMP into the chemotactic signaling pathway. **mBio**, v. 4, n. 2, p. e00001-13, 19 mar. 2013.

SAMBROOK, J. et al. **Molecular cloning: a laboratory manual**. Cold Spring Harbor Laboratory Press, n. 2. 1989

SANT'ANNA, F. H. et al. The PII Superfamily Revised: A Novel Group and Evolutionary Insights. **Journal of Molecular Evolution**, v. 68, n. 4, p. 322–336, 19 abr. 2009.

SCHIRMER, T. C-di-GMP Synthesis: Structural Aspects of Evolution, Catalysis and Regulation. **Journal of Molecular Biology**, v. 428, n. 19, p. 3683–3701, 25 set. 2016.

SCHMIDT, A. J.; RYJENKOV, D. A.; GOMELSKY, M. The Ubiquitous Protein Domain EAL Is a Cyclic Diguanylate-Specific Phosphodiesterase: Enzymatically Active and Inactive EAL Domains. **Journal of Bacteriology**, v. 187, n. 14, p. 4774–4781, 15 jul. 2005.

SCHUTT, H.; HOLZER, H. Biological Function of the Ammonia-Induced Inactivation of Glutamine Synthetase in Escherichia coli. **European Journal of Biochemistry**, v. 26, n. 1, p. 68–72, 15 mar. 1972.

SEDLYAROVA, N. et al. sRNA-Mediated Control of Transcription Termination in E. coli. **Cell**, v. 167, n. 1, p. 111- 121.e13, 22 set. 2016.

SEEFELDT, L. C.; HOFFMAN, B. M.; DEAN, D. R. Mechanism of Mo-dependent nitrogenase. **Annual review of biochemistry**, v. 78, p. 701–22, 2009.

SERGANOV, A.; NUDLER, E. A Decade of Riboswitches. **Cell**, v. 152, n. 1–2, p. 17–24, jan. 2013.

SHAPIRO, B. M. Glutamine synthetase deadenylylating enzyme system from Escherichia coli. Resolution into two components, specific nucleotide stimulation, and cofactor requirements. **Biochemistry**, v. 8, n. 2, p. 659–670, fev. 1969.

SHASHNI, R. et al. Redundancy of primary RNA-binding functions of the bacterial transcription terminator Rho. **Nucleic Acids Research**, v. 42, n. 15, p. 9677–9690, 2 set. 2014.

SILVA, I. J. et al. SraL sRNA interaction regulates the terminator by preventing premature transcription termination of rho mRNA. **Proceedings of the National Academy of Sciences of the United States of America**, v. 116, n. 8, p. 3042–3051, 2019.

SKORDALAKES, E.; BERGER, J. M. Structure of the Rho transcription terminator: mechanism of mRNA recognition and helicase loading. **Cell**, v. 114, n. 1, p. 135–46, 11 jul. 2003.

SOTOMAIOR, P. et al. Effect of ATP and 2-oxoglutarate on the in vitro interaction between the NifA GAF domain and the GlnB protein of Azospirillum brasilense. **Brazilian Journal of Medical and Biological Research**, v. 45, n. 12, p. 1135–1140, dez. 2012.

SULTAN, S. Z. et al. Analysis of the HD-GYP Domain Cyclic Dimeric GMP Phosphodiesterase Reveals a Role in Motility and the Enzootic Life Cycle of Borrelia burgdorferi. **Infection and Immunity**, v. 79, n. 8, p. 3273–3283, ago. 2011.

SRIVASTAVA, D. et al. Cyclic di-GMP inhibits *Vibrio cholerae* motility by repressing induction of transcription and inducing extracellular polysaccharide production. **Molecular Microbiology**, v. 90, n. 6, p. 1262–1276, dez. 2013.

STASOLLA, C. et al. Purine and pyrimidine nucleotide metabolism in higher plants. **Journal of Plant Physiology**, v. 160, n. 11, p. 1271–1295, jan. 2003.

STEINMETZ, E. J.; PLATT, T. Evidence supporting a tethered tracking model for helicase activity of Escherichia coli Rho factor. **Proceedings of the National Academy of Sciences**, v. 91, n. 4, p. 1401–1405, 15 fev. 1994.

STITT, B. L. Escherichia coli transcription termination protein rho has three hydrolytic sites for ATP. **The Journal of biological chemistry**, v. 263, n. 23, p. 11130–7, 15 ago. 1988.

STITT, B. L. *Escherichia coli* Transcription Termination Factor Rho Binds and Hydrolyzes ATP Using a Single Class of Three Sites †. **Biochemistry**, v. 40, n. 7, p. 2276–2281, 20 fev. 2001.

STITT, B. L.; XU, Y. Sequential hydrolysis of ATP molecules bound in interacting catalytic sites of Escherichia coli transcription termination protein Rho. **The Journal of biological chemistry**, v. 273, n. 41, p. 26477–86, 9 out. 1998.

SUNDRIYAL, A. et al. Inherent Regulation of EAL Domain-catalyzed Hydrolysis of Second Messenger Cyclic di-GMP. **Journal of Biological Chemistry**, v. 289, n. 10, p. 6978–6990, 7 mar. 2014.

TAGLIABUE, L. et al. The diguanylate cyclase YddV controls production of the exopolysaccharide poly-N-acetylglucosamine (PNAG) through regulation of the PNAG biosynthetic pgaABCD operon. **Microbiology**, v. 156, n. 10, p. 2901–2911, 1 out. 2010.

TAL, R. et al. Three cdg operons control cellular turnover of cyclic di-GMP in Acetobacter xylinum: genetic organization and occurrence of conserved domains in isoenzymes. **Journal of bacteriology**, v. 180, n. 17, p. 4416–25, set. 1998.

TCHIGVINTSEV, A. et al. Structural Insight into the Mechanism of c-di-GMP Hydrolysis by EAL Domain Phosphodiesterases. **Journal of Molecular Biology**, v. 402, n. 3, p. 524–538,

24 set. 2010.

THOMAS, G.; COUTTS, G.; MERRICK, M. The glnKamtB operon. A conserved gene pair in prokaryotes. **Trends in genetics : TIG**, v. 16, n. 1, p. 11–4, jan. 2000.

THOMPSON, J. D.; HIGGINS, D. G.; GIBSON, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Research**, v. 22, n. 22, p. 4673–4680, 11 nov. 1994.

THOMSEN, N. D.; BERGER, J. M. Running in Reverse: The Structural Basis for Translocation Polarity in Hexameric Helicases. **Cell**, v. 139, n. 3, p. 523–534, 30 out. 2009.

THOMSEN, N. D. et al. Molecular mechanisms of substrate-controlled ring dynamics and substepping in a nucleic acid-dependent hexameric motor. **Proceedings of the National Academy of Sciences of the United States of America**, v. 113, n. 48, p. E7691–E7700, 29 nov. 2016.

TOKER, A. S.; MACNAB, R. M. Distinct regions of bacterial flagellar switch protein FliM interact with FliG, FliN and CheY 1 1Edited by M. F. Moody. **Journal of Molecular Biology**, v. 273, n. 3, p. 623–634, 31 out. 1997.

TOMAR, S. K.; ARTSIMOVITCH, I. NusG-Spt5 proteins-Universal tools for transcription modification and communication. **Chemical reviews**, v. 113, n. 11, p. 8604–19, 13 nov. 2013.

TONG, L. Structure and function of biotin-dependent carboxylases. **Cellular and Molecular Life Sciences**, v. 70, n. 5, p. 863–891, 7 mar. 2013.

TREMBLAY, L.; DUNAWAY-MARIANO, D.; ALLEN, K. Structure and Activity Analyses of Escherichia coli K-12 NagD Provide Insight into the Evolution of Biochemical Function in the Haloalkanoic Acid Dehalogenase Superfamily†,‡. 2006.

TRUAN, D. et al. A New PII Protein Structure Identifies the 2-Oxoglutarate Binding Site. **Journal of Molecular Biology**, v. 400, n. 3, p. 531–539, 16 jul. 2010.

TRUAN, D. et al. Structure and Thermodynamics of Effector Molecule Binding to the Nitrogen Signal Transduction PII Protein GlnZ from Azospirillum brasilense. **Journal of Molecular Biology**, v. 426, n. 15, p. 2783–2799, 29 jul. 2014.

TUCKERMAN, J. R. et al. An Oxygen-Sensing Diguanylate Cyclase and Phosphodiesterase Couple for c-di-GMP Control. **Biochemistry**, v. 48, n. 41, p. 9764–9774, 20 out. 2009.

URBANSKI. A. H. **Investigação e caracterização da interação i*n vitro* entre as proteínas PII, GlnZ e GlnB com uma diguanilato ciclase (AZOBR_140132) e uma fosfodiesterase (AZOBR_p1130052) de *Azospirillum brasilense* SP245**. 97 f. Dissertação (Mestrado em Ciências - Bioquímica) - Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, 2018.

VALABHOJU, V.; AGRAWAL, S.; SEN, R. Molecular Basis of NusG-mediated Regulation of Rho-dependent Transcription Termination in Bacteria. **The Journal of biological chemistry**, v. 291, n. 43, p. 22386–22403, 21 out. 2016.

VAN HEESWIJK, W. C. et al. **An alternative P II protein in the regulation of glutamine synthetase in *Escherichia coli* . Molecular Microbiology**, v. 21, n. 1, p. 133–146, 31 jul. 1996.

VAN HEESWIJK, W. C. et al. The Escherichia coli signal transducers PII (GlnB) and GlnK

form heterotrimers in vivo: Fine tuning the nitrogen signal cascade. **Proceedings of the National Academy of Sciences**, v. 97, n. 8, p. 3942–3947, 11 abr. 2000.

VOROBIEV, S. M. et al. Crystal structure of a catalytically active GG(D/E)EF diguanylate cyclase domain from Marinobacter aquaeolei with bound c-di-GMP product. **Journal of Structural and Functional Genomics**, v. 13, n. 3, p. 177–183, 29 set. 2012.

WALSTROM, K. M. et al. Kinetics of the RNA−DNA Helicase Activity of *Escherichia coli* Transcription Termination Factor Rho. 1. Characterization and Analysis of the Reaction †. **Biochemistry**, v. 36, n. 26, p. 7980–7992, 1 jul. 1997.

WALSTROM, K. M.; DOZONO, J. M.; VON HIPPEL, P. H. Kinetics of the RNA−DNA Helicase Activity of *Escherichia coli* Transcription Termination Factor Rho. 2. Processivity, ATP Consumption, and RNA Binding †. **Biochemistry**, v. 36, n. 26, p. 7993–8004, 1 jul. 1997.

WASHBURN, R. S.; GOTTESMAN, M. E. Transcription termination maintains chromosome integrity. **Proceedings of the National Academy of Sciences**, v. 108, n. 2, p. 792–797, 11 jan. 2011.

WASSMANN, P. et al. Structure of BeF3−-Modified Response Regulator PleD: Implications for Diguanylate Cyclase Activation, Catalysis, and Feedback Inhibition. **Structure**, v. 15, n. 8, p. 915–927, 14 ago. 2007.

WEI, R. R.; RICHARDSON, J. P. Identification of an RNA-binding Site in the ATP Binding Domain of *Escherichia coli* Rho by H_2O_2/Fe-EDTA Cleavage Protection Studies. **Journal of Biological Chemistry**, v. 276, n. 30, p. 28380–28387, 27 jul. 2001.

WEINHOUSE, H. et al. c-di-GMP-binding protein, a new factor regulating cellulose synthesis in *Acetobacter xylinum*. **FEBS Letters**, v. 416, n. 2, p. 207–211, 20 out. 1997.

WILD, J. R.; LOUGHREY-CHEN, S. J.; CORDER, T. S. **In the Presence of CTP, UTP Becomes an Allosteric Inhibitor of Aspartate TranscarbamoylaseProceedings of the National Academy of Sciences of the United States of America**National Academy of Sciences, , 1989.

WINKLER, F. K. Amt/MEP/Rh proteins conduct ammonia. **Pflügers Archiv - European Journal of Physiology**, v. 451, n. 6, p. 701–707, 5 mar. 2006.

XU, Y. et al. GlnK, a PII-homologue: structure reveals ATP binding site and indicates how the T-loops may be involved in molecular recognition. **Journal of Molecular Biology**, v. 282, n. 1, p. 149–165, 11 set. 1998.

XU, Y. et al. The structures of the PII proteins from the cyanobacteria *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803. **Acta Crystallographica Section D Biological Crystallography**, v. 59, n. 12, p. 2183–2190, 1 dez. 2003.

XU, Y.; KOHN, H.; WIDGER, W. R. Mutations in the Rho Transcription Termination Factor That Affect RNA Tracking. **Journal of Biological Chemistry**, v. 277, n. 33, p. 30023–30030, 16 ago. 2002.

YAN, D.; LENZ, P.; HWA, T. Overcoming Fluctuation and Leakage Problems in the Quantification of Intracellular 2-Oxoglutarate Levels in Escherichia coli. **Applied and Environmental Microbiology**, v. 77, n. 19, p. 6763–6771, 1 out. 2011.

YU, X. et al. Three-dimensional reconstruction of transcription termination factor rho:

orientation of the N-terminal domain and visualization of an RNA-binding site. **Journal of Molecular Biology**, v. 299, n. 5, p. 1279–1287, 23 jun. 2000.

YUAN, J. et al. Metabolomics-driven quantitative analysis of ammonia assimilation in E. coli. **Molecular Systems Biology**, v. 5, p. 302, 18 ago. 2009.

YUTAKA MIZUNO et al. Crystal Structure of Arabidopsis PII Reveals Novel Structural Elements Unique to Plants†,‡. 2007.

ZHANG, C.; WEI, Z.-H.; YE, B.-C. Quantitative monitoring of 2-oxoglutarate in Escherichia coli cells by a fluorescence resonance energy transfer-based biosensor. **Applied Microbiology and Biotechnology**, v. 97, n. 18, p. 8307–8316, 28 set. 2013.

ZHANG, Y. et al. Mutagenesis and Functional Characterization of the Four Domains of GlnD, a Bifunctional Nitrogen Sensor Protein. **Journal of Bacteriology**, v. 192, n. 11, p. 2711–2721, 1 jun. 2010.

ZHULIN, I. B. et al. Oxygen taxis and proton motive force in Azospirillum brasilense. **Journal of bacteriology**, v. 178, n. 17, p. 5199–204, set. 1996.

ZHULIN, I. B.; NIKOLSKAYA, A. N.; GALPERIN, M. Y. Common extracellular sensory domains in transmembrane receptors for diverse signal transduction pathways in bacteria and archaea. **Journal of bacteriology**, v. 185, n. 1, p. 285–94, jan. 2003.