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Letícia Miranda Lery Santos

Análise proteômica quantitativa da interação entre a bactéria endofítica *Gluconacetobacter diazotrophicus* e cana-de-açúcar

Rio de Janeiro, 2010

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Tese de doutorado a ser submetida ao Instituto de Biofísica Carlos Chagas Filho da Universidade Federal do Rio de Janeiro, visando a obtenção do grau de Doutora em Ciências Biológicas - Biofísica

Orientadores: Paulo Mascarello Bisch e Wanda Maria Almeida von Krüger

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26 de Abril de 2010

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Professor Doutor Paulo Mascarello Bisch – Orientador

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Professora Doutora Wanda Maria Almeida von Krüger – Orientadora

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Professora Doutora Lúcia Mendonça Previato –Revisora

_____

Professor Doutor Fábio Oliveira Pedrosa - Membro da Banca Examinadora

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Pesquisador Doutor Dario Eluan Kalume - Membro da Banca Examinadora

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Professora Doutora Ana Maria Abrantes Coelho - Membro da Banca Examinadora

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Resumo

Este é o primeiro estudo sobre proteínas envolvidas diretamente na interação entre *G. diazotrophicus* e cana-de-açúcar. *G. diazotrophicus* é uma bactéria endofítica e promotora do crescimento vegetal, encontrada em plantas como cana-de-açúcar. Com o intuito de conhecer moléculas envolvidas no estabelecimento dessa associação, foram analisadas proteínas diferencialmente expressas por *G. diazotrophicus* e dois genótipos de cana-de-açúcar (SP-70 e Chunee), após 24 de horas de interação. Para esta finalidade, foi otimizado um protocolo pioneiro de análise proteômica quantitativa. O protocolo consistiu na marcação metabólica de células em cultura e em co-cultura com sais contendo $^{15}N/^{14}N$, métodos rápidos de extração de proteínas, eletroforese unidimensional, cromatografia líquida e espectrometria de massas. Um dos aspectos mais inovadores deste estudo foi a comparação das proteínas diferencialmente expressas durante a interação de *G. diazotrophicus* com dois genótipos de cana-de-açúcar, que apresentam contribuições distintas da fixação biológica de nitrogênio (FBN). A comparação entre células de *G. diazotrophicus* controle e co-cultivadas com cada genótipo da planta revelou 77 proteínas diferencialmente expressas pela bactéria na presença da planta. Na interação com ambos os genótipos de cana-de-açúcar, a bactéria expressou diferencialmente antígenos de superfície, proteínas de mecanismos de adaptação celular e sinalização. Esse resultado sugere que *G. diazotrophicus* percebe e responde de forma similar à ambos os genótipos de cana-de-açúcar. A comparação entre plantas de cana-de-açúcar controle e plantas co-cultivadas com *G. diazotrophicus* revelou 7 proteínas diferencialmente expressas pelo genótipo eficiente em FBN (SP-70), tais como receptores de membrana e proteínas associadas a mecanismos de adaptação celular à presença da bactéria. Contudo,

Chunee, o genótipo com pouca contribuição de FBN, expressou 9 proteínas exclusivamente durante a interação com *G. diazotrophicus.* Chunee respondeu à bactéria através da alteração global da expressão gênica, com aumento da produção de proteínas de mecanismos de degradação protéica e defesa, sugerindo que esta variedade não reconhece *G. diazotrophicus* como uma bactéria benéfica. Ainda, foram identificadas 30 proteínas de *G. diazotrophicus* em extratos das raízes de cana-de-açúcar co-cultivadas com a bactéria. Essas proteínas foram expressas por *G. diazotrophicus* que está em contato muito próximo com a planta. Dentre essas, 9 foram exclusivamente expressas nessas amostras de raízes, ou seja, não foram identificadas nas células em suspensão no co-cultivo com a cana-de-açúcar nem em estudos anteriores. Assim, sugerimos que a expressão dessas proteínas seja regulada por sinais do hospedeiro. Todos esses resultados contribuem para um maior entendimento das relações entre a cana-de-açúcar e *G. diazotrophicus*.

Abstract

This is the first study regarding proteins directly involved in *G. diazotrophicus* interaction with sugarcane. *G. diazotrophicus* is an plant growth promoting bacterium found in sugarcane plants. In order to understand the molecular details of this interaction we identified proteins differentially expressed by both bacteria and plant, after 24 hours of co-cultive. For this purpose we have optimized a pioneer quantitative proteomic workflow, including: stable isotopic labeling of cells in culture and in co-culture with $^{15}N/^{14}N$ salts, broad and rapid protein extraction, one-dimensional electrophoresis, liquid chromatography and mass spectrometry. One of the innovative aspects of this study was the comparison between the interaction of *G. diazotrophicus* with two sugarcane genotypes with distinct contribution of the biological nitrogen fixation (BNF). The differential analysis between *G. diazotrophicus* control cells and bacteria co-cultivated with each sugarcane genotype revealed 77 proteins differentially expressed by the bacterium under interaction with the host. In both experiments, *G. diazotrophicus* presented increased expression of surface antigens and proteins involved in mechanisms of adaptation and cellular signalling. This result suggests that *G. diazotrophicus* perceives and responds similarly to both sugarcane genotypes. In another hand, the comparison between control and inoculated plants revealed 7 proteins upregulated in SP-70, the plant genotype more efficient in BNF. These included, receptors and other proteins involved in the adaptation to the presence of *G. diazotrophicus*. However, Chunee, the genotype with low BNF contribution, expressed 9 proteins exclusively upon bacterial interaction. Chunee altered gene expression and activated protein degradation complexes and defense mechanisms, suggesting that this genotype is not able to recognize *G. diazotrophicus* as a beneficial bacterium. Additionally, 30 *G.*

diazotrophicus proteins were identified from sugarcane roots samples. The bacterial cells in intimate contact with the plants expressed such proteins. Among them, 9 were identified for the first time in this experiment. So, these results suggest that their expression is regulated by plant signals. Altoghether these results contribute to a better understanding of the sugarcane interaction with *G. diazotrophicus*.

Lista de abreviaturas e siglas

1D-GE: eletroforese em gel unidimensional

2D-GE: eletroforese em gel bidimensional

ESI: *electronspray ionization* (ionização por *electronspray*)

FBN: fixação biológica de nitrogênio

IAA: *indol acetic acid* (ácido indol acético)

Kegg: *Kyoto Encyclopedia of Genes and Genomes*

MS: *mass spectrometry* (espectrometria de massas)

MS/MS: *tandem mass spectrometry* (espectrometria de massas em seqüência)

RMSD: *root mean square deviation* (desvio quadrático médio)

SDS-PAGE: eletroforese em gel de poliacrilamida com dodecil sulfato de sódio

Q: quadrupolo

TOF: *time of flight* (tempo de vôo)

Sumário

1- INTRODUÇÃO

1.1- A *Gluconacetobacter diazotrophicus*

A *Gluconacetobacter diazotrophicus* é uma bactéria Gram-negativa e aeróbica que pertence à classe das α-proteobactérias (1; 2). *G. diazotrophicus* foi isolada pela primeira vez por Cavalcante e Döbereiner em 1988 (1), a partir de raízes lavadas e caules de diferentes variedades de cana-de-açúcar, oriundas de diversas regiões do Brasil, e descrita como “uma nova bactéria fixadora de nitrogênio, tolerante ao meio ácido”.

Uma das propriedades desta espécie que permitiu sua caracterização (1) é sua capacidade de fixar o nitrogênio atmosférico. O nitrogênio gasoso, N_2, embora constitua quase 80% da atmosfera terrestre, é quimicamente inerte à temperatura ambiente. Diferentemente de outros elementos que ocorrem na natureza, suas reservas minerais são relativamente raras e o conteúdo de matéria orgânica nitrogenada no solo é limitado, podendo ser esgotado (3). Além disso, em regiões tropicais, as condições de temperatura e umidade do solo aceleram os processos de decomposição da matéria orgânica e perdas de nitrogênio, resultando em solos com baixos teores desse nutriente, entre 0,05 e 0,30% (4). A fixação de nitrogênio permite a disponibilização deste elemento para plantas e outros seres vivos, através da redução do nitrogênio atmosférico (N_2) a amônia (NH_3), um processo que consome muita energia.

A fixação biológica do N_2 mediada por microrganismos ocorre graças a presença de um complexo enzimático denominado nitrogenase (3). A estequiometria da reação proposta para redução de N_2 a duas moléculas NH_3 é a seguinte :

$$N_2 + 16\ ATP + 8\ e^- + 8H^+ \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ Pi.$$

(onde e^- simboliza elétron e Pi simboliza o fosfato inorgânico).

Visto que os organismos eucariontes são incapazes de assimilar o N_2 e convertê-lo a uma forma assimilável, a maioria das plantas obtém o nitrogênio do solo sob a forma de íon nitrato (NO_3^-), havendo algumas que o absorvem sob a forma de íon amônio (NH_4^+). Porém, estas duas formas são extremamente instáveis no solo, representando o principal agravante da utilização de fertilizantes nitrogenados, ou seja uma baixa eficiência de aproveitamento pelas plantas, raramente ultrapassando 50% (5). Isto significa um aumento do custo da produção pois metade dos fertilizantes nitrogenados aplicados à agricultura é perdida por lixiviação dos solos (lavagem no perfil do solo), denitrificação (transformação do NO_3^- em N_2 e NO_2^{-2}) e pela volatilização do NH_3. Portanto, o processo de fixação biológica do nitrogênio (FBN) é de suma importância para a nutrição nitrogenada das plantas e fertilidade dos solos.

Diversas metodologias, tais como a diluição de ^{15}N e a abundância natural de ^{15}N, comprovaram os altos índices de fixação biológica de nitrogênio em plantas gramíneas, tais como cana-de-açúcar, arroz e grama forrageira (6). A fixação de nitrogênio por *G. diazotrophicus* tem sido assunto de diversos estudos (7; 8; 9; 10; 11). Alguns dos efeitos da inoculação de *G. diazotrophicus* em plantas de cana-de-açúcar também já foram observados (Fig. 1), e incluem o aumento acentuado do crescimento radicular e da superfície foliar (12; 13). Tais efeitos são dependentes do isolado de *G. diazotrophicus,* assim como da variedade de cana-de-açúcar (12; 13), sendo a cepa PAL5 de *G. diazotrophicus* a mais estudada até o momento (13).

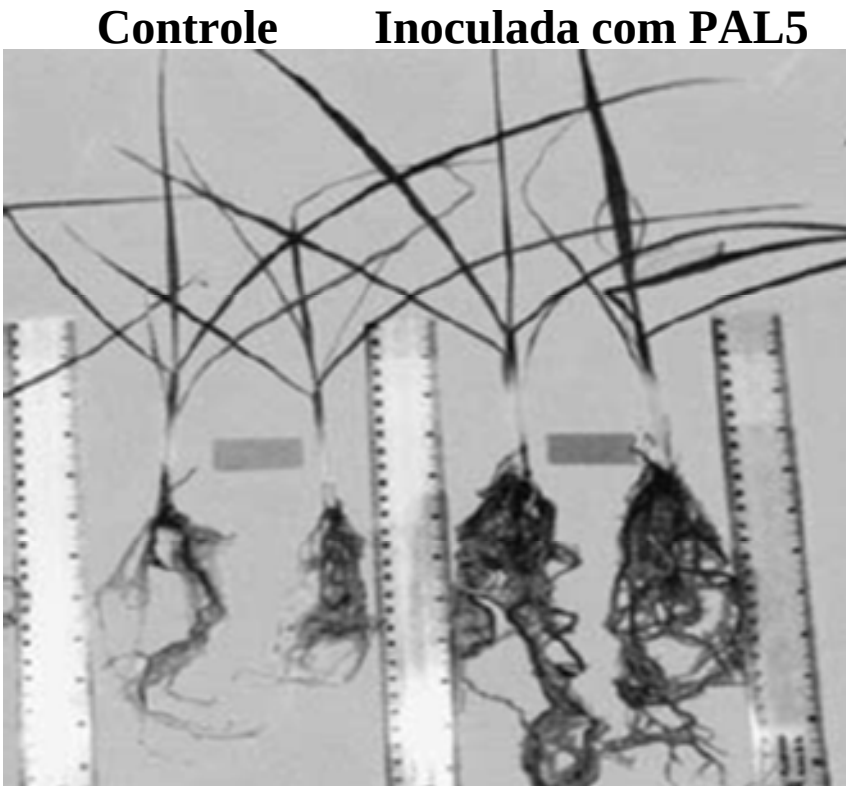


Figura 1: Efeito da inoculação de *G. diazotrophicus* PAL5 em plantas de cana-de-açúcar da variedade MEX 57-473. A comparação entre plantas controles e inoculadas com a cepa PAL5, mostra que a cepa PAL5 estimulou o crescimento radicular e da superfície foliar desta variedade de cana-de-açúcar (13).

No entanto, tais efeitos resultam de várias estratégias utilizadas pela *G. diazotrophicus* na colonização da planta, indo além do simples processo de fixação de nitrogênio. Em 2002, Piñon e colaboradores (14) identificaram uma proteína produzida e secretada pela *G. diazotrophicus* em cultura líquida, capaz de impedir o crescimento de *Xanthomonas albilineans* – o agente causador da queimaduras de folhas, uma doença bacteriana vascular da cana-de-açúcar (15) - em meio sólido. É sugerido que esta proteína atue na destruição da parede celular da *Xanthomonas* e consequente perda de conteúdo citoplasmático para o meio (14). Esta proteína foi identificada como sendo uma “bacteriocina tipo lisozima” de 12,5 kDa, ligeiramente

menor do que outras lisozimas bacterianas que possuem massa molecular em torno de 14 kDa.

Um terceiro fator que contribui para o maior crescimento das plantas inoculadas com *G. diazotrophicus* é a produção de fito-hormônios pela bactéria. Em 1993, Fuentez-Ramirez e colaboradores (16) descreveram a produção de ácido-indol-acético (IAA), um hormônio que estimula o crescimento vegetal, por cepas de *G. diazotrophicus* isoladas de cultivares de cana-de-açúcar do México (16). Bastian e colaboradores caracterizaram fitohormônios produzidos *in vitro* por esta bactéria, confirmando a produção do IAA. Mostraram ainda a produção de giberelinas GA1 e GA3 por *G. diazotrophicus* crescida em meio definido contendo 10 a 15% de sacarose (17).

A característica de *G. diazotrophicus* descrita mais recentemente, que também deve contribuir para a promoção do crescimento de plantas, é a solubilização de compostos de zinco. O zinco é um micronutriente essencial para o crescimento de plantas, sendo cofator de diversas enzimas. Seu suprimento em grande parte é devido ao uso de fertilizantes agrícolas, embora no solo o zinco rapidamente seja transformado em formas insolúveis. Assim, a solubilização de compostos de zinco por microrganismos é fundamental para manutenção deste habitat. As cepas PAL5 e L3 de *G. diazotrophicus* solubilizam com eficiência compostos insolúveis de zinco (18). Tal eficiência de solubilização é dependente da fonte de carbono, sendo mais pronunciada a 10% de glucose. Nessa condição, foi observada a presença de ácido 5-cetoglucônico no meio extracelular, que é um mediador da solubilização do zinco (7; 18; 19). Esta habilidade de *G. diazotrophicus* traz vantagens ecológicas importantes, primeiro por tornar o cátion disponível para as plantas em solos

deficientes deste metal, e ainda pela possibilidade de usar esta bactéria em processos de fitoextração e fitoremediação de solos contaminados por zinco (18).

1.2- Habitat e hospedeiros

Organismos endofíticos são aqueles que vivem no interior de plantas sem lhes causar danos, apresentando localização intracelular ou intercelular, colonizando principalmente raízes, colmos e folhas ou o tecido vascular do hospedeiro (20). Este modo de associação apresenta vantagens competitivas em relação as bactérias rizosféricas (vivem na superfície das raízes), considerando que a disponibilidade de nutrientes é maior no interior das plantas e existe uma menor competição, pois o número de espécies endofíticas é menor do que o das rizosféricas (21). Além disto, as bactérias endofíticas encontram-se mais protegidas das condições adversas do meio ambiente e por estabelecerem um contato mais íntimo com seus hospedeiros, podem usufruir de benefícios mais diretos em comparação com bactérias rizosféricas (20; 21).

O habitat endofítico de *G. diazotrophicus* foi confirmado pelo seu isolamento de tecidos internos de diferentes variedades de cana-de-açúcar, cultivadas no Brasil, na Austrália e no México (16; 22; 23), assim como de plantas de batata-doce (*Ipomoea batatas*), grama *Pennisetum pururеum* var Cameroon (24), abacaxi (*Ananas comosus*), café (*Coffea arabica*) (25), arroz (26) e do cereal *Eleusine coracana* (27) (tab. 1). Ocasionalmente, *G. diazotrophicus* é detectada na rizosfera de cana-de-açúcar (22) e isolada do inseto cochonilha (*Saccharococcus sacchari*) (28), praga comum nas regiões onde se cultiva cana-de-açúcar (Tabela 1).

O modo de interação endofítico entre esta bactéria e seus hospedeiros naturais representa uma vantagem, do ponto de vista da troca de compostos nitrogenados com a planta, em relação a interação com os organismos de vida livre.

Tabela 1: Hospedeiros naturais de *G. diazotrophicus*

Hospedeiro	***Região***	***Referências***
Cana-de-açúcar	Raízes, caules, folhas	1, 4, 7, 14
Batata doce	Raízes, caules	13
Café	Raízes, caules, rizosfera	9
Abacaxi	Fruto	8
Arroz	Raízes	10
Cochonilhas	Interno	5, 16
Chá	Raízes	15
Grama Cameroon	Raízes, caules	6
Ragi	Raízes, caules, rizosfera	11

1.3- A interação entre *G. diazotrophicus* e cana-de-açúcar

A inoculação de *G. diazotrophicus* em plantas de cana-de-açúcar promove o crescimento das raízes e folhas, incremento na altura da planta e na fixação de nitrogênio e, consequentemente, aumento da produtividade (12; 29). Efeitos similares foram observados para plantas de arroz geradas a partir de sementes inoculadas com *G. diazotrophicus,* em comparação a sementes não inoculadas (29). Ainda, foi demonstrado um efeito sinergístico da co-inoculação de *G. diazotrophicus* com outros diazotrofos ou esporos de fungos micorrízicos (30).

Diversos estudos utilizando técnicas de microscopia e co-cultura confirmaram a localização endofítica de *G. diazotrophicus* em plantas de cana-de-açúcar (31; 32). Cerca de 10^6-10^7 células de *G. diazotrophicus* por grama de tecido fresco foram recuperadas de plantas em co-cultivo com a bactéria, mesmo após a esterilização da

superfície de folhas, caules e raízes de cana-de-açúcar, confirmando a localização da bactéria no interior da planta (33; 34) . Especula-se que a entrada da bactéria na planta acontece principalmente através das junções laterais das raízes, assim como ocorre para diversas outras bactérias diazotróficas endofíticas (20). Frequentemente, *G. diazotrophicus* forma monocamadas na superfície e nas junções laterais das raízes (32).

No interior da planta, *G. diazotrophicus* é encontrada dispersa aleatoriamente ou em biofilme (Fig. 2, (32)). Esses biofilmes, observados na epiderme radicular de plântulas de cana-de-açúcar, sete dias após a inoculação com *G. diazotrophicus*, são compostos por células bacterianas polimórficas imersas em uma matriz fibrilar heterogênea (36). Foi observado que células de *G. diazotrophicus* alongadas, enfileiradas e aderidas à parede celular vegetal são capazes de induzir uma polarização das células da cana-de-açúcar (36).

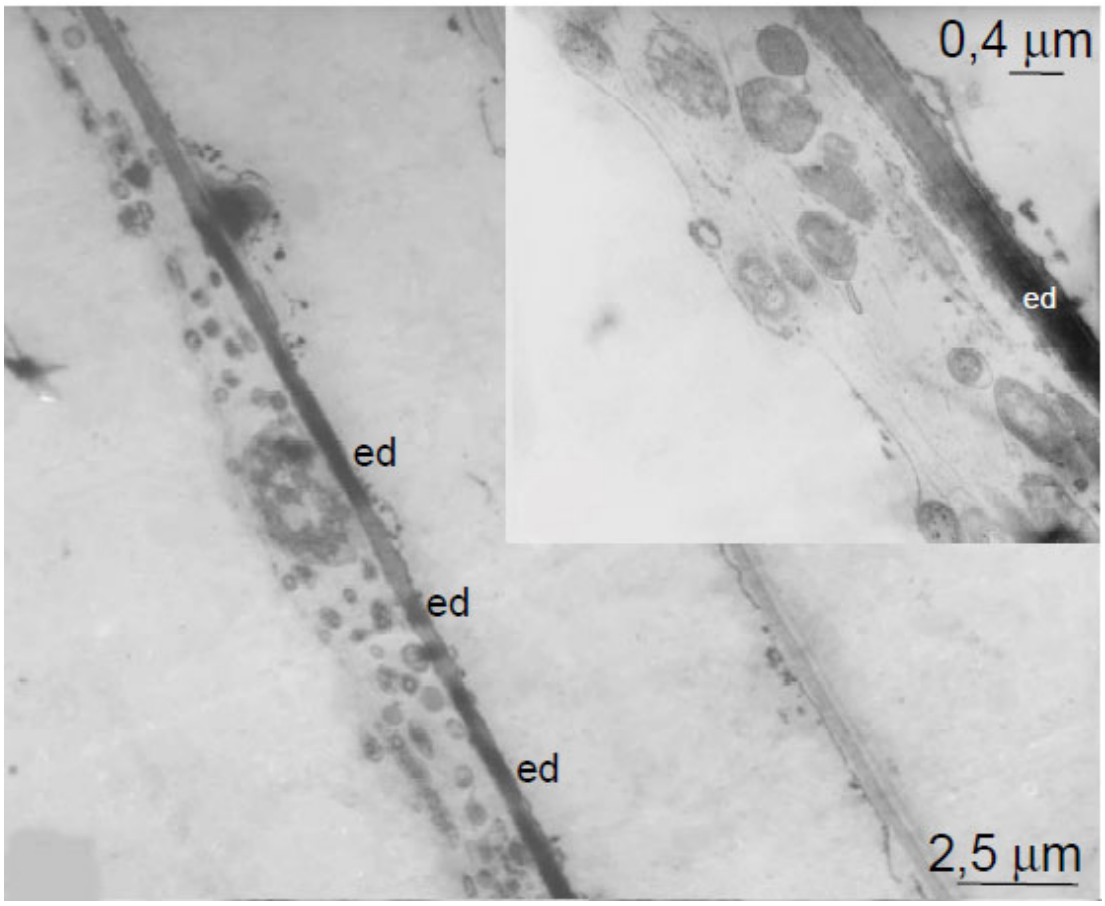


Figura 2: Microscopia eletrônica de transmissão, mostrando células de *G. diazotrophicus* PAL5, polimórficas, com aspecto frágil e formando biofilme na epiderme radicular de cana-de-açúcar RB72454, 7 dias após a inoculação (adaptado de Da Silva, 2005 (36)).

Além dos estudos estruturais e fisiológicos, recentemente foi demonstrado que células de *G. diazotrophicus* co-cultivadas por 7 dias com cana-de-açúcar aumentam a expressão de suas proteínas de estresse (37). Ainda, nosso grupo estabeleceu um banco de dados proteômico da *G. diazotrophicus* PAL5, contendo quase 600 proteínas, incluindo diversas com possíveis funções reguladoras e em processos de adaptação celular (38). Em outro estudo, identificamos proteínas envolvidas no processo de fixação de nitrogênio e demonstramos que o metabolismo do envoltório celular e produção de polihidroxialcanoatos (PHA) são processos co-regulados com

a FBN (39). Contudo, proteínas de *G. diazotrophicus* envolvidas especificamente na interação com seus hospedeiros, ainda não foram estudadas.

Por outro lado, alguns estudos sobre alterações moleculares na cana-de-açúcar induzidas por bactérias endofíticas têm sido realizados (40; 41; 42). Grande parte desses estudos usou um sistema de plantas micropropagadas desenvolvido por Reis e colaboradores (32). Eles concluíram que, para a melhor eficiência da interação, *G. diazotrophicus* deve ser inoculada ao término do período de enraizamento, em um meio de cultivo sem hormônios e vitaminas e com a concentração de açúcares de nutrientes minerais reduzidas por um fator de 10 (meio MS/10) (32).

Entre os diversos estudos morfológicos sobre a interação entre *G. diazotrophicus e* cana-de-açúcar, demonstrou-se que células da epiderme radicular de cana-de-açúcar secretam inúmeras vesículas, de composição desconhecida, e uma matriz diferenciada em resposta a interação endofítica (36; 43). Tais estudos sugerem um papel ativo da planta no processo de colonização (36). Com a intenção de tentar entender o papel da cana-de-açúcar na associação com a *G. diazotrophicus*, Nogueira e colaboradores (41) compararam os perfis de expressão gênica de plantas, através de bibliotecas de cDNA de tecidos e órgãos de cana-de-açúcar em condições fisiológicas, com uma biblioteca de cDNA de tecidos e órgãos de cana-de-açúcar inoculada *in vitr*o com *G. diazotrophicus.* Entre os RNAs expressos exclusivamente na biblioteca de plantas inoculadas, encontram-se diversos que codificam proteínas relacionadas ao metabolismo de nitrogênio, divisão celular, produção de hormônios, nodulinas e moléculas envolvidas em processos de sinalização celular, tais como serina e treonina cinases (41). Esses dados reforçam o papel da cana-de-açúcar na interação com *G. diazotrophicus*, respondendo a diversos processos metabólicos durante a associação.

Uma evidência adicional da importância da participação da planta no estabelecimento da interação com bactérias diazotróficas resultou de experimentos de diluição isotópica do nitrogênio, que estimaram a contribuição da FBN para diversos cultivares de cana-de-açúcar *Saccharum sp*. Neste estudo os autores estimaram o percentual do nitrogênio total da planta que era proveniente da FBN. Este trabalho revelou que a FBN contribui com 35% do nitrogênio total da planta nas variedades SP-70-1143, um híbrido comercial, e genótipo Krakatan de *S. spontaneum*, enquanto que para variedade Chunee de *S. barberi* a FBN não foi tão eficiente, contribuindo em apenas 12% (6). Foi demonstrado também que plantas cultivadas em solos pobres de nitrogênio têm maior contribuição da FBN do que solos fertilizados (6; 12). Embora esses experimentos não tenham avaliado a contribuição específica da *G. diazotrophicus* para a FBN em cana-de-açúcar, os dados sugerem que a interação de *G. diazotrophicus* com as variedades SP-70/Krakatan e Chunee possa apresentar características diferentes.

Contudo, nenhum trabalho até o momento explorou aspectos comparativos da interação de *G. diazotrophicus* com diferentes genótipos de cana-de-açúcar. Com o intuito de conhecer os processos moleculares que determinam a eficiência da interação entre *G. diazotrophicus* e cana-de-açúcar, este estudo visa comparar proteínas diferencialmente expressas pela bactéria e pela planta durante a interação entre *G. diazotrophicus* com os genótipos SP-70 (grande contribuição da FBN) e Chunee (pouca contribuição da FBN).

1.4- Análise proteômica comparativa

A proteômica tem sido utilizada como uma ferramenta para a análise do perfil de expressão de proteínas e peptídeos em diversos tipos celulares. Tal análise requer a combinação de preparo de amostras protéicas de boa qualidade, resolução e

identificação de proteínas. Geralmente, os métodos de preparação de amostras incluem diversas etapas de solubilização diferencial das proteínas e remoção de contaminantes, como lipídeos, ácidos nucléicos e polissacarídeos. Esses métodos (extração com detergentes, precipitação, diálise, uso de inibidores de proteases, agentes redutores, desnaturantes, entre outros) precisam ser otimizados para cada modelo de estudo e adaptado de acordo com as moléculas presentes na amostra. Esse aprimoramento é notável na literatura e de uma forma geral, tem-se a idéia de que quanto mais "pura" a amostra estiver, melhores serão os resultados obtidos. Contudo, é evidente que esses protocolos incluem muitas etapas de manipulação e exposição da amostra. Dessa forma, eles estão sujeitos a uma quantidade maior de artefatos e erros experimentais (44; 45; 46; 47).

Os métodos mais utilizados para resolver proteínas ou peptídeos das amostras são: a eletroforese em gel de poliacrilamida (GE) e a cromatografia líquida (LC). Ambos as técnicas já estão bem estabelecidas e diversas variações e protocolos estão disponíveis na literatura, de acordo com o tipo de amostra a ser analisada (38; 39; 47; 48; 49). Em relação às técnicas de identificação de proteínas, a espectrometria de massas (MS) tem se destacado no cenário mundial, com o desenvolvimento de equipamentos rápidos, de alta precisão e grande resolução (50; 51; 52; 53).

Contudo, a reprodutibilidade dos experimentos de proteômica vem sido discutida na literatura e em sociedades especializadas, com isto surgem questionamentos entre pesquisadores da área (54; 55). Atualmente é consenso que dois pontos fundamentais para a credibilidade dos experimentos são a descrição detalhada dos métodos de preparação das amostras e os parâmetros utilizados para processamento dos dados obtidos por MS.

O outro ponto que vem sendo discutido diz respeito à descrição dos experimentos em artigos científicos (54; 56). Nem sempre os protocolos utilizados em um laboratório são reproduzidos apropriadamente em outro laboratório para análise de uma determinada amostra, devido, principalmente, à falta de detalhamento dos métodos nas publicações. Com o intuito de evitar este problema a HUPO (*Human Proteomics Organization*) recrutou um grupo de especialistas para padronizar os requerimentos mínimos necessários para a descrição de um experimento de proteoma (MIAPE: *Minimum Information About* a *Proteomic Experiment*) (56; 57). Esta iniciativa visa solicitar aos pesquisadores que sejam objetivos sobre todas as etapas do procedimento realizado com a amostra – desde seu cultivo até a análise estatística dos resultados. O MIAPE não pretende definir como os experimentos devem ser realizados, mas sim como eles devem ser descritos para publicação.

Uma descrição detalhada dos métodos de preparação das amostras e dos parâmetros utilizados para processamento dos dados por MS são ainda mais relevantes quando relacionados a experimentos de análise proteômica comparativa. Nesses casos, deseja-se obter informações sobre proteínas expressas diferencialmente entre duas condições. Portanto, é extremamente importante que variações experimentais que possam originar artefatos sejam evitadas. Tendo em vista que a espectrometria de massas é uma técnica de grande sensibilidade (capaz de detectar fentogramas de proteínas), pequenas diferenças de processamento poderão dar origem a alterações detectáveis por MS, que, no entanto não representam variações biológicas reais.

Recentemente a técnica de DIGE (Differential Gel Electrophoresis), que utiliza marcadores fluorecentes e eletroforese bidimensional, foi apontada como uma alternativa a análise tradicional por géis bidimensionais para análises proteômicas

comparativas. Nesse método as proteínas de cada amostra a ser comparada são marcadas com uma determinada cianina fluorecente. Com isso, as amostras a serem comparadas podem ser misturadas e separadas em um mesmo gel de eletroforese. Ao final da separação, o gel contendo a mistura das duas amostras pode ser lido num *scaner* de fluorescência, nos comprimentos de onda de emissão dos fluoróforos utilizados, gerando duas ou mais imagens a partir de um mesmo gel. Este procedimento facilita a etapa de comparação dos géis. Contudo, artefatos experimentais na etapa de extração das proteínas não são evitadas e as limitações de géis bidimensionais em relação a separação de proteínas básicas, hidrofóbicas e de grande peso molecular permanecem.

Nos últimos anos foram descritos novos métodos para análise quantitativa da expressão de proteínas por MS (58; 59; 60; 61). Entre os mais usados estão a marcação química das amostras, a marcação metabólica e métodos independentes de marcação ong(62; 63; 64; 65). Dentre esses métodos quantitativos, os que lidam com a marcação metabólica de células em cultura apresentam a vantagem de permitir a mistura das duas ou mais amostras a serem comparadas no início do processamento. A marcação metabólica é realizada através da incorporação de isótopos estáveis (C^{14}, N^{15}, O^{18}, H^{3}, entre outros) durante o crescimento das células no meio de cultura. A incorporação desses isótopos nas moléculas biológicas, durante o cultivo celular, causa um aumento previsível na massa de todas as proteínas, em princípio sem alterar suas propriedades físico-químicas. Dessa forma, as amostras a serem comparadas podem ser cultivadas cada uma na presença de um isótopo diferente e misturadas antes da análise. Após a extração, separação e digestão das proteínas, a amostra de origem de cada peptídeo pode ser facilmente detectada pela análise do espectro de MS: peptídeos oriundos de células crescidas

na presença de um isótopo pesado estarão deslocados para valores de massa maiores do que peptídeos oriundos de células crescidas na presença de um isótopo leve (62; 66; 67).

Dessa forma, uma das grandes vantagens dos métodos de marcação metabólica é possibilitar todo o processamento de duas amostras em conjunto, incluindo a aquisição dos espectros de massa, visando a minimização de artefatos e variações experimentais. Os primeiros protocolos de marcação metabólica para análise proteômica descrito utilizaram a marcação isotópica através de aminoácidos (65). Como uma alternativa mais barata aos aminoácidos marcados com isótopos pesados, tem sido utilizados sais contendo os isótopos, principalmente sais nitrogenados (68).

Por todos esses motivos, a primeira parte do estudo descrito a seguir foi a otimização de cada etapa da análise proteômica, de forma a estabelecer um protocolo de marcação metabólica das proteínas, rápido, com o menor número de etapas possível, minimizando as perdas e evitando erros experimentais. Em seguida, o procedimento considerado o mais adequado foi aplicado à análise proteômica comparativa da interação entre *G. diazotrophicus* e cana-de-açúcar dos genótipos SP-70 e Chunee.

2- OBJETIVOS

2.1- Objetivo geral

Estudar as respostas induzidas durante a interação entre a bactéria *G. diazotrophicus* e dois genótipos de cana-de-açúcar, cada um com distinta contribuição da fixação biológica de nitrogênio, pela expressão diferencial de proteínas.

2.2- Objetivos específicos

1- Estabelecer um método eficiente para a incorporação metabólica de isótopos estáveis (sais contendo ^{14}N ou ^{15}N) durante o co-cultivo de células de *G. diazotrophicus* e plantas de cana-de-açúcar para a quantificação de peptídeos por espectrometria de massas;

2- Identificar proteínas de *G. diazotrophicus* diferencialmente expressas durante a interação com cana-de-açúcar do genótipo SP-70

3- Identificar proteínas de *G. diazotrophicus* diferencialmente expressas durante a interação com cana-de-açúcar do genótipo Chunee

4- Comparar as respostas induzidas na *G. diazotrophicus* pelos genótipos SP-70 e Chunee de cana-de-açúcar

5- Identificar proteínas diferencialmente expressas pela cana-de-açúcar do genótipo SP-70 durante a interação com *G. diazotrophicus*

6- Identificar proteínas diferencialmente expressas pela cana-de-açúcar do genótipo Chunee durante a interação com *G. diazotrophicus*

7- Comparar as respostas induzidas nos genótipos SP-70 e Chunee de cana-de-açúcar pela *G. diazotrophicus*

8- Identificar proteínas de *G. diazotrophicus* expressas nas amostras das raízes de ambos os genótipos de cana-de-açúcar

3– MATERIAIS E MÉTODOS

3.1- Cepas bacterianas, meios e condições de cultivo

A linhagem PAL5 de *G. diazotrophicus* (BR11281) foi cultivada em meio de cultura LGI-P: 0,2 g/L K_2HPO_4, 0,6 g/L KH_2PO_4, 0,2 g/L $MgSO_4.7H_2O$, 0,02 g/L $CaCl_2.2H_2O$, 0,002 g/L $Na_2MoO_4.2H_2O$, 0,01 g/L $FeCl_3.6H_2O$, 5 ml/L de azul de bromotimol a 0,5 % em 0,2 M KOH, 10 g/L sacarose contendo 10 mM de $(NH_4)_2SO_4$ por 36 horas a 30 ºC, que neste caso corresponde ao início da fase exponencial de crescimento. A cultura foi centrifugada a 18,000 g, 10 min, 4 ºC e as células foram lavadas com tampão PBS. Cerca de 5 x 10^6 células foram inoculadas no mesmo volume de meio MS diluído dez vezes: MS/10: 0,75 mM $MgSO_4$, 0,65 mM KH_2PO_4, 1,5 mM $CaCl_2$, 0,05 mM $CoCl_2.6H_2O$, 0,16 mM $CuSO_4.5H_2O$, 45 mM FeNaEDTA, 23 mM H_3BO_3, 2,5 mM KI, 50 mM $MnSO_4.H_2O$, 0,26 mM $Na_2MoO_4.2H_2O$, 0,36 mM $ZnSO_4.7H_2O$, 4,6 mM $MnCl_2.4H_2O$, 0,05 mM NH_4NO_3, 2 mM KNO_3, 2 g/l sacarose e incubadas por 8 horas a 30 °C e 400 rpm.

3.2- Crescimento de plantas de cana-de-açúcar *in vitro* e co-cultivo com *G. diazotrophicus*

Os genótipos de cana-de-açúcar SP70-1143 (grande contribuição da FBN) e Chunee (pouca contribuição da FBN) de cana-de-açúcar (6) foram utilizados neste estudo. Plantas de cana-de-açúcar livres de microorganismos foram obtidas a partir do cultivo do meristema apical e micropropagadas de acordo com o método de Hendre *e* colaboradores (69). Todas as plantas foram mantidas a 30°C com irradiação de 60 µmol de fótons por m^{-2} s^{-1} por 12 h por dia. Plantas de cana-de-açúcar enraizadas foram inoculadas com *G. diazotrophicus* como descrito por Reis e colaboradores (70) usando 0.1 ml de uma suspensão contendo 10^6 células. Plantas controles foram inoculadas apenas com o meio de cultivo. Vinte e quatro horas após, o meio de co-cultivo e as raízes das plantas foram coletadas e as amostras

processadas. Um conjunto de seis plantas constituiu cada amostra, que foi coletada em duplicata. Ainda, as plantas foram utilizadas para a quantificação da colonização bacteriana pelo Método do Número mais Provável (MPN; de acordo com Reis e colaboradores (31)).

3.3- Marcação metabólica de células da bactéria e da planta por incorporação metabólica de isótopos $^{14}N/^{15}N$

A marcação metabólica ocorreu pela incorporação de isótopos $^{14}N/^{15}N$ fornecidos na forma de sais durante o crescimento celular. Amostras crescidas em meio ^{14}N (isótopo natural mais abundante de nitrogênio) foram denominadas amostras não-marcadas e as crescidas em meio contendo ^{15}N enriquecido em 98 % foram denominadas marcadas. Proteínas bacterianas e da cana-de-açúcar foram marcadas por incubação em meio contendo os sais $^{15}NH_4{}^{15}NO_3$ e $K^{15}NO_3$ (*Cambridge Isotope Laboratories Inc*) em quantidades iguais as de NH_4NO_3 e KNO_3 (seção 3.1). A marcação das proteínas das plantas aconteceu durante os períodos de enraizamento, multiplicação e interação (não apenas nas 24 horas de interação com a bactéria). Bactérias e plantas não marcadas (^{14}N) foram cultivadas em paralelo às amostras marcadas com ^{15}N, nas mesmas condições. Foram realizados dois experimentos independentes, além da marcação reversa em cada um deles.

A estratégia geral utilizada neste estudo está esquematizada na Figura 3.

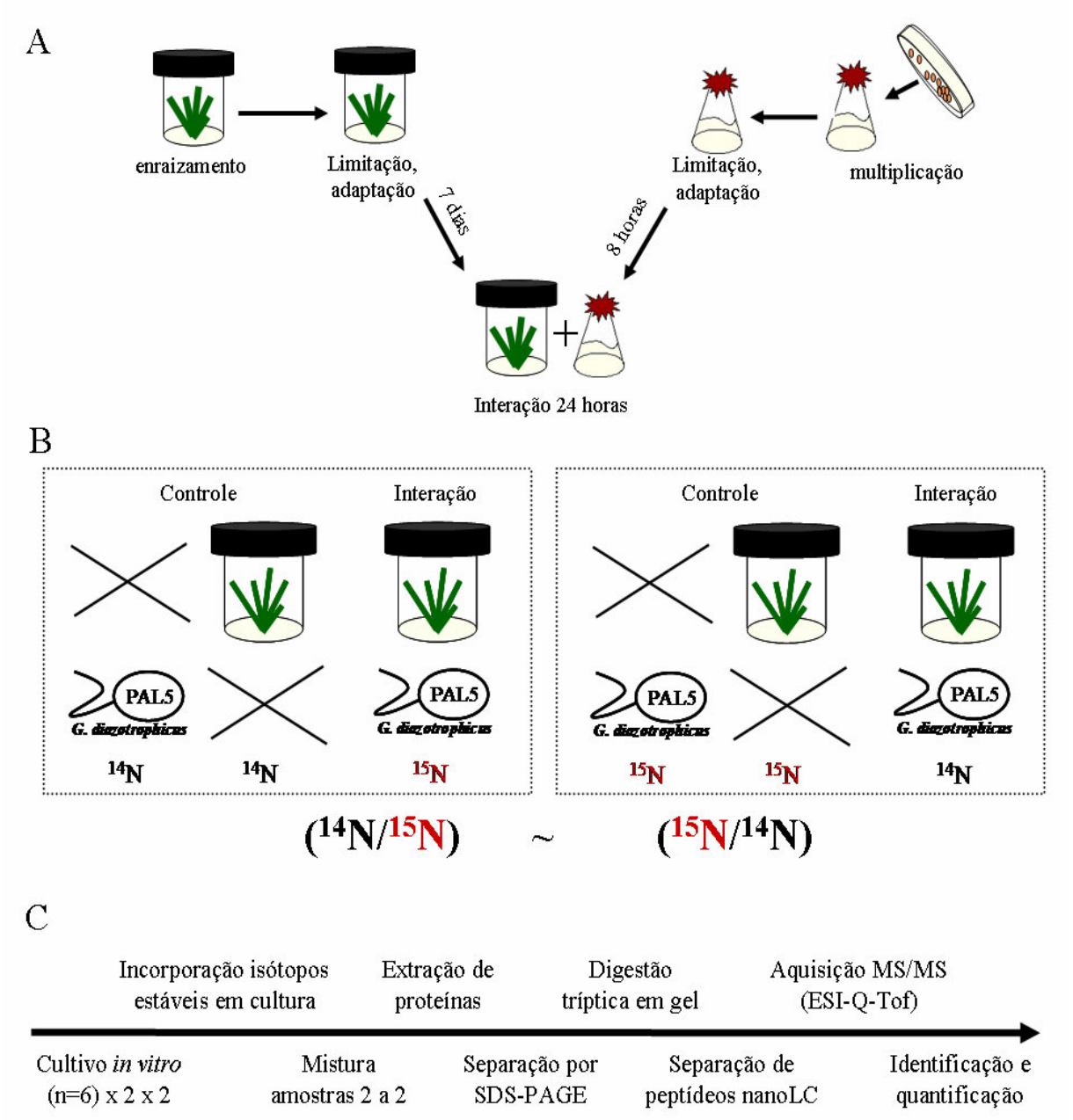


Fig. 3: Procedimentos realizados para obtenção das amostras para a análise proteômica diferencial da interação entre *G. diazotrophicus* e cana-de-açúcar SP-70 e Chunee. A) Cultivo da bactéria, da planta e co-cultivo. Para a obtenção dos controles foi utilizado o mesmo protocolo substituindo o co-cultivo pelo cultivo independente. B) Cultivos isolados (controles) e co-cultivo em meio de cultura definido contendo sais nitrogenados marcados (^{15}N) ou não-marcados (^{14}N). Foi utilizada também a estratégia de marcação reversa. C) Etapas realizadas para a análise da expressão diferencial de proteínas.

3.4- Métodos de extração de proteínas

A densidade ótica a 600 nm das suspensões de células bacterianas coletadas no experimento foi obtida por análise espectrofotométrica. Volumes normalizados das suspensões bacterianas das amostras controle e em co-cultivo com a planta (uma marcada e outra não-marcada) foram misturados. A mistura foi centrifugada por 10 min a 10000 g e o sedimento de células ressuspendido em tampão Laemmli para SDS-PAGE (71) e aquecidas por 10 minutos a 100 ºC. Um sobrenadante foi obtido por centrifugação a 14000 g por 45 min a 4 ºC.

As plantas coletadas foram lavadas em água Mili-Q estéril por 5 min e em seguida em etanol 75% por 5 min, com agitação, através do uso de barras magnéticas. O excesso de líquido das plantas foi escorrido em papel de filtro. Com o auxílio de pinças e tesoura, as raízes foram coletadas e pesadas. 100 mg de tecido das amostras controle e inoculada com *G. diazotrophicus* (uma marcada e outra não-marcada) foram misturadas, congeladas em N_2 líquido e maceradas com um pilão até que o tecido estivesse despedaçado (2 a 3 min). O material resultante desta maceração inicial foi transformado a um pó fino com o *Sample Grinding Kit* (GE *Healthcare*). A seguir, as proteínas foram extraídas com o *Plant Total Protein Extraction Kit* (Sigma) e a solução contendo as proteínas solúveis foi obtida por centrifugação a 14000 g por 30 min a 4 ºC.

3.5- Separação de proteínas e digestão tríptica em gel

A separação por SDS-PAGE (71) foi realizada em gel a 12,5 % no sistema Mini Protean II (Bio-Rad), utilizando 20 µg de proteínas por amostra. Os géis foram corados com 0,1 % de azul brilhante de Coomasie G-250 em 30 % de etanol e as imagens foram adquiridas no *LabScan* 5 (GE *Healthcare*). Os géis de SDS-PAGE foram divididos em 5 a 10 pedaços de mesmo tamanho. Cada um desses pedaços

de gel foi reduzido, alquilado e digerido com tripsina como descrito anteriormente (38).

3.6- Análise por ESI-Q-TOF

A cromatografia líquida seguida de espectrometria de massas (LC-MSMS) foi realizada no equipamento Q-TOF Ultima API (*Waters*), do Laboratório Nacional de Luz Síncroton, para analisar os peptídeos trípticos das proteínas separadas por SDS-PAGE. Uma fonte de *nanoelectronspray* (ESI) foi utilizada e medidas do calibrante ácido fosfórico a 0,1 % foram obtidas durante toda a corrida cromatográfica, com intervalos de 30 s. As proteínas digeridas foram inicialmente dessalinizadas diretamente usando a coluna Waters Opti-Pak C18. A mistura de peptídeos foi separada por eluição com um gradiente de 10 a 90 % de acetonitrila em água e 0.1 % ácido fórmico em uma coluna capilar Nanoease C18 (75 µm ID). Os espectros de massas foram adquiridos no modo de "aquisição dependente dos dados" (DDA: *data dependent aquisition*) e peptídeos multi-carregados (+2 e +3) foram automaticamente selecionados e dissociados em experimentos de MS/MS. Condições típicas da LC e ESI foram: fluxo de 200 nl/min, voltagem no capilar de 3 kV, temperatura do bloco 100 °C e voltagem do cone a 100 V. Os espectros foram visualizados no programa *MassLynx* versão 4.1 (Waters).

3.7- Identificação de proteínas

Os parâmetros padrões do arquivo Q-Tof.opt do *Mascot Distiller* (disponível em www.matrixscience.com.br em Julho de 2009) foi usado para processamento dos espectros. A calibração de massa foi baseada na média de 3 aquisições da massa 784,8230, com tolerância de +- 0,3 Da. Os espectros de MS/MS foram processados usando o software Mascot Distiller versão 2.3.0 (*MatrixScience*) e as listas de picos

geradas foram comparadas ao banco de proteínas de *G. diazotrophicus* e de *Arabdopsis thaliana* usando o MASCOT *search engine tool* (*MatrixScience*).

As buscas em bancos de dados foram realizadas considerando a especificidade da digestão tríptica, uma clivagem perdida e tolerância de massa de 0,1 Da para peptídeos e fragmentos de peptídeos. Oxidação de resíduos de metionina e carbamidometilação de cisteínas foram consideradas modificações varáveis; outras modificações foram procuradas apenas nos casos explicitados ao longo do texto. Todos os peptídeos com $p < 0,05$ na identificação foram considerados para inspeção manual. A identificação das proteínas foi baseada em pelo menos um peptídeo único (uma sequência de peptídeo exclusiva de uma sequência protéica do banco de dados). No caso de uma proteína aparecer na lista de identificação com nomes ou números de acesso distintos, a entrada com maior cobertura de sequência foi selecionada.

3.8- Quantificação relativa e análise estatística

Para a quantificação relativa baseada na marcação metabólica $^{14}N/^{15}N$, o pacote de quantificação do *Mascot Distiller* (*Matrix Science*) foi utilizado. O arquivo *quantitation.xlm* (disponível no site da *MatrixScience* em Julho de 2009) foi modificado de acordo com as necessidades específicas desta análise: o percentual de incorporação de ^{15}N foi ajustado para 25% (amostras bacterianas) e 50% (amostras de plantas), a integração quadrática da intensidade do pico precursor foi realizada em todo o envelope isotópico, o mínimo de 1 peptídeo único, a razão $^{14}N/^{15}N$ para uma proteína foi calculada com base na média geométrica das razões $^{14}N/^{15}N$ de cada um de seus peptídeos e normalização foi feita em relação a média. O desvio padrão do valor de quantificação para a razão $^{14}N/^{15}N$ de uma proteína foi calculado de forma a verificar a presença de valores anormais e a consistência das

medidas para uma distribuição normal. Variações significativas nos níveis de expressão de uma proteína foram informadas quando a razão calculada foi significativamente diferente de 1. O teste de comparação realizado pelo programa *Mascot Distiller* foi: $|x-\mu| \leq t\,(s/\sqrt{N})$, onde x a média das razões dos peptídeos, μ é o valor da razão $^{14}N/^{15}N$ a ser testado, t é o valor do teste de *student t* , bilateral, para N-1 graus de liberdade e 95 % de confiabilidade, s é o desvio padrão e N é o número de razões de peptídeos. O desvio padrão é um fator que permite calcular o intervalo de confiança dos dados. Este é calculado através da divisão e multiplicação da média pelo valor do desvio padrão.

3.9- Anotação de seqüências de proteínas por ferramentas de bioinformática

Proteínas anotadas como hipotéticas, putativas ou de função desconhecida foram analisadas por bioinformática para tentar prever alguma função ou para obter alguma característica estrutural que pudesse relacioná-la a uma possível função. A busca por sequências similares ou homológas foi realizada através do Blastp (72). A busca por domínios funcionais foi feita no *Protein Families database* (PFAM; (73)) e no TIGR CMR (https://cmr.jcvi.org). A presença de peptídeo sinal e regiões transmembranares foi investigada com os programas SignalP (74) e TMHMM (75) e PredTMBB (76), respectivamente.

3.10- Modelagem molecular comparativa

Para a construção do modelo da estrutura tridimensional de uma proteína foi escolhida uma seqüência molde através de buscas de similaridade com as seqüências depositadas no PDB (77), com o programa *Blastp* (72). O alinhamento entre a sequência da proteína molde e a sequência alvo foi feito com o programa *ClustalW* (78). Utilizando este alinhamento, 100 modelos tridimensionais foram gerados por modelagem comparativa através do programa *Modeller* (79). Os valores

do *Dope score* e as restrições estereoquímicas (também gerados pelo *Modeller*) de cada um desses modelos foram avaliados e o modelo que apresentou melhor qualidade de acordo com estas análises – menor energia e maior percentual de resíduos em áreas permitidas do gráfico de Ramachandran - foi utilizado nos passos seguintes. O potencial eletrostático da superfície do molde e do modelo foi calculado com o pacote APBS (80) do programa *Pymol* versão 1.2 (81).

4– RESULTADOS E DISCUSSÃO

Os experimentos realizados inicialmente visaram verificar a viabilidade de cada uma das etapas do projeto e o ajuste das condições necessárias à obtenção de dados confiáveis. Após a etapa de otimização, foram realizados experimentos de interação entre *G. diazotrophicus* e cana-de-açúcar dos genótipos SP-70 e Chunee e análise proteômica diferencial entre essas amostras e seus respectivos controles. Os resultados obtidos serão apresentados a seguir, de acordo com a lista dos oito objetivos específicos.

4.1- Estabelecimento de uma estratégia proteômica quantitativa para o estudo da interação bactéria-hospedeiro

4.1.1- Justificativa da estratégia utilizada

O entendimento da interação microrganismo-hospedeiro é importante não apenas para as ciências básicas, mas também para aplicações em medicina, saúde pública, agricultura, entre outros. Por isso, diversas moléculas envolvidas em mecanismos de sinalização e reconhecimento de patógenos humanos por células eucariotas já foram descritas (82; 83; 84; 85; 86). No entanto, existem poucos estudos direcionados a proteínas envolvidas na interação entre plantas e bactérias simbiontes (37; 40; 42). Por exemplo, proteínas envolvidas na interação de *G. diazotrophicus* com cana-de-açúcar dos genótipos SP-70 e Chunee ainda não foram descritas. Assim, usando estes organismos como modelo, a análise proteômica em larga escala a que este trabalho se propõe visa preencher esta lacuna.

Um dos métodos mais confiáveis para quantificação relativa de proteínas combina a marcação metabólica de proteínas de células em cultura e sua quantificação por espectrometria de massas (62; 87; 88). Os resultados acurados obtidos com esta técnica são possíveis devido à possibilidade de misturar as duas amostras a serem comparadas (uma marcada e outra não-marcada) imediatamente após a sua coleta

e processá-las em conjunto para minimizar os erros. Os principais passos num protocolo de quantificação compreendem a incorporação dos isótopos durante o cultivo celular, métodos eficientes de extração, separação e digestão de proteínas, separação de peptídeos por cromatografia líquida de alta resolução e aquisição de dados de MS/MS com grande acurácia (89).

Até o momento, a utilização dos sais $^{15}N/^{14}N$ tem sido a estratégia mais bem sucedida para a marcação metabólica com isótopos em amostras de plantas (66; 68; 90; 91). Esta estratégia também já foi utilizada para o estudo de bactérias e leveduras (92; 93). No entanto, esta metodologia nunca foi utilizada para a análise de sistemas complexos como, por exemplo, o co-cultivo de uma bactéria com seu hospedeiro. Por esta razão, fez-se necessária uma otimização dos protocolos disponíveis na literatura para o estudo da interação entre *G. diazotrophicus* e cana-de-açúcar. De uma forma geral, este estudo utilizou as técnicas de cultivo bacteriano, cultivo de plantas *in vitro*, marcação isotópica com sais $^{15}N/^{14}N$, eletroforese unidimensional (1D-GE), cromatografia líquida e espectrometria de massas (LC-ESI-Q-Tof) para quantificação relativa de proteínas. A estratégia de marcação reversa também foi utilizada para evitar desvios causados por uma possível incorporação diferencial desses isótopos. A estratégia geral utilizada neste estudo está esquematizada na Figura 3.

4.1.2- Avaliação de cada etapa dos métodos

Cada uma das etapas desse procedimento foi testada através de pequenas variações (descritas logo abaixo) dos métodos descritos na seção 3 (Material e Métodos). Esses testes foram realizados para escolher os protocolos mais adequados às análises pretendidas. Assim, nesta seção estão descritas as razões

das escolhas feitas, na tentativa de esclarecer esses métodos, assim como a qualidade dos resultados obtidos.

Para avaliar a etapa de incorporação de isótopos estáveis nas amostras da bactéria e da planta, a incorporação de ^{15}N foi estimada como uma média da incorporação de ^{15}N (de acordo com variações no padrão de distribuição do envelope isotópico) de aproximadamente 20 peptídeos diferentes em cada amostra. As amostras de bactérias e plantas cultivadas em ^{15}N apresentaram aproximadamente 25 % e 50 % de incorporação de ^{15}N, respectivamente. Esses níveis de marcação asseguram uma quantificação confiável, pois os envelopes isotópicos de amostras marcadas e não-marcadas são bastante distintos (ver exemplo na Figura 7. Para uma revisão sobre a quantificação relativa por incorporação metabólica de ^{15}N e como calcular o percentual de ^{15}N efetivamente incorporado em uma amostra, recomenda-se ler a referência (66)). Além disso, essas taxas de incorporação foram alcançadas com pouco tempo de cultivo e pouca manipulação das amostras, possibilitando a obtenção de organismos mais saudáveis (Fig. 4). Os valores de incorporação parcial calculados foram utilizados como fator de correção para o cálculo de quantificação (93) no *Mascot Distiller*.

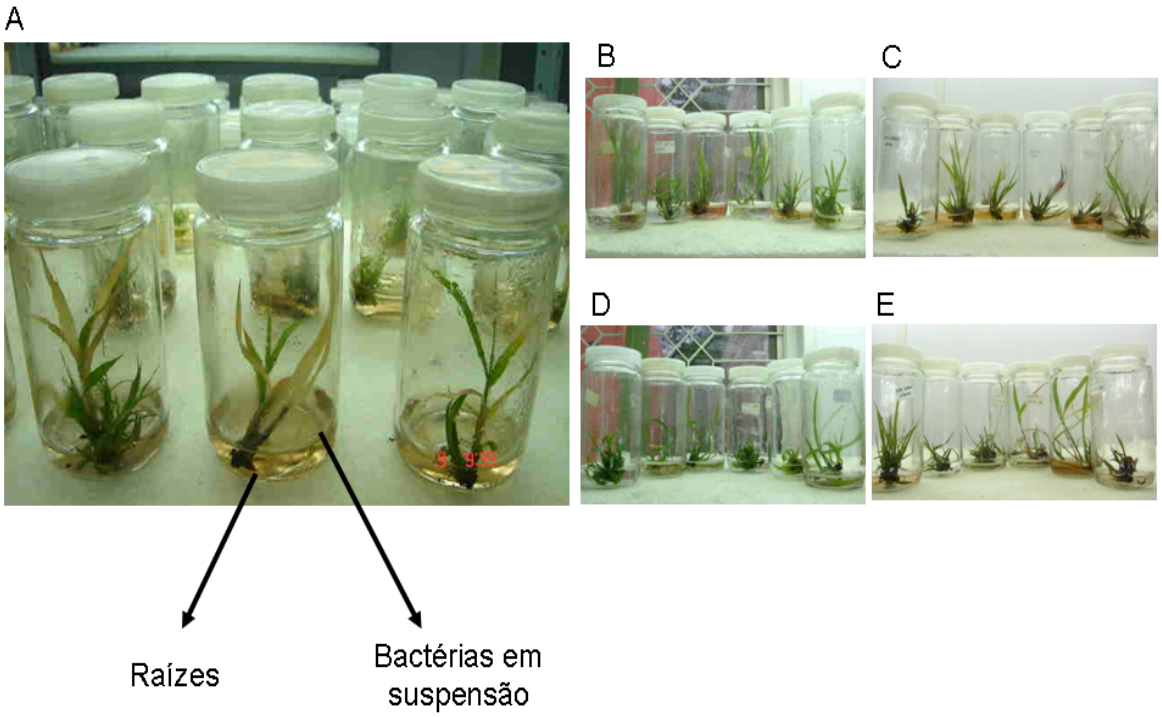


Fig. 4: Representação das amostras coletadas. Os experimentos de interação entre *G. diazotrophicus* e plantas de cana-de-açúcar SP-70 e Chunee foram realizado em meio líquido e potes estéreis (A). Cada amostra foi constituída por um conjunto de 6 plantas posicionadas de forma aleatória na bancada durante o período de cultivo, com o intuito de obter quantidade de material suficiente e minimizar variações individuais. B e C representam as amostras coletadas nos experimentos de marcação e marcação reversa da interação de PAL5 com SP-70 e D e E representam as amostras coletadas nos experimentos de marcação e marcação reversa da interação de PAL5 com Chunee. Para cada amostra, foram coletados o meio de cultura contendo bactérias em suspensão e raízes. Esse experimento completo foi realizado 2 vezes.

Em seguida, a eficiência dos métodos de separação e extração de proteínas foi determinada em géis de SDS-PAGE. Os métodos de extração utilizados (seção 3.4) foram simples e rápidos, minimizando a degradação e perdas de proteínas. As amostras de bactérias e raízes das plantas apresentaram bandas com massa molecular relativa entre 10 to 120 kDa bem separadas em géis de poliacrilamida 12,5 % (Fig. 5). Conforme descrito na seção 3.4, para a análise proteômica diferencial proposta, as amostras a serem comparadas (controle e de co-cultivo)

foram misturadas após a coleta e processadas juntas – inclusive a etapa de extração de proteínas. Dessa forma, os géis de eletroforese unidimensional mostram, em cada coluna, a combinação de uma amostra controle com uma amostra de co-cultivo. Portanto, as amostras a serem comparadas estão numa mesma coluna e a comparação entre colunas de um gel não indica expressão diferencial.

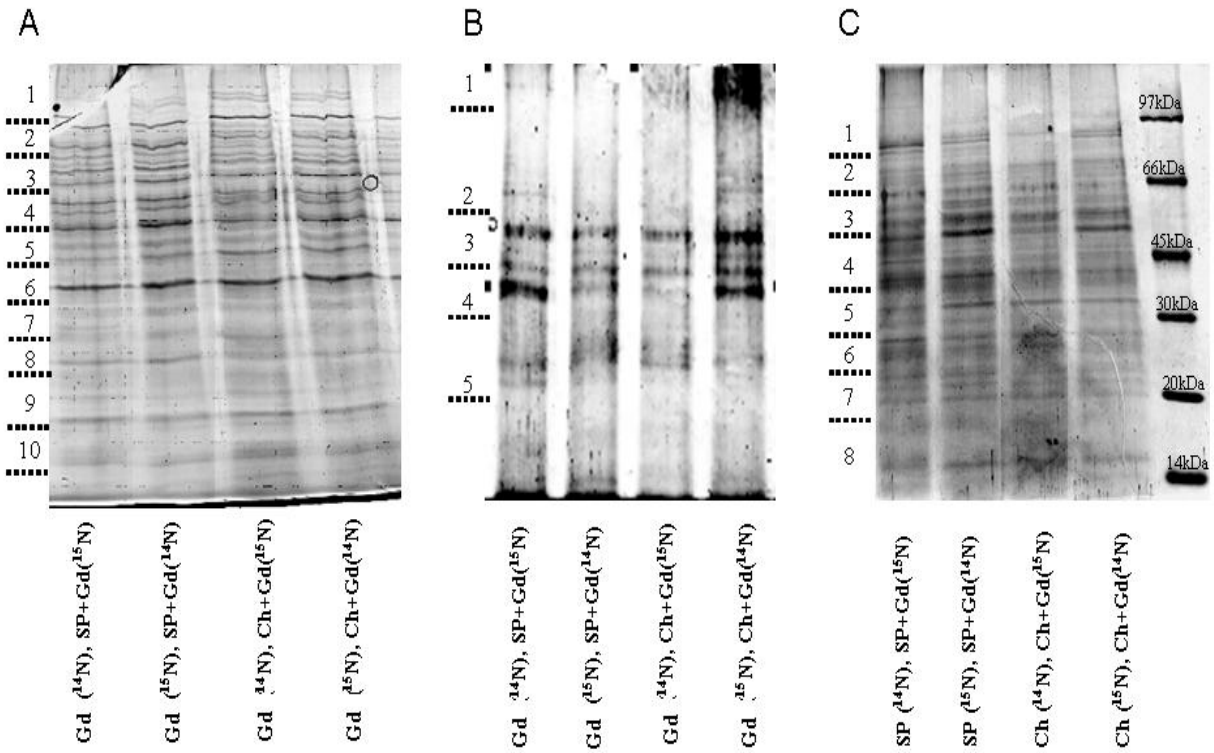


Fig. 5: Separação de proteínas por SDS-PAGE em gel a 12,5 %. (A) Lisado total de *G. diazotrophicus* e (B), sedimento do lisado de *G. diazotrophicus*. Em (C) são as amostras de raízes de cana-de-açúcar. Cada amostra controle foi misturada a uma amostra do co-cultivo em quantidades iguais, ver seção 3.4, de forma que cada coluna no gel mostra o conjunto de proteínas dessas misturas. Cada coluna do gel foi dividida em partes (indicadas por números) antes da digestão tríptica, totalizando 92 frações.

Da mesma forma foi importante avaliar a resolução dos picos cromatográficos, ou seja, o método de separação dos peptídeos. Embora o programa de processamento de espectros utilizado, o *Mascot Distiller*, seja capaz de corrigir alguns desvios,

quanto maior a resolução dos picos cromatográficos, ou seja, menor o tempo de eluição de um peptídeo, e co-eluição dos peptídeos ^{14}N e ^{15}N, melhor para a quantificação relativa de proteínas. Uma outra característica que nos permite avaliar a eficiência da separação dos peptídeos é a dispersão dos peptídeos obtidos por digestão tríptica ao longo da separação cromatográfica. Assim, o fluxo, o gradiente, o método de seleção dos peptídeos para MS/MS, critérios de exclusão, entre outros foram parâmetros ajustados de forma a se obter cromatogramas de alta resolução e espectros de grande intensidade. Os peptídeos analisados, em todas as amostras, apresentaram perfis variados de hidrofobicidade, visto que foram separados ao longo dos 60 minutos da cromatografia numa coluna de C18 com um gradiente linear de acetonitrila entre 10 e 90 %. A Figura 6 ilustra a qualidade dos dados obtidos por LC-MS/MS. A obtenção de cromatogramas com picos (peptídeos) bem distribuídos ao longo do tempo é importante para permitir que o maior número possível de peptídeos seja fragmentado e que o seu espectro de MS/MS seja adquirido durante o ápice de eluição cromatográfica de cada pico.

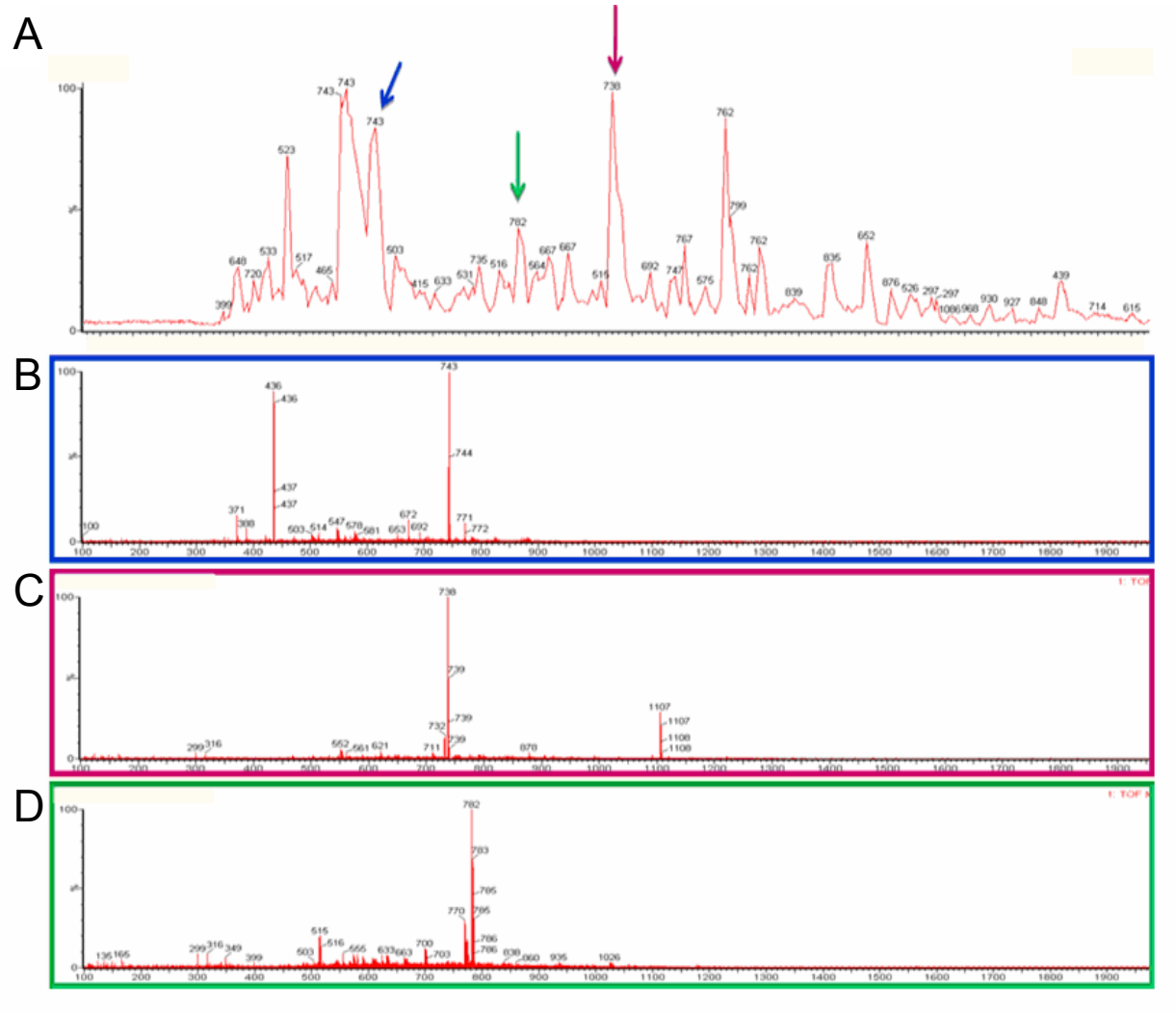


Fig. 6: Resolução cromatográfica. A) Cromatograma de uma das 92 amostras analisadas, representativo da qualidade de todas elas. As setas coloridas apontam três picos cromatográficos. (B), (C) e (D) Espectros de MS adquiridos nos tempos indicados pelas setas e cores. Notar que cada espectro de MS apresenta poucos picos (peptídeos), o que demonstra que a separação cromatográfica foi eficiente.

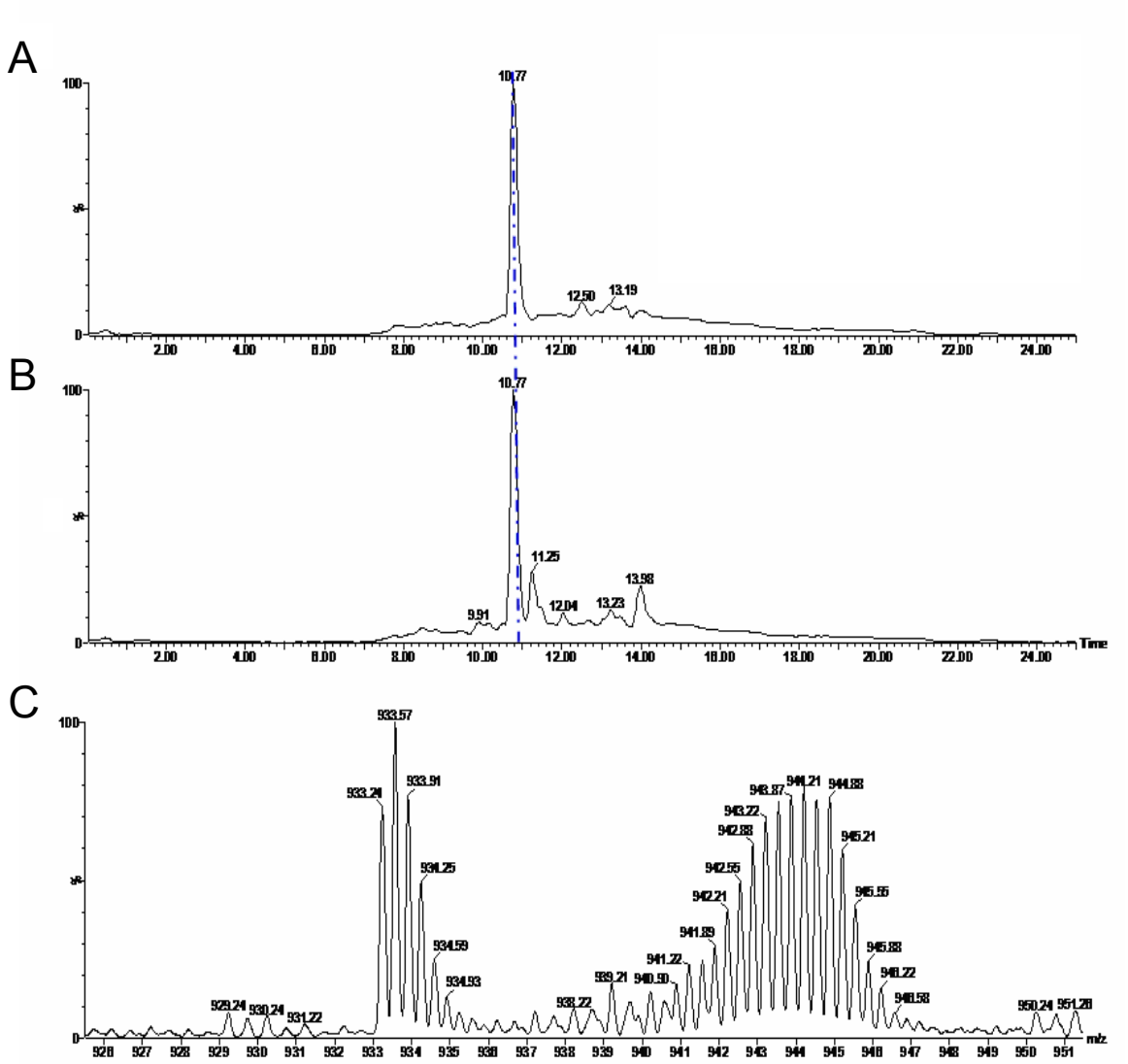


Fig. 7: Co-eluição de um par de peptídeos marcado (^{15}N) e não marcado (^{14}N). O perfil de eluição cromatográfica de cada um desses peptídeos foi registrado separadamente no programa MassLynx e estão representados em (A) e (B), respectivamente. Em (C) está representada uma região de um espectro de MS que apresenta um par de peptídeos marcado e não marcado. À esquerda (m/z em torno de 933 Da) está o envelope isotópico do peptídeo não marcado e, à direita (m/z em torno de 944 Da) o envelope isotópico do peptídeo marcado.

Após a aquisição dos espectros com as especificações descritas acima, foi realizada a quantificação relativa dos peptídeos. A comparação da intensidade dos picos correspondentes a diferentes peptídeos em um espectro de MS não revela

nenhuma informação quantitativa, pois peptídeos com sequência distinta de aminoácidos apresentam diferentes propriedades de ionização. No entanto, a marcação com ^{15}N não modifica essa característica do peptídeo. Assim, o método quantitativo que utiliza amostras marcadas com ^{15}N é baseado num incremento da massa do peptídeo marcado em relação ao mesmo peptídeo não-marcado. E neste caso, a intensidade dos picos pode ser comparada, refletindo a abundância de cada um nas amostras originais (94).

Uma característica muito interessante deste método é que cada peptídeo é detectado em diversos espectros de MS ao longo da sua eluição por LC. O Q-TOF utilizado para aquisição desses dados adquire um espectro de MS por segundo. Como, em geral, o tempo de eluição de cada peptídeo é maior que um segundo - depende de características instrínsecas do peptídeo e de sua abundância - cada peptídeo é detectado em vários espectros de MS. Como a quantificação é baseada no pico do peptídeo no espectro de MS, e não no MS/MS, é possível calcular a razão entre a intensidade dos peptídeos leve e pesado para cada espectro de MS. O gradiente da reta que representa o mínimo quadrado deste gráfico é a melhor estimativa possível da razão ^{15}N /^{14}N (89). Esta estratégia é tão robusta que pequenas variações podem ser detectadas com alta confiabilidade. Além disso, foram avaliados os valores de desvio padrão entre as razões calculadas para cada peptídeo de uma mesma proteína. As proteínas que apresentaram valores de desvio padrão altos não foram consideradas como diferencialmente expressas. Por fim, a análise de marcação reversa aumentou a confiabilidade dos dados, visto que foi observada uma correspondência de aproximadamente 95 % entre os experimentos I ($^{15}N/^{14}N$) e II ($^{14}N/^{15}N$), como observado visualmente no exemplo da Figura 8.

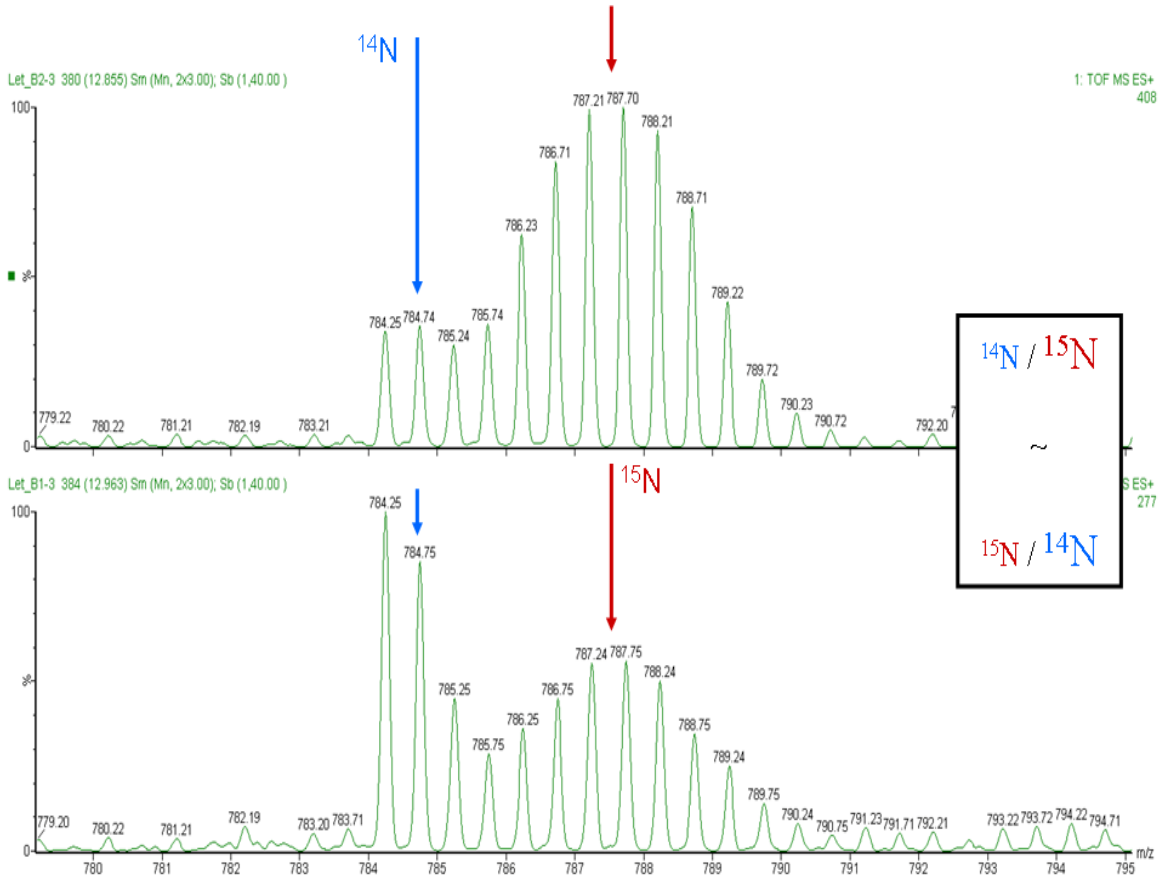


Fig. 8: Correspondência entre os experimentos I (marcação) e II (marcação reversa). Na parte superior da figura é possível observar a baixa intensidade do peptídeo de razão massa / carga 784,7 e grande intensidade dos picos correspondentes a este mesmo peptídeo marcado com ^{15}N, para uma amostra do experimento I. Na parte inferior da figura, está representada a mesma região do espectro, para uma amostra do experimento II (marcação reversa) e é possível observar o peptídeo não-marcado mais intenso do que o peptídeo marcado.

4.2- Proteínas de *G. diazotrophicus* diferencialmente expressas durante a interação com cana-de-açúcar SP-70

Como descrito na seção 3.5 do Material e Métodos e Fig. 3, para identificar proteínas de *G. diazotrophicus* envolvidas no estabelecimento de uma interação supostamente eficiente com a cana-de-açúcar, comparamos o perfil de expressão de proteínas de células bacterianas co-cultivadas por 24 horas com cana-de-açúcar do genótipo SP-70 com o da bactéria na condição controle. A suspensão de células do

co-cultivo foi misturada à cultura bacteriana controle, e a amostra foi processada para análise proteômica, conforme descrito na seção 3.4 e Fig.3. Este procedimento foi realizado para a quadriplicata: 2 replicatas biológicas, cada uma com a marcação e a marcação reversa.

De uma forma geral, essas análises permitiram a identificação de 278 proteínas diferentes expressas por *G. diazotrophicus*. Após a verificação da consistência dos dados entre as replicatas biológicas, isto é, quando a razão da quantificação da amostra controle / amostra da interação for aproximadamente a mesma entre as replicatas, e nos experimentos de marcação reversa, a análise quantitativa revelou a expressão diferencial de 34 proteínas (cerca de 12 % das proteínas identificadas). Dessas, 32 tiveram sua expressão aumentada significativamente (de acordo com os procedimentos descritos na seção 3.8) nas células bacterianas durante a interação com a planta e 2 estavam mais abundantes nas células da condição controle.

A Tabela 2 resume as proteínas e as funções assinaladas para cada uma delas. Entre as proteínas mais expressas nas células de *G. diazotrophicus* durante a interação, 3 principais grupos foram detectados: proteínas envolvidas no metabolismo celular (15 proteínas), proteínas envolvidas em mecanismos de adaptação celular (14 proteínas) e participantes de mecanismos de sinalização (5 proteínas).

Tab. 2: Proteínas de *G. diazotrophicus* expressas diferencialmente durante a interação com cana-de-açúcar SP-70.

Gi	Locus	Nome da Proteína	MM	Escore Pid	NP	razão C/I	DP %
		Proteínas com expressão aumentada durante a interação com a cana-de-açúcar					
162147150	GDI1355	aconitate hydratase	97	490	7	0.57	1.1
162146316	GDI0491	fructose-1,6-bisphosphate aldolase	33	265	2	0.76	1.0
162147711	GDI1927	enolase protein	45	126	5	0.59	1.1
162147402	GDI1617	putative phosphoketolase	90	114	9	0.51	1.1
162146116	GDI0286	bifunctional transaldolase/phosoglucose isomerase	102	1238	46	0.6	1.4
162146115	GD10285	transketolase	83	489	11	0.7	1.1
162148355	GDI2572	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	85	236	66	0.5	1.1
162146350	GDI0525	glutamine synthetase	53	296	18	0.55	1.2
162147166	GDI1371	polynucleotide phosphorylase/polyadenylase	76	415	11	0.62	1.0
162149109	GDI3339	carbamoyl phosphate synthase large subunit	116	358	4	0.6	1.1
162149180	GDI3410	DNA-directed RNA polymerase subunit beta	153	733	36	0.66	1.1
162149228	GDI3460	30S ribosomal protein S1	62	346	20	0.64	1.3
162146518	GDI0694	F0F1 ATP synthase subunit alpha	55	452	10	0.59	1.0
162146853	GDI1036	ATP-dependent Clp protease proteolytic subunit	24	104	2	0.76	1.0
162148966	GDI3196	cell division protein FtsZ	50	107	8	0.45	1.1
209544264	GDIA2119	2-oxoglutarate dehydrogenase, E2 subunit, dihydrolipoamide succinyltransferase	44	28	5	0.55	1.0
162146117	GDI0287	6-phosphogluconate dehydrogenase-like protein	35	129	20	0.7	1.1
162147824	GDI2040	alcohol dehydrogenase [acceptor] precursor	80	227	3	0.57	1.1
162147075	GDI1280	electron transfer flavoprotein subunit alpha	31	65	6	0.71	1.3
162147775	GDI1991	isocitrate dehydrogenase	36	60	5	0.75	1.1
162146401	GDI0576	peroxiredoxin protein	20	62	3	0.76	1.0
209545461	GDIA3348	translation elongation factor Ts	32	120	3	0.82	1.0
162147058	GDI1263	chaperone binding	29	28	4	0.76	1.2
162147057	GDI1262	chaperone protein DnaK	67	30	20	0.82	1.1
162147833	GDI2049	chaperonin GroEL	58	33	41	0.66	1.3
162147969	GDI2185	Outer membrane lipoprotein	40	47	22	0.68	1.2
162147928	GDI2144	Outer membrane protein	31	108	3	0.71	1.1
162149477	GDI3715	TonB-dependent receptor	94	302	9	0.43	1.2
162147266	GDI1471	TonB-dependent receptor protein	117	53	40	0.75	1.1
209544645	GDIA2515	hypothetical protein Gdia_2515	22	49	3	0.90	1.0
162146599	GDI0777	surface antigen protein	24	34	2	*	-
162147251	GDI1456	two-component response regulator	25	60	4	*	-
209543371	GDI3420	two component transcriptional regulator	26	50	4	*	-
162147158	GDI1363	transcription elongation factor NusA	58	100	2	0.70	1.0
		Proteínas com expressão diminuída durante a interação com a cana-de-açúcar					
162149580	GDI3819	phosphate-binding periplasmic protein precursor	34	172	2	1.2	1.0
162147102	GDI1307	peptidase	80	28	2	*	-

MW: massa molecular teórica; PId: escore calculado pelo Mascot para identificação da proteína; PM: número de peptídeos quantificados; C/I: razão entre controle e amostra de interação; DP: desvio padrão do valor de C/I em percentual. *: proteína detectada em apenas uma das condições.

4.2.1- Proteínas envolvidas na manutenção do metabolismo celular

A identificação de tantas proteínas relacionadas ao metabolismo, incluindo proteínas do processo de divisão celular e via das pentoses fosfato indica que as células de *G. diazotrophicus* estavam ativas e replicando no momento em que foram coletadas. Esse dado é reforçado por dados de contagem da viabilidade celular de *G. diazotrophicus* cultivadas em meio MS/10 por 72 horas na presença ou não da planta. Neste experimento, as culturas de *G. diazotrophicus* que estiveram em contato com plantas de cana-de-açúcar SP-70 apresentaram um aumento populacional de três ordens de grandeza (de 5,5 x 10^4 células / ml para 5,5 x 10^7 células / ml), enquanto que as células controle morreram após 3 dias (de 5,4 x 10^4 células / ml para 3,2 x 10^2 células / ml). De fato, da Silva e colaboradores observaram por microscopia eletrônica de transmissão células de *G. diazotrophicus* em processo de divisão celular, quando colonizando raízes de cana-de-açúcar, sete dias após a inoculação (36). Estes dados demonstram o papel fundamental da cana-de-açúcar para a sobrevivência e replicação de *G. diazotrophicus* durante a limitação de nutrientes.

Esse grupo inclui proteínas envolvidas no metabolismo de carboidratos (como aconitato hidratase, frutose-1,6-bisfosfato aldolase, enolase, fosfocetolase, transaldolase/fosfoglucose isomerase bifuncional e transcetolase), metabolismo de energia (F0F1 ATP sintase), metabolismo de aminoácidos (5-metiltetrahidropteroiltriglutamato-homocisteína metiltransferase e glutamina sintetase), metabolismo de nucleotídeos (polinucleotídeo fosforilase), transcrição (RNA polimerase DNA-dependente subunidade beta, proteína ribosomal S1), degradação de proteínas (Clp protease ATP dependente) e replicação (proteína de divisão celular FstZ).

4.2.2- Proteínas envolvidas em processos de adaptação celular

Além dessas proteínas, diversas outras relacionadas à resposta a processos de adaptação celular foram diferencialmente expressas, sugerindo que em 24 horas *G. diazotrophicus* percebe sinais da planta e inicia uma resposta geral a ela. Algumas dessas proteínas realizam funções de chaperonas nas células (proteína chaperona DnaK, chaperonina GroEL, fator Ts de alongamento da tradução e proteína ligadora de chaperona) (37; 95). A função de proteínas que ajudam o enovelamento protéico está intimamente relacionada com a resposta a estresses, visto que diversos agentes estressantes podem interferir diretamente com o enovelamento das proteínas e consequentemente com o seu correto funcionamento. Por isso, o aumento dos níveis de expressão dessas proteínas durante a adaptação celular a uma condição de estresse é um processo necessário para o funcionamento adequado da célula (96). Conforme discutido anteriormente, a interação entre uma bactéria endofítica e seu hospedeiro constitui um estresse biótico para ambos os organismos (97). Assim, a identificação dessas 4 chaperonas moleculares é coerente com o experimento realizado e sugere que embora essas células de *G. diazotrophicus* não tenham entrado na planta, elas sentem a presença do hospedeiro no meio. Um trabalho anterior mostrou que proteínas de estresse são induzidas em *G. diazotrophicus* após 7 dias de interação com plantas de cana-de-açúcar, reforçando esses resultados (37).

Um mecanismo adicional de adaptação celular ocorre durante a interação de *G. diazotrophicus* com SP-70, através de alterações no envoltório celular. Conforme já citado, *G. diazotrophicus* apresenta polimorfismo celular quando colonizando cana-de-açúcar e é sugerido que este processo esteja intimamente ligado ao remodelamento do seu envoltório celular (36). A membrana externa e o LPS

funcionam como uma barreira protetora e seletiva das células bacterianas. Por isso, alterações nestes componentes são reguladas de acordo com estímulos ambientais (98). A identificação de um receptor dependente de TonB, de uma proteína TonB, da proteína de membrana externa OmpH e de uma lipoproteína de membrana externa expressas diferencialmente por *G. diazotrophicus na* presença da planta sugere que estas proteínas fazem parte deste mecanismo adaptativo. Os receptores da família TonB frequentemente funcionam em cooperação com lipoproteínas que controlam o transporte de nutrientes para dentro da célula (99). Assim, a identificação desse conjunto de proteínas nas células de *G. diazotrophicus* que interagiram com a cana-de-açúcar SP-70 reforça a importância deste mecanismo para a sinalização bactéria-planta. Além disso, os receptores dependentes de TonB são proteínas antigênicas, envolvidas na utilização de ferro e na virulência de diversas de bactérias (98; 100; 101). Como alguns peptídeos de TonB são muito conservados é possível que eles representem um padrão para reconhecimento pelas plantas. Adicionalmente, foi descrito que um homólogo de TonB em *Ralstonia solanacearum*, PrhA, se liga à parede celular de células do seu hospedeiro, iniciando uma cascata reguladora que induz respostas de defesa da planta cruciais para a interação (102; 103). Com tudo isso, nossos resultados sugerem que receptores da família TonB superexpressos durante a interação da *G. diazotrophicus* com a cana-de-açúcar sejam elementos essenciais para induzir processos na cana-de-açúcar SP-70 que permitam a colonização por *G. diazotrophicus.*

Da mesma forma, a proteína de membrana externa OmpH que pertence a uma família de porinas imunogênicas, que permitem o fluxo de pequenas moléculas pela membrana externa, também pode atuar como receptor ou chaperona (104; 105;

106). Assim, a função desta proteína em *G. diazotrophicus* pode ser tanto estrutural quanto relacionada a mecanismos de adaptação.

Além do remodelamento da membrana celular e do enovelamento protéico, a resposta geral de estresses induzida em bactérias visa também evitar danos oxidativos (107; 108). Dessa forma, proteínas envolvidas em processos de oxidação e redução e em cadeias de transporte de elétrons fazem parte da resposta geral de estresse, como uma tentativa de resistir a danos oxidativos. Durante a interação com SP-70, *G. diazotrophicus* expressou especificamente uma álcool desidrogenase, 2-oxoglutarato desidrogenase, uma proteína similar a 6-fosfogluconato desidrogenase, subunidade alfa de uma flavoproteína transportadora de elétrons, isocitrato desidrogenase e peroxiredoxina. Essas 6 proteínas devem fazer parte deste sistema de resposta. Entre elas, a álcool desidrogenase deve desempenhar um papel fundamental, visto que seu homólogo em *E. coli,* constitui a principal barreira celular contra danos oxidativos (109). De uma forma geral, esses mecanismos de adaptação metabólica mostram que *G. diazotrophicus* se "protege" do estresse e, portanto está adaptada a interação com cana-de-açúcar.

4.2.3- Proteínas com funções reguladoras e possivelmente envolvidas na interação da bactéria com o hospedeiro

Além das alterações metabólicas descritas acima, *G. diazotrophicus* apresentou expressão aumentada de proteínas com funções reguladoras durante a interação com SP-70. Três reguladores foram identificados: dois reguladores transcricionais (Gdi3420, um regulador transcricional de um sistema de dois componentes; e Gdi1456 regulador de resposta de um sistema de dois componentes) e o fator de alongamento da transcrição NusA (Gdi1363). Esta última proteína é uma moduladora da RNA polimerase, estando, portanto, envolvida na regulação da

expressão gênica (110). O regulador transcricional do sistema de dois componentes Gdi3420 compartilha 70 % de identidade de seqüência com a proteína KdpE de *Acetobacter pasteurianum*. Em *E. coli,* KdpE é ativador transcricional do operon Kdp que codifica um sistema de transporte de potássio de alta afinidade, dependente de ATPase (111). Foi sugerido que o sistema Kdp é regulado pela concentração de potássio, pressão de turgência e estresse salino (112). De fato, a regulação osmótica é um processo essencial para a sobrevivência de *G. diazotrophicus* na cana-de-açúcar, um ambiente muito rico em açúcares. O outro regulador de resposta de um sistema de dois componentes (Gdi1456) identificado pertence à família TIGR 01387. A maioria dos membros desta família compõe um sistema sensor de metais pesados, como cobre, prata, cádmio e zinco (113). Curiosamente, *G. diazotrophicus* é uma bactéria que metaboliza compostos insolúveis de zinco e é sugerido que este processo contribui para o crescimento da planta hospedeira, em virtude do aumento na disponibilidade de nutrientes para a planta (114).

Duas outras proteínas identificadas foram: uma proteína de função hipotética (Gdia2515) e um antígeno de superfície (Gdi0770). A proteína Gdia2515 pertence à família YfdX de função desconhecida. Este resultado é bastante relevante, pois proteínas hipotéticas geralmente são organismo-específicas, podendo realizar funções fundamentais para estas células. Além disso, foi possível confirmar que esta proteína é realmente expressa em *G. diazotrophicus*. Da mesma forma, a proteína predita como antígeno de superfície não apresenta similaridade com nenhuma outra proteína conhecida, mas também parece fazer parte do mecanismo inicial de reconhecimento bactéria-planta.

Em contraste com o grande número de proteínas cuja expressão foi aumentada durante a interação de *G. diazotrophicus* com cana-de-açúcar SP-70, apenas duas

proteínas foram significativamente diminuídas: o precursor da proteína periplasmática ligadora de fosfato Gdi3819 e uma peptidase Gdi1307.

4.3- Proteínas de *G. diazotrophicus* diferencialmente expressas durante a interação com cana-de-açúcar Chunee

Como descrito na seção 3.4 e Fig. 3, para identificar proteínas de *G. diazotrophicus* envolvidas no estabelecimento de uma interação pouco eficiente com a cana-de-açúcar, comparamos o perfil de expressão de proteínas de células bacterianas co-cultivadas por 24 horas com cana-de-açúcar do genótipo Chunee com o das células cultivadas sob condição controle. A suspensão de células do co-cultivo foi misturada à cultura controle e a amostra foi processada para análise proteômica, conforme descrito na seções 3.4 e 3.5 e na Fig. 3. Este procedimento foi realizado em quadriplicata: 2 replicatas biológicas, cada uma com a marcação e a marcação reversa.

Essa análise comparativa entre amostra de *G. diazotrophicus* que interagiu com a cana-de-açúcar Chunee com seu controle permitiu a identificação de 264 proteínas. Deste total, 43 (aproximadamente 16%) foram diferencialmente expressas entre as amostras controle e em co-cultivo (42 proteínas com expressão aumentada e 1 com a expressão diminuída durante a interação bactéria-planta; Tab. 3). Conforme observado na interação com SP-70, a maioria das proteínas com expressão aumentada durante a interação pode ser agrupada em proteínas relacionadas com o metabolismo, adaptação e sinalização celular.

Tab. 3: Proteínas de *G. diazotrophicus* expressas diferencialmente durante a interação com cana-de-açúcar do genótipo Chunee.

Gi	Locus	Nome da Proteína	MM	Escore Pid	NP	razão C/I	DP %
		Proteínas com expressão aumentada durante a interação com a cana-de-açúcar					
162146316	GDI0491	fructose-1,6-bisphosphate aldolase	33	348	16	0.73	1.1
162146116	GDI0286	bifunctional transaldolase/phosoglucose isomerase	102	705	66	0.76	1.1
162146115	GD10285	transketolase	83	286	16	0.68	1.1
209543968	GDIA1822	Phosphoketolase	306	91	6	0.74	1.2
162146526	GDI0702	inosine-guanosine kinase	36	36	2	*	-
162147744	GDI1960	phosphoribosylformylglycinamidine synthase II	77	179	2	0.66	1.0
162147166	GDI1371	polynucleotide phosphorylase/polyadenylase	76	415	6	0.71	1.1
162147682	GDI1898	UTP--glucose-1-phosphate uridylyltransferase	32	194	4	0.83	1.2
162149109	GDI3339	carbamoyl phosphate synthase large subunit	116	358	14	0.89	1.1
162146350	GDI0525	glutamine synthetase	53	296	6	0.66	1.1
162149150	GDI3380	DNA-directed RNA polymerase subunit alpha	37	247	11	0.75	1.2
162149179	GDI3409	DNA-directed RNA polymerase subunit beta'	155	201	4	0.81	1.1
162149228	GDI3460	30S ribosomal protein S1	62	346	6	0.76	1.1
162149173	GDI3403	50S ribosomal protein L4	22	156	3	0.59	1.1
162146520	GDI0696	F0F1 ATP synthase subunit beta	53	452	7	0.76	1.0
162147262	GDI1467	aminopeptidase	95	401	6	0.69	1.3
162147775	GDI1991	isocitrate dehydrogenase	36	182	10	0.78	1.1
162146401	GDI0576	peroxiredoxin protein	20	95	2	0.60	1.0
162149047	GDI3277	putative quinoprotein glucose dehydrogenase	86	83	2	0.75	1.0
162146117	GDI0287	6-phosphogluconate dehydrogenase-like protein	36	344	28	0.76	1.1
162145866	GDI0024	ketol-acid reductoisomerase	36	180	9	0.69	1.0
162145915	GDI0079	catalase	55	370	9	0.69	1.1
162147057	GDI1262	chaperone protein DnaK	67	854	20	0.61	1.2
162147833	GDI2049	chaperonin GroEL	58	394	46	0.66	1.1
162147665	GDI1881	cold shock-like protein cspE	23	157	13	0.68	1.0
162148836	GDI3065	elongation factor G	77	358	13	0.66	1.3
162148789	GDI3018	elongation factor Ts	32	160	3	0.64	1.1
162149176	GDI3406	elongation factor Tu	43	375	23	0.71	1.1
162147969	GDI2185	Outer membrane lipoprotein	40	382	38	0.69	1.2
162146629	GDI0807	Outer membrane lipoprotein omp16	16	161	3	0.68	1.0
162147070	GDI1275	Outer membrane protein	28	188	6	0.58	1.1
162149477	GDI3715	TonB-dependent receptor	94	375	5	0.78	1.1
162147266	GDI1471	TonB-dependent receptor protein	117	857	21	0.63	1.2
162146240	GDI0415	bacteriocin protein	29	49	1	*	-
162146281	GDI0456	chemoreceptor McpA	57	45	2	*	-
162147469	GDI1685	putative chemotaxis protein CheA	79	38	1	*	-
162146452	GDI0628	inositol-3-phosphate synthase	40	75	3	*	-
162147515	GDI1731	putative large-conductance mechanosensitive channel	16	30	2	0.77	1.2
162147529	GDI1745	putative signal transduction histidine kinase	66	35	1	*	-
162147963	GDI2179	IclR family transcriptional regulator	33	37	2	*	-
162148926	GDI3156	transcriptional regulator LysR	32	40	2	*	-
162149456	GDI3694	bacterioferritin	19	32	2	*	-
		Proteínas com expressão diminuída durante a interação com a cana-de-açúcar					
162146117	GDI0287	6-phosphogluconate dehydrogenase	35	344	10	1.4	1.1
162148355	GDI2572	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	85	160	7	2.2	1.0

MW: massa molecular teórica; PId: escore calculado pelo *Mascot* para identificação da proteína; PM: número de peptídeos quantificados; C/I: razão entre controle e amostra de interação; DP: desvio padrão do valor de C/I em percentual. *: proteína detectada em apenas uma das condições.

4.3.1- Proteínas envolvidas na manutenção do metabolismo celular

As 16 proteínas de metabolismo especificamente expressas por *G. diazotrophicus* durante a interação com a cana-de-açúcar do genótipo Chunee incluem: proteínas envolvidas na transcrição (subunidades alfa e beta' da RNA polimerase dirigida por DNA), na tradução (proteína S1 da subunidade ribossomal 30S e proteína L4 da subunidade ribossomal 50S), no metabolismo de energia (subunidade beta da F0F1 ATP sintase), no metabolismo de carboidratos (frutose-1,6-bisfosfato aldolase, transaldolase/fosfoglucose isomerase bifuncional, transcetolase, fosfocetolase e inosina-guanosina cinase), no metabolismo de aminoácidos (carbamoil fosfato sintase e glutamina sintetase), no metabolismo de nucleotídeos (polinucleotídeo fosforilase, fosforibosilformilgiycinamidina sintase II e UTP--glucose-1-fosfato uridiltransferase) e outros (aminopeptidase). Essas identificações sugerem que *G. diazotrophicus* apresentou metabolismo ativo mesmo durante a interação com Chunee, que apresenta pouca contribuição da FBN.

4.3.2- Proteínas envolvidas em processos de adaptação celular

Como discutido na seção 4.2, a resposta adaptativa é requerida para a proteção das células e sobrevivência em estresse biótico. De acordo, 17 proteínas envolvidas em mecanismos de adaptação celular foram identificadas e entre elas, 6 são relacionadas a enovelamento protéico (chaperona DnaK, chaperonina GroEL, similar a proteína induzida por choque frio CspE, fatores de alongamento G, Ts e Tu), 6 a estresse oxidativo (isocitrato desidrogenase, peroxiredoxina, quinoproteína glucose desidrogenase putativa, similar à proteína 6-fosfogluconato desidrogenase, cetol-ácido redutoisomerase e catalase) e 5 ao remodelamento de membrana (lipoproteína de membrana externa Gdi2185 e Omp16, proteína de membrana externa Gdi1275, receptores dependentes de TonB Gdi3715 e Gdi1471). Entre

essas proteínas vale ressaltar que a quinoproteína glucose desidrogenase (GDH-PQQ) é a enzima responsável pela oxidação extracelular da glucose em ácido glucônico. Através da acidificação do meio, este ácido é responsável pela solubilização de compostos de fosfato e zinco, tornando esses elementos mais disponíveis para a nutrição das células e, consequentemente, contribui para o crescimento do hospedeiro de *G. diazotrophicus* (115).

4.3.3- Proteínas com funções reguladoras e possivelmente envolvidas na interação da bactéria com o hospedeiro

Contudo, as identificações mais interessantes estão relacionadas com quimiotaxia (proteínas McpA e CheA) e metabolismo de inositol (inositol-3-fosfato sintase). A quimiotaxia é um processo de sinalização molecular, que pode ocorrer na interação entre dois organismos. Embora este processo ainda não tenha sido demonstrado na interação de *G. diazotrophicus* com seus hospedeiros, é provável que McpA e CheA sejam moléculas fundamentais na comunicação entre *G. diazotrophicus* e cana-de-açúcar. Quando o sinal de quimiotaxia é percebido pelo seu receptor, uma cascata de sinalização é ativada e resulta no movimento bacteriano na direção das condições favoráveis. Em relação aos derivados de inositol, em bactérias, ao contrário dos eucariotos, eles não participam de vias de sinalização. No entanto, sugere-se que derivados do inositol em bactérias funcionam como reguladores osmóticos e âncoras lipídicas para moléculas importantes na interação entre células (116). Curiosamente, uma proteína do metabolismo de inositol (inositol-3-fosfato-sintase) teve sua expressão aumentada em *G. diazotrophicus* durante eventos iniciais da interação com cana-de-açúcar, sugerindo um papel para o inositol 3-fosfato na interação.

Adicionalmente, moléculas envolvidas em sinalização e regulação da expressão gênica foram detectadas. A histidina cinase Gdi1745 e os reguladores transcricionais IciR e LysR podem participar de cascatas de fosforilação e modulação do metabolismo celular.

Além destas, três outras proteínas foram aumentadas na interação com Chunee: uma bacterioferritina, uma bacteriocina e um canal mecanosensível. A bacterioferritina é uma proteína envolvida no armazenamento de ferro. Estudos posteriores serão necessários para averiguar se há alguma sobreposição entre metabolismo de ferro e o estabelecimento da colonização endofítica. A bacteriocina Gdi0415 pertence à família linocina M18 que atua como peptídeo antimicrobiano. De fato, já foi demonstrado que *G. diazotrophicus* é capaz de antagonizar o crescimento de alguns patógenos de cana-de-açúcar em meio de cultura (14; 117; 118). No entanto, este é o primeiro relato da expressão de uma bacteriocina de *G. diazotrophicus* induzida pela presença da cana-de-açúcar. A função do canal mecanosensível será discutida na próxima seção.

Ao todo, a identificação dessas proteínas de expressão aumentada durante a interação *G. diazotrophicus* com Chunee indica que a bactéria sente e responde a estímulos de Chunee, uma variedade de cana-de-açúcar com baixa contribuição da FBN. Por outro lado, apenas uma proteína teve sua expressão diminuída durante a interação: 5-metiltetrahidropteroiltriglutamato-homocisteina metiltransferase. Essa proteína é componente do metabolismo de metionina. Uma possível função para esta via metabólica no estabelecimento da interação entre *G. diazotrophicus* e cana-de-açúcar será discutida na seção 4.4.

4.4- *G. diazotrophicus* responde aos dois genótipos de cana-de-açúcar (SP-70 e Chunee) por mecanismos similares

A interação de *G. diazotrophicus* com a cana-de-açúcar foi descrita pela primeira vez em 1988 (1). Devido às aplicações biotecnológicas promissoras, diversos estudos foram conduzidos de forma a explorar aspectos morfofisiológicos desta interação. Em 1992 Urquiaga e colaboradores (119) determinaram o percentual de contribuição da FBN em diversos genótipos de cana-de-açúcar. Como descrito na seção 1 (Introdução), eles observaram que o genótipo comercial SP-70 apresentou uma das maiores contribuições detectadas (34 %), enquanto que genótipos como Chunee apresentaram pouca influência da FBN (12 %). Embora a quantificação da contribuição da FBN relatada por estes autores não se refira exclusivamente à contribuição de *G. diazotrophicus*, foi sugerido que a interação de *G. diazotrophicus* com SP-70 poderia ser uma relação mais adaptada do que a interação de *G. diazotrophicus* com Chunee. Por esta razão, a maioria dos estudos sobre a interação *G. diazotrophicus* com cana-de-açúcar foram realizados utilizando o genótipo SP-70 (37; 40; 41; 42). No entanto, uma comparação a nível molecular entre a interação de *G. diazotrophicus* com SP-70 *versus* a interação com Chunee não foi descrita anteriormente. Como discutido acima, *G. diazotrophicus* expressou diferencialmente proteínas envolvidas no metabolismo de vários compostos, em mecanismos adaptativos e reguladores e de sinalização celular durante a interação com ambos os genótipos de cana-de-açúcar. Um padrão similar de expressão diferencial foi detectado nos dois experimentos (SP-70 e Chunee, Fig. 9). Em ambos os casos, células bacterianas expressam antígenos, alteram o metabolismo energético e aumentam a expressão de proteínas que ajudam a célula a sobreviver em condições de estresse. Esta estratégia de interação, envolvendo as classes de proteínas citadas, já foi descrita em outros modelos, tais como na interação de *Rhizobium* com leguminosas (21; 120). *G. diazotrophicus* também apresentou expressão aumentada

de algumas proteínas das membranas interna e externa e da regulação osmótica. Portanto, os resultados aqui apresentados sugerem que *G. diazotrophicus* percebe ambos os genótipos de cana-de-açúcar e responde a eles através de mecanismos similares.

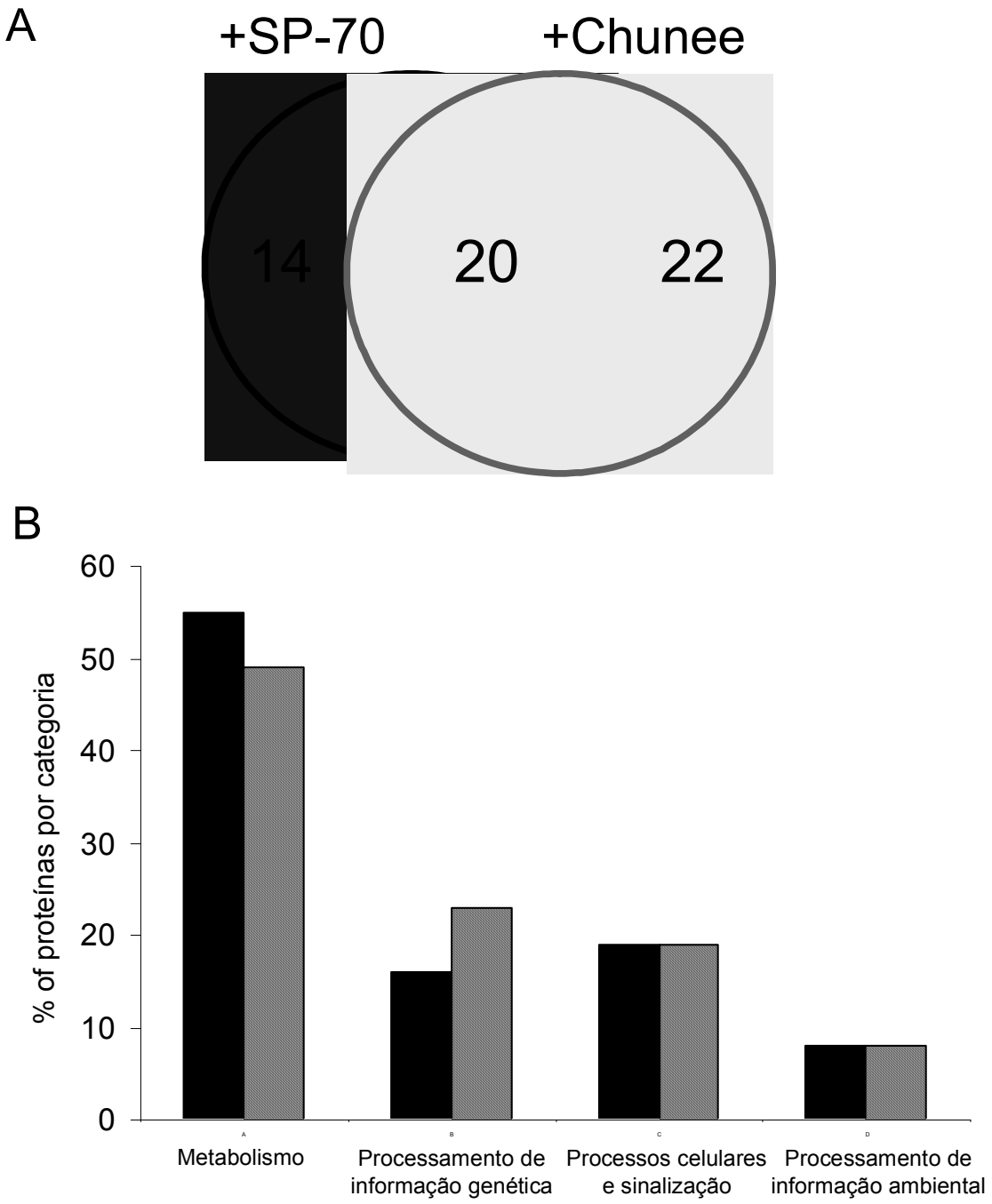


Fig. 9: Análise comparativa entre as proteínas diferencialmente expressas por *G. diazotrophicus* durante a interação com cana-de-açúcar SP-70 (barras pretas) e com Chunee (barras listradas). (A) Representação esquemática do número de proteínas

diferencialmente expressas por *G. diazotrophicus* durante a interação com cana-de-açúcar SP-70 e com Chunee, mostrando o número de proteínas identificadas comuns às duas condições. (B) As proteínas foram agrupadas de acordo com a sua função metabólica no Kegg e os valores do gráfico se referem ao percentual de proteínas diferencialmente expressas por categoria em relação ao total de proteínas diferencialmente expressas em cada amostra.

Contudo, algumas proteínas diferencialmente expressas foram identificadas em apenas uma das interações (somente com SP-70 ou com Chunee). Entre essas proteínas, os fatores de alongamento G, Ts e Tu – que também auxiliam no enovelamento protéico e, portanto fazem parte de um mecanismo adaptativo – foram diferencialmente expressos por *G. diazotrophicus* apenas durante a interação com Chunee. Esse nível aumentado de proteínas envolvidas em um mecanismo de proteção celular sugere que *G. diazotrophicus* está em uma condição mais estressante durante a interação com Chunee do que com SP-70.

No entanto, a principal diferença observada quando comparamos as proteínas diferencialmente expressas por *G. diazotrophicus* durante a interação com SP-70 com aquelas reguladas durante a interação com Chunee, foi a expressão da proteína 5-metiltetrahidropteroiltriglutamato homocisteína metiltransferase. Esta proteína teve sua expressão aumentada por *G. diazotrophicus* na interação com SP-70 e diminuída com Chunee. De acordo com o banco de vias metabólicas Kegg, esta enzima participa do metabolismo de metionina. A função do metabolismo de metionina em interações simbióticas não é conhecida.

Contudo, o metabolismo de metionina forma um ciclo muito importante para algumas espécies de bactéria (121). A homocisteína é convertida em metionina pela enzima homocisteína metiltransferase. Em seguida, a metionina da célula é transformado em S-adenosilmetionina (SAM), que por sua vez é considerada a

principal molécula doadora de metil para reações de metilação que regulam o metabolismo celular. Quando a SAM doa um metil é convertida em S-adenosilhomocisteína (SAH) e posteriormente em ribosilhomocisteína (RH). Este ciclo se fecha quando a enzima LuxS converte RH em homocisteína, liberando o precursor do autoindutor 2 (AI-2). AI-2 é uma molécula que faz parte do sistema de *quorum-sensing*, ou seja, que, de uma forma geral, depende da densidade de células bacterianas e atua como um sistema de comunicação celular entre as células vizinhas de uma comunidade (121). Assim, embora as outras enzimas envolvidas no ciclo da metionina/homocisteína não tenham sido quantificadas, a expressão diferencial da 5-metiltetrahidropteroiltriglutamato-homocisteína metiltransferase de *G. diazotrophicus* quando em contato com genótipos de cana-de-açúcar sugere que sistemas de *quorum-sensing* estejam envolvidos no estabelecimento da interação endofítica. De acordo com esta hipótese, o contato com SP-70 poderia induzir em *G. diazotrophicus* a formação de AI-2, através do ciclo da metionina, aumentando a comunicação entre as células, de forma a facilitar o processo de colonização. Por outro lado, em contato com Chunee, o sistema de *quorum sensing* de *G. diazotrophicus*, via AI-2, não estaria ativo, dificultando o estabelecimento da interação eficiente.

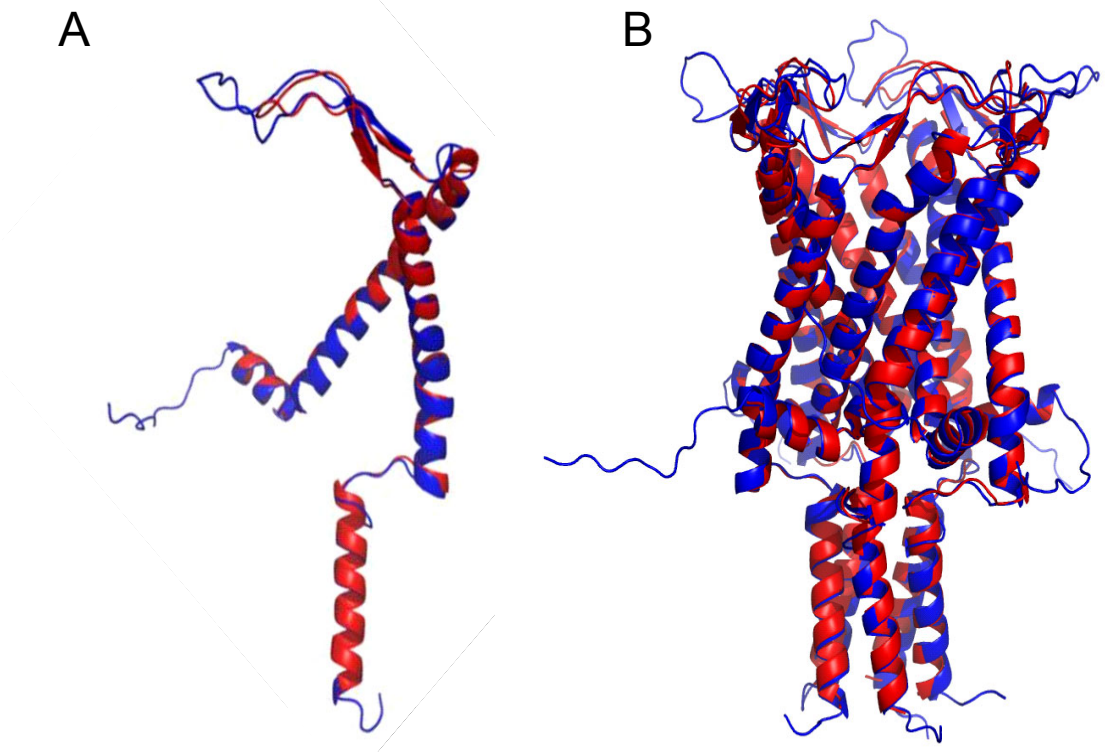
Assim, *G. diazotrophicus* responde a interação com ambos os genótipos de cana-de-açúcar - eficiente e não-eficiente em FBN – através de mecanismos adaptativos parecidos. Porém, é possível que o sistema de *quorum-sensing* via autoindutor AI-2 seja importante para permitir uma sinalização celular que leve à interação com este hospedeiro.

4.5- O canal mecanosensível de *G. diazotrophicus* pode apresentar uma função central no processo de adaptação celular à interação com cana-de-açúcar

Uma das proteínas diferencialmente expressas por células de *G. diazotrophicus* que foram co-cultivadas por 24 horas com plantas de cana-de-açúcar Chunee em relação à amostra controle foi um canal mecanosensível de grande condutância (MscL). A MscL é uma proteína pentamérica que se insere na membrana interna de bactérias Gram-negativas, apresentando uma porção voltada para o periplasma e outra região no citoplasma (122; 123). Dados da literatura mostram que essa proteína é capaz de perceber alterações na membrana celular e as variações da tensão da membrana fazem o canal alternar entre uma forma aberta e uma forma fechada (124). A abertura deste canal permite a passagem de íons e pequenas moléculas denominadas de osmólitos, envolvidas na regulação osmótica da célula (125; 126; 127).

Assim, o envolvimento deste canal nos processos de remodelamento do envoltório e regulação osmótica em outras bactérias coincide com algumas observações feitas para *G. diazotrophicus* (36). Dados anteriores mostraram que *G. diazotrophicus* apresenta morfologia variada quando coloniza plantas de cana-de-açúcar (36) e os dados descritos nas seções 4.2 e 4.3 desta tese, sobre a expressão diferencial de diversas proteínas de membranas e transportadores de íons, sugerem que em *G. diazotrophicus* os processos de remodelamento de envoltório celular e de regulação osmótica parecem ser fundamentais para o estabelecimento da interação entre a bactéria e a planta.

Tendo em vista esses dados, sugerimos que é possível que o canal mecanosensível de *G. diazotrophicus* apresente um papel central no início da interação com o hospedeiro. Com o intuito de entender a função de MscL em *G.*



diazotrophicus utilizamos a modelagem molecular comparativa para construir um modelo tridimensional deste canal. Vale a pena lembrar que, até o momento, não foi resolvida a estrutura tridimensional de nenhum canal mecano-sensível de bactérias diazotróficas. Através de buscas por similaridade sequencial entre a MscL de *G. diazotrophicus* e sequências depositadas no banco de estruturas tridimensionais, selecionamos a proteína MscL de *Mycobacterium tuberculosis* como molde (código PDB: 2oar). O alinhamento entre a seqüência molde e alvo cobre quase 90% dos resíduos da MscL de *G. diazotrophicus* e as duas proteínas apresentam 39% de identidade e 50% de similaridade. Dessa forma, foi possível construir um modelo de alta qualidade estereoquímica, confiável para o estudo de domínios funcionais. O modelo (Fig. 8) apresenta um pequeno desvio em relação ao molde, o que pode ser constatado pelo valor de RMSD igual a 1,8 angstrons. A maior diferença observada entre o molde e o modelo está relacionada aos 16 aminoácidos iniciais do N-terminal da sequência de *G. diazotrophicus* que não alinham com a do molde (Fig. 10) e provavelmente nem estão presentes na forma madura dessas proteínas.

Fig. 10: Sobreposição entre a estrutura cristalográfica do canal MscL de *M. tuberculosis* (em vermelho) e o modelo tridimensional de MscL de *G. diazotrophicus* (azul). Em (A) está representado o monômero e em (B) o pentâmero.

A determinação da estrutura cristalográfica de MscL de *M. tuberculosis* revelou que o canal é formado por 5 subunidades idênticas, cada uma apresentando duas hélices transmembranares e uma hélice citoplasmática (Fig. 9; (128). Além disso, foram apontados dez resíduos voltados para a face interna do canal e que podem ser cruciais para o transporte de moléculas (128). A estrutura secundária predita de MscL de *G. diazotrophicus* apresenta as mesmas características do molde – 2 hélices transmembranares e 1 hélice citoplasmática. Contudo, observamos que dos 10 resíduos voltados para o poro no cristal de MscL, apenas 3 são conservados em *G. diazotrophicus* e nas outras 7 posições, apenas 1 apresenta uma substituição conservada (troca de aminoácidos com as mesmas características físico-quimicas). Conforme ilustrado na Fig. 11, foram observadas duas trocas de resíduos polares neutros por apolares (T por A e T por V), duas trocas de resíduos apolares por polares neutros (A por G), uma troca de resíduo polar básico por resíduo polar neutro (K por S) e uma troca de resíduo polar ácido por polar básico (D por K), sendo todas essas substituições semi-conservadas (troca por outro resíduo que não altera muito as características físico-químicas da proteína).

Além desses resíduos, foram apontados 5 resíduos da hélice citoplasmática que possivelmente estão envolvidos na função de transporte deste canal em *M. tuberculosis* (128). Destes 5, apenas um é conservado em *G. diazotrophicus* e os outros 4 apresentam substituições não conservadas.

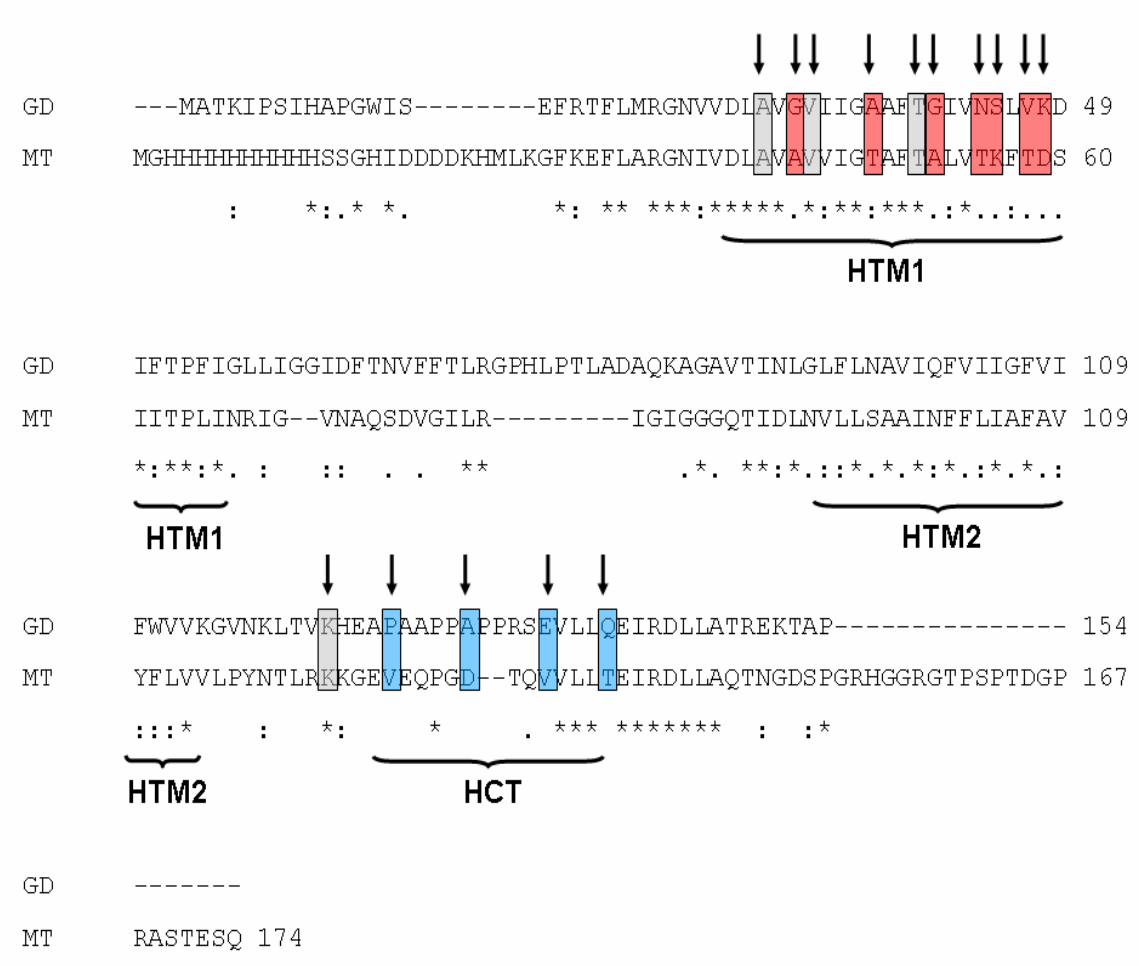


Fig. 11: Alinhamento entre as seqüências de MscL de *G. diazotrophicus* (GD) e de *M. tuberculosis* (MT). Os asteriscos indicam aminoácidos idênticos no alinhamento, os “dois pontos” indicam substituições conservadas e “um ponto” as substituições semi-conservadas. As hélices transmembranares estão indicadas pela sigla HTM e a hélice citoplasmática por HCT. Os resíduos voltados para o interior do canal, assim como os resíduos citoplasmáticos determinantes da função de transporte deste canal estão indicados por setas. Esses 15 resíduos foram destacados em cores para realçar as substituições conservadas (vermelho), não-conservadas (azul) e identidade (cinza).

Assim, embora o modelo construído apresente boa qualidade e sobreposição ao molde, as substituições destacadas, em posições funcionais importantes, sugerem que as propriedades do canal de *G. diazotrophicus* possam ser diferentes das do canal de *M. tuberculosis*. Para melhor entender essa diferença foi calculado o potencial eletrostático da superfície acessível ao solvente, tanto na estrutura

cristalográfica do molde quanto no modelo proposto. A Fig. 12 mostra, a partir da visão superior dos canais MscL., que a MscL de *M. tuberculosis* apresenta uma superfície eletrostática predominantemente neutra (branco) com algumas regiões negativas (vermelha) na face interna do canal. Contudo, a MscL de *G. diazotrophicus* apresenta um perfil fortemente positivo (azul) nessa mesma região. Essa diferença na superfície eletrostática pode refletir em alterações na seletividade dos compostos que podem ser transportados por esse canal e deve ser mais explorada no futuro.

No entanto, essa diferença no perfil de cargas na superfície do canal entre a proteína de *G. diazotrophicus* e de *M. tuberculosis* pode ainda explicar uma diferença ao nível da expressão dessas proteínas. Em *M. tuberculosis* foi observado que o canal mecanosensível é mais expresso em condições de baixa osmolaridade. Neste estudo, nós detectamos o aumento da expressão do canal mecanosensível MscL de *G. diazotrophicus* na presença da cana-de-açúcar, em relação às células que não tiveram contato com a planta. Tendo em vista que a cana-de-açúcar apresenta grande concentração de sacarose, sugere-se portanto que as células de *G. diazotrophicus* co-cultivadas com a planta estavam em um ambiente hiperosmótico. Assim, o aumento da expressão de MscL em *G. diazotrophicus* acontece provavelmente em condição oposta a de *M. tuberculosis*. Com isso, sugerimos que a diferença na superfície eletrostática dessas duas proteínas possa estar relacionada com funções diferentes dessas proteínas.

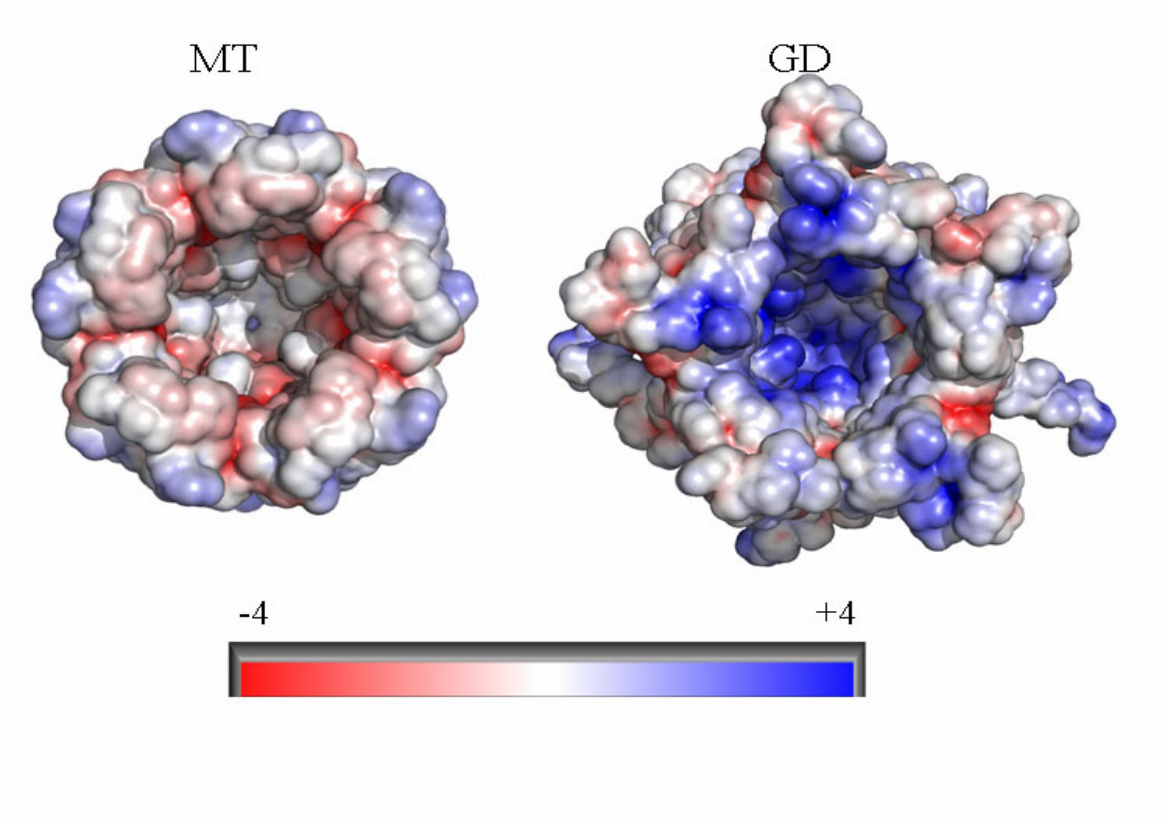


Fig. 12: Potencial eletrostático da superfície dos canais mecano-sensíveis MscL de *M. tuberculosis* e de *G. diazotrophicus*. Em azul estão representadas as regiões de potencial positivo, em vermelho as negativas e em branco as neutras, considerando uma força iônica de 100 mM de NaCl.

4.6- Proteínas de cana-de-açúcar do genótipo SP-70 exclusivamente expressas durante a interação com *G. diazotrophicus*

A análise comparativa, descrita acima, das proteínas diferencialmente expressas por *G. diazotrophicus* mostrou que esta bactéria percebe e responde, essencialmente através dos mesmos mecanismos, a interação com os genótipos SP-70 e Chunee. Tendo em vista esses resultados, as distintas contribuições da FBN entre esses genótipos devem ser explicadas através de outros fatores. Dessa forma, resolvemos analisar a expressão diferencial de proteínas da planta durante a interação e verificar como os genótipos SP-70 e Chunee respondem a *G. diazotrophicus.* As amostras de plantas coletadas correspondem apenas às finas

raízes das plantas de cana-de-açúcar cultivadas *in vitro*. A coleta (24 hs de interação com *G. diazotrophicus* e controle) foi feita de forma a restringir bastante essa região, que estaria em contato mais direto com *G. diazotrophicus*. As buscas para identificação das proteínas da planta foram realizadas no banco de *A. thaliana*, visto que seu genoma já foi seqüenciado, anotado e está disponível publicamente. Contudo, a utilização de um banco de dados não específico para o organismo estudado causa a diminuição do número total de proteínas identificadas.

Ao todo foram identificadas 61 proteínas das raízes de cana-de-açúcar SP-70. Embora o número total de proteínas identificadas não seja grande, foi possível detectar 7 proteínas (aproximadamente 7 % das observadas) exclusivamente expressas por SP-70 durante a interação com *G. diazotrophicus* (Tab. 4), a saber: L-ascorbato peroxidase, ATP citrato liase, proteína TUA6, glutamato amonia liase, proteína 14-3-3, uma proteína cinase ligadora de ATP e uma proteína similar a um receptor cinase.

Tabela 4: Proteínas exclusivamente expressas por cana-de-açúcar SP-70 durante a interação com *G. diazotrophicus*.

Gi	Nome da Proteína	Possível Função	MM	Sequência dos peptídeos
4733972	14-3-3 protein (grf15), putative	Sinalização	9	DSTLIMKILR
15226452	ATP binding / protein kinase/ protein tyrosine kinase	Sinalização	70	RPVTSHK, LGLDWCK
99698	glutamate-ammonia ligase (EC 6.3.1.2), cytosolic	Metabolismo de nitrogênio	41	HKEHIAAYGEGNER
16173	L-ascorbate peroxidase	Resposta adaptativa	27	SGFEGAWTSNPLIFDNSYFK
4262228	putative receptor-like protein kinase	Sinalização	78	VVSLSIPR
9759429	ATP citrate lyase	Metabolismo de lipídeos	66	AGKDLVSSLVSGLLTIGPR
30683070	TUA6; structural constituent of cytoskeleton	Estrutural	47	SVLKVVTMR, LVSQVISSLTASLR, AVFVDLEPTVIDEVR, AVFVDLEPTVIDEVRTGTYR

A L-ascorbato peroxidase é uma proteína envolvida na resposta a estresse oxidativo, um mecanismo de proteção comumente requerido por células que enfrentam algum tipo de estresse biótico (129). Outro mecanismo de resposta a estresse detectado em SP-70 esta relacionado ao metabolismo de lipídeos. A enzima ATP citrato liase é fundamental para a regulação da biossíntese de lipídeos, indicando que SP-70 possa alterar a composição de lipídeos da membrana celular em resposta a interação com *G. diazotrophicus*. A modulação da composição lipídica é muito importante para o processo de colonização de plantas por outras bactérias e está de acordo com a morfologia variada observada em células de cana-de-açúcar 7 dias após a inoculação com bactérias endofíticas (130). Além disso, foi demonstrado que uma ATP citrato liase citosólica de batata doce está envolvida na proteção de células da planta contra patógenos (131). Portanto, esta ATP citrato liase parece fazer parte da resposta protetora de cana-de-açúcar à bactéria.

Ainda, TUA6 é uma proteína que apresenta regiões transmembranares, cuja função ainda não foi definida, mas é uma evidência adicional de que a membrana celular é um componente essencial para a interação bactéria-hospedeiro.

Além da resposta adaptativa, SP-70 apresentou proteínas que podem estar diretamente envolvidas no estabelecimento da interação com a bactéria. Umas delas é a proteína 14-3-3, que pertence a uma família de proteínas reguladoras

conservadas com sítios de interação com diversas proteínas de sinalização celular (132; 133). O nível de expressão desta proteína é aumentado em plantas submetidas a diferentes estresses abióticos e bióticos e diversos ligantes parceiras foram identificados, incluindo proteínas diretamente envolvidas na resposta a estresse, cinases e fosfatases (134). Curiosamente, duas proteínas cinases (uma tirosina cinase putativa e uma proteína similar a um receptor cinase) também tiveram sua expressão aumentada significativamente em SP-70 durante a interação com *G. diazotrophicus*. A identificação dessas proteínas indica que vias de sinalização celular através de cascatas de fosforilação possivelmente são ativadas pela interação da planta com a bactéria. Contudo, mais estudos serão necessários para determinar as funções dessas 3 proteínas e investigar uma possível interação entre elas.

Por fim, foi identificada a proteína glutamato amônia liase, sugerindo que o metabolismo de nitrogênio nas plantas de cana-de-açúcar SP-70 está aumentado. Como já discutido anteriormente, *G. diazotrophicus* é uma bactéria fixadora de nitrogênio, de forma que aumenta a disponibilidade de nitrogênio para a planta. Curiosamente, SP-70 é uma das variedades de cana-de-açúcar que apresenta maior contribuição da FBN.

4.7- Proteínas de cana-de-açúcar do genótipo Chunee diferencialmente expressas durante a interação com *G. diazotrophicus*

A análise proteômica de raízes de Chunee colonizadas por *G. diazotrophicus* em comparação com raízes não inoculadas, revelou 9 proteínas diferencialmente expressas, dentre 82 identificadas. Entre elas, foram detectadas duas subunidades do complexo proteasoma, 3 histonas, um regulador de ciclo celular, um receptor de etileno e duas outras proteínas de membrana (Tab. 5).

Tabela 5: Proteínas diferencialmente expressas por cana-de-açúcar Chunee durante a interação com *G. diazotrophicus*.

Gi	Nome da Proteína	Possível Função	MM	Sequência dos peptídeos
6652882	26S proteasome AAA-ATPase subunit RPT3	Degradação de proteínas	45	ENAPAIIFIDEVDAIATAR
6056388	26S proteasome ATPase subunit	Degradação de proteínas	47	TMLEIVNQLDGFDAR
6630743	CDC48-like protein	Ciclo celular e mecanismos de defesa	90	KGDLFLVR, LDEVGYDDVGGVR, IVSQLLTLMDGLK, AHVIVMGATNRPNSIDPALR, ETVVEVPNVSWEDIGGLENVKR, AFEEAEKNAPSIIFIDEIDSIAPK
2407802	histone H2B	Regulação da expressão gênica	16	LVLPGELAK, QVHPDIGISSK, SVETYKIYIFK, KTAAEKPVEENK
9759615	histone H3	Regulação da expressão gênica	15	STEILIR
145334271	histone H3.2	Regulação da expressão gênica	19	STELLIR, VLSHSSLK
3687654	putative ethylene receptor; ETR2	Sinalizaçãi	85	GRGGYGGCSVSMEDLDVVR, EIASWLLILSMVVFVSPVLAINGGGYPR
6671939	putative T-complex protein 1, ETA subunit	Membrana	60	LAIGDLATQYFADRDIFCAGR
396218	transmembrane protein TMP-B	Membrana	30	GSGLGAEIIGTFVLVYTVFSATDAKR, DSHVPILAPLPIGFAVFLVHLATIPITGTGINPAR

A identificação do receptor de etileno ETR2 é um resultado interessante, visto que já foi demonstrada uma relação cruzada entre vias que respondem ao hormônio etileno e estresses bióticos. De uma forma geral, a presença de etileno livre culmina com o aumento de respostas de estresse e mecanismos de defesa das plantas, através de cascatas de sinalização celular (40). Ao contrário, a ligação do etileno em seu receptor não desencadeia essas respostas de defesa, possibilitando a colonização bacteriana (40). Assim, a detecção desse receptor nas células de Chunee durante o período inicial de interação com *G. diazotrophicus*, indica que Chunee percebeu a bactéria e aumentou a expressão deste receptor, como uma possível forma de controlar o processo de colonização bacteriana. Ainda, a expressão deste receptor de etileno por plantas de cana-de-açúcar Chunee colonizadas por *G. diazotrophicus,* sugere que este genótipo também permite que esta bactéria estabeleça um contato muito próximo com suas raízes. Esse resultado é corroborado por um estudo anterior que verificou níveis do mRNA do receptor de etileno *er1* de cana-de-açúcar aumentados em células desafiadas pela interação com bactérias endofíticas (40).

Além da expressão diferencial deste receptor celular, outros mecanismos moleculares de defesa foram detectados em Chunee. O aumento observado nos níveis de componentes do proteasoma, como as subunidades RPT3 AAA-ATPase e a ATPase do 26S, sugere que o processo de degradação de proteínas seja acentuado nas raízes de Chunee durante a interação com *G. diazotrophicus*. O aumento na expressão deste complexo é frequentemente associado com mecanismos de defesa de plantas, que às vezes, estão associados a processos de morte celular programada (135). Esta estratégia ocorre em diversas interações entre plantas e patógenos, como uma forma de controlar o processo de infecção. A degradação de proteínas é também parte de um mecanismo adaptativo, no qual a célula degrada as proteínas que não são importantes naquela situação e passa a expressar novas proteínas. Portanto, esse resultado sugere que as raízes de cana-de-açúcar do genótipo Chunee, em 24 horas de contato com *G. diazotrophicus*, não são capazes de reconhecer esta bactéria como um organismo benéfico e, ao contrário, parece que a planta lida com *G. diazotrophicus* como se ela fosse um patógeno.

Contudo, a extensão desse processo de degradação na interação entre cana-de-açúcar Chunee e *G. diazotrophicus* ainda não foi estabelecida, mas sugerimos que a degradação de moléculas através do complexo proteasoma seja um mecanismo pontual de defesa contra a colonização bacteriana. Esta hipótese é reforçada pela identificação de uma proteína reguladora do ciclo celular similar a Cdc48. Cdc48 é uma proteína envolvida no controle do ciclo celular, cuja expressão é aumentada em células em divisão celular durante processos de crescimento tecidual (136). Além disso, em *A. thaliana* Cdc48 localiza-se em regiões de fusão/liberação de vesículas da membrana plasmática, sugerindo um papel no transporte e fusão dessas

vesículas (136). Em cana-de-açúcar é possível que esta proteína também esteja envolvida nesses processos, visto que já foi observada a formação de inúmeras vesículas a partir da parede celular vegetal de células da epiderme de raízes da planta quando inoculadas com *G. diazotrophicus* (36). Dessa forma sugere-se que a formação dessas vesículas seja parte dos mecanismos de defesa da cana-de-açúcar Chunee ao estresse biótico da interação com *G. diazotrophicus*.

Por fim, a identificação das histonas H2B, H3 e H3.2 sugerem que a expressão gênica é regulada a nível transcricional, possivelmente através de mecanismos de silenciamento gênico. As histonas são proteínas que se ligam ao DNA e alteram seu enovelamento, assim como a acessibilidade de outras moléculas a ele. Essas proteínas apresentam muitas modificações pós-traducionais determinantes de sua atividade funcional. Curiosamente, um dos peptídeos identificados da histona H2B, KTAAEKPVEENK, foi detectado com 3 acetilações (nos resíduos K1, K6 e K12) e 2 metilações (nos resíduos E9 e E10). A função dessas modificações e sua possível relação com mecanismos de regulação gênica em Chunee serão avaliadas posteriormente.

4.8- Os genótipos SP-70 e Chunee expressaram proteínas diferentes em resposta à interação com *G. diazotrophicus*

As proteínas de cana-de-açúcar identificadas mostraram um mecanismo interessante de resposta à interação com *G. diazotrophicus*. Embora as proteínas expressas diferencialmente por cada genótipo sejam distintas, todas elas mostram que ambos os genótipos da planta percebem e respondem à bactéria.

Por um lado, SP-70 expressou proteínas que conferem resistência ao estresse e envolvidas em cascatas de sinalização celular que permitem o processo de colonização bacteriana. Por outro lado, Chunee alterou completamente seu

metabolismo regulando a expressão de genes através das histonas e de uma proteína importante no controle do ciclo celular. Como discutido acima, os proteasomas são complexos envolvidos na degradação de proteínas com enovelamento incorreto e o aumento da expressão de membros deste complexo sugere que Chunee não foi capaz de responder de forma adequada ao estresse causado pela interação com *G. diazotrophicus*. Ainda, a identificação de histonas metiladas e acetiladas sugere que essas proteínas atuem como reguladores globais da expressão gênica. Tendo em vista esses resultados, sugere-se que SP-70 reconhece eficientemente *G. diazotrophicus* como uma bactéria benéfica enquanto Chunee apresenta dificuldades para lidar com esta bactéria e dispara alterações metabólicas maiores na tentativa de responder, controlar ou até mesmo impedir a continuidade desta interação.

4.9- Proteínas de *G. diazotrophicus* identificadas a partir de extratos de raízes de plantas de cana-de-açúcar inoculadas com a bactéria

Uma área de conhecimento muito interessante diz respeito aos mecanismos de adaptação de microorganismos ao seu ambiente natural. Os avanços recentes nos métodos de biologia molecular e técnicas de bioquímica permitiram a detecção de moléculas presentes em baixo número de cópias em uma amostra, revelando novos aspectos da adaptação ao meio ambiente (137; 138; 139; 140). Contudo, o estudo da interação de microorganismos com um hospedeiro eucarioto ainda é desafiador do ponto de vista metodológico, visto que as moléculas de microorganismos são encontradas muito diluídas numa enorme quantidade de moléculas provenientes do eucarioto. Em alguns poucos casos, como o de bactérias que formam nódulos nas raízes de plantas, existem métodos relativamente simples para separar o microorganismo do hospedeiro. No entanto, bactérias endofíticas como *G.*

diazotrophicus dificilmente são removidas intactas de amostras de plantas. Por esta razão, existem poucos estudos proteômicos que tenham identificado proteínas de microorganismos expressas dentro de seu hospedeiro.

Como uma tentativa de identificar proteínas expressas por células de *G. diazotrophicus* que estejam em contato próximo com cana-de-açúcar, os peptídeos derivados de extratos protéicos de raízes de cana-de-açúcar SP-70 e Chunee, inoculadas com *G. diazotrophicus*, foram analisados usando o banco de dados de proteínas de *G. diazotrophicus*. Ao todo, 8 proteínas de *G. diazotrophicus* foram identificadas a partir das raízes de SP-70 e 25 proteínas a partir das amostras de Chunee. Ao todo, 30 proteínas diferentes foram identificadas. Entre elas, 9 não foram identificadas em nenhum estudo anterior de *G. diazotrophicus* cultivadas em diversas condições, sugerindo que a expressão dessas proteínas seja induzida pelo contato direto com as raízes da cana-de-açúcar.

4.9.1- *G. diazotrophicus* expressou proteínas envolvidas em regulação osmótica, quimiotaxia e remodelamento da membrana celular nas raízes de cana-de-açúcar

As nove proteínas identificadas, expressas por *G. diazotrophicus* exclusivamente nas raízes da cana-de-açúcar e não detectadas em outras condições são: um componente de um sistema de transporte de potássio, proteína A reguladora do volume celular, uma metiltransferase envolvida em quimiotaxia, uma cistationa gama sintase, uma glutationa S-transferase, UDP glucose 6-desidrogenase, a nucleosídeo 2-deoxiribosiltransferase, uma proteína de membrana externa e um transportador putativo (Tab. 6).

Tab. 6: Proteínas de *G. diazotrophicus* identificadas exclusivamente em células em contato próximo com raízes de cana-de-açúcar.

Gi	Locus	Proteína	MW	Escore	PM	Peptídeos	Amostra
162148168	GDI2385	potassium transport system protein	23	70	2	GLIMNRQK, IPQGGWVPVLLGIALTLMMTTWK	SP-70 e Chunee
162147597	GDI1813	putative cell volume regulation protein A	25	65	1	LGCPVMLVDTCTGALAPASRAGIPILK	SP-70
162147471	GDI1687	putative chemotaxis protein methyltransferase	31	31	1	NHFTHLKEFVVPR	Chunee
162146602	GDI0780	putative cystathionine gamma-synthase	28	45	1	AYRPATRLLHSGVER	SP-70
162148550	GDI2774	putative glutathionine S-transferase	23	25	1	TPEFLR	Chunee
162147594	GDI1810	putative nucleoside 2-deoxyribosyltransferase	21	31	1	IAIAELAR	Chunee
162146935	GDI1140	putative UDP-glucose 6-dehydrogenase	46	23	1	QIARAMTDYAVIVTK	Chunee
162149225	GDI3457	putative transporter protein	34	34	1	VVATTVPR	Chunee
209544487	GDIA2347	putative outer membrane protein	38	22	1	LLSILR	Chunee

MW: massa molecular teórica; PM: numero de peptídeos sequenciados.

O potássio é um íon muito abundante em células bacterianas e apresenta uma função importante na regulação osmótica e homeostasia celular (141). As concentrações de potássio e outros íons, como sódio e hidrogênio, são rigorosamente controladas pelas células, pois esses íons apresentam diversas funções reguladoras, inclusive em resposta a estresses ambientais (142). Assim, a identificação de dois componentes de sistemas transportadores de íons em células de *G. diazotrophicus* em interação com cana-de-açúcar sugere que esta bactéria encontra um ambiente osmótico diferente dentro da planta e que mecanismos de transporte de íons sejam essenciais para a adaptação celular. Adicionalmente, foi demonstrado que o sistema de transporte de potássio Kup (homólogo de GDI2385) é fundamental para a colonização de alfafa por *Sinorhizobium meliloti* (143). Todos esses motivos indicam que os transportadores codificados por Gdi2385 e Gdi3457 são importantes para uma associação de *G. diazotrophicus* com seu hospedeiro.

Além da resposta ao estresse osmótico, *G. diazotrophicus* expressou um mecanismo para compensar estresse oxidativo, através da proteína glutationa S-transferase. Essa enzima pertence a uma família de proteínas envolvidas na resposta ao estresse oxidativo, mas que também apresentam atividade de detoxificação de xenobióticos (144). Outra proteína que pode fazer parte deste

sistema de regulação redução-oxidação é a cistationa gama sintase, que faz parte do metabolismo de enxofre, cisteína e metionina. A identificação desta proteína é ainda mais interessante visto que já foi demonstrado que outra proteína da via de síntese de metionina é regulada durante a interação de *G. diazotrophicus* com cana-de-açúcar (ver seções 4.2 e 4.3).

A resposta de estresse de *G. diazotrophicus* à interação com cana-de-açúcar também implica em alterações da composição da membrana celular. Duas proteínas de membrana (GDI3457 e GDI2347) foram expressas exclusivamente por células da bactéria em contato íntimo com a planta e podem estar envolvidas na percepção de sinais do ambiente e remodelamento do envoltório bacteriano. Em conjunto, essas proteínas sugerem que a regulação do volume celular e remodelamento do envoltório celular são necessários para a interação entre *G. diazotrophicus* e cana-de-açúcar. Essa proposição é ainda sustentada por observações microscópicas anteriores (36) que revelaram células de *G. diazotrophicus* de morfologia alterada 7 dias após inoculação em plantas de cana-de-açúcar, de acordo com as evidências descritas a nível celular.

Por fim, foram identificadas duas proteínas relacionadas ao metabolismo de nucleotídeos (GDI1810 e GDI1140) e uma ao processo de quimiotaxia (GDI1687). Uma delas, a UDP glucose 6-desidrogenase é essencial para *V. fischeri* colonizar seu hospedeiro (145), sugerindo que seu homólogo em *G. diazotrophicus* possa estar diretamente relacionado com a etapa inicial de interação com a cana-de-açúcar. Da mesma forma, a metiltransferase do sistema de quimiotaxia é uma proteína que realiza o acoplamento entre a percepção do sinal de quimiotaxia e a regulação da motilidade bacteriana. Certamente a quimiotaxia é um processo

requerido para o estabelecimento da interação, como um dos eventos iniciais na comunicação bactéria-planta.

4.9.2- *G. diazotrophicus* expressa proteínas envolvidas em metabolismo, quimiotaxia e resposta a estresses durante associação com raízes de cana-de-açúcar

Além das proteínas bacterianas que foram identificadas exclusivamente a partir de amostras de raízes, outras 21 proteínas de *G. diazotrophicus* foram detectadas nessas mesmas amostras de raízes de cana-de-açúcar co-cultivadas com *G. diazotrophicus* (Tab. 7). Contudo, essas 21 proteinas já foram identificadas em *G. diazotrophicus* cultivada em outras condições de crescimento ou mesmo em bactérias co-cultivadas com a planta. Entre elas foram identificadas 6 proteínas envolvidas no metabolismo celular, como proteínas ribossomais S1 e L9, transaldolase/fosoglucose isomerase bifuncional, subunidade beta da F0F1 ATP sintase, gliceraldeído-3-fosfato desidrogenase e glutamina sintetase. A expressão dessas proteínas sugere que *G. diazotrophicus* está metabolicamente ativa após 24 horas de co-cultivo com o hospedeiro.

Tab. 7: Proteínas de *G. diazotrophicus* expressas por células em contato próximo com raízes de cana-de-açúcar.

Gi	Locus	Proteína	MW	Escore	PM	Peptídeos	Amostra
162145880	GDI0041	outer membrane protein	40	65	3	SSPNFPDGR, IEVDGYTDNSAAHPGPR, RVEIILH	SP-70 e Chunee
162145915	GDI0079	catalase	55	63	1	LVSNIVGHVLQGVEEPVLSR	Chunee
162146116	GDI0286	bifunctional transaldolase/phosoglucose isomerase	102	26	1	ASAASVPPALNPGVVLGTVLGVAATQFGR	Chunee
162146281	GDI0456	chemoreceptor mcpA	57	23	2	ISASFARRPR, MTKESQIVER	SP-70
162146350	GDI0525	glutamine synthetase	53	118	3	LIPGFEAPVLLAYSAR, HHHEVAQSQHELGTK, RLIPGFEAPVLLAYSAR	Chunee
162146401	GDI0576	peroxiredoxin protein	20	46	1	ATFLVDPEGIIR	Chunee
162146520	GDI0696	F0F1 ATP synthase subunit beta	53	58	1	TVIIQELINNIAK	Chunee
162147070	GDI1275	outer membrane protein	28	24	1	FNPYVGVGATLAFFHNESPAGGLVK	Chunee
162147085	GDI1290	pyruvate kinase	51	58	1	TVETVVDQAIESTVASSLAR	Chunee
162147166	GDI1371	polynucleotide phosphorylase/polyadenylase	76	75	2	TADFGAFVNFLGAR, EAVLAALATEGLDVTAAKPILKDLEADVVR	Chunee
162147179	GDI1384	biopolymer transport exbB protein	36	53	1	NFAADLQSILLGGFR	Chunee
162147833	GDI2049	chaperonin GroEL	58	141	4	VGGSTEVEVK, TALQDASSVAGLLITTEAMVAEKPEKK, TALQDASSVAGLLITTEAMVAEKPEK, VLESNDYNYGFDAQIGDYKDLVAAGIIDPTK	Chunee
162147969	GDI2185	outer membrane lipoprotein	40	78	2	TYLVFFDWDR, TYLVFFDWDRSDLTAR	Chunee
162148085	GDI2302	glyceraldehyde-3-phosphate dehydrogenase	36	38	1	GILAYNTAPLVSSDFNHSIASSTFDATETALVDG GK	Chunee
162148355	GDI2572	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	85	131	5	ETTCRQIAYAIR, LLPVYVAILR, GGGFDPLTLLPR, GMLTGPVTILNWSFVR, AQGIETRPVLLGPVSYLLLGK	SP-70 e Chunee
162148832	GDI3061	hypothetical protein GDIA_3307	57	26	1	IRDSLPR	SP-70 e Chunee
162149101	GDI3331	50S ribosomal protein L9	21	94	2	TQVILENPIKQLGLYDVR, SAVELILLQRVENLGQMGDVVK	Chunee
162149176	GDI3406	elongation factor Tu	43	65	4	GVVNVGDEIEIVGLR, QVGVPALVVFLNK, ELLSAYQFPGDDIPIIK, DLMDAVDAYIPQPERPVDRPFLMPIEDVFSISGR	SP-70 e Chunee
162149228	GDI3460	30S ribosomal protein S1	63	30	1	DVTPLMGVPQPFQILK	Chunee
162149428	GDI3666	hypothetical protein GDI_3666	42	154	2	NWNIVLTNIIR, TLIPLSFQFASFDGR	Chunee
209542569	GDIA0387	Superoxide dismutase	22	32	1	AFELPTLPFQTNALAAR	Chunee

MW: massa molecular teórica; PM: numero de peptídeos seqüenciados. *: proteína detectada em apenas uma das condições.

Como esperado, proteínas de estresse também foram visualizadas (catalase, fator de alongamento Tu, peroxiredoxina, superóxido dismutase, proteína de membrana externa e lipoproteína), indicando que *G. diazotrophicus* lida com o hospedeiro através de respostas adaptativas.

Entretanto, moléculas com funções mais específicas na interação, também foram descritas. O quimioreceptor McpA é uma proteína de quimiotaxia receptora de grupamento metil que pode ser metilada por uma metiltransferase (identificada exclusivamente em células bacterianas nas raízes de cana-de-açúcar) e acoplada a

proteína de quimiotaxia CheA (identificada em células de *G. diazotrophicus* co-cultivadas com plantas de cana-de-açúcar). Essas 3 proteínas compõem um sistema de transdução de sinal do hospedeiro para a bactéria.

Em contraste com esses mecanismos reguladores pós-traducionais citados anteriormente, foi identificada também a polinucleotídeo fosforilase/poliadenilase (PNPase), que pode participar da regulação da pós-transcrição. A PNPase integra o metabolismo de nucleotídeos, mas também descrita como reguladora da estabilidade de mRNAs, através da modificação pós-transcricional deles (146). A possibilidade de regular a expressão gênica em diversos níveis deve conferir maior versatilidade a *G. diazotrophicus*, aumentando a eficiência da colonização.

Ainda, a identificação de duas proteínas de função desconhecida representa um sinal espécie específico relacionado a esta interação. Por um lado, a proteína codificada por GDI3666 não apresenta similaridade a nenhuma proteína ou domínio funcional conhecido, nem mesmo apresenta regiões transmembranares preditas. Embora não seja possível especular a função de GDI3666 em *G. diazotrophicus* no momento, este resultado é bastante instigante por se tratar de uma proteína que pode ser específica desta espécie bacteriana. Por outro lado, a proteína hipotética GDIA3307 apresenta duas hélices transmembranares preditas (entre os resíduos 129 e 151 e entre 509 e 531) e um domínio conservado entre proteínas de função desconhecida (Pfam0445). Por ser uma proteína de membrana expressa por células de *G. diazotrophicus* que estão em contato próximo com as raízes de cana-de-açúcar, é razoável supor que ela participe de algum mecanismo de percepção de sinais da planta.

Mais uma vez, a membrana celular parece apresentar um papel importante para o sucesso da interação célula a célula. A proteína ExbB de *G. diazotrophicus*, também

identificada a partir das amostras de raízes, é requerida para o funcionamento adequado de receptores dependentes de TonB e consequentemente para o transporte de nutrientes, ferro, vitamina B12 e outras moléculas que não atravessam facilmente a membrana externa bacteriana (147). Como discutido na 4.2, foi sugerido que um mecanismo dependente de TonB seja fundamental para *G. diazotrophicus*.

Porém, a identificação da proteína 5-metiltetrahidropteroiltriglutamato-homocisteína metiltransferase, uma enzima do metabolismo de metionina é a mais notável. Esta proteína foi identificada a partir de amostras de raízes de SP-70, mas não de raízes de Chunee. Coerentemente, esta proteína teve sua expressão aumentada mais de duas vezes em células de *G. diazotrophicus* co-cultivadas com SP-70, e a expressão reduzida a menos da metade em células co-cultivadas com Chunee (seções 4.2 e 4.3). Nas amostras de raízes de cana-de-açúcar foram identificadas 8 proteínas de *G. diazotrophicus*, contra 25 identificadas das amostras de Chunee, sugerindo que a expressão seja regulada diferencialmente também quando *G. diazotrophicus* está em contato muito próximo desses genótipos de cana-de-açúcar. A função do metabolismo de metionina em interações simbióticas ainda não foi esclarecida, mas como descrito na seção 3.4, esta proteína pode estar envolvida em mecanismos de *quorum-sensing*.

5– CONCLUSÕES

A *G. diazotrophicus* é uma bactéria promotora do crescimento de seus hospedeiros vegetais, como por exemplo, a cana-de-açúcar. Tendo em vista o grande interesse econômico em aumentar a produtividade deste cultivar e, ao mesmo tempo, utilizar recursos sustentáveis na agricultura, *G. diazotrophicus* e outras bactérias fixadoras de nitrogênio têm sido caracterizadas pelo seu potencial uso biotecnólogico.

Embora o objetivo deste trabalho não esteja diretamente relacionado com possíveis aplicações desta bactéria em plantações de cana-de-açúcar, o detalhamento molecular, descrito nesta tese, sobre a interação entre *G. diazotrophicus* e duas variedades de cana-de-açúcar, gerou uma enorme quantidade de informação (cerca de 500 proteínas identificadas e 70 proteínas diferencialmente expressas) que, no futuro, poderão ser utilizadas para o aprimoramento desta associação.

Os três pontos mais importantes deste trabalho foram: 1) o estabelecimento de um método de proteômica quantitativa que permitiu a detecção de proteínas diferencialmente expressas com alta confiabilidade, durante a interação bactéria-planta; 2) a comparação entre a interação de *G. diazotrophicus* com SP-70 e Chunee, duas variedades que apresentam contribuições distintas da FBN, permitindo relacionar a presença de determinadas moléculas com a eficiência da interação; 3) a identificação de proteínas de *G. diazotrophicus* expressas quando esta encontra-se em contato muito próximo da planta.

O estabelecimento de um método de análise proteômica diferencial confiável foi um passo fundamental para o desenvolvimento de todo o trabalho. Pela primeira vez, ao nosso conhecimento, foi utilizada a marcação metabólica simultânea de uma planta e uma bactéria em interação. Conforme discutido ao longo desta tese, cada

etapa do procedimento foi escolhida e otimizada de forma a permitir a realização de toda a análise em passos simples e rápidos, minimizando as perdas e erros experimentais. Esse cuidado na escolha dos métodos utilizados permitiu uma análise sensível e acurada da expressão diferencial de proteínas.

Ao todo foram analisadas quase 400 proteínas de *G. diazotrophicus* e 100 proteínas de cana-de-açúcar, possibilitando a visualização do panorama geral do estado metabólico dessas células. Do ponto de vista da bactéria, foram identificadas 50 proteínas diferencialmente expressas durante a interação com a planta (células em suspensão) e, entretanto, a comparação da resposta bacteriana a interação aos dois genótipos de cana-de-açúcar não revelou diferenças significativas. Este resultado indicou que *G. diazotrophicus* é capaz de perceber e responder, de forma similar, à interação com SP-70 e Chunee.

Por outro lado, as proteínas diferencialmente expressas por SP-70 durante a interação com *G. diazotrophicus* sugerem que esta variedade de cana-de-açúcar reconhece *G. diazotrophicus,* apresenta respostas de estresse e possibilita uma interação eficiente com a bactéria. Contudo, Chunee apresenta aumento nos níveis de proteínas do complexo proteasoma, de histonas e indução de mecanismos de defesa contra patógenos, sugerindo alterações drásticas na expressão gênica e metabolismo das células das raízes, como uma tentativa de lidar com *G. diazotrophicus*. Dessa forma, sugere-se que a diferença na contribuição da FBN entre esses genótipos seja em parte explicada pela habilidade de cada genótipo responder a interação com *G. diazotrophicus*.

Ainda, foram identificadas 30 proteínas expressas por *G. diazotrophicus* nas amostras de raízes de cana-de-açúcar. Essas proteínas são oriundas de células bacterianas em contato muito próximo com raízes da planta. Nove dessas proteínas

não foram identificadas em nenhuma das outras condições de cultivo de *G. diazotrophicus* já analisadas, sugerindo que a expressão dessas proteínas seja estimulada por algum sinal da cana-de-açúcar. Esses resultados mostraram que *G. diazotrophicus* deve encontrar um ambiente osmótico diferenciado nas proximidades da planta e se adapta a essa condição através de alterações no envoltório celular e regulação osmótica.

Assim, os resultados apresentados aqui sugerem que *G. diazotrophicus* inicia o processo de interação com a cana-de-açúcar através da expressão de proteínas de quimiotaxia e antígenos de superfície. Para o estabelecimento eficiente desta interação, *G. diazotrophicus* responde a variações osmóticas, regulando seu volume celular e alterando a expressão de proteínas e lipoproteínas de membrana e transportadores de íons. Há indícios que a planta reconhece e estabelece a interação com *G. diazotrophicus* através de alguns receptores e proteínas cinases, modele componentes do citoesqueleto e não dispare respostas de defesa contra *G. diazotrophicus*. Esses eventos parecem cruciais para o estabelecimento de uma interação efetiva entre *G. diazotrophicus* e seu hospedeiro, através da qual ambos são beneficiados.

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7- ANEXOS

Nesta seção estão anexados os manuscritos referentes a três artigos produzidos durante o período de doutoramento. O primeiro desses artigos (anexos 1) descreve os resultados acima apresentados e portanto diretamente relacionado aos resultados desta tese. Este manuscrito está em fase final de revisão antes de ser submetido. O anexo 2 corresponde ao estudo computacional de uma proteína envolvida em um mecanismo de proteção conformacional da nitrogenase – o complexo responsável pela fixação de nitrogênio – em *G. diazotrophicus*. Este trabalho foi realizado em conjunto com a aluna de iniciação científica Mainá Bitar e em colaboração com o doutorando Maurício Costa. Este artigo foi submetido à revista BMC Genomics e estamos aguardando um parecer do editor.

Por outro lado, o anexo 3 é referente a um artigo produzido em colaboração com todos os demais autores do manuscrito sobre a relação entre o gene *pho*B de *Vibrio cholerae* e respostas gerais de estresse. Neste artigo também descrevemos uma análise proteômica, através da abordagem de géis bidimensionais, mas também diversas outras técnicas de biologia molecular, tais como *western blot,* dosagem de atividade enzimática, cromotografia, entre outras. Este artigo está em fase final de elaboração e será submetido nos próximos meses.

Bacterial osmotic regulation and plant genotype are essential *for G. diazotrophicus*-sugarcane interaction

Leticia M. S. Lery[1], Adriana S. Hemerly[2], Eduardo M. Nogueira[2], Wanda M. A. von Krüger[1], Paulo M. Bisch[1]

[1]Unidade Multidisciplinar de Genômica, Universidade Federal do Rio de Janeiro, Brasil; [2]Laboratório de Biologia Molecular de Plantas, Instituto de Pesquisas do Jardim Botânico do Rio de Janeiro, Brasil

Corresponding author: Leticia Miranda Lery Santos, e-mail: llery@biof.ufrj.br

Avenida Carlos Chagas Filho, CCS, bloco G. Instituto de Biofísica Carlos Chagas Filho, sala G1-043. Ilha do Fundão. Rio de Janeiro, RJ – Brasil. CEP 21949-902.

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Abstract

G. diazotrophicus is a plant growth promoting bacterium that colonizes sugarcane. In order to investigate molecular details of *G. diazotrophicus*-sugarcane interaction we performed a quantitative MS-based proteomic analysis of both bacteria and root samples. In overall, more than 400 proteins were analyzed and 78 were differentially expressed between the plant-bacterium interaction model and control cultures. A comparative analysis of the *G. diazotrophicus* interaction with two distinct genotypes of sugarcane, SP-70 and Chunee, revealed proteins with fundamental roles for cellular recognition. *G. diazotrophicus* presented proteins involved in adaptation to atypical conditions and signaling systems under the interaction with both genotypes. However, SP-70 and Chunee, sugarcane varieties with high and low contribution of biological nitrogen fixation (BNF), respectively, showed divergent responses in contact with *G. diazotrophicus*. SP-70 overexpressed proteins of signaling cascades and lipid metabolism whilst Chunee induced chromatin remodeling and protein degradation pathways. In addition we have identified 30 bacterial proteins expressed “in planta” and 9 specifically induced by plant signals. This is the first insight on early signaling of *G. diazotrophicus*-sugarcane interaction, as well as the first quantitative proteomic analysis of a bacterium-plant interaction. Thus, these results could also help to increase our knowledge on microorganism-host relationship.

Introduction

Plant-bacteria interactions are widely known (148; 149; 150; 151). Both pathogenic and beneficial interactions have been studied and revealed complex mechanisms of recognition and signaling. For example, symbiotic organisms interact intimately with plants and provide them metabolic advantages (120; 152; 153). Therefore, the plant host might be able to recognize these organisms and to signal for pathways that allow them to grow nearby or even inside the plant. The most studied example of bacteria-plant symbiosis is the *Rhizobium*-legume interaction (154). The plant perceives the *Rhizobium* cells and a plant signaling cascade culminates in the proliferation of plant cells around the bacterial infection site. These symbiotic bacteria contribute to plant growth while the plant develops an organ (the Nodule) that supports bacteria growth (153; 154).

In contrast to this nodulation system described (152; 155; 156), there are a few studies regarding molecular aspects of the interaction between endophytic bacteria and non-leguminous plants (157; 158; 159). In the last decades, were described new species of nitrogen-fixing bacteria responsible for high contribution of Biological Nitrogen Fixation (BNF) in sugarcane plants (1; 20; 24; 35).

Gluconacetobacter diazotrophicus is a N_2-fixing bacterium found in roots, stems and leaves of plants such as sugarcane and sweet potato (1; 16; 25; 26; 27; 29; 33; 160; 161). Its contributions to a profitable association with the host-plants include nitrogen fixation, production of plant growth promoting hormones, secretion of bacteriocins and solubilization of phosphate and zinc compounds (12; 13; 114; 118; 162; 163). In overall, these mechanisms increase root and foliar growth (13). Previous studies have pointed out *G. diazotrophicus* proteins involved in nitrogen metabolism and

bacterial metabolism and adaptation (37; 38; 39). However, proteins directly involved in bacteria-host interaction were not yet described.

In another hand, analysis of sugarcane mRNAs differentially represented between ESTs libraries of plantlets inoculated with *G. diazotrophicus* and control samples revealed that signaling pathways involved in plant growth promotion and nitrogen metabolism are activated under the interaction (42; 164). Additionally, it is shown that distinct sugarcane varieties present different contribution of BNF, suggesting that the plant genotype is somehow determining the efficiency of bacteria colonization. The commercial sugarcane genotype SP-70 present high contribution of BNF(~34%), thus suggesting that its interaction with diazotrophic bacteria is efficient. However, the sugarcane variety Chunee does not benefit much from BNF (~12%) indicating that diazotrophs might not be able to establish the interaction.

Sugarcane crop production is economically important for food and biofuel production. As *G. diazotrophicus* is a naturally occurring bacterium that improves sugarcane growth, the knowledge of the molecules specifically involved in the establishment of an efficient interaction is a promising strategy for biotechnological developments. Therefore, this study aimed to identify both plant and bacterial proteins involved in early bacterium-host signaling. In order to achieve this aim we have designed a comparative analysis of *G. diazotrophicus* interaction with the sugarcane genotypes SP-70 (high BNF contribution) and Chunee (low BNF contribution) through a quantitative proteomic analysis.

One of the most reliable methods of quantitative proteomic analysis combines metabolic labeling of cells in culture and relative quantification by mass spectrometry (62; 87; 88). The accurate results obtained with this technique are mainly due to the possibility of mixing labeled and non-labeled samples immediately after they are

collected. Therefore, all processing steps are identically performed for both samples avoiding experimental variations. The main steps in such a protocol comprise the homogeneous incorporation of the isotopes during cell growth, broad protein extraction procedures, efficient protein separation and digestion, high resolution LC for peptides separation and high accuracy MS/MS data acquisition (89).

Although such recent advances in molecular techniques have provided the possibility to detect specific low copy molecules in a tissue sample (137; 138; 139; 140) the analysis of the microorganisms interaction with its eukaryote host is still challenging. Usually microorganism molecules are found “diluted” in the vast amount of eukaryote material. In a few specific samples such as nodulating bacteria, it is relatively simple to isolate bacteria from plant components. However, endophytic bacteria are not easily removed as intact cells from plant samples. For this reason, there are few proteomic studies disclosing proteins of microorganisms expressed inside its hosts. Thus, in this study we have optimized such reliable comparative proteomic strategy for the analysis of a complex system, such as the co-culture of *G. diazotrophicus* and sugarcane.

Summarizing, this is the first report of the metabolic labeling of a bacterium-plant co-culture system for a differential proteomic analysis, as well as the first comparative study regarding molecular aspects of *G. diazotrophicus* interaction with the sugarcane genotypes SP-70 and Chunee and the first description of *G. diazotrophicus* proteins expressed in planta.

Materials and Methods

Bacterial strains, media and growth conditions

G. diazotrophicus, lineage PAL5 (BR11281), was pre-cultured in LGI-P medium (0.2 g/L K_2HPO_4, 0.6 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.02 g/L $CaCl_2.2H_2O$, 0.002 g/L

$Na_2MoO_4.2H_2O$, 0.01 g/L $FeCl_3.6H_2O$, 5 ml/L bromothymol blue 0.5 % in 0.2 M KOH, 10 g/L sacarose) containing 10 mM $(NH_4)_2SO_4$ for 36 hours (exponential phase). Cells were harvested by centrifugation (18,000 g, 10 min, 4 ºC) and washed in PBS buffer. Bacteria were ressuspended in the same volume of a ten fold diluted MS medium (MS/10: 0.75 mM $MgSO_4$, 0.65 mM KH_2PO_4, 1.5 mM $CaCl_2$, 0.05 mM $CoCl_2.6H_2O$, 0.16 mM $CuSO_4.5H_2O$, 45 mM FeNaEDTA, 23 mM H_3BO_3, 2.5 mM KI, 50 mM $MnSO_4.H_2O$, 0.26 mM $Na_2MoO_4.2H_2O$, 0.36 mM $ZnSO_4.7H_2O$, 4.6 mM $MnCl_2.4H_2O$, 0.05 mM NH_4NO_3, 2 mM KNO_3, 2 g/l sucrose) for 8 hours at 30 °C and 400 rpm.

In vitro plant growth and bacterial co-cultivation

The sugarcane genotypes SP70-1143 (high inputs of N from BNF) and Chunee (low inputs of N from BNF) were used throughout this work. Sugarcane plantlets free of microorganisms were obtained by sterile meristem culture and micropropagated according to the method of Hendre et al. (69).

In vitro-grown rooted sugarcane plantlets were inoculated as described before (70). Briefly, rooted plantlets were starved in the MS/10 medium for 7 days and then inoculated with 0.1 ml of a suspension containing 10^5 bacterial cells. Controls were inoculated with medium only. All plants were maintained at 30°C with an irradiance of 60 µmol photons m^{-2} s^{-1} for 12 h d^{-1}. 24 hours after the inoculation, plants were harvested and and both the roots and culture medium were collected in duplicates. A pool of six plantlets constituted each sample.

Examination of bacterial colonization was estimated by the Most Probable Number (MPN, according to the methods of Reis *et al.* (31)).

Metabolic labeling of bacterial and plant cells by incorporation of $^{14}N/^{15}N$ isotopes

Metabolic labeling with $^{14}N/^{15}N$ was performed through the incorporation of $^{14}N/^{15}N$ isotopes during cellular growth in medium containing ^{14}N or ^{15}N salts. Samples grown in ^{14}N media (natural abundance of nitrogen isotopes) are called “non-labeled” and those grown in 98 % enriched ^{15}N media are called “labeled”. For bacterial labeling cells were grown as described above with the replacement of NH_4NO_3 and KNO_3 by $^{15}NH_4{}^{15}NO_3$ and $K^{15}NO_3$ in equal amounts of ^{14}N media (described in section above). ^{15}N-labeled plants were obtained by substitution of NH_4NO_3 and KNO_3 by $^{15}NH_4{}^{15}NO_3$ and $K^{15}NO_3$ in equal amounts of ^{14}N media, during multiplication and rooting periods. ^{14}N-labeled bacterium and plants were grown in parallel under the same conditions in the presence of salts containing ^{14}N and ^{15}N isotopes at their natural abundance. Reverse-labeling and two independent experiments were performed. ^{15}N salts were purchased from Cambridge Isotope Laboratories Inc.

Protein extraction methods

Optical density at 600 nm of the bacterial suspension collected after the experiment were obtained by spectrophotometrical analysis and normalized volumes of bacterial cells from test and control samples (labeled and non-labeled) were mixed, ressuspended in SDS-PAGE Laemmli buffer (71) and heated for 10 minutes at 100 ºC. A clear lysate was obtained after centrifugation at 14,000 g for 1 hour at 4 ºC. Plant samples were weighted immediately after the experiment. 100 mg of tissue from test and control samples (labeled and non-labeled) were mixed, frozen in N_2 liquid and macerated. The resulting tissue was disrupted to a fine powder with the Sample Grinding Kit (GE Healthcare). Proteins were further extracted with the Plant Total Protein Extraction Kit (Sigma). A clear lysate was obtained after centrifugation at 14,000 g for 30 min at 4 ºC.

Protein separation and in gel digestion

SDS-PAGE was performed on a 12.5 % polyacrylamide gel in a mini Protean II system (Bio-Rad). About 20 µg of protein per lane were applied onto the gels. Gels were stained with Coomasie Brilliant Blue G-250 and images were acquired in LabScan 5 (GE Healthcare). SDS-PAGE gels were sliced in 5 to 10 regular pieces. Excision and processing of protein spots from SDS-PAGE gels were performed as previously described (38).

ESI-Q-TOF analysis

Liquid chromatography followed by tandem mass spectrometry (LC-MSMS) was performed in a Q-TOF Ultima API mass spectrometer (Micromass, Manchester, UK) to analyze tryptic peptides from proteins separated by SDS-PAGE. A nanoflow ESI source was used with a lockspray source for lockmass measurements during all the chromatographic run. Digested proteins were desalted online using a Waters Opti-Pak C18 trap column. The mixture of trapped peptides was then separated by elution with a water/ACN 0.1 % formic acid gradient through a Nanoease C18 (75µm ID) capillary column. Data were acquired in data-dependent mode (DDA), and multiple-charged peptides ions (+2 and +3) were automatically mass selected and dissociated in MS/MS experiments. Typical LC and ESI conditions were a 200 nl/min flow, a 3 kV nanoflow capillary voltage, a 100 °C block temperature and a 100 V cone voltage.

Protein identification

Mascot Distiller file Q-Tof.opt default parameters (available at www.matrixscience.com.br in July, 2009) was used for spectra processing and lock spray mass 784.8230 over 3 scans and tolerance of +- 0.3 Da was used for calibration. The MS/MS spectra were processed using the Mascot Distiller software version 2.3.0 (MatrixScience) and the peaklists generated were searched against the *G. diazotrophicus* or NCBI database using the MASCOT search engine tool

(MatrixScience). Protein identifications from root samples were performed with a taxonomy filter for *Arabdopsis thaliana* entries, due to high level of database annotation.

Database searches were performed with tryptic specificity allowing one missed cleavage and a mass tolerance of 0.1 Da for parent and fragment ions. Oxidation of methionine residues and carbamidomethylation at cysteine residues were considered as variable modifications; other modifications were included if clearly described below. Every peptide with $p < 0.05$ was considered for manual inspection. Protein identification was based on at least one unique peptide (a peptide sequence exclusive of one protein hit of the database). If a protein appeared under different names and/or accession numbers, the entry with the highest sequence coverage was selected.

Relative quantification and statistical analysis

For relative protein quantification based on metabolic $^{14}N/^{15}N$ labeling, the Mascot Distiller quantification package (Matrix Science) was used. The quantitation.xlm file (available at Matrix Science homepage in July 2009) was modified according to the specific requirements. Briefly: the percent of ^{15}N incorporation was set to 25% (bacterial samples) or 50% (plant samples), a quadratic integration of precursor peak intensities across a range of survey scans was performed over the entire isotopic envelope, a minimum of 1 unique peptide was required, protein ratio was calculated as the geometric average of peptide ratios, outliers were automatically removed and normalization was done in relation to the average. The standard deviation of the quantitation value for the protein ratio was performed in order to check for outliers and the consistency of the measures for a normal distribution. Significant changes in protein expression levels were reported if the protein ratio is significantly different

from unity. The comparison test is: $|x-\mu| \leq t * s/\sqrt{N}$, where N is the number of peptide ratios, s is the standard deviation and x the mean of the peptide ratios, both numbers calculated in log space. The true value of the ratio, μ, is 0 in log space. t is students t for N-1 degrees of freedom and a two-sided confidence level of 95%. Unpaired peptides indicated a protein expressed exclusive of one condition and therefore they were also included in the differential expression list.

Bioinformatic analysis of proteins

A series of computational tools were used in an attempt to assign potential functions to hypothetical proteins. The BLASTp program and Pfam database were used, respectively, to search for sequence similarities, protein domains or conserved protein regions (72; 73). TMHMM Server v2.0 was used for prediction of transmembrane helices in proteins (75). SignalP Server v3.0 predicted the presence and location of signal peptide cleavage sites in amino acid sequences (165).

Results and Discussion

A reliable quantitative proteomic workflow was established for the study of bacteria-plant interaction

Until now $^{15}N/^{14}N$ salts have been the most successful approach for metabolic isotopic labeling of plants (66; 68; 90; 91). Such strategy has also been applied for the study of bacteria and yeast cells (92; 93). However, this approach has never been employed for a complex system such as the co-culture of bacterium and host. In order to investigate the differential proteome expression of *G. diazotrophicus* co-cultivated in vitro with sugarcane the available protocols for quantitative proteomic analysis were optimized in this work. Then, we have combined $^{15}N/^{14}N$ metabolic labeling of proteins, 1D-GE and LC-ESI-Q-Tof to obtain a relative quantification measure of protein expression levels. A reverse labeling approach was performed in

order to avoid bias on isotopic labeling. The overall strategy chosen for this study is shown in Fig. 1.

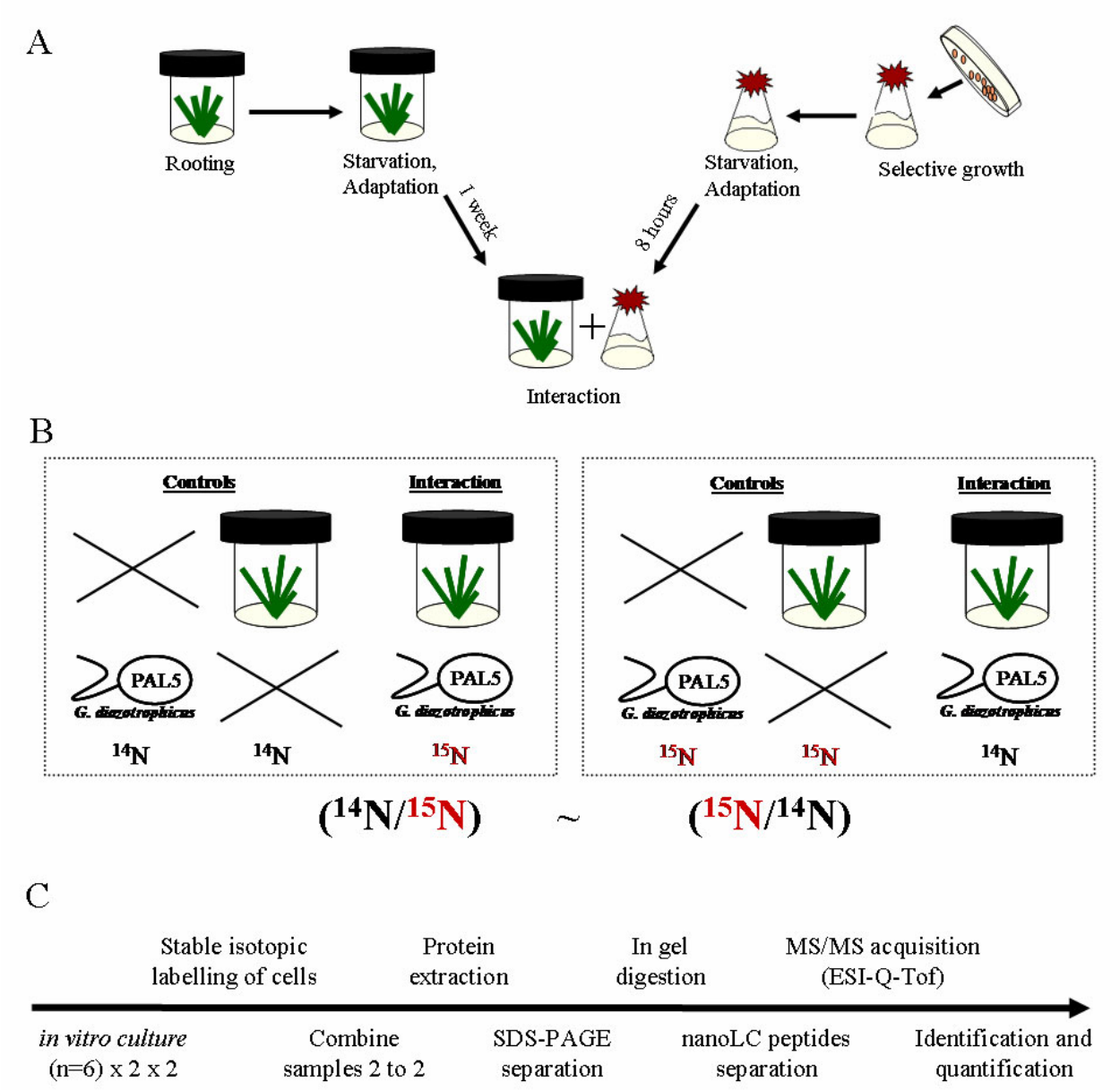

Fig. 1: Schematic representation of the methodology followed in this study. A) Bacterium and plant were initially grown in multiplication media and then starved in a ten fold diluted MS medium before the interaction. B) Control and interaction samples were grown in minimal media containing ^{14}N (non-labeled sampels) or ^{15}N (labeled samples) for the metabolic incorporation of these isotopes in proteins. The reverse

labeling strategy was used in order to increase the reliability of the results. C) Steps required for a reliable measure of protein expression.

Following the optimization, an evaluation of each step of the protocol was carried out. For this purpose, we have estimated the incorporation of ^{15}N as an average of the ^{15}N incorporation rate (according to variations at the isotopic envelope distribution) of 20 different random peptides in each sample. Bacterial and plant cells from ^{15}N labeled samples counted about 25% and 50% of ^{15}N incorporation, respectively. These values obtained assure a reliable quantification approach since at these rates labeled and non-labeled isotopic envelopes are clearly dissimilar (66). Besides, such low incorporation levels were achieved in a few weeks for plants and few hours of bacteria growth, allowing less manipulated and healthier biological samples. Furthermore, the partial incorporation rates were accounted as a correction factor for the quantification calculi (93).

Further, we have guaranteed the efficiency of the protein extraction and separation methods on SDS-PAGE gels. The simple and fast methods of protein extraction employed promised minimal protein loss and degradation. Bacterial and root samples presented several bands widely separated on 12.5 % bis-acrylamide gels. Most of proteins presented molecular weight ranging from 10 to 100 kDa. Accordingly, the peptides obtained after a tryptic digestion spread over the 60 min range of the LC separation on a C18 collumn if eluted in a linear gradient of 10 to 90 % of acetonitrile. Such high resolution of the chromatographic peaks in LC is a pre-requisite for a consistent relative quantification. The comparison of the peak intensities from different peptides in a simple MS spectrum does not reveal any quantitative information, because peptides with different aminoacid sequences present different ionization properties. However, the ^{15}N labeling of a peptide does not modify its

ionization properties. The quantification method using ^{15}N labeling is based on a shift of mass of the ^{15}N labeled peptide in relation to the same peptide sequence from a non-labeled (^{14}N) sample. Thus, the peak intensity of a ^{15}N labeled peptide can be compared to the intensity of the ^{14}N peptide revealing their abundances in the original samples (94).

A very interesting feature of this methodology that we have employed is that each peptide is detected in several MS spectra, according to its elution profile from LC. As the quantitative measure depends exclusively on the MS (and not MS/MS) data it is possible to plot the intensities of the heavy and light peptide for each MS scan of the survey run along the chromatographic peak. The gradient of a least square fit of this plot represents the best estimate ratio of ^{15}N /^{14}N (89). This approach is such robust that small variations in peptide abundance might be detected with high confidence. A further issue considered was the standard deviation between ratios calculated for different peptides belonging to the same protein. Obviously, high deviations among the measures for the same protein represent an unreliable value and were not considered.

Finally, the reverse labeling approach provided an additional aspect of trustworthy, since it was verified a 95 % correspondence between experiment I ($^{15}N/^{14}N$) and experiment II ($^{14}N/^{15}N$).

G. diazotrophicus expressed proteins involved in stress response and signaling cascades under interaction with sugarcane SP-70

In order to discover proteins involved in the interaction between *G. diazotrophicus* and sugarcane, we have compared the protein expression profile of *G. diazotrophicus* control samples with *G. diazotrophicus* co-cultivated with SP-70. As the co-cultivation system implies that the bacterial cells are under plant signaling

stimuli and SP-70 present a high contribution of BNF, this comparison reveals bacterial proteins important for the establishment of an efficient interaction.

In overall, these samples allowed the identification of 278 different bacterial proteins. The quantitative analysis, including replicate verification and reverse labeling, revealed the differential expression of 34 proteins (about 12%). Of these, 32 were significantly upregulated and 2 were downregulated during interaction with sugarcane.

Table 1 summarizes the proteins and putative functions assigned for them.

Regarding the general functions of proteins more abundant in *G. diazotrophicus* cells in contact with sugarcane SP-70, three main groups of proteins were differentially expressed: proteins involved in metabolism (15 proteins), general stress response (14 proteins) and signaling systems (5 proteins).

The identification of so many metabolism related proteins, including cell division process and pentose phosphate pathway, indicate that *G. diazotrophicus* cells are active and replicating. They include proteins involved in carbohydrate metabolism (such as aconitate hydratase, fructose-1,6-bisphosphate aldolase, enolase protein, phosphoketolase, bifunctional transaldolase/phosoglucose isomerase and transketolase), energy metabolism (F0F1 ATP synthase), aminoacids metabolism (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase and glutamine synthetase), nucleotides metabolism (polynucleotide phosphorylase), transcription (DNA-directed RNA polymerase subunit beta, ribosomal protein S1) and others (ATP dependent Clp protease, cell division protein FtsZ). This result is in agreement with previous studies that observed dividing *G. diazotrophicus* cells inside sugarcane plants (36).

Besides these proteins, several molecules involved in cellular adaptation were differentially expressed, indicating that *G. diazotrophicus* cells sensed plant signals and presented a general response to the interaction in 24 hours. Some of these proteins are related to stress response (chaperone protein DnaK, chaperonin GroEL, translation elongation factor Ts and chaperone binding protein) and perform chaperone functions in the cell (37; 95). Protein folding assistance is intimately involved in stress response since several stress agents directly interfere with protein folding. Therefore, the increase of chaperone expression levels during cellular adaptation to a stress situation is a necessary process for proper cell functioning (96). As discussed above, the interaction between an endophytic bacterium with its host is a biotic stress source to both organisms (97). Therefore, the identification of these 4 chaperone related proteins is coherent with the experiment performed and suggest that although these bacterial cells did not enter the plant in 24 hours they are already sensing the presence of the sugarcane. Accordingly, stress related proteins of *G. diazotrophicus* are also increased after 7 days of interaction with sugarcane plantlets (37).

An additional mechanism of cellular adaptation occurs during *G. diazotrophicus* interaction with SP-70. The plasma membrane and LPS function as a protective and selective barrier to bacterial cells. Thus, alterations in these components are regulated according to environmental changes and implies in activation/deactivation of signaling systems (98). The identification of a TonB-dependent receptor protein, TonB protein, Outer membrane protein OmpH and Outer membrane lipoprotein differentially expressed by *G. diazotrophicus* in the presence of the plant host suggest they are part of this regulatory process. The TonB receptors frequently function in cooperation with lipoproteins to control the transport of nutrients to the cell

(99). Thus, the identification of both a TonB receptor protein and a lipoprotein during *G. diazotrophicus* interaction with sugarcane reinforce the importance of this mechanism for bacterium-plant signaling. Moreover, TonB-dependent receptors are antigenic proteins involved in iron utilization and virulence in several bacteria (98; 100; 101). As some TonB peptides are well conserved, it is reasonable that they might represent a pattern for plant recognition of bacterial cells. Additionally, it was described that the TonB homologue of *Ralstonia solanacearum*, PrhA, binds the plant host cell wall and initiates a regulatory cascade that induces plant defense responses crucial for plant-bacteria interaction (102; 103). Therefore, it is reasonable that receptors from TonB family are overrepresented in *G. diazotrophicus* cells during interaction with sugarcane and could instigate a sugarcane cascade that allows bacterial colonization.

The outer membrane protein OmpH belongs to a protein family of immunogenic porins that allows small molecules flow through the outer membrane, but also act as receptors or chaperones (104; 105; 106). Thus, its role in *G. diazotrophicus* might be both structural and regulatory.

Besides membrane remodeling and protein folding, general stress responses also deal with avoiding oxidative damage (107; 108). Thus, proteins involved in redox reactions and electron transfer chains are also related to stress responses, as an attempt to resist to oxidative injure. Under interaction with SP-70 *G. diazotrophicus* specifically expressed an alcohol dehydrogenase, 2-oxoglutarate dehydrogenase, 6-phosphogluconate dehydrogenase-like protein, electron transfer flavoprotein subunit alpha, isocitrate dehydrogenase and peroxiredoxin protein. These proteins might take part of this system and demonstrate that *G. diazotrophicus* evolved mechanisms of protection under interaction with the host. Thus, it suggests that *G. diazotrophicus*

is adapted to the interaction with sugarcane SP-70. Among them, the alcohol dehydrogenase might perform a fundamental role, as an *E. coli* homologue constitutes the main barrier against oxidative damage (109).

Besides the metabolic alterations, *G. diazotrophicus* displayed regulatory functions under interaction with SP70. Three transcriptional regulators were identified: a two-component transcriptional regulator (Gdi3420), a two component response regulator (Gdi1456), and the transcription elongation factor NusA (Gdi1363). The transcription elongation factor NusA is a modulator of RNA polymerase, thus involved in the regulation of gene expression (110). The two component transcriptional regulator (Gdi3420) identified is 70% identical to *Acetobacter pasteurianum* KdpE protein. KdpE is the transcriptional activator of the *kdp* operon in *E. coli* that codes for an ATPase transport system of high affinity for potassium (111). It is suggested that the Kdp system is regulated by potassium concentrations, turgor pressure and salt stress (112). In fact, the osmotic regulation might be a key process for *G. diazotrophicus* to survive inside sugarcane. The other two component response regulator identified (Gdi1456) belongs to TIGR family 01387. Most members of this family are part of a system for sensing heavy metals, such as copper, silver, cadmium, and zinc (113). Accordingly, *G. diazotrophicus* is a bacterium that metabolizes insoluble zinc compounds and is suggested that the availability of zinc favors *G. diazotrophicus* host growth (114).

Two other interesting proteins identified were a hypothetical protein (Gdia2515) and a surface antigen protein (Gdi0770). The hypothetical protein Gdia2515 belongs to YfdX protein family of unknown function. This is a very interesting result since hypothetical proteins are organism-specific. Although annotated as hypothetical we have confirmed that this protein is really expressed by *G. diazotrophicus*. Likewise,

the predicted surface antigen protein does not present high similarity to any other known protein, but seems to be part of the early plant-bacteria recognition process. In contrast with the high number of proteins upregulated during *G. diazotrophicus* interaction with SP-70, only two proteins were significantly downregulated. They are: a phosphate-binding periplasmic protein precursor (Gdi3819) and a peptidase (Gdi1307).

G. diazotrophicus **expressed proteins involved in adaptation to atypical conditions under interaction with sugarcane Chunee**

In order to discover proteins involved in the interaction between *G. diazotrophicus* and sugarcane, we have compared the protein expression profile of *G. diazotrophicus* control samples with *G. diazotrophicus* co-cultivated with Chunee. As the co-cultivation system implies that the bacterial cells are under plant signaling stimuli and Chunee presents low contribution of BNF, this comparison might reveal bacterial proteins that deals with the establishment of a non-efficient interaction.

The comparative analysis of *G. diazotrophicus*-Chunee interaction and control samples revealed about 264 protein identifications. Of these, 44 (16%) were differentially expressed between control and test samples (42 upregulated and 1 downregulated during plant-bacterium interaction; Table 2). As observed in interaction with SP-70, many *G. diazotrophicus* proteins were upregulated during interaction and comprehend three major categories of proteins: metabolism, stress-related and signaling.

The 16 metabolism proteins specifically expressed by *G. diazotrophicus* during interaction with Chunee include transcription (DNA-directed RNA polymerase subunit alpha, DNA-directed RNA polymerase subunit beta'), translation (30S ribosomal protein S1 and 50S ribosomal protein L4), energy metabolism (F0F1 ATP synthase

subunit beta), carbohydrate metabolism (fructose-1,6-bisphosphate aldolase, bifunctional transaldolase/phosoglucose isomerase, transketolase and phosphoketolase, inosine-guanosine kinase), aminoacids metabolism (carbamoyl phosphate synthase and glutamine synthetase), nucleotides metabolism (polynucleotide phophorylase, phosphoribosylformylglycinamidine synthase II and UTP--glucose-1-phosphate uridylyltransferase) and others (aminopeptidase). These protein identifications also indicate that *G. diazotrophicus* present an active metabolism under these conditions.

As discussed above, the general stress response is required for bacterial cells to adapt and survive under a biotic stress. Accordingly, 17 proteins responsive to stress were identified. Among them, 6 are related to protein folding (chaperone protein DnaK, chaperonin GroEL, cold shock-like protein CspE, elongation factor G, elongation factor Ts and elongation factor Tu), 6 to oxidative stress (isocitrate dehydrogenase, peroxiredoxin protein, putative quinoprotein glucose dehydrogenase, 6-phosphogluconate dehydrogenase-like protein, ketol-acid reductoisomerase and catalase) and 5 to membrane remodeling (Outer membrane lipoprotein Gdi2185, Outer membrane lipoprotein Omp16, Outer membrane protein Gdi1275, TonB-dependent receptor Gdi3715 and TonB-dependent receptor protein Gdi1471). It is interesting to note that the quinoprotein glucose dehydrogenase (GDH-PQQ) is the enzyme that converts glucose to gluconic acid. It was shown that gluconic acid is responsible for solubilization of phosphate and zinc compounds, increasing the availability of these elements for both bacterium and plant nutrition (115). Therefore, the increased expression of this protein under interaction of *G. diazotrophicus* with sugarcane, suggest that this mechanism might also occur in vivo.

The most interesting protein identifications are related to chemotaxis (McpA and CheA proteins) and Inositol metabolism (inositol-3-phosphate synthase). Chemotaxis is a process of molecular attraction between two interacting organisms (166; 167; 168). Although it was not yet shown to occur in *G. diazotrophicus* interaction with its hosts, it is quite suitable that McpA and CheA are key *G. diazotrophicus* molecules on the communication interplay between *G. diazotrophicus* and sugarcane. If the chemotaxis signal is perceived by the receptor, signaling cascades might be activated and result in bacterial cell movement towards the host (166). In contrast with eukaryotes, in bacteria the inositol derivatives are not related to signal transduction pathways. They are suggested to function as osmolytes and lipid anchors that are critical for the interaction with host cells (116). Curiously, one protein belonging to inositol metabolism (inositol-3-phosphate-synthase) was also upregulated by *G. diazotrophicus* under early events of the interaction with Chunee, suggesting that it might be part of this specific signaling pathway.

Additionally, molecules involved in signaling and regulation of gene expression were identified. The histidine kinase Gdi1745 and the transcriptional regulators IciR and LysR might be related to signal transduction and modulation of cellular metabolism. Besides, two other proteins were upregulated during *G. diazotrophicus* interaction with Chunee: a bacterioferritin and a bacteriocin protein. The bacterioferritin is a protein involved in iron storage (169). Further studies are required to determine the overlap between bacterial colonization in plants and iron metabolism. The bacteriocin Gdi0415 is linocin M18 family member that act as antimicrobial peptides. In fact, it is already shown that *G. diazotrophicus* antagonizes the growth of some sugarcane pathogens in culture medium (118; 162; 170). However, this is the first report of the expression of a *G. diazotrophicus* bacteriocin instigated by sugarcane.

Altogether, the identification of these proteins upregulated during *G. diazotrophicus* interaction with Chunee, indicate that the bacterial cell sense and respond to the interaction with this low BNF contribution genotype. In another hand, two additional proteins were downregulated under the same conditions: 6-phosphogluconate dehydrogenase and 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase. These proteins are components of sugar and aminoacids metabolism, respectively. Therefore, it seems that *G. diazotrophicus* decreased cellular catabolism in order to adapt and interact with the new environment.

G. diazotrophicus respond to both sugarcane genotypes (SP-70 and Chunee) through similar mechanisms

As introduced above, sugarcane genotypes present distinct contribution of BNF (119). Thus, a previous study suggested that *G. diazotrophicus* interaction with SP-70 could be a more adapted relation than *G. diazotrophicus* interaction with Chunee (119). However, a molecular comparison between *G. diazotrophicus* interaction with SP-70 versus Chunee was not previously described. In order to disclose proteins involved in the establishment of a successful interaction between *G. diazotrophicus* and sugarcane, we compared the bacterial proteins differentially expressed under the interaction with each genotype.

As discussed above, *G. diazotrophicus* differentially expressed proteins involved in metabolism, adaptative responses and signaling during the interaction with both sugarcane genotypes (Tables 1 and 2). From the 34 proteins upregulated by *G. diazotrophicus* during interaction with sugarcane SP-70, 20 were also differentially expressed by bacterial cells under interaction with sugarcane Chunee (Fig. 2A). This result indicates that *G. diazotrophicus* present similar mechanisms of interaction with both sugarcane genotypes. In order to further explore these differences and

similarities observed, proteins were grouped according to Kegg categories (Fig. 2B) and the percent of proteins per category was compared between *G. diazotrophicus* interaction with SP-70 and Chunee.

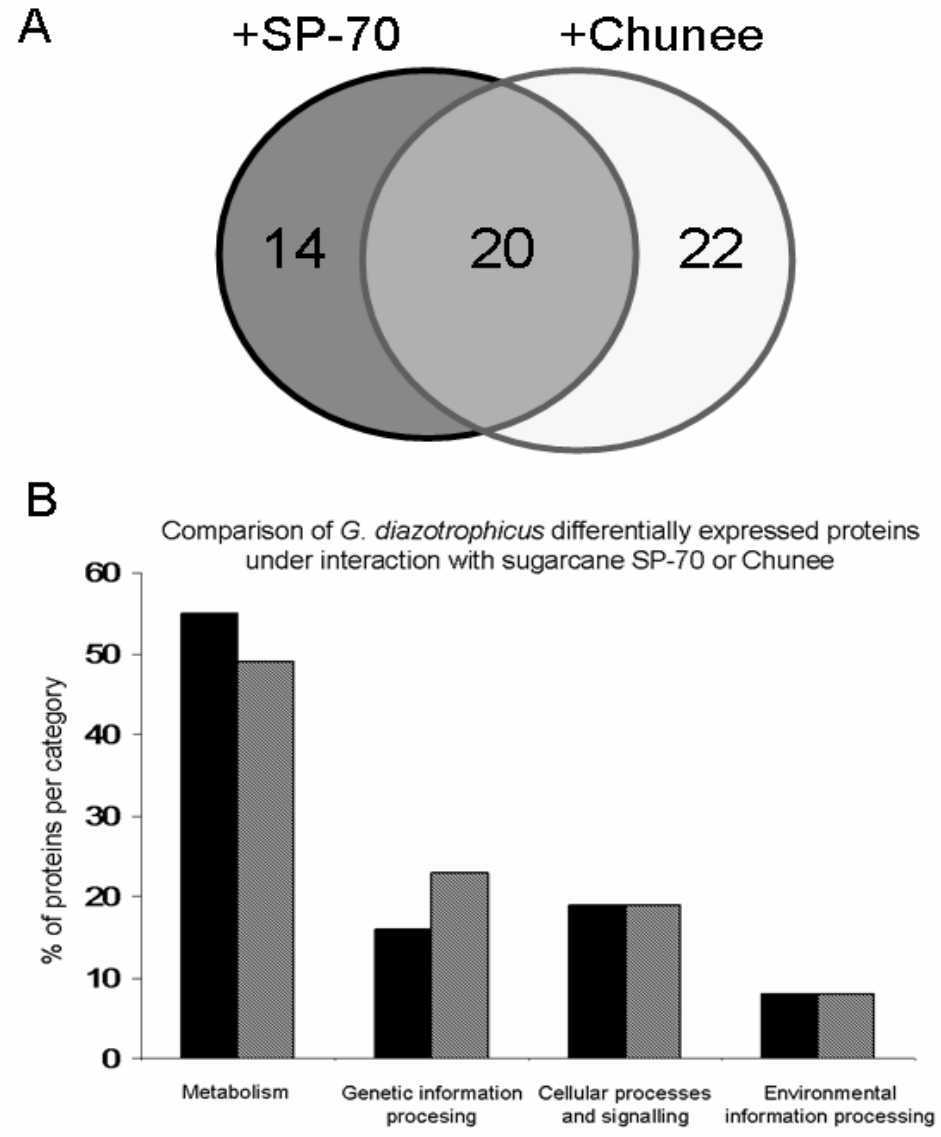

Fig. 2: A comparison of the differentially expressed proteins by *G. diazotrophicus* under interaction with SP-70 or Chunee. A) A Venn diagram showing the intersection (number of proteins) between the differentially expressed proteins by *G. diazotrophicus* under interaction with SP-70 and Chunee. B) Grouping of the differentially expressed proteins according to Kegg categorization. Proteins

upregulated under interaction with SP-70 are shown in black bars and those increased on interaction with Chunee are in gray.

The Fig. 2 present a similar differential expression profile of proteins in both experiments analyzed. In both situations, bacterial cells increased metabolism and signaling mechanisms that improves cell survival and adaptation to a different environment. Such strategy is a mechanism described to occur during the interaction of *Rhizobium* species with its hosts and might also be part of *G. diazotrophicus* general response to the interaction with sugarcane (37; 120). Our results suggest that *G. diazotrophicus* perceives both sugarcane genotypes and initially respond through similar general mechanisms to both interactions.

However, a few differentially expressed proteins were found exclusively during *G. diazotrophicus* interaction with SP-70 or Chunee. For example, it is noteworthy that *G. diazotrophicus* interaction with Chunee upregulated the expression of more chaperone proteins (elongation factors G, Tu and Ts) than *G. diazotrophicus* interaction with SP-70. Thus, such identifications indicates that *G. diazotrophicus* is under higher stress level and corroborates the hypothesis that *G. diazotrophicus* might be less adapted to Chunee than SP-70 interaction.

Another remarkable aspect is the increased expression (about 2 fold) of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MetH) under interaction with SP-70 and decreased level (reduced 2 fold) under interaction with Chunee (Tables 1 and 2). MetH is a protein involved in methionine metabolism, responsible for the homocysteine conversion in methionine. The role of methionine metabolism under symbiotic interactions was not described. However, it is shown that the homocysteine – methionine cycle directly influences the production of autoinducer molecule AI-2. AI-2 is known as a quorum sensing molecule involved in

bacterial cell to cell signaling. Thus, the differential expression of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase might suggest that *G. diazotrophicus* present a more efficient communication system during interaction with SP-70 than with Chunee, leading to a promising interaction with the high BNF contribution genotype SP-70.

Summarizing, the comparative analysis of *G. diazotrophicus* proteins presented above revealed that it perceives and respond essentially through the same mechanisms to interaction with SP-70 and Chunee. Thus, the distinct BNF contribution of SP-70 and Chunee might be explained also by other factors. In order to unveil the role of the plant genotype as a determinant factor of the establishment of the plant-bacterium interaction we analyzed the proteins differentially expressed by SP-70 and Chunee plant roots during interaction with *G. diazotrophicus.*

Sugarcane genotype SP-70 altered lipid composition and phosphorylation cascades in response to *G. diazotrophicus* interaction

In order to disclose plant proteins involved in the interaction between *G. diazotrophicus* and sugarcane, we have compared the protein expression profile of SP-70 roots that were inoculated with *G. diazotrophicus* and control samples. As SP-70 presents a high contribution of BNF, it is expected that inoculated SP-70 roots present signaling mechanisms important for the establishment of an efficient interaction.

In overall, 61 proteins were confidently identified from sugarcane SP-70 root samples. Although not so many proteins were identified, it was possible to quantify their expression and detect 7 proteins (about 11%) ecxclusively expressed by SP-70 roots during interaction with *G. diazotrophicus* (Table 3). The differentially expressed proteins were: L-ascorbate peroxidase, an ATP citrate lyase, TUA6 protein,

glutamate ammonia lyase, 14-3-3 protein, ATP binding protein kinase and a receptor like kinase.

The L-ascorbate peroxidase is involved in oxidative stress response, a known protective mechanism required under biotic stress challenges (129). Another adaptative mechanism detected under this condition is related to lipids metabolism. The ATP citrate lyase is a key enzyme for the regulation of lipids biosynthesis, thus indicating that SP-70 changes lipids in the membrane in order to respond to bacterial infection. The modulation of lipid composition is very important for the colonization process of other bacteria and agrees with the altered morphology of plant cells previously observed (130). Additionally, it was demonstrated that cytosolic ATP citrate lyase of sweet potato is involved in protection of plant cells against pathogen infection, thus, indicating the involvement of this protein as a response to bacterial interaction (131). Hence, TUA6 is a membrane protein whose function is not yet clear, but its identification is additional evidence that the cell membrane is an essential component for bacterial-host interaction.

Besides the adaptative response, SP-70 presented proteins that might function specifically in the interaction. 14-3-3 proteins are conserved regulatory proteins with a motif of interaction with several signaling proteins (132; 133). It is upregulated in several plant biotic and abiotic stresses (134). Further studies are required for the correct assignment of its function. Furthermore, SP-70 upregulated the expression of two protein kinases (a putative tyrosine kinase and a putative receptor like kinase), further indicating the activation of a signaling cascade supported by *G. diazotrophicus* interaction.

Finally, the identification of glutamate ammonia lyase indicates an increased nitrogen metabolism in SP-70 plants grown with *G. diazotrophicus*. It is far known that *G. diazotrophicus* raises nitrogen inputs for plant growth.

Sugarcane genotype Chunee activated protein degradation systems and chromatin silencing pathways in response to *G. diazotrophicus* interaction

In order to disclose plant proteins involved in the interaction between *G. diazotrophicus* and sugarcane, we have compared the protein expression profile of Chunee roots that were inoculated with *G. diazotrophicus* and control samples. As Chunee genotype does not benefit much of BNF, it is expected that inoculated Chunee roots present mechanisms that do not support he establishment of an efficient interaction with *G. diazotrophicus.*

The proteomic analysis of Chunee roots infected by *G. diazotrophicus* revealed 9 proteins differentially expressed. Among them, there are 2 distinct subunits of the proteasome complex, 3 histones, a cell cycle regulator, an ethylene receptor and 2 other membrane proteins (Table 4).

The increased level of proteasome components suggests that protein degradation occurs in infected Chunee roots cells. Increased levels of proteasome complexes are frequently associated with plant defense mechanisms that sometimes might trigger plant cells programmed death (135). Such strategy is described for pathogen-host interactions. Therefore, it suggests that Chunee roots might not have been able to recognize *G. diazotrophicus* as a benefic bacterium, fired defense mechanisms and deals with it as if it was a pathogen.

Additionally, the identification of Cdc48-like protein, a cell cycle regulator also important for vesicles trafficking and fusion in membrane, suggests additional efforts concerned in plant defense against *G. diazotrophicus* (136). Accordingly, previous

studies have demonstrated the synthesis of several vesicles from epidermal cells of sugarcane roots under interaction with *G. diazotrophicus*. Putting these results together we suggest that such membrane vesicles are also part of the plant defense system.

The identification of histones H2B, H3 and H3.2 also indicates that gene expression is being regulated and it is feasible that gene silencing mechanisms are involved. Histones are proteins known to be highly post-translational modified and such modifications are crucial for its activity. Curiously, we have identified the peptide KTAAEKPVEENK of histone H2B with 3 acetylations (K1, K6, K12) and 2 methylations (E9, E10). The role of these modifications will be further explored.

Finally, the identification of the ethylene receptor ETR2 is very intriguingly since is described a clear correlation between ethylene responsive pathways and biotic stresses. The activation of ethylene receptors culminates with a decrease of stress responses and plant defense mechanisms, among others. Thus, it favors bacterial-plant interaction. Accordingly, a previous study has shown that a sugarcane ethylene receptor mRNA is induced by *G. diazotrophicus* stimuli, thus our results for protein levels are in agreement with the previous results for mRNA quantification (40).

Sugarcane genotypes SP-70 and Chunee expressed different proteins in response to *G. diazotrophicus* interaction

The sugarcane proteins identified showed a very interesting mechanism of response to an endophytic microorganism. The differential expressed proteins by each genotype during interaction with *G. diazotrophicus* are quite different, although they demonstrate that both genotypes respond to *G. diazotrophicus* interaction.

In one hand, SP-70 mainly expressed proteins for stress resistance and signaling cascades that allow for bacterial colonization. In another hand, Chunee completely

altered its metabolism and increased defense resposes. As discussed above proteasomes are complexes involved in degradation of incorrect folded proteins, suggesting that Chunee was not able to properly respond to the biotic stress of *G. diazotrophicus*. Additionally, the identification of the histones suggest that they might act as global regulators of gene expression. Altogether, these results indicate that SP-70 efficiently recognized *G. diazotrophicus* as a benefic bacterium whereas Chunee manage to deal with the bacterium and initiates broader metabolic changes and defense responses.

Identification of *G. diazotrophicus* proteins from sugarcane root samples

As an attempt to identify *G. diazotrophicus* proteins expressed by *G. diazotrophicus* cells in intimate contact with sugarcane, roots of sugarcane SP-70 and Chunee varieties after 24 hours of bacterial inoculation were analyzed for the presence of bacterial proteins. Thus, we have searched all the MS/MS spectra acquired from root samples against *G. diazotrophicus* database. We have identified 8 *G. diazotrophicus* proteins from SP-70 roots and 25 from Chunee samples. In overall 30 different bacterial proteins were identified (Table 5). This result is extremely exciting since *G. diazotrophicus* proteins expressed “in planta” were not previously described. Among them, 9 were not previously detected under high throughput proteomic analysis of *G. diazotrophicus* grown under several conditions, indicating that their expression is exclusively induced under direct interaction with sugarcane.

These nine proteins are: a component of potassium transport system, the putative cell volume regulator protein A, a chemotaxis protein methyltransferase, a cystathione gamma synthase, a glutathione S-transferase, the UDP glucose 6-dehydrogenase, a nucleoside 2-deoxyribosyltransferase, an outer membrane protein and a putative transporter.

Potassium is an abundant ion in bacterial cells and it has an essential role in osmotic regulation and cellular homeostasis (141). In addition to potassium, sodium and hydrogen ions concentrations are also strictly regulated by cells, as they perform regulatory roles mainly in response to environmental stresses (142). Thus, the identification of two components of ion transport in *G. diazotrophicus* cells suggest that during interaction with sugarcane, bacterial cells faces a new osmotic environment and ion transport is a key mechanism for cellular adaptation. Additionally, it was shown that the potassium uptake system Kup (homologue of GDI2385) is essential for *Sinorhizobium meliloti* colonization of alfafa plants (143). Therefore, it reinforces the relevance of these proteins for a fruitful association of *G. diazotrophicus* with sugarcane.

Besides the osmotic stress response, *G. diazotrophicus* expressed a mechanism to avoid oxidative stress. This mechanism is pointed out by the identification of a glutathione S-transferase. It belongs to a family of proteins involved in avoiding oxidative damage, but can either perform roles in detoxification of xenobiotics (144). Another protein that might be part of a redox regulation system is cystathione gamma synthase. This enzyme participates on sulfur, cysteine and methionine metabolism. Such protein is very interesting since methionine pathway was previously shown to be regulated during *G. diazotrophicus* interaction with sugarcane (see discussion below).

G. diazotrophicus stress response to sugarcane interaction is further verified by the identification of two membrane proteins (GDI3457 and GDI2347), that might be involved in signal perception and membrane remodeling. Altogether, the identification of these proteins suggests that cell volume regulation and membrane remodeling are required for proper *G. diazotrophicus* interaction with sugarcane. Accordingly,

previous studies have demonstrated that *G. diazotrophicus* present altered morphology during endophytic colonization, thus the data present herein explains at molecular level the evidence described before.

Moreover, we have identified two proteins involved in nucleotides metabolism (GDI1810 and GDI1140). One of them, UDP glucose 6-dehydrogenase is essential for *V. fischeri* colonization of its host (145), suggesting that its homologue in *G. diazotrophicus* might even play a direct role during interaction with sugarcane.

Finally, we have identified a chemotaxis protein methyltransferase that is involved in coupling the chemotaxis signal perception and bacterial motility. Certainly, chemotaxis is a process directly involved in early signaling of *G. diazotrophicus* and therefore indicates a mechanism of plant-bacterium signaling.

Concluding remarks

In this paper we describe for the first time a quantitative MS-based proteomic approach applied to the study of a microorganism-host interaction. The workflow proposed is quite fast and simple, avoiding experimental artifacts and assuring high quality data. Thus, we were able to identify and provide relative quantification data for more than 300 proteins of *G. diazotrophicus* and almost 100 proteins of sugarcane roots. From these, 58 bacterial proteins and 16 plant proteins were differentially expressed under the interaction of *G. diazotrophicus* with sugarcane. Accordingly, this is the first report regarding molecular characterization of early events (24 hours) of *G. diazotrophicus*-sugarcane interaction (Fig. 3). Additionally, we have compared the differential protein expression profiles *G. diazotrophicus* interaction with two genotypes of sugarcane. The two genotypes used for the experiments present very distinct contributions of BNF (SP-70: ~34 % and Chunee ~12 %). Accordingly, we have shown that *G. diazotrophicus* perceives and respond similarly to the interaction

with both of them, but each genotype presented quite dissimilar molecular responses. SP-70 expressed proteins involved in cellular adaptation and signaling systems that allow the bacterial colonization. However, Chunee roots instigated defense mechanisms that do not support the establishment of bacterial interaction. As discussed above, we propose that the proteins pointed in this study are related to a specific response and determine the success – or not - of bacterial colonization of plant tissues and interaction benefits.

Moreover, this is the first report on proteins expressed by *G. diazotrophicus* under close contact with sugarcane plants. 30 different bacterial proteins were described to be expressed by bacterial cells during the initial 24 hours of the process of sugarcane roots colonization. Among them, 9 have not been described in prior proteomic analysis of *G. diazotrophicus*. Therefore, these proteins are suggested to be regulated by a host signal. They comprise functions involved in chemotaxis, cell differentiation and osmotic and oxidative stress regulation. Whereas these molecular processes occur, bacterial morphology is significantly altered, corroborating microscopy data published before

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Table 1: Proteins differentially expressed by *G. diazotrophicus* cells co-cultivated with sugarcane SP-70.

Gi	Locus	Protein name	MW	Pld Score	PM	I/C ratio	Std
		Metabolism-related					
162147150	GDI1355	aconitate hydratase	97	490	7	1.75	1.1
162146316	GDI0491	fructose-1,6-bisphosphate aldolase	33	265	2	1.32	1.0
162147711	GDI1927	enolase protein	45	126	5	1.70	1.1
162147402	GDI1617	putative phosphoketolase	90	114	9	1.96	1.1
162146116	GDI0286	bifunctional transaldolase/phosoglucose isomerase	102	1238	46	1.66	1.4
162146115	GD10285	transketolase	83	489	11	1.43	1.1
162148355	GDI2572	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	85	236	66	2.0	1.1
162146350	GDI0525	glutamine synthetase	53	296	18	1.81	1.2
162147166	GDI1371	polynucleotide phosphorylase/polyadenylase	76	415	11	1.61	1.0
162149109	GDI3339	carbamoyl phosphate synthase large subunit	116	358	4	1.66	1.1
162149180	GDI3410	DNA-directed RNA polymerase subunit beta	153	733	36	1.51	1.1
162149228	GDI3460	30S ribosomal protein S1	62	346	20	1.56	1.3
162146518	GDI0694	F0F1 ATP synthase subunit alpha	55	452	10	1.69	1.0
162146853	GDI1036	ATP-dependent Clp protease proteolytic subunit	24	104	2	1.32	1.0
162148966	GDI3196	cell division protein FtsZ	50	107	8	2.22	1.1
		Adaptative responses and signalling systems					
209544264	GDIA2119	2-oxoglutarate dehydrogenase, E2 subunit, dihydrolipoamide succinyltransferase	44	28	5	2.20	1.0
162146117	GDI0287	6-phosphogluconate dehydrogenase-like protein	35	129	20	1.42	1.1
162147824	GDI2040	alcohol dehydrogenase [acceptor] precursor	80	227	3	1.75	1.1
162147075	GDI1280	electron transfer flavoprotein subunit alpha	31	65	6	1.41	1.3
162147775	GDI1991	isocitrate dehydrogenase	36	60	5	1.33	1.1
162146401	GDI0576	peroxiredoxin protein	20	62	3	1.32	1.0
209545461	GDIA3348	translation elongation factor Ts	32	120	3	1.22	1.0
162147058	GDI1263	chaperone binding	29	28	4	1.31	1.2
162147057	GDI1262	chaperone protein DnaK	67	30	20	1.22	1.1
162147833	GDI2049	chaperonin GroEL	58	33	41	1.51	1.3
162147969	GDI2185	Outer membrane lipoprotein	40	47	22	1.47	1.2
162147928	GDI2144	Outer membrane protein	31	108	3	1.40	1.1
162149477	GDI3715	TonB-dependent receptor	94	302	9	2.33	1.2
162147266	GDI1471	TonB-dependent receptor protein	117	53	40	1.33	1.1
209544645	GDIA2515	hypothetical protein Gdia_2515	22	49	3	1.12	1.0
162146599	GDI0777	surface antigen protein	24	34	2	*	-
162147251	GDI1456	two-component response regulator	25	60	4	*	-
209543371	GDI3420	two component transcriptional regulator	26	50	4	*	-
162147158	GDI1363	transcription elongation factor NusA	58	100	2	1.43	1.0
		Decreased during plant-bacterium interaction					
162149580	GDI3819	phosphate-binding periplasmic protein precursor	34	172	2	0.83	1.0
162147102	GDI1307	peptidase	80	28	2	*	-

Pid score: Mascot protein identification score; MW: protein theoretical molecular weight; PM: number of peptides matched; I/C ratio: relative quantification of protein expression between Interaction and Control samples; Std: standard deviation of the ratio; *: protein exclusively expressed in one sample.

Table 2: Proteins differentially expressed by *G. diazotrophicus* cells co-cultivated with sugarcane Chunee.

Gi	Locus	Protein name	MW	Pld Score	PM	I/C ratio	Std
		Metabolism-related					
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162146116	GDI0286	bifunctional transaldolase/phosoglucose isomerase	102	1238	46	1.66	1.4
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162146350	GDI0525	glutamine synthetase	53	296	18	1.81	1.2
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162149180	GDI3410	DNA-directed RNA polymerase subunit beta	153	733	36	1.51	1.1
162149228	GDI3460	30S ribosomal protein S1	62	346	20	1.56	1.3
162146518	GDI0694	F0F1 ATP synthase subunit alpha	55	452	10	1.69	1.0
162146853	GDI1036	ATP-dependent Clp protease proteolytic subunit	24	104	2	1.32	1.0
162148966	GDI3196	cell division protein FtsZ	50	107	8	2.22	1.1
		Adaptative responses and signalling systems					
209544264	GDIA2119	2-oxoglutarate dehydrogenase, E2 subunit, dihydrolipoamide succinyltransferase	44	28	5	2.20	1.0
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162146401	GDI0576	peroxiredoxin protein	20	62	3	1.32	1.0
209545461	GDIA3348	translation elongation factor Ts	32	120	3	1.22	1.0
162147058	GDI1263	chaperone binding	29	28	4	1.31	1.2
162147057	GDI1262	chaperone protein DnaK	67	30	20	1.22	1.1
162147833	GDI2049	chaperonin GroEL	58	33	41	1.51	1.3
162147969	GDI2185	Outer membrane lipoprotein	40	47	22	1.47	1.2
162147928	GDI2144	Outer membrane protein	31	108	3	1.40	1.1
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162147266	GDI1471	TonB-dependent receptor protein	117	53	40	1.33	1.1
209544645	GDIA2515	hypothetical protein Gdia_2515	22	49	3	1.12	1.0
162146599	GDI0777	surface antigen protein	24	34	2	*	-
162147251	GDI1456	two-component response regulator	25	60	4	*	-
209543371	GDI3420	two component transcriptional regulator	26	50	4	*	-
162147158	GDI1363	transcription elongation factor NusA	58	100	2	1.43	1.0
		Decreased during plant-bacterium interaction					
162149580	GDI3819	phosphate-binding periplasmic protein precursor	34	172	2	0.83	1.0
162147102	GDI1307	peptidase	80	28	2	*	-

Pid score: Mascot protein identification score; MW: protein theoretical molecular weight; PM: number of peptides matched; I/C ratio: relative quantification of protein expression between Interaction and Control samples; Std: standard deviation of the ratio; *: protein exclusively expressed in one sample.

Table 3: Proteins exclusively expressed by sugarcane SP-70 under interaction with *G. diazotrophicus*.

Gi	Protein name	Putative function	MW	Peptide sequence
4733972	14-3-3 protein (grf15), putative	Signalling	9	DSTLIMKILR
15226452	ATP binding / protein kinase/ protein tyrosine kinase	Signalling	70	RPVTSHK, LGLDWCK
99698	glutamate-ammonia ligase (EC 6.3.1.2), cytosolic	Nitrogen Metabolism	41	HKEHIAAYGEGNER
16173	L-ascorbate peroxidase	Stress response	27	SGFEGAWTSNPLIFDNSYFK
4262228	putative receptor-like protein kinase	Signalling	78	VVSLSIPR
9759429	ATP citrate lyase	Lipids metabolism / Stress	66	AGKDLVSSLVSGLLTIGPR
30683070	TUA6; structural constituent of cytoskeleton	Membrane protein	47	SVLKVVTMR, LVSQVISSLTASLR, AVFVDLEPTVIDEVR, AVFVDLEPTVIDEVRTGTYR

Table 4: Proteins exclusively expressed by sugarcane SP-70 under interaction with *G. diazotrophicus*.

Gi	Protein name	Putative function	MW	Peptide Sequence	PTM
6652882	26S proteasome AAA-ATPase subunit RPT3	Protein degradation	45	ENAPAIIFIDEVDAIATAR	-
6056388	26S proteasome ATPase subunit	Protein degradation	47	TMLEIVNQLDGFDAR	-
6630743	CDC48-like protein	Cell cycle	90	KGDLFLVR, LDEVGYDDVGGVR, IVSQLLTLMDGLK, AHVIVMGATNRPNSIDPALR, ETVVEVPNVSWEDIGGLENVKR, AFEEAEKNAPSIIFIDEIDSIAPK	-
2407802	histone H2B	Regulation of gene expression	16	LVLPGELAK, QVHPDIGISSK, SVETYKIYIFK, KTAAEKPVEENK	K13 (Ac), K18 (Ac), K24 (Ac), E21 (Me), E22 (Me)
9759615	histone H3	Regulation of gene expression	15	STEILIR	-
145334271	histone H3.2	Regulation of gene expression	19	STELLIR, VLSHSSLK	-
3687654	putative ethylene receptor; ETR2	Signalling	85	GRGGYGGCSVSMEDLDVVR, EIASWLLILSMVVFVSPVLAINGGGYPR	-
6671939	putative T-complex protein 1, ETA subunit	Membrane component	60	LAIGDLATQYFADRDIFCAGR	-
396218	transmembrane protein TMP-B	Membrane component	30	GSGLGAEIIGTFVLVYTVFSATDAKR, DSHVPILAPLPIGFAVFLVHLATIPITGTGI NPAR	-

Table 5: Proteins expressed by *G. diazotrophicus* cells in sugarcane roots

Gi	Locus	Protein	MW	Score	PM	Peptides	Sample
			Exclusively expressed in planta				
162148168	GDI2385	potassium transport system protein	23	70	2	GLIMNRQK, IPQGGWVPVLLGIALTLMMTTWK	SP-70 e Chunee
162147597	GDI1813	putative cell volume regulation protein A	25	65	1	LGCPVMLVDTCTGALAPASRAGIPILK	SP-70
162147471	GDI1687	putative chemotaxis protein methyltransferase	31	31	1	NHFTHLKEFVVPR	Chunee
162146602	GDI0780	putative cystathionine gamma-synthase	28	45	1	AYRPATRLLHSGVER	SP-70
162148550	GDI2774	putative glutathionine S-transferase	23	25	1	TPEFLR	Chunee
162147594	GDI1810	putative nucleoside 2-deoxyribosyltransferase	21	31	1	IAIAELAR	Chunee
162146935	GDI1140	putative UDP-glucose 6-dehydrogenase	46	23	1	QIARAMTDYAVIVTK	Chunee
162149225	GDI3457	putative transporter protein	34	34	1	VVATTVPR	Chunee
209544487	GDIA2347	putative outer membrane protein	38	22	1	LLSILR	Chunee
			Others				
162145880	GDI0041	outer membrane protein	40	65	3	SSPNFPDGR, IEVDGYTDNSAAHPGPR, RVEIILH	SP-70 e Chunee
162145915	GDI0079	catalase	55	63	1	LVSNIVGHVLQGVEEPVLSR	Chunee
162146116	GDI0286	bifunctional transaldolase/phosoglucose isomerase	102	26	1	ASAASVPPALNPGVVLGTVLGVAATQFGR	Chunee
162146281	GDI0456	chemoreceptor mcpA	57	23	2	ISASFARRPR, MTKESQIVER	SP-70
162146350	GDI0525	glutamine synthetase	53	118	3	LIPGFEAPVLLAYSAR, HHHEVAQSQHELGTK, RLIPGFEAPVLLAYSAR	Chunee
162146401	GDI0576	peroxiredoxin protein	20	46	1	ATFLVDPEGIIR	Chunee
162146520	GDI0696	F0F1 ATP synthase subunit beta	53	58	1	TVIIQELINNIAK	Chunee
162147070	GDI1275	outer membrane protein	28	24	1	FNPYVGVGATLAFFHNESPAGGLVK	Chunee
162147085	GDI1290	pyruvate kinase	51	58	1	TVETVVDQAIESTVASSLAR	Chunee
162147166	GDI1371	polynucleotide phosphorylase/polyadenylase	76	75	2	TADFGAFVNFLGAR, EAVLAALATEGLDVTAAKPILKDLEADVVR	Chunee
162147179	GDI1384	biopolymer transport exbB protein	36	53	1	NFAADLQSILLGGFR	Chunee
162147833	GDI2049	chaperonin GroEL	58	141	4	VGGSTEVEVK, TALQDASSVAGLLITTEAMVAEKPEKK, TALQDASSVAGLLITTEAMVAEKPEK,	Chunee
162147969	GDI2185	outer membrane lipoprotein	40	78	2	TYLVFFDWDR, TYLVFFDWDRSDLTAR	Chunee
162148085	GDI2302	glyceraldehyde-3-phosphate dehydrogenase	36	38	1	GILAYNTAPLVSSDFNHSIASSTFDATETALVDG GK	Chunee
162148355	GDI2572	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	85	131	5	ETTCRQIAYAIR, LLPVYVAILR, GGGFDPLTLLPR, GMLTGPVTILNWSFVR, AQGIETRPVLLGPVSYLLLGK	SP-70 e Chunee
162148832	GDI3061	hypothetical protein GDI3061	57	26	1	IRDSLPR	SP-70 e Chunee
162149101	GDI3331	50S ribosomal protein L9	21	94	2	TQVILENPIKQLGLYDVR, SAVELILLQRVENLGQMGDVVK	Chunee
162149176	GDI3406	elongation factor Tu	43	65	4	GVVNVGDEIEIVGLR, QVGVPALVVFLNK, ELLSAYQFPGDDIPIIK, DLMDAVDAYIPQPERPVDRPFLMPIEDVFSISGR	SP-70 e Chunee
162149228	GDI3460	30S ribosomal protein S1	63	30	1	DVTPLMGVPQPFQILK	Chunee
162149428	GDI3666	hypothetical protein GDI3666	42	154	2	NWNIVLTNIIR, TLIPLSFQFASFDGR	Chunee
209542569	GDIA0387	Superoxide dismutase	22	32	1	AFELPTLPFQTNALAAR	Chunee

Unraveling the molecular mechanisms of nitrogenase conformational protection against oxygen in diazotrophic bacteria

Letícia MS Lery[1§*], Mainá Bitar[1*], Mauricio GS Costa[1*], Shaila CS Rössle[2], Paulo M Bisch[1]

[1]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21949-901, Rio de Janeiro, Brasil

[2]Department of Earth and Environmental Sciences, Center for Nanoscience, Ludwig-Maximilians-Universität München, 80333 Munich, Germany

[§]Corresponding author

* These authors contributed equally to this work

Email addresses:

LMLS: llery@biof.ufrj.br

MB: maina@biof.ufrj.br

MGSC: maucosta@biof.ufrj.br

SCSR: shaila.roessle@lrz.uni-muenchen.de

PMB: pmbisch@biof.ufrj.br

Abstract

Background

G. diazotrophicus and *A. vinelandii* are aerobic nitrogen-fixing bacteria. Although oxygen is essential for the survival of these organisms, it irreversibly inhibits nitrogenase, the complex responsible for nitrogen fixation. Both microorganisms deal with this paradox through compensatory mechanisms. In *A. vinelandii* a conformational protection mechanism occurs through the interaction between nitrogenase complex and the FeSII protein. Previous studies suggested the existence of a similar system in *G. diazotrophicus*, but the putative protein involved was not yet described. This study intends to identify the protein coding gene in the recently sequence genome of *G. diazotrophicus* and also provide detailed structural information of nitrogenase conformational protection in both organisms.

Results

Genomic analysis of *G. diazotrophicus* sequences revealed a protein coding ORF (Gdia0615) enclosing a conserved "fer2" domain, typical of the ferredoxin family and found in *A. vinelandii* FeSII. High quality comparative models of both FeSII and Gdia0615 disclosed a conserved beta-grasp fold. Cysteine residues that coordinate the 2[Fe-S] are in conserved positions towards the metallocluster. Analysis of solvent accessible residues and electrostatic surfaces unveiled an hydrophobic dimerization interface. Dimers assembled by docking presented a stable behaviour and a proper accommodation of regions possibly involved in binding of FeSII to nitrogenase throughout molecular dynamics simulations in aqueous solution.

Molecular modeling of the nitrogenase complex of *G. diazotrophicus* was performed and compared to the crystal structure of *A. vinelandii* nitrogenase. Docking experiments of FeSII and Gdia0615 with its corresponding nitrogenase complex pointed out in both systems a

putative binding site presenting shape and charge complementarities at the Fe-protein/MoFe-protein complex interface.

Conclusions

The identification of the putative FeSII coding gene in *G. diazotrophicus* genome represents a large step towards the understanding of the conformational protection mechanism of nitrogenase against oxygen. In addition, this is the first study regarding the structural complementarities of FeSII-nitrogenase interactions in diazotrophic bacteria. The combination of bioinformatic tools for genome analysis, comparative protein modelling, docking calculations and molecular dynamics provided a powerful strategy for the elucidation of molecular mechanisms and structural features of FeSII-nitrogenase interaction.

Background

Nitrogen is a component of nucleic acids, proteins and many other biological molecules thus, it is an essential element for all living organisms. Although the N_2 gas is abundant in the atmosphere, it can not be readily used by most organisms. Nitrogen fixation is a key process in which molecular nitrogen is reduced to form ammonia, which is the form used by living systems for the synthesis of many organic compounds (171). Biological nitrogen fixation is catalyzed by the oxygen-sensitive enzyme nitrogenase present in some microorganisms known as diazotrophs, mainly bacteria (172; 173; 174).

The most abundant and extensively studied nitrogenase is the molybdenum (Mo)-dependent enzyme (173). It is composed of two metalloproteins: the MoFe-protein (also called dinitrogenase or component I) and the Fe-protein (also called dinitrogenase reductase or component II) (172). Both the MoFe and Fe-protein are irreversibly damaged by oxygen

(175). O_2 exposure leads to inappropriate oxidation of the metalloclusters, decrease of protein secondary structure and further degradation (176).

Yet, several nitrogen-fixing bacteria are aerobic and demand high O_2 flux for proper cellular metabolism. Therefore these bacteria present mechanisms to solve this apparent paradox. Under high O_2 concentration, they increase metabolism and consume more carbon source than needed to satisfy its energy requirement for growth in order to decrease O_2 pressure (177; 178). Subtle adjustments in the composition and functioning of the respiratory chain, mainly differential expression of cytochromes, were described (179; 180; 181). Additionally, diazotrophs grown in air secrete abundant extracellular polysaccharides and live inside colonies that help to produce a local microaerobic environment (182; 183).

Besides the respiratory protection mechanism, it was shown that a few nitrogen-fixing bacteria present a conformational protection mechanism of nitrogenase against O_2 (184; 185; 186; 187). Such system involves the interaction of a ferredoxin protein (FeSII or Shethna protein) with the nitrogenase complex that results in stabilization of a reversibly inactive complex. The ferredoxin protein is found as an homodimer that contains a 2[Fe-S] cluster and interacts specifically and reversibly with the nitrogenase complex (188).

Similar mechanisms were described in *Azotobacter chroococcum* (184), *Azotobacter vinelandii* (185), *Clostridium pasteurianum* (189; 190) and *Klebsiella pneumoniae* (187). The FeSII protein was mainly studied in *A. vinelandii* (188; 191; 192). Site-directed mutagenesis and crosslinking experiments revealed two residues possibly with fundamental roles on the interaction of FeSII with nitrogenase (190; 193).

Previous studies have shown evidences of a similar mechanism of conformational protection in *Gluconacetobacter diazotrophicus*, however the putative FeSII protein was not yet identified (186).

Due to the great importance of biological nitrogen fixation for sustainable crop production, advances have been achieved in genetics and biochemistry, culminating in the determination of the crystallographic structures of both nitrogenase components (194; 195; 196). However, studies on protein regulation and dynamics need to be carried out to a complete understanding of the molecular nature of the process.

In order to gain knowledge on structure and function of the proteins involved in the conformational protection of nitrogenase, we performed bioinformatic analysis on the whole genome sequence of *G. diazotrophicus* and identified a putative FeSII protein. In addition, molecular modeling, dynamics and docking studies on both *A. vinelandii* and *G. diazotrophicus* FeSII proteins and nitrogenases were carried out, elucidating molecular aspects of protein-protein interaction.

Results and Discussion

Gdia0615 codes for a putative FeSII protein of G. diazotrophicus

C. pasteurianum and *A. vinelandii* FeSII proteins were target of several theoretical and experimental studies (185; 189; 190; 191; 192; 193; 195). Thus, its primary sequences were used to search for an homologue in *G. diazotrophicus*. Searches were performed on both versions of *G. diazotrophicus* complete genome sequence available, containing 3852 (Riogene; (197)) and 3501 (DOE) predicted protein coding genes. However, similarity searches on predicted protein sequences or raw genomic sequence did not return any hit with high sequence similarity (>30% identity) and coverage (>70%).

In order to follow up the search for a putative FeSII in *G. diazotrophicus* the functional properties of FeSII proteins were analyzed. For instance, *A. vinelandii* FeSII protein presents 13 kDa and a fer2 domain (191), found in ferredoxins that are electron carrier proteins with a 2Fe-2S cofactor acting in a wide variety of metabolic reactions. The members of this family

are proteins of approximately one hundred aminoacids with four conserved cysteine residues that coordinate the 2[Fe-S] cluster and have a general core structure consisting of beta(2)-alpha-beta(2) (198). Accordingly, Ureta et al, 2002 (186) have shown the co-precipitation of a 14 kDa protein with nitrogenase of *G. diazotrophicus* cells grown diazotrophically under high oxygen concentration. This result suggested a conformational protection mechanism of nitrogenase in *G. diazotrophicus*, involving a 14 kDa protein.

In a second attempt to identify the *G. diazotrophicus* FeSII protein, we have analyzed the whole genome sequences for ferredoxin proteins. From the 7353 ORFs analyzed, a search for text annotation with the "ferredoxin" or "2Fe-2S" syntax revealed 16 putative ferredoxins. From these, only 8 present predicted molecular weight between 9 and 20 kDa, around the expected size for a putative 2[Fe-S] Shethna protein. Analysis of these 8 ferredoxins for the presence of functional domains disclosed two ORFs in *G. diazotrophicus* genome with high score for the fer2 domain (Protein family PF00111; Gdi2370 and Gdia0615both presented an e-value of $7.2e^{-17}$). However, they correspond to the same protein, as they come from one of each complete genome sequence available. They are 99% identical and the only difference is that Gdia0615 is 3 aminoacids longer (initial MPH sequence) than Gdi2370. Thus, Gdia0615 was chosen for further analysis.

In order to assert the putative function of Gdia0615, prediction of secondary structure of *A. vinelandii* FeSII was compared to the predicted secondary structure of Gdia0615. Both sequences form 2 beta sheets followed by an alpha helix and 2 subsequent beta-sheets (Fig. 1). *A. vinelandii* FeSII presents a 13 residues larger loop in the region 77-90. In summary, the secondary structure profile of both proteins is highly similar and the only structure discrepancy is the mentioned loop region (Fig. 1).

Following this analysis, the functional conserved residues from *A. vinelandii* FeSII were investigated for corresponding residues on the putative *G. diazotrophicus* sequence. Lou et al

1999 (193) described the importance of two lysine residues (K14 and K15) crucial for FeSII interaction with nitrogenase. Interestingly, Gdia0615 present two arginine residues (R13 and R14) in a conserved position (Fig. 1). K and R are both basic residues and might encompass same function in the protein context. Besides, *A. vinelandii* FeSII present 4 cysteine residues at positions 42, 47, 50 and 102, that coordinate the metal cluster, essential for plenty function of FeSII (193). Gdia0615 also presents this 4 cysteine residues well conserved in structure (C39, C45, C48 and C85; Fig. 1). Altogether, these results strongly suggest that Gdia0615 is the gene that codes for the putative FeSII in *G. diazotrophicus*.

FeSII molecular models revealed a conserved β-grasp core structure

The results discussed above strongly suggest that Gdia0615 is the putative FeSII protein of *G. diazotrophicus*. However, there are no structural information available for both *A. vinelandii* FeSII and Gdia0615. It is far known that protein structure is a determinant of protein function and three-dimensional structure is more conserved than protein sequence. Thus, as a support to the above predictions, 3D comparative models for Gdia0615, as well as for *A. vinelandii* FeSII, were constructed.

As a support to these predictions, a 3D comparative model for Gdia0615, as well as for *A. vinelandii* FeSII, was constructed. The first step in comparative modeling is the definition of a template sequence. The template sequence must have its 3D structure experimentally determined and present sequence similarity with the target. The quality of 3D structure (resolution of the template structure) and the alignment between template and target sequences are crucial for the model quality. Therefore, several alignments were generated for the FeSII sequences, using information from sequence database searches, secondary structure prediction and available homologues with resolved 3D structures. Alignments were then manually optimized in order to minimize gaps and assert that the conserved domain residues

are aligned. Although Gdia0615 and *A. vinelendii* FeSII might both perform the conformational protection of nitrogenase, they share low sequence similarity (18% identity and 42% similarity). Therefore, template structures chosen for comparative modeling of each of them were different. Gdia0615 chosen template is FdvI protein (48% identity and 67% similarity), a 2[Fe-S] ferredoxin essential for growth of *Rhodobacter capsulatus* (198), also a diazotrophic bacterium. *A. vinelandii* FeSII chosen template is *E. coli* Fdx (20% identity and 36% similarity), an adrenodoxin-type 2[Fe-S] ferredoxin with an essential role in the maturation of various iron-sulfur (Fe-S) proteins (199). This two template proteins share some structural similarity and both coordinate a 2[Fe-S] cluster.

The *A. vinelandii* FeSII model (deposited at Protein Model DataBase at identification number PM0075978) exhibited high stereochemical quality (97% of residues in the allowed regions of Ramachandran plot) and a high probability to represent a native-like conformation (Dope-score -3.7493 and ProSa Z-score -5.06). The average distance deviation between backbone equivalent atoms (RMSD - Root Mean Square Deviation) measures the similarity between 3D structures after optimal superposition; the RMSD score for FeSII proposed model vs. the crystal structure of Fdx was 0.53Å, a further indication of the high quality of the model (200). A remarkable feature of this model is a large loop region, comprising aminoacids 77-90, as expected from the observed gap in sequence alignment. In overall, this represents a structure of high quality.

The Gdia0615 model (deposited at PMDB at identification number PM0075980) also presented high stereochemical quality (100% of residues in the allowed regions of Ramachandran plot) and is a highly probable native-like structure (Dope-score -4.0447 and ProSa Z-score -6.57). The RMSD between Gdia0615 model and FdvI crystal structure is 0.46Å, representing the overall quality of the model.

Although FeSII and Gdia0615 models were built from different template structures, they share many structural features. The RMSD between their structures is only 1.46 Å (Fig. 2A). This reflects the protein structure conservation, the most required feature for proper protein function maintenance. Both models present 4 well defined beta-sheets and 1 structural alpha-helix (Fig. 1). The specific arrangement of these structural elements is a conserved motif typical of the general core structure characteristic of ferredoxin proteins, known as beta-grasp fold (Fig. 2). The beta-grasp fold supplies an effective scaffold for binding iron-sulfur clusters in the case of the 2Fe-2S ferrodoxins (201).

Additionally, the 4 cysteine residues are positioned towards the 2[Fe-S] cluster, as was expected, in both models (Fig. 2). The 2 lysine (K14 and K15) residues of *A. vinelandii* FeSII and the corresponding 2 arginine (R13 and R14) of *G. diazotrophicus* are positioned at exposed surface and might be involved in nitrogenase interaction, as suggested before. Altogether these results provide evidence that FeSII and Gdia0615 could perform same molecular function.

FeSII dimer is the stable functional unit

Previous studies detected that *C. pasteurianum* and *A. vinelandii* FeSII binds to nitrogenase complex as an homodimer. Analyses of electrostatic potential and solvent exposed surface of the monomer molecular models were performed to identify a possible dimerization interface. Electrostatic potential of *A. vinelandii* FeSII revealed a predominant positive structure. However, protein-protein dimerization surfaces are typically neutral, as hydrophobic residues are usually found enclosed inside the complex structure. Charged residues largely exposed at protein surface may also contribute to protein-protein interactions through salt bridges and hydrogen bonds. Careful analysis of solvent accessible residues showed a possible region of dimerization comprising hydrophobic and basic residues accessible to solvent comprising L9, M10, G116, L80 and P81. Therefore, these residues were set as obligatory contacts for

docking calculations. Additionally, the large loop region (residues 77-94) presents some hydrophobic aminoacids and consequently was set as a semi-flexible region for docking experiments. The resulting dimer (Fig. 3) presented high quality (ProSa Z-score -6.08), a symmetric structure (each monomer is horizontally rotated in approximately 180^{o} in relation to the other) and complementarities of shape and charge in the dimerization interfaces. The loop region seems to strengthen protein dimerization. A 10 ns molecular dynamics simulation in aqueous solution revealed that the dimer is stable (RMSD = 3.08 A^{o}; standard deviation = 0.041 A^{o}), supporting the hypothesis that the dimer is the functional unit.

Gdia0615 showed a similar pattern of charge distribution in comparison to FeSII (Fig. 2). Mainly positive residues are found in the surface. From the solvent exposed surface residues, the most notable ones (V51, L73, F75, G76, R99, P101, H102 and K103) were selected as obligatory contacts for docking experiment. Calculations resulted in dimer structure with native-like structure (ProSa Z-score -4.52) that close resembles the *A. vinelandii* FeSII dimer model (Fig. 3). Shape and charge complementarities of the surface interface of the proposed dimer are according to physicochemical restrictions. Gdia0615 dimeric orientation is very similar to FeSII (Fig. 3) and showed stable behavior after 10 ns of dynamics simulation (RMSD = 2.45 A^{o} +- 0.043 A^{o}).

G. diazotrophicus nitrogenase complex structure is close related to A. vinelandii nitrogenase

In order to better understand the conformational protection mechanism of nitrogenase from oxygen damage, analysis of FeSII-nitrogenase interaction is required. The protection is due to the formation of an oxygen-tolerant three-component nitrogenase complex (Fe-protein + MoFe-protein + FeSII protein) (186; 202). Crystal structures of *A. vinelandii* nitrogenase complex were already determined and presented insights on multiple docking sites of nucleotides (203; 204). Mutant forms of nitrogenase proteins were also studied and revealed

key residues for nucleotide-protein interaction (194). In contrast, FeSII interaction with nitrogenase complex was not yet studied in respect to structural aspects. In addition, it lacks information on 3D structure of *G. diazotrophicus* nitrogenase complex.

As a strategy to fulfill this gap, a molecular model of *G. diazotrophicus* nitrogenase complex was built through comparative modeling. The Fe-protein presents 70% identity and 85% positivity with the *A. vinelandii* homologue. The MoFe-protein α-subunit shows 71% identity and 81% similarity with the *A. vinelandii* counterpart. The β-subunit of MoFe-protein is 52% identical and 70% similar to the *A. vinelandii* protein. In summary, all components of nitrogenase complex from *G. diazotrophicus* and *A. vinelandii* share high sequence similarity. Therefore, the template structures for both components of nitrogenase complex were the *A. vinelandii* subunits.

In consequence of this high degree of similarity between target and template sequences, the molecular model of *G. diazotrophicus* nitrogenase complex presented high stereochemical quality. Molecular models of Fe-protein (deposited at PMDB at identification number PM0075981), MoFe-protein α and β-subunits (deposited at PMDB at identification numbers PM0075982 and PM0075983, respectively) presented 99%, 98% and 99% of residues in allowed regions of Ramachandran plot. The overall RMSD between each crystal structure and the proposed model is 0.41, 0.31 and 0.54 A^{o}, respectively. Such a high quality model is suitable for protein-protein interaction analysis.

A negative pocket anchors the FeSII positive region near the Fe-protein/MoFe-protein interface in the nitrogenase complex

As mentioned before, a few studies reported the interaction of FeSII with nitrogenase. Nitrogenase protein is found as an oligomer (two Fe-protein homodimers + one heterotetramer of MoFe-protein). The binding site of FeSII in nitrogenase is not clearly described. It was shown that a 2[Fe-S] ferredoxin of *C. pasteurianum* binds the MoFe-protein

at a site involving both subunits of the MoFe-protein (205). However, in *A. vinelandii* and *A. chroococcum* it was demonstrated that FeSII binds to the nitrogenase complex, but not to each individual subunit (193; 202; 206). Apparently, the formation of a three-component nitrogenase complex (Fe-protein + MoFe-protein + FeSII) is essential for nitrogenase oxygen tolerance and suggests that FeSII interacts near the subunits interface of nitrogenase complex (202; 206).

Furthermore, in *A. vinelandii*, several site-directed mutants were constructed and tested for its FeSII capability of interaction with nitrogenase (193). Two of those (K14 and K15) lost ability of interaction. This experiment showed the importance of these two K residues for interaction. However, it was not shown if is due to direct contact or allosteric effect.

The oxygen-stable three-component complex is very large to perform blind docking calculations in a feasible computational time. In order to elucidate this mechanism, electrostatic surface, shape and physicochemical complementarities of both FeSII and nitrogenase complex were exhaustively inspected. *A. vinelandii* Fe-protein presented 3 glutamic acid residues (E69, E112 and E113) at Fe-protein surface near the interface with MoFe-protein. This negative area visually fits in shape and charge the FeSII region around K14 and K15, the two lysine that may have an important role in the initial steps of recognizing the nitrogenase component residues (Fig. 4; (193)). In addition, the histidine 55 (H55) is in this same structural interface. It was suggested that H55 might modulate the FeSII protein's affinity for nitrogenase in a redox state-dependent manner (193).

G. diazotrophicus Gdia0615-nitrogenase interaction was also analyzed. The surface electrostatic potential and structural shape of both were manually inspected. As observed in *A. vinelandii* proteins, there is a small negative pocket on the interface of Fe-protein and MoFe-protein. This region is completely complementary in shape and charge to the unique positive region of Gdia0615, corresponding to R13 and R14 neighborhood. Such suspicious analysis

suggests these arginine residues are directly involved in the nitrogenase oxygen-stable three-component complex formation in *G. diazotrophicus* (Fig. 4).

In order to support these analyses, docking calculations of *A. vinalendii* FeSII protein and nitrogenase complex were performed. According to the evidences described above, nitrogenase E69, E112 and E113 residues and FeSII K14, K15 and H55 were set as active residues for docking calculation.

From the 1000 complexes generated, 49 are clustered in the same docking site and show a very similar conformational structure. They also represent the lowest energy complexes obtained and the best one is shown on figure 4 (Haddock score = -59.8 with standard deviation of 2.6). The complex structure presented energy values of: - 763.5 +/- 41.8 (electrostatic contribution), -26.8 +/- 6.3 (van de Waals), 102.8 +/- 12.4 (desolvation energy) and 169.4 +/- 47.81 (restraints penalty energy). Analysis of these energetic components of FeSII-nitrogenase complex confirmed that interaction is mainly due to electrostatic potential. This result explains previous experimental data which shows that an increase in ionic strength disrupts the FeSII-nitrogenase complex (202; 206).

Further, the docking complex structure revealed that FeSII K14 and K15 are directly involved in the complex formation, supporting the earlier mutagenesis data (193). Fe-protein residues engaged on interaction are E68, E71, E73, D74 and E112. MoFe-protein main aminoacids are E288 and D385 (Fig. 5). Additional residues also participate on molecular interactions, either through hydrogen bonds, saline contacts or electrostatic interaction (Tab. 1).

Conclusions

The nitrogenase conformational protection mechanism against oxygen is a bacterial strategy to avoid the enzyme degradation during a sudden increase in oxygen concentration. FeSII protein (Shethna protein) interacts with nitrogenase complex (189; 191). The three-component

complex keeps nitrogenase inactive but transiently protected from damage by oxygen (185). This oxygen-stable nitrogenase complex is formed only under conditions of low ionic strength in the presence of $MgCl_2$, and requires all three components to be present in an oxidized state (202; 206).

A. vinelandii FeSII was target of a few studies regarding its function and regulation. This study contributed for the understanding of this mechanism with structural aspects. In contrast, *G. diazotrophicus* protein involved in nitrogenase conformational protection was not yet known. The results described herein present for the first time a putative FeSII protein for *G. diazotrophicus*. A detailed analysis of all predicted ORFs of complete genomic sequence revealed only one possible protein coding gene (Gdia0615) for the putative FeSII. Although Gdia0615 present low sequence similarity with *A. vinelandii* FeSII, all functional residues are well conserved.

A comparative analysis of the 3D molecular models of Gdia0615 and FeSII support the hypothesis of Gdia0615 protective function. Both form a beta-grasp folding and present similar electrostatic properties. In addition, they are functional as symmetric homodimers that interact through a hydrophobic interface.

A. vinelandii FeSII docking with nitrogenase complex revealed a putative binding site near the Fe-protein/MoFe-protein interface, corroborating previous data on the three-component complex. This interaction is dependent of shape and charge complementarities. Two FeSII lysine residues (K14 and K15), as well as an histidine H55 participate actively on interaction. On the other hand, 3 glutamic acid aminoacids of Fe-protein enclose a negative cavity for FeSII binding. In *G. diazotrophicus* we suggest that arginine residues R13 and R14, histidine H56 and adjacent regions compose the main region for Gdia0615 interaction with nitrogenase complex.

In overall, this study provided the first molecular insights on structural properties of the conformational protection mechanism of nitrogenase against oxygen. Such study will certainly contribute to a better understanding of the biological nitrogen fixation process.

Methods

Genomic analysis

A. vinelandii FeSII (gi: 451865) and *C. pasteurianum* FeSII (gi: 119942) sequences are available. These sequences were used to scan the two public available complete genome sequences of *G. diazotrophicus* (gi: 162145846 and 209542188) for a putative FeSII protein. Both genome sequences present essentially the same genes. Therefore, most of the genes in one version have a matching in the other. Sequence similarity searches were performed using BLAST algorithms (72), for both aminoacids and nucleotides. Analysis of functional domains was performed in NCBI Conserved Domains (version 2.17; 31608 PSSMs; (207)) and Pfam databases (version 23.0; 10340 families; (73)). Protein secondary structure prediction based on position-specific scoring matrices was performed on PSI-PRED server (208).

Comparative Modeling

The 3D molecular models of the FeSII (*A. vinelandii),* the putative FeSII, Fe-protein and MoFe-protein (*G. diazotrophicus*) were built by comparative modeling. The search for candidate template sequences was performed at the PDB database (77). Templates used for modeling were the *Escherichia coli* Fdx, (1I7H), the *Rhodobacter capsulatus* FdvI (1E9M) and *A. vinelandii* nitrogenase complex (1G20), respectively. Templates and target sequences were aligned using Promals3D (209) and manually optimized with the support of the DNATagger (210) by monitoring the alignment of cysteine residues and conservation of physicochemical properties of aligned residues.

Molecular models were generated using the program Modeller (version 9.7; (79)), considering the presence of heteroatoms (2 [Fe-S] cluster) if suitable. Additionally, loop refinement of *A.*

vinelandii FeSII loop region, comprising the residues 77 to 90 was performed. 100 candidate models were generated for each protein system and all of them were evaluated using stereochemical quality Ramachandran plots generated by Procheck (version 3.5.4; (211)) and energy values according to Modeller Dope-score (212) and Prosa (ProSa 2003; (213)). RMSD calculations, visualization and manipulation of molecular images were performed with Pymol (version 1.2; (81)).

Docking

Prediction of solvent accessible surface area (Naccess version 2.1.1; (214)) and evaluation of the electrostatic potential on the protein surface (APBS software package; (80)) were accounted to determine potential protein-protein binding sites. These results, together with literature information, were introduced as restraints to drive docking calculations with Haddock (215). The Haddock advanced guru interface provided full control over parameters and supported the setting of active and passive residues. A set of probable complexes were generated and the results were analyzed through stereochemical, cluster-sizes and energy evaluations.

Molecular Dynamics

Molecular dynamics (MD) simulations, energy minimization and trajectory analyses were carried out with GROMACS 4.05 package (216), using GROMOS96 (G53a6) force field (217). Explicit SPC water molecules (218) were used in all simulations, in which a 14 Å layer of water molecules were added around the solute molecules, within a cubic water box, using periodic boundary conditions. Counter ions were inserted for system neutralization. LINCS (219) and SETTLE (220) were applied to constraint solute and solvent bond, respectively. Temperature was kept at 298 K by rescaling velocities with a stochastic term (221) and pressure at 1 atm using the Berendsen approach (222). Electrostatic interactions were

corrected with PME method (223), using non-bonded cutoffs of 1.0 nm for Coulomb and 1.2 nm for van der Waals. MD integration time was 2 fs.

A 3-steps energy minimization protocol was used to avoid artifacts in atomic trajectories due to conversion of potential into kinetic energies: firstly, applying the steepest-descent algorithm: *i.* 5000 steps with solute heavy atom positions restrained to their initial positions using an harmonic constant of 1 kJ/mol.nm in each Cartesian direction, allowing free water and hydrogen movements; and *ii.* 5000 steps with all atoms free to move. Subsequently, the conjugated gradient algorithm was applied for further energy minimization until an energy gradient of 42 KJ/mol.nm. A preliminary MD (1 ns), with heavy atom positions restrained, was performed for achieving solvent equilibration and system heating until 298 K. In this step, the initial velocities were generated once for each simulation. Then we performed a 10 ns production MD.

Authors' contributions

LMLS designed the study, participated on genomic analysis, comparative modeling and docking and drafted the manuscript. MB designed the study and carried out the genomic analysis, comparative modeling and docking. MGSC designed the study and carried out comparative modeling, docking and molecular dynamics. SCSR conceived the study. PMB designed and coordinated the study.

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Figures

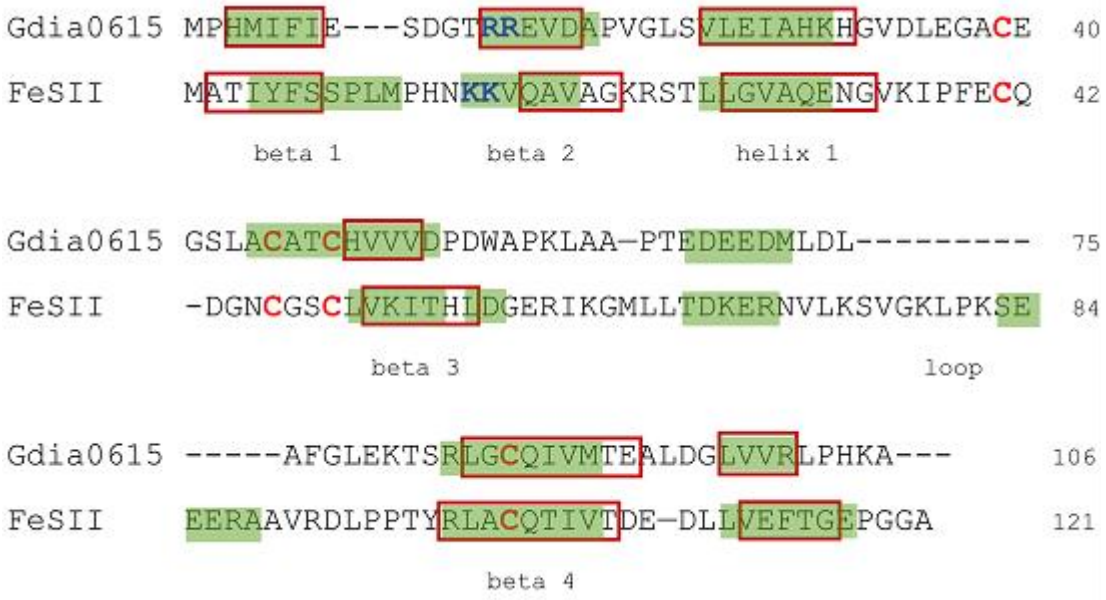

Figure 1 - Alignment of primary sequences of* A. vinelandii *FeSII and Gdia0615 (putative* G. diazotrophicus *FeSII protein)

The predicted beta-sheets and alpha-helices are shaded in green . Beta-sheets and alpha-helices present in the comparative models are indicated inside red boxes. Conserved lysine and arginine residues (K and R), important for FeSII interaction with nitrogenase complex are indicated in blue. Cysteines involved in 2[Fe-S] cluster coordination are shown in red.

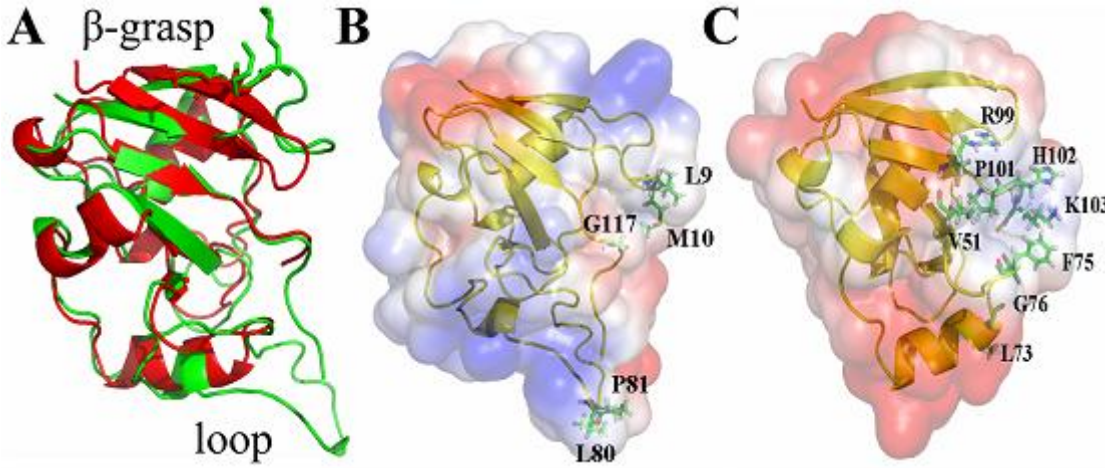

Figure 2 - The β-grasp fold and electrostatic surface of molecular models of FeSII and Gdia0615

The β-grasp structure is shown in yellow. A) *A. vinelandii* FeSII (green) fitted to *G. diazotrophicus* Gdia0615 (putative FeSII; red). B) Electrostatic surface of both 3D molecular models. Blue indicates positive charged residues, red represents negative areas and white are neutral regions. Active residues for docking calculations are numbered.

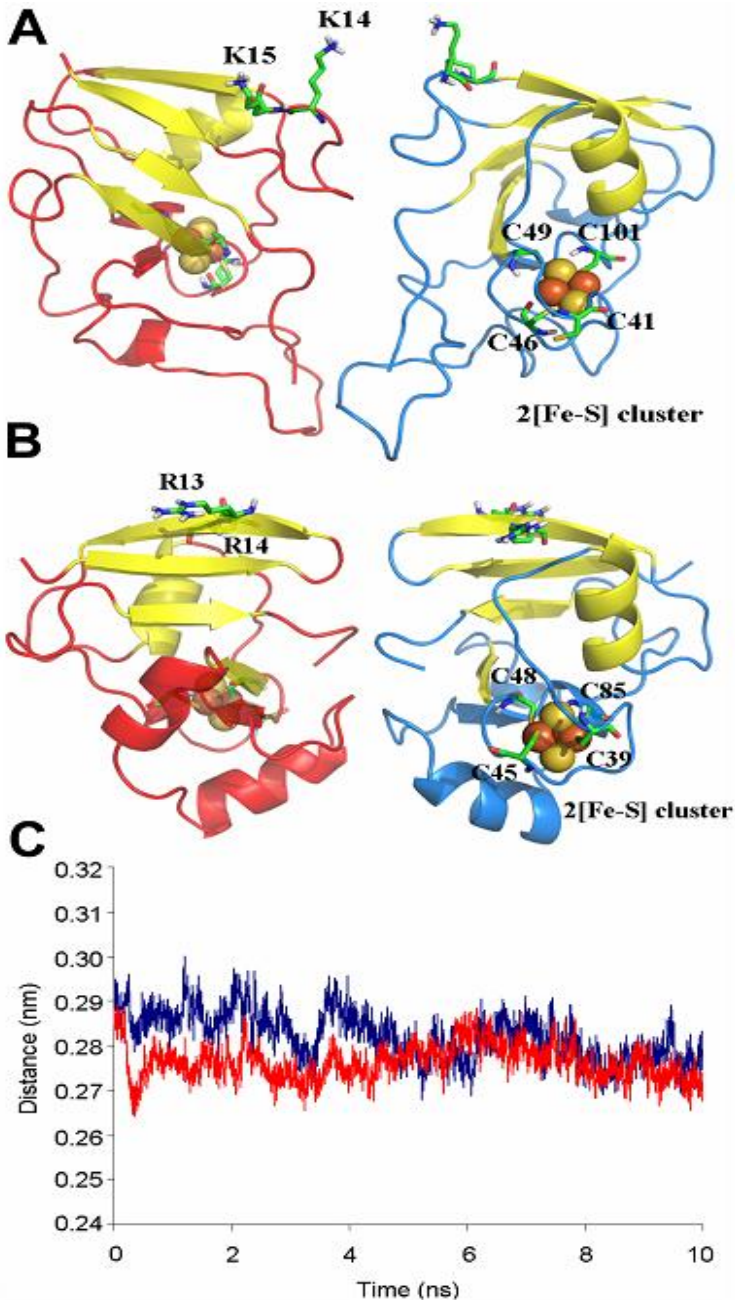

Figure 3 - Conserved core structure of the dimer complexes of FeSII and Gdia0615 are stable

A) *A. vinelandii* FeSII dimer. B) *G. diazotrophicus* Gdia0615 (putative FeSII) dimer. C) Distance of center of mass between each subunit of *A. vinelandii* FeSII (blue) or *G. diazotrophicus* Gdia0615 (red) throughout 10ns of molecular dynamics simulations

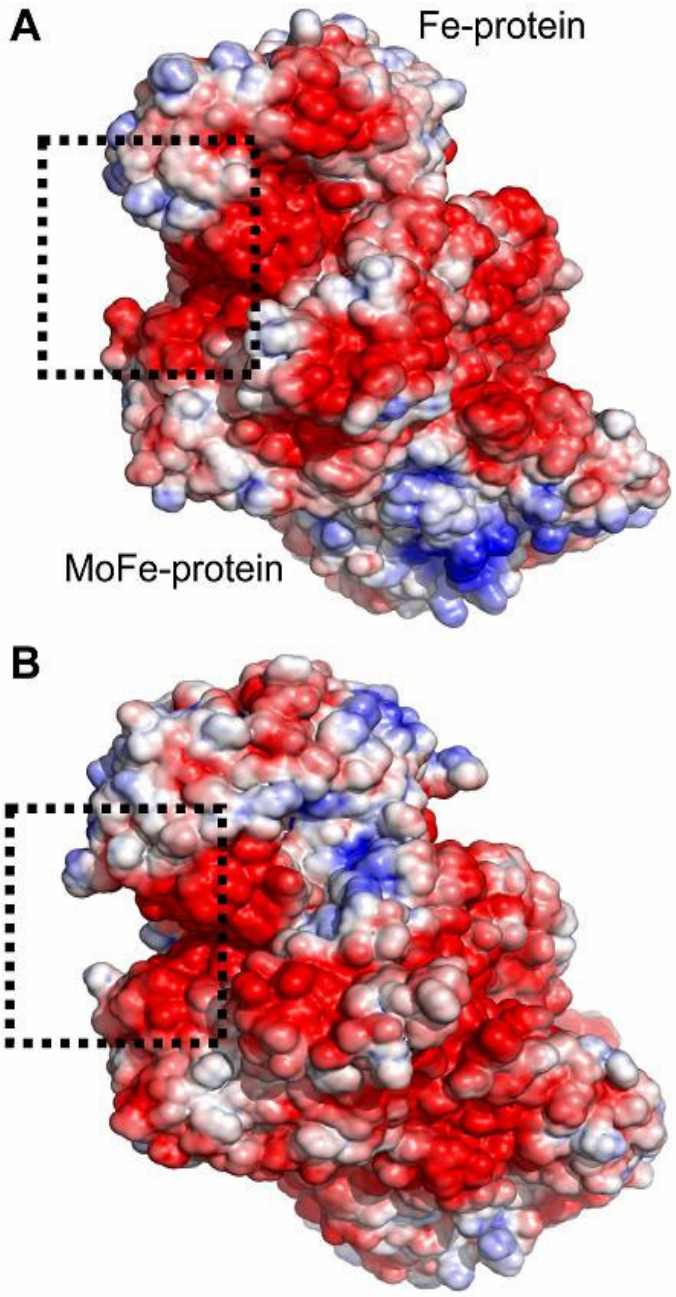

***Figure 4 - Electrostatic surface of nitrogenase complex of* A. vinelandii *and* G. diazotrophicus**

The positive regions are indicated in blue and the negative regions in red. The squares indicate the interaction site of FeSII. A) *A. vinelandii* nitrogenase complex. B) *G. diazotrophicus* nitrogenase complex.

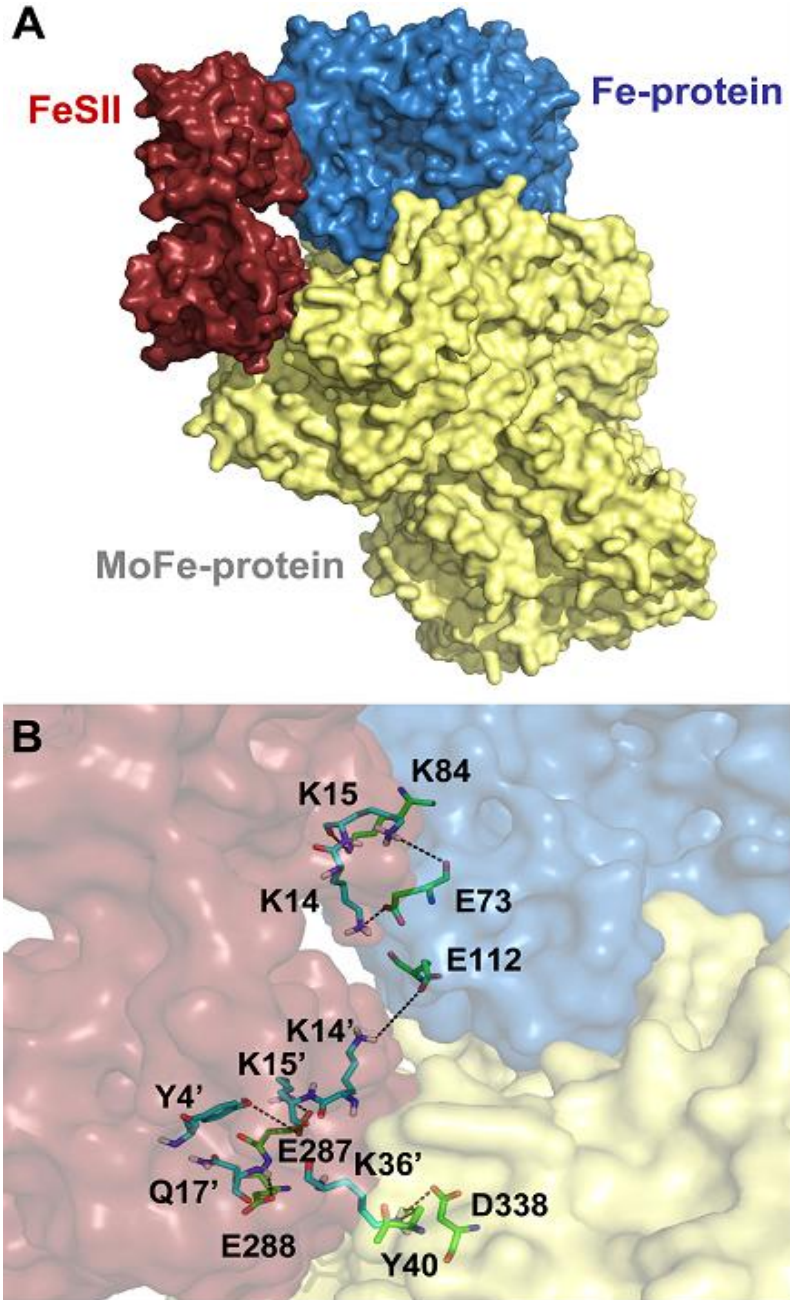

***Figure 5 - The oxygen stable three-component complex of* A. vinelandii**

A) Surface representation of FeSII (red), Fe-protein (blue) and MoFe-protein (yellow). B) A closer view of the interaction interface. Residues involved in interaction are highlighted.

Table

Table 1 - FeSII and nitrogenase interacting residues

FeSII β-chain are indicated by an (’). Nitrogenase residues marked with an asterisk (*) belong to the Fe-protein and those without are from MoFe-protein. Polar interactions were classified in salt bridges and hydrogen bonds. Atoms participating on these contacts were separated in backbone or side-chain atoms.

Table 1

FeSII	Nitrogenase	Distance (A^o)	Type
K14	E71*	6.5	Salt bridge
K14	E73*	2.5	H-bond (side chain-side chain)
K15	D74*	6.6	Salt bridge
K15	E73*	4.6	Salt bridge
K15	K84*	3.8	H-bond (side chain-backbone)
Y4'	E288*	4.0	H-bond (backbone-side chain)
K14'	E112*	5.9	Salt bridge
K15'	E288	2.7	H-bond (side chain-side chain)
Q17'	E287	2.6	H-bond (side chain-backbone)
K36'	D388	3.2	H-bond (side chain-side chain)

Vibrio cholerae PhoB regulates RpoS expression and stress survival

Letícia MS Lery[1], Carolina L Goulart[1], Felipe R Figueiredo[1], Karine S Verdoorn[2], Marcelo E Lamas[2], Fabio M Gomes[3], Ednildo A Machado[3], Paulo M Bisch[1], Wanda MA von Kruger[1]

1- Unidade Multidisciplinar de Genômica- Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

2- Laboratório de Físico-Química Biológica - Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

3- Laboratório de Entomologia Médica- Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Brazil

Corresponding author: Letícia Miranda Lery Santos, e-mail: llery@biof.ufrj.br

Avenida Carlos Chagas Filho, CCS, bloco G. Instituto de Biofísica Carlos Chagas Filho, sala G1-043. Ilha do Fundão. Rio de Janeiro, RJ – Brasil. CEP 21949-902.

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Abstract

PhoB/PhoR is a two-component system originally described to be involved with phosphate levels sensing, transport and metabolism in several bacteria. In order to investigate further roles of this system, we performed a high throughput proteomic analysis of *V. cholerae* 569BSR and its *phoB* mutant under high phosphate levels. Protein expression patterns disclosed further PhoB/PhoR-dependent functions not related to phosphate metabolism. Most of the proteins expressed differentially by 569BSR have roles in energy production and conversion and aminoacid transport and metabolism. In contrast, the mutant expressed proteins related to adaptation to atypical conditions, protein synthesis, folding and stabilization and DNA metabolism. The expression of several stress related proteins in the mutant was correlated with the lack of DksA, RpoS and catalase in these cells and increase of pppGpp and PoliP levels. *pho*B mutant is more sensitive to acid shock and more resistant to termic and osmotic stresses than wild type strain under high phosphate growth. Besides, it was shown that PhoB is essential for RpoS expression and required for the full growth of *V. cholerae*. The majority of the genes identified have not been described before, thus, they could also help to increase our knowledge on *V. cholerae* O1 physiology.

Introduction

Many bacterial species respond to inorganic phosphate (Pi) starvation by expressing genes with roles in transport and metabolism of phosphorus compounds (Pho regulon) (224; 225; 226; 227; 228; 229; 230; 231). In *V. cholerae* and other bacteria the expression of Pho regulon relies on the PhoB/PhoR two-component system (225; 226; 229). A previous differential proteomic analysis of *V. cholerae* O1 569BSR versus its isogenic *pho*B mutant under Pi starvation showed that 569BSR expressed mainly the Pho regulon genes involved in the adaptation to the new environment. The *pho*B mutant, on the other hand, expressed genes related to the general stress response as a strategy to survive (232).

Interestingly, not all proteins expressed differentially by the 569BSR under Pi limitation were related to Pi metabolism. Genes with putative roles in virulence, amino acid and nucleic acid metabolisms were found among those whose expression were affected by mutation in *pho*B, suggesting additional functions for PhoB/PhoR in *V. cholerae* (232). For instance, the influence of PhoB in *V. cholerae* biofilm formation and motility, both pathogenesis related processes, once more substantiated the importance of PhoB/PhoR additional functions (233; 234).

Accordingly, in *Eschericihia coli*, the PhoB/PhoR system also regulates the expression of a number of genes involved in distinct metabolic processes, such as de novo biosynthesis of NAD (235), initiation of chromosome replication (236), acid shock resistance (237), metal ions and general stress response triggered by the alternative σ factor RpoS (238; 239). In *V. cholerae*, RpoS plays a role in colonization and virulence (240) and also confers environmental stress resistance even in *in vivo* animal models (241). Stress tolerance is particularly important for *V. cholera*e once it

faces distinct stimuli throughout its life cycle in aquatic reservoirs or inside human host. Such diverse conditions require modulation of gene expression and cellular adaptation (242) and, as a major stress response regulator, RpoS plays a critical role for bacterial survival.

In a previous work we suggested a link between PhoB and several RpoS-mediated responses in *V. cholerae* (232). Controversially, a comparison of *pho*B and *rpo*S mutants derived from an El Tor strain withstanding environmental conditions revealed divergent behaviors and the authors suggested that *V. cholerae* PhoB mediates stress response in a RpoS independent manner (233). In order to elucidate the involvement of PhoB with RpoS and other stress signaling molecules, we have performed a comparative analysis of *V. cholerae* classical 569BSR and a *pho*B mutant (WK3). Protein expression, RpoS, pppGpp and PolyP levels were taken into account and our results clearly demonstrated that PhoB is required for RpoS expression in *V. cholerae*, as it was observed for *E. coli*.

Materials and Methods

Bacterial strains, plasmid and culture conditions

V. cholerae O1 strain 569BSR, a classical Inaba, and its isogenic *pho*B mutant WK3 were used (226). For complementation in trans of WK3, the low-copy number plasmid pWK20, that contains the entire *phoB/phoR* operon including its regulatory region, was used. In some tests, *E. coli* K12A15 was used as control. Strains were routinely grown at 37°C in Luria-Bertani broth (LB) (243) or on solid medium (LA, 1.5% agar in LB). For growth under defined Pi concentrations, TG medium (244) supplemented with KH_2PO_4 at final concentrations of 6.5 mM (TGHP) or 65 μM

(TGLP) were used. For *E. coli*, TG was also supplemented with casaminoacids 0.02%. Antibiotics were added to the medium at the following concentrations: streptomycin [100 μg ml^{-1}], kanamycin [50 μg ml^{-1}] and ampicilin [100 μg ml^{-1}].

Sample preparation and protein analysis

Whole cell lysates were prepared and fractionated by ultracentrifugation as previously described (226). The supernatant fraction contained the Triton X-100 soluble proteins and the pellet, a membrane protein-enriched fraction, was further solubilized with 7 M urea, 2 M thiourea, 2 % (w/v) DTT, 0.5 % (v/v) Pharmalyte 3-10, 2 % (w/v) ASB-14**.** Periplasmic proteins were obtained by centrifugation of a suspension of spheroplasts prepared as reported in our previous work (226). Protein concentration was estimated by the method of Bradford (245).

One and two-dimensional (1D or 2D) SDS-polyacrylamide gel electrophoresis (PAGE) were performed as previously described (232). For 2D analysis, samples containing 200, 400 or 500 μg of proteins were loaded onto the strips of 7 (membrane enriched fraction), 11 (periplasmic proteins) and 18 (whole cell lysate) cm and proteins were focused using Multiphor (Amersham Biosciences), according to the manufacturer's instructions. After focusing, each strip was reduced and alkylated, positioned on top of 12-14% gradient SDS-polyacrylamide gels and ran at 600 V for 3.5 hours. Gels were stained and scanned with the ImageScanner v5.0 and analyzed with the Image Master 2D Platinum software v5.0 (Amersham Biosciences). pI values of the proteins of interest were determined using a linear 4-7 distribution and the relative molecular mass (MMr) determined based on protein low MM markers (Amersham Biosciences).

Protein identification by mass spectrometry

Proteins cut from 2D gels were digested by trypsin as described before (246). Samples were analyzed by tandem mass spectrometry on a 4700 Proteomics Analyzer TOF/TOF (Applied Biosystems).Calibration mixtures 1 and 2 (Sequazyme Peptide Mass Standard kit, PerSeptive Biosystems, Foster City, CA, USA) were used as external standards. The MASCOT program interface (Matrix Science, www.matrixscience.com) was used to search in public available databases of *Vibrio cholerae* and other *Vibrio* sequences. The minimum criteria for identification were: two peptides matched, at least one peptide unique for the protein identified and $p<0.05$.

Search for putative Pho box sequences

Regions extending 200 bp upstream the putative translation start sites of hypothetical Pho regulon genes were analyzed for the presence of a conserved sequence motif, employing the Hidden Markoff Model-based computer programs MEME/MAST (247) and the program BLASTn (72). Selected DNA regions were compared to the conserved domains of Pho boxes observed in bacteria, yeast and plants: direct repeats of GTCAT, GCCAT, TCCAT and/or GCCAAT separated by a string of 4-5 A/T (232; 248; 249; 250). MAST searched the regions and selected putative Pho box with around 18 nucleotides, defined when p-value was less than 0.0001. Blast searches were performed varying the width between the two domains of Pho boxes.

Evaluation of *rpoS* mRNA

RNA was isolated from *V. cholerae* strains grown in TGHP, TGLP or LB media for 5 (exponential growth) or 16 hours (stationary phase) with Trizol reagent (Invitrogen).

RNA was purified following treatment with RNase Out and DNase RQ1 (Promega). cDNA was synthesized from 3µg RNA by using ImPromII reverse transcriptase enzyme (Promega). Reverse transcription PCR (RT-PCR) analysis was performed using the following primers: 5'-CCTACGCAACATGGTGGATC-3' and 5'-GCAGACGACGTAGACCTTCC-3'.

Detection of RpoS protein expression

Protein samples were boiled in SDS-polyacrylamide gel electrophoresis loading buffer and 40 µg of protein was loaded into each well in a 12.5% SDS-PAGE. The acrylamide gel was electroblotted onto a nitrocellulose membrane and RpoS protein was detected with a rabbit anti-RpoS serum (kindly donated by Dra. F. Nörel, Pasteur Institute, France) and peroxidase-conjugated anti-rabbit immunoglobulin G (KPL) (251).

Detection of catalase activity

Catalase was assayed by measuring decomposition of H_2O_2 and recording the decrease in absorbance at 240 nm (252). One unit of catalase activity was defined as the amount of enzyme required to decompose 1 µmol of H_2O_2 per minute.

PolyP extractions and quantification

Long and short chain PolyP samples were differentially extracted as previously described (253). Aliquots of long or short chain PolyP extracts were incubated for 15 min at 37^{o}C with 5000 units of the enzyme. Relative PolyP levels were determined as a function of the amount of Pi released upon treatment with an excess of recombinant exopolyphosphatase purified from *Sacharomyces cerevisiae*.

Determination of intracellular (p)ppGpp

The pppGpp concentration was measured in cells grown in TGHP or TGLP minimal media for 16 hours containing 0.25 µCi ^{32}P as described previously (254). Nucleotides were extracted by adding ice-cold formic acid to 6.5 M. Samples were frozen and thawed at least twice before being spotted on PEI cellulose plates (Merck). One-dimensional chromatography was carried out in 1.5 M KH_2PO_4. Quantification was with a Storm PhosphorImager.

Growth and Survival assays

Growth curves were constructed by measuring OD_{600nm} and after serial dilutions and plating onto LB agar. For pH (pH 4.5), temperature (42°C), salinity (1.5M NaCl), oxidative (1mM H_2O_2), ethanol (7%) and ultraviolet (2 or 4J) stresses, wild-type and *pho*B mutant *V. cholerae* strains were grown for 16 hours (stationary phase) in LB. The cells were centrifuged and ressuspended in the appropriate medium in the presence of the specific environmental stressor. Acid shock was performed in LB pH 4.5 and adaptation to acid was performed for 1 hour at pH 5.7 (255). At different times, viability was assessed by dilution plating onto LB agar. Data are representative of at least 6 measures of 2 independent experiments.

Results and Discussion

The involvement of PhoB/PhoR system is Pi-independent stress responses

A previous comparative proteomic analysis of 569BSR and its isogenic *pho*B mutant, WK3, grown under low Pi supply, revealed several members of the Pho regulon of *V. cholerae* O1 and suggested roles for the PhoB/PhoR regulatory system unrelated to

Pi transport and metabolism (232; 233; 234). In order to better exploit those PhoB functions, in the present study, we analyzed the proteomes of 569B and *pho*B mutant WK3 under high Pi concentration (TGHP). Protein products of genes differentially induced under this condition are those whose expression might be dependent of PhoB/PhoR, but independent of the phosphate starvation stimulus.

Proteins in whole cell lysates (Fig. 1A), in the periplasms (Fig. 1B) and membrane enriched fractions (Fig. 1C) of cells grown in TGHP were focused on immobilized pH gradient (pH 4-7) strips and separated in 12-14% linear gradient gels by SDS-PAGE. Most of the protein spots on the gels were visible in the pH range 4.5 -7.0 and presented molecular weight varying from10-90 kDa. In addition, the 2D-gel patterns for all samples closely resembled those obtained in similar experiments and published elsewhere (232; 246; 256). A remarkable feature of this analysis was the high resolution of the membrane protein two-dimensional maps, achieved with a combination of 7 M urea, 2 M thioureia and 1% ASB-14, which successfully solubilized the membrane-enriched fractions.

In total 1546 different protein spots were visualized on the gels by the Coomassie brilliant blue staining. The great majority was not differentially expressed, but 209 presented relative volumes that varied at least 3 fold between wild type and mutant samples. From these, 108 were found at higher levels in the wild type 569B and 101 in the *phoB* mutant, WK3. The quantitative distribution of the spots in the subcellular fractions is detailed in Table 1.

Of the 209 differential protein spots, 60 (about 30%) were identified by tandem mass spectrometry. Some of them represent isoforms of a single gene product, but the majority of the proteins (43) are coded by distinct genes. All proteins identified in the

current study and their putative roles are listed in Tables 2-3 and grouped into categories according to TIGR CMR classification of cellular functions.

The main group of proteins induced in the wild type cells in TGHP (6 proteins, 32%) were those involved in energy production and conversion, which represented only 4% (1 protein) of the highly expressed in WK3.

Groups of amino acid transporters and general metabolism proteins were also better represented in 569B (3 or16%) than from WK3 (2 or 8%). In contrast, proteins of metabolisms of DNA (3 or 13%) and lipopolyssacharide (1 or 4%) were only induced in WK3 cells.

Proteins of the following groups were distributed as described: adaptation to atypical conditions, 1 protein or 5% in 569B against 3 proteins or 13% in WK3; protein synthesis, 1 protein or 5% in 569B against 2 proteins or 8% in WK3; folding and stabilization, 1 protein or 5% in 569BSR against 3 proteins 13% in WK3; transporters and binding proteins, 2 proteins or 10% in 569BSR against 3 proteins or 13% in WK3. The majority of them are stress-related proteins and were expressed preferentially in WK3 cells. Proteins with hypothetical functions and those of cell surface were equally represented in both strains.

WK3 under high phosphate: dealing with stress

The differential up-regulation of genes whose products are involved in processes such as adaptation to adverse conditions, protein folding and stabilization, DNA and lipopolyssacharide metabolisms in the mutant WK3 is an indication that PhoB deficient cells, independently of the extracellular Pi level, are more sensitive to stress than the wild-type, confirming previous observations (232). Nevertheless, WK3 cells

survived well at the stationary-phase culture in a defined medium, thanks to their ability to activate innumerous anti-stress defense mechanisms.

The higher sensitivity of WK3 was also observed by comparing its growth kinetics with that of 569B in both, TGHP and in TGLP (Fig. X). In TGHP, for instance, although the cultures OD_{600} varied similarly over time (232) the corresponding values of viable cell counts showed lower growth rate and smaller viable cell numbers for WK3, observed mainly in the stationary growth phase (data not shown). These facts corroborate the assumption that the parental strain is more fit than the *phoB* mutant, which strongly supports a protective role for the system PhoB/PhoR in *V. cholerae* under any culture condition.

Therefore, it was of great interest to investigate the functions of the proteins differentially induced in the *phoB* mutant as an attempt to improve our understanding of the stress response in *V. cholerae* O1 and of its dependence on the bacterium PhoB/PhoR system in abundance of Pi.

Roles for some of the proteins expressed preferentially in cells of WK3 in the stationary phase of the culture in TGHP are discussed below.

Among the differentially expressed proteins more abundant in WK3, three seem to present roles in adaptation to atypical conditions: the 16 KDa heat shock protein (HtpA; VC0018), the heat shock protein G (HtpG; VC0985) and the cold shock DNA binding domain protein (Csp; VCA0184). The former was previously shown to negatively regulate the expression of ToxR, the central coordinator of *V. cholerae* virulence factors (257). Therefore, the upregulation of HtpG in WK3 might decrease the expression of ToxR and could contribute to the low colonization levels observed for this *pho*B mutant (226) although the expression of cholera toxina is equivalent in

both strains (226). The latter is one of the four cold shock proteins predicted to be coded by *V. cholerae* genome. However, it seems that VCA0184 is not expressed at low temperatures (258) and might respond to other stimuli than cold.

Interestingly, the 16 KDa heat shock protein A (HspA; VC0018) was detected in both wild type and mutant strains grown at 37°C in similar levels but at distinct isoforms. WK3 presented a more acidic form of HspA than 569BSR. This protein is described to be one of the three major Hsp in *V. cholerae* MAK757 but detected at low levels in strain 569B (259). Its expression is induced upon hypoxic conditions (260) and it seems to be negatively regulated by PhoB when the bacterium is grown aerobically under Pi limitation (232). The synthesis of HspA and the two other proteins mentioned above in response to diverse stressful stimuli besides heat/cold shock might protect cells from general stress (261).

Another related mechanism to compensate for injurious stimuli is the differential expression of outer membrane proteins (OMPs). Accordingly, WK3 expressed OmpA (VC2213), OmpT (VC1854) and OmpV (VC1318) as different isoforms than those identified in wild type cells. The isoform patterns of proteins are easily resolved in 2D gels and for these OMPs they clearly differ from 569BSR and WK3 cells. However, the identification of post-translational modifications current in these proteins was not yet assessed by mass spectrometry. OmpA was also upregulated in WK3 under low Pi growth (232). As discussed previously, it might confer resistance to environmental stresses and plays a vital role in the maintenance of cellular structure (262). It is proposed that OmpA is the structural and organizational component of the outer membrane of Gram-negative bacteria, interacting in a specific manner with a number of other membrane components (262). Thus, it is reasonable that a protein with so

many roles is found as post-translational modified isoforms. OmpT is one of the most studied *V. cholerae* porins. It is believed to be repressed by ToxR inside the host, as it increases cell sensitivity to environmental stresses, such as bile acids (263; 264). OmpV, on the other hand, is not sufficiently characterized but it might have a primary role in the interaction of the bacterium with the environment as well as in the regulation of hydrophilic compounds flow (265; 266; 267).

Besides OMPs, lipopolyssacharide (LPS) composition is also altered according to diverse stimuli. The LPS of Gram-negative organisms is the most abundant molecule on the cell surface and provides a protective barrier to hydrophobic agents (268). RfbT protein (VC0258) is a member of the *rfb* gene cluster involved in the biosynthesis of O-antigen (269; 270). The specific function of RfbT was not yet determined, but it might be a modifying enzyme of the B-determinant of O-antigen (269). Its identification in WK3 might represent an additional mechanism expressed by the mutant cells in order to circumvent adverse conditions.

An alcohol/acetaldehyde dehydrogenase (AdhE homologue; VC2033) was differentially expressed in WK3. In *E. coli*, AdhE constitutes the most important barrier against low levels of oxidative stress (109; 271). Thus, it seems that the *pho*B mutant expresses a system for dealing with the oxidative stress, even during HP growth (no evident oxidative stress). It corroborates the hypothesis that *pho*B mutant senses a stress situation even in non stressing conditions and initiates mechanisms of defense. In the other hand, we have identified a putative *pho* box sequence (GACACaagaatGACAC), at position -33 from the translation start site of *adh*E. The pho box upstream *adh*E, might suggest a binding site of PhoB and direct regulation

of PhoB in the oxidative stress response. Whether *pho*B could play a direct or indirect role in oxidative stress response will be further investigated.

In addition, WK3 expressed 2 proteases and a peptidase, involved in protein degradation, and the elongation factors G (VC2342) and TU (VC0321), which are related to general stress and may be acting as molecular chaperones. In conjunction with other known chaperones products up-regulated in WK3, these proteins are important for proper protein folding and protection from the stress (272; 273). The expression of a mechanism for removal of damaged proteins suggest that even in HP, WK3 present a stressed metabolism and alterations of its cellular components. Both subunits of protease Hsl, HslV (VC2675) and HslU (2674), as well as a peptidyl-prolyl cis trans isomerase might be part of this signaling system.

A possible involvement of PhoB in regulation of aminoacid biosynthesis is suggested by the identification of 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase (VC1704), 5,10 methyltetrahydrofolate reductase (MetF, VC2685) and phospho 2 dehydro 3 deoxyheptonate aldolase (VC1507), upregulated in the *pho*B mutant. Interestingly, we have found a putative *pho* box sequence at position -48 from transcription start site of *met*F (GACATtctacaGCCAT), supporting an involvement of PhoB with methionine synthesis pathway.

Other proteins identified were: C4-dicarboxylate binding protein (VC1929), an immunogenic protein (VC0430) member of TRAP family of secondary transporters; polyribonucleotide nucleotidyltransferase (PNPase, VC0647), Ribonuclease PH (VC0210), Vibriobactin protein (ViuB, VC2210) and a conserved hypothetical protein (VC0006).

569B presented active metabolism under high phosphate conditions

HspA, OmpA, OmpT, OmpV, ViuB and phospho 2 dehydro 3 deoxyheptonate aldolase were differentially expressed in 569BSR and WK3 cells. They were expressed in both strains as different isoforms. As discussed above, the differential regulation of HspA and OMPs is required for the bacterial adaptation to many conditions.

While WK3 expressed additional proteins that signal for stress response in HP growth and managed to survive, 569BSR expressed proteins involved in general metabolism, such as energy production and conversion. Proteins upregulated in 569B were: histidinol phosphate (VC1134) and aspartate (VC1293) aminotransferase from aminoacids metabolism; tyrosyl tRNA synthethase (VC0631) involved in protein synthesis; oxaloacetate decarboxylase (VC0550), phosphoglycerate kinase (VC0477), fructose biphosphate aldolase (VC0478), acetate kinase (VC1098), 2 oxoglutarate dehydrogenase, E2 component (VC2086) and phosphoenolpyruvate carboxylase (VC2646) from energetic metabolism; aminoacid periplasmic ABC transporter (VC1362); and protease DO (VC0566).

The identification of these proteins might shed light on further functions for the *V. cholerae* PhoB/PhoR, beyond those involved in Pi metabolism. The majority of the genes identified have not been described, thus, they could also help to increase our knowledge on *V. cholerae* O1 physiology. 569BSR presented an active metabolism, and the presence of PhoB in basal levels allowed the cells to grow dynamically. The comparison of 569BSR and WK3 expressed proteins makes clear that *pho*B is essential for a full growth of *V. cholerae*, even in a high phosphate medium.

In order to demonstrate this hypothesis, we have grown both strains in HP for 24 hours and accompanied growth through both optical density and counts of viable cells in agar plates. Although cultures OD_{600nm} varied similarly over time for wild-type and mutant strains in agreement to what was observed before (data not shown and (226)), values of colony forming units (CFU)/ml showed that the mutant strain has a growth defect when compared to the wild type. In the first 5 hours, both strains presented equivalent growth rates. However, during the next 9 hours, WK3 presented viable cell counts 40% smaller then 569B. After 16 hours, the mutant strain showed only 20% of the CFU/ml observed for the wild type. The reduced growth of the *pho*B mutant in comparison to the parental strain in TGHP corroborates that full growth of the 569B cells relies on a functional PhoB/PhoR, even under high levels of Pi. In the absence of a functional PhoB/PhoR cells managed to survive by expressing general stress responses.

PhoB positively regulates RpoS expression

The comparative proteomic analysis presented here at high Pi levels and before at Pi limitation (232) revealed that some stress responsive proteins are either directly or indirectly regulated by PhoB. One of the genes up-regulated in 569B under low Pi conditions was *vc0596* (232), whose product DksA is required for intestinal colonization of *V. cholerae* O1 (274). In *E. coli*, DksA in concert with (p)ppGpp up regulates RpoS transcription and increases RpoS mRNA stability and therefore induces global changes in transcription initiation by altering the utilization of alternative sigma factors (275). RpoS is the major regulator of responses to a variety of stress conditions, mainly through the stationary phase inducible genes (276). The differential expression of DksA lead us to analyze the *rpo*S mRNA and RpoS protein

levels in the wild-type cells and *pho*B mutants at both, the exponential and stationary phase of growth by RT-PCR and Western blot with polyclonal RpoS antibodies.

RT-PCR analysis of *rpo*S was carried out in *V. cholerae* 569BSR and WK3 grown under TGLP or TGHP culture media, for 5 h (exponential) or 16 h (stationary phase). The *rpo*S mRNA was detected in all conditions, except in WK3 cells under stationary phase of growth (Fig. 2A). This result indicates that PhoB might affect *rpo*S mRNA stability through DksA protein activity.

As RpoS expression is regulated at several levels, namely transcription, mRNA stability, translation, post-translational and so on (277), we decided to further investigate the involvement of PhoB in RpoS regulation and validate the RT-PCR results by analyzing RpoS protein expression. RpoS was detected in wild-type stationary phase cells grown in all tested media, but not detected in WK3 (Fig. 2B). RpoS detection was performed using an antibody raised against *Salmonella thyphimurium* (278) but it was successfully used to recognition of *V. cholerae* RpoS before (233). As a control, *E. coli* K12A15 grown in LB till stationary phase was used (Fig. 2C) indicating that this antibody is able to recognize RpoS from different Enterobacteria. The complementation of WK3 with wild-type operon *pho*B/*pho*R *in trans* restored the wild type phenotype with respect to the production of RpoS (Fig. 2C). Taken together, these results suggest that PhoB is required for the proper expression of RpoS.

In summary, it is likely that in the *pho*B mutant the lack of RpoS is the major signal of stress that induced the expression of the chaperones, proteases, elongation factors and other proteins involved in survival and adaptation.

PhoB negatively affects pppGpp expression.

As described above, basal levels of PhoB positively correlates with RpoS expression. The *pho*B null mutant overexpressed the stationary phase genes needed for survival in TGHP. If those genes are not activated by RpoS in WK3, it is clear that another signaling mechanism must be involved. In several bacteria, global changes associated with the modulation of metabolic activities in response to starvation (stringent response) are mainly triggered by the intracellular accumulation of two alarmones derivate of guanosine called ppGpp and pppGpp (collectively denominated (p)ppGpp) (275; 279; 280).

In *E. coli*, ppGpp nucleotide synthesis is controlled by SpoT and RelA (281). Mutants in these genes are unable to activate the Pho regulon genes and it was suggested a regulatory link between ppGpp and the phosphate limitation response (282). Further, it was shown that ppGpp induces RpoS accumulation, mediated by IraP (283). In order to examine the tripartite relationship between PhoB, RpoS and (p)ppGpp in *V. cholerae*, we have analyzed the occurrence of these alarmones in *pho*B mutant compared to wild type. TLC analysis of nucleotides in *V. cholerae* 569BSR and WK3 cells grown under LP or HP for 16 h (stationary phase) was performed. *E. coli* K12 culture sample was used as a control as its nucleotides profile is well established. pppGpp was detected in WK3 cells grown in LP and HP media and in 569BSR cells only under LP condition (Fig. 3A). As expected for a stringent condition, pppGpp levels detected under LP were higher than under HP growth. However, the detection of pppGpp in WK3 even in HP, suggest a negative involvement between PhoB and the synthesis of pppGpp. In *V. cholerae*, pppGpp levels are regulated by RelA/SpoT

or RelV pathways (284; 285). Whether PhoB affects the synthesis or function of these regulatory proteins remains to be studied.

Surprisingly, pppGpp levels do not correlate positively with RpoS in *V. cholerae* 569BSR, as observed for *E. coli* and suggested novel intersections among the Pho (phosphate limitation) and RpoS (stationary phase) regulons and the stringent response mediated by pppGpp.

pppGpp and PolyP levels are correlated in *V. cholerae* cells

Within this overlap between the phosphate limitation and stringent responses is a key compound, the polyphosphate (PolyP). PolyP is a known stress responsive molecule, synthesized upon nutrients starvation and high salt concentrations (253; 282; 286; 287; 288). The synthesis and break of PolyP is regulated by polyphosphate kinase (Ppk) and exopolyphosphatase (Ppx), respectively. In *E. coli*, these enzymes are located in an operon. Therefore, it is evident that PolyP levels and the sizes of PolyP chains are regulated by a balance between Ppk and Ppx, through post-translational mechanisms of regulation. It was shown that pppGpp somehow inhibits Ppx, inducing the accumulation of PolyP (282).

In *V. cholerae*. *ppk*/*ppx* are also organized in an operon (289) and, under low Pi conditions, the bacterium remarkably accumulates PolyP (290). We have searched for a putative *pho* box sequence in *V. cholerae* N16961 genome and found a GTCATaatattGCGGT motif, at position -32, close to the consensus sequence proposed for *V. cholerae pho* box, suggesting once more that PhoB and Pi levels are key regulators of PolyP metabolism

Thus, we have quantified long and short chains of PolyP in 569BSR and WK3 cells grown in both LP and HP to stationary phase. Long chain PolyPs were only detected in 569BSR and WK3 cells grown in LP (Fig. 3B). Short chain PolyP was preferentially expressed in *pho*B mutant cells, independent of Pi levels (Fig. 3B).

Our measures revealed PolyP levels lower than previously observed (289; 290). However, the high levels of PolyP detected in *V. cholerae* were observed up to 10 hours of growth and the experiments described herein were performed after 16 hours. The usual methods of PolyP measurements refer to long chain PolyP extractions. Therefore, ours results are in agreement with others published that low Pi levels induce PolyP accumulation, but also revealed an additional Pi-independent system that controls Ppx activity.

PolyP is always synthesized as long chains and the formation of short chains are a result of partial activity of phosphatases. The *pho*B mutant under TGHP presented higher levels of pppGpp and short chain PolyP than the wild type. Hence, it seems that pppGpp positively regulates the activity of Ppx in *V. cholerae*. PolyP might be acting as both energy source and phosphate reservoir for the *pho*B mutant.

PhoB coordinate a general stress response

As described above, the product of VC0647, a polyribonucleotide nucleotidyltransferase (EC: 2.7.7.8), also known as polynucleotide phosphorylase (PNPase), was up-regulated in 569BSR under both low and high levels of Pi (232) suggesting its constitutive expression is PhoB-dependent. It has a role in acid tolerance response (ATR), a strategy required for successful *V. cholerae* O1

intestinal colonization. The differential expression of this protein indicates that PhoB could be a regulator of acid stress.

We have tested this hypothesis through an acid shock survival assay. The acid tolerance resistance (ATR) of 569BSR and WK3 grown in LB, previously adapted or not at pH 5.7, was measured. Viability of adapted and unadapted cells was assayed immediately upon acid shock at pH4.5, after 15, 30 and 60 min by plating onto LB agar. Unadapted cells of 569BSR and WK3 were very sensitive to acid shock. All adapted cells showed higher ATR than unadapted ones. Moreover, after 60 min in LB pH 4.5, adapted WK3 cells showed about 1/3 of the acid-resistance of 569BSR (Fig. 4A). The lack of a functional PhoB in WK3 and subsequently absence of PNPase lead to a slightly acid sensitive phenotype in the *pho*B mutant.

This interconnection between phosphate starvation and acid stress response was recently suggested to occur in *E. coli* also (231) and our results corroborate the proposed hypothesis, establishing a positive relationship between PhoB, PNPase and acid tolerance.

The importance of a functional PhoB for stress resistance under low phosphate conditions is already described (226; 232; 233). However the role of PhoB independent of Pi levels was not unveiled yet. As discussed above, proteins involved in oxidative and termic stresses were also differentially expressed between the two strains. As well as PolyP, which also confers resistance to osmotic and nutritional stress (290). Therefore, we further investigated the survival of 569BSR and WK3 to several stresses.

As observed in Fig. 4, 569BSR and WK3 presented the same behavior under oxidative, ethanol and UV stresses in LB growth. The *pho*B mutant was not more sensitive to these challenges than the wild type. As WK3 do not express RpoS, it was expected that the mutant presented higher sensitivity to oxidative stress than wild type strain, as catalase expression is dependent of RpoS. This intriguingly result lead us to measure catalase acitivity. While 569BSR presented high levels of catalase activity at stationary phase in TGHP (69.28±29.71 U/μg protein), the WK3 showed only basal levels (1.33±1.06 U/μg protein). However, AdhE protein is expressed only in the *pho*B mutant. As discussed above, AdhE function as a protective barrier to low oxidative stress and thus might be responsible for WK3 resistance to oxidative stress.

Surprisingly, the *pho*B mutant was a little bit more resistant to termic and osmotic stresses than 569BSR (Fig. 4). The proteomic analysis revealed an induction of heat shock proteins expression in WK3. The activity of these proteins as molecular chaperones might have protected WK3 cells from elevated temperatures in a more efficient approach than 569BSR. Accordingly, the high levels of PolyP accumulated by WK3 might also be a protective mechanism against osmotic variations.

Probably, the absence of a functional PhoB leads to a stressed cellular metabolism that through signaling molecules, such as pppGpp and PolyP) initiates a stress response.

The wild type under TGHP present high levels of RpoS and respond to the various stresses through the RpoS-dependent response pathways. In overall, WK3 and 569BSR presented distinct pathways of cellular resistance. The existence of redundant mechanisms of cellular protection confers evolutive advantage for *V. cholerae* to persist under environmental stress.

Concluding remarks

This work showed for the first time that *V. cholerae* PhoB affects RpoS expression (RT-PCR and Western blot). This result is a hallmark o the study of the Pho regulon, indicating further roles for PhoB on stress response other than phosphate limitation. The direct effect of PhoB in RpoS levels might be mediated by DksA (proteomic analysis) and enhances the resistance of bacterial cells to acid shock stress (survival assay).

The lack of a functional PhoB mimics a constitutive stress signal and cellular metabolism is straight to survival responses (growth curve and proteomic data). As the mutant do not express RpoS in none of the conditions tested, it adapted cell metabolism (proteomic data) and established other mechanisms for survival (survival assays), mediated by pppGpp (TLC) signaling and PolyP (Ppx enzymatic assay). PolyP accumulations seem an efficient mechanism of resistance to osmotic stress, as the mutant was more resistant than the wild type (survival assay).

In conclusion, we have reported the interrelationship of PhoB, RpoS, pppGpp and PolyP in *V. cholerae* 569BSR (Fig. 5). Two mechanisms of adaptative response, RpoS-dependent and PolyP-dependent were suggested to occur. RpoS-dependent mechanism is supposed to take place in cells $PhoB^+$ and PolyP under $PhoB^-$ background.

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Authors' contributions

LMLS designed the study, carried out proteomic analysis, *rpo*S and RpoS detection, PolyP measures and survival assays and drafted the manuscript. CLG designed the study, participated on proteomic analysis and survival assays. FRF carried out pppGpp chromatography. KSV and MEL participated on pppGpp assay. FMG and EAM participated on PolyP measures. PMB coordinated the study. WMAVK conceived, designed and coordinated the study.

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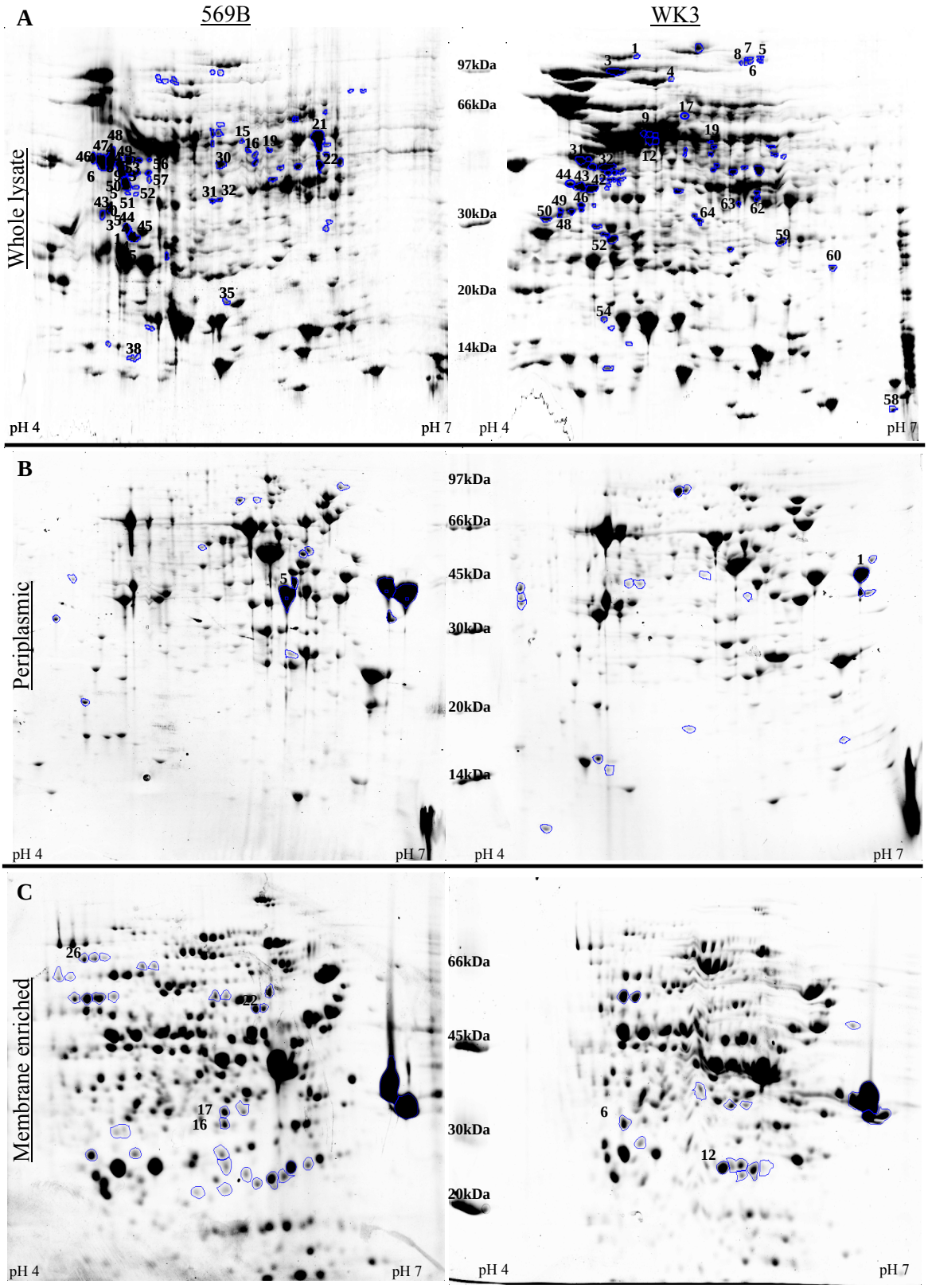

Fig. 1: 2D electrophoresis maps of whole cell lysate (A), periplasmic fractions (B) and membrane enriched samples (C) from *V. cholerae* 569BSR and WK3 mutant. Differentially expressed spots are blue outlined. Numbered spots were identified by mass spectrometry, according to tables 2 and 3.

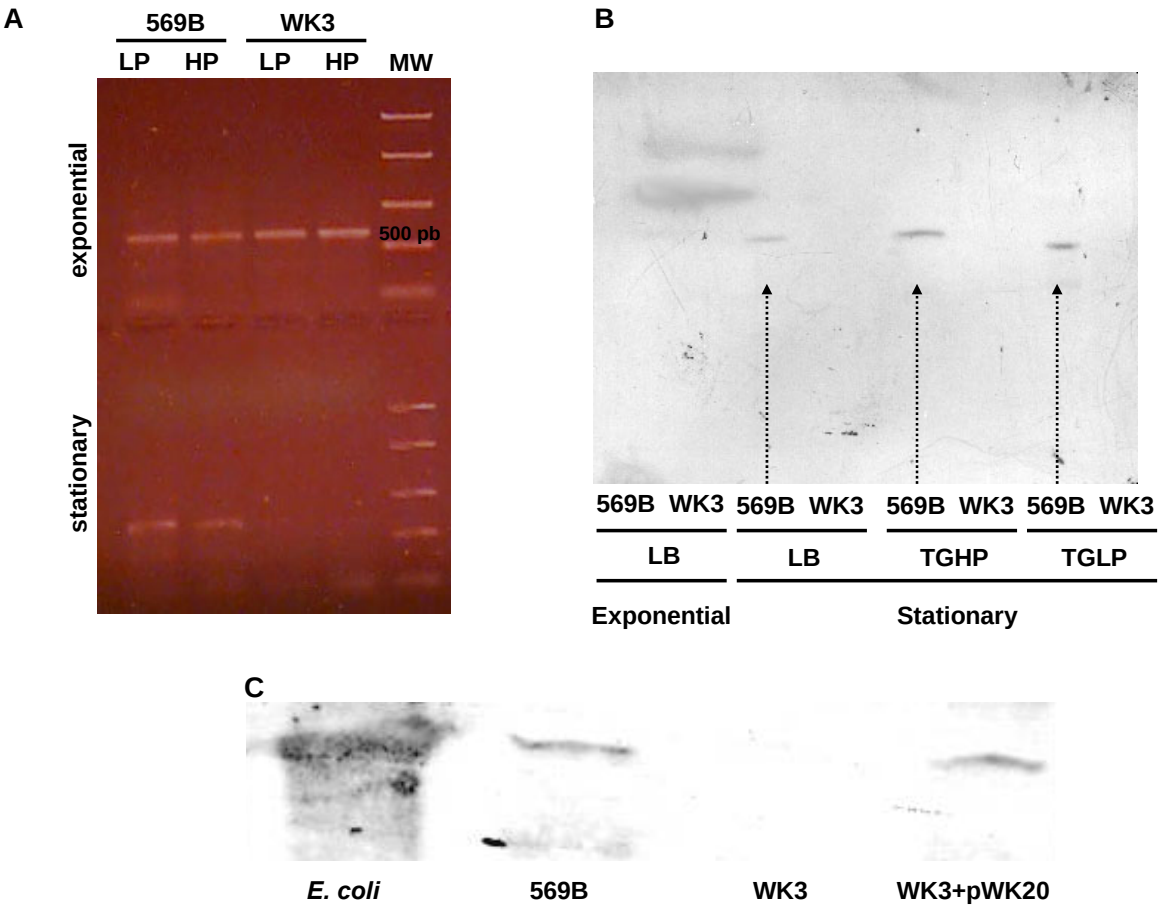

Fig. 2: Detection of *rpo*S mRNA and RpoS protein. A) RT-PCR analysis of *rpo*S in *V. cholerae* 569BSR and WK3 grown under low (LP) or high (HP) phosphate culture media, for 5h (exponential) or 16hs (stationary phase). B) RpoS detection by Western blotting analysis. *V. cholerae* 569BSR and WK3 were grown under LB medium, low (LP) or high (HP) phosphate culture media, for 5h (exponential) or 16hs (stationary phase) and RpoS in the lysates were detected with specific antibody. C) Western blotting analysis of RpoS in *V. cholerae* 569BSR, WK3 and WK3 complemented with pWK20 (plasmid containing a wild type copy of *pho*B/*pho*R operon), grown under high (HP) phosphate culture medium for 16hs (stationary).

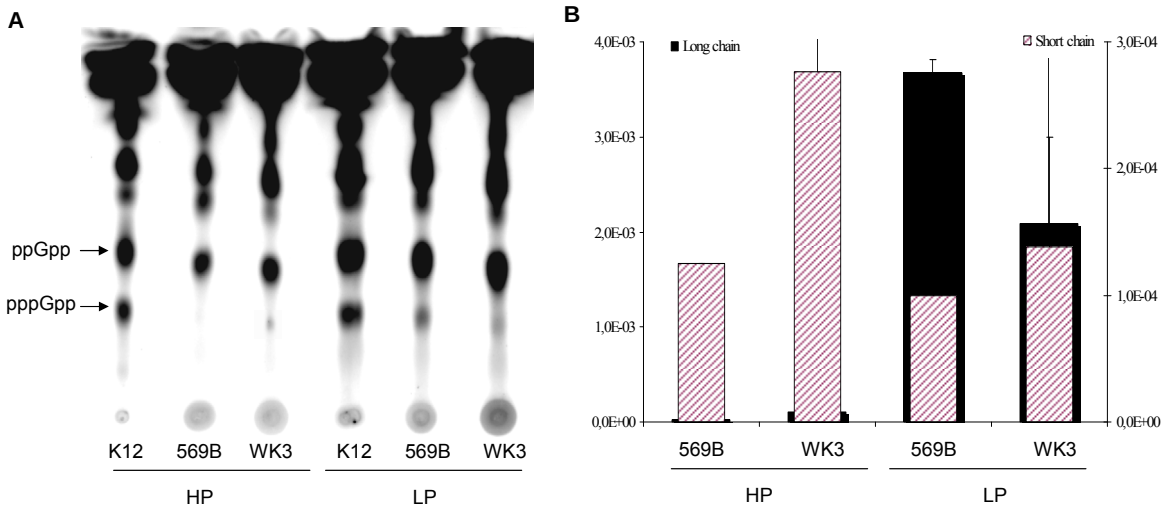

Fig. 3: (p)ppGpp and PolyP accumulation. A) Occurrence of (p)ppGpp. TLC of nucleotides in *V. cholerae* 569BSR and WK3 cells grown under low (LP) or high (HP) Pi culture media for 16hs (stationary phase). *E. coli* K12A15 was used as a control. B) PolyP quantification. Long and short PolyP chains were extracted from *V. cholerae* 569BSR and WK3 cells, grown under low (LP) or high (HP) phosphate culture media, for 16hs (stationary phase). Solid black bars represent the amount of Pi released after 15 min incubation of long chain PolyP with Ppx. Hatched bars represent the amount of Pi obtained from the short chain PolyP, under the same treatment. Relative PolyP amounts were determined as a function of the Pi released upon treatment with an excess of exopolyphosphatase (Ppx). Average and standard deviation of three independent experiments are shown.

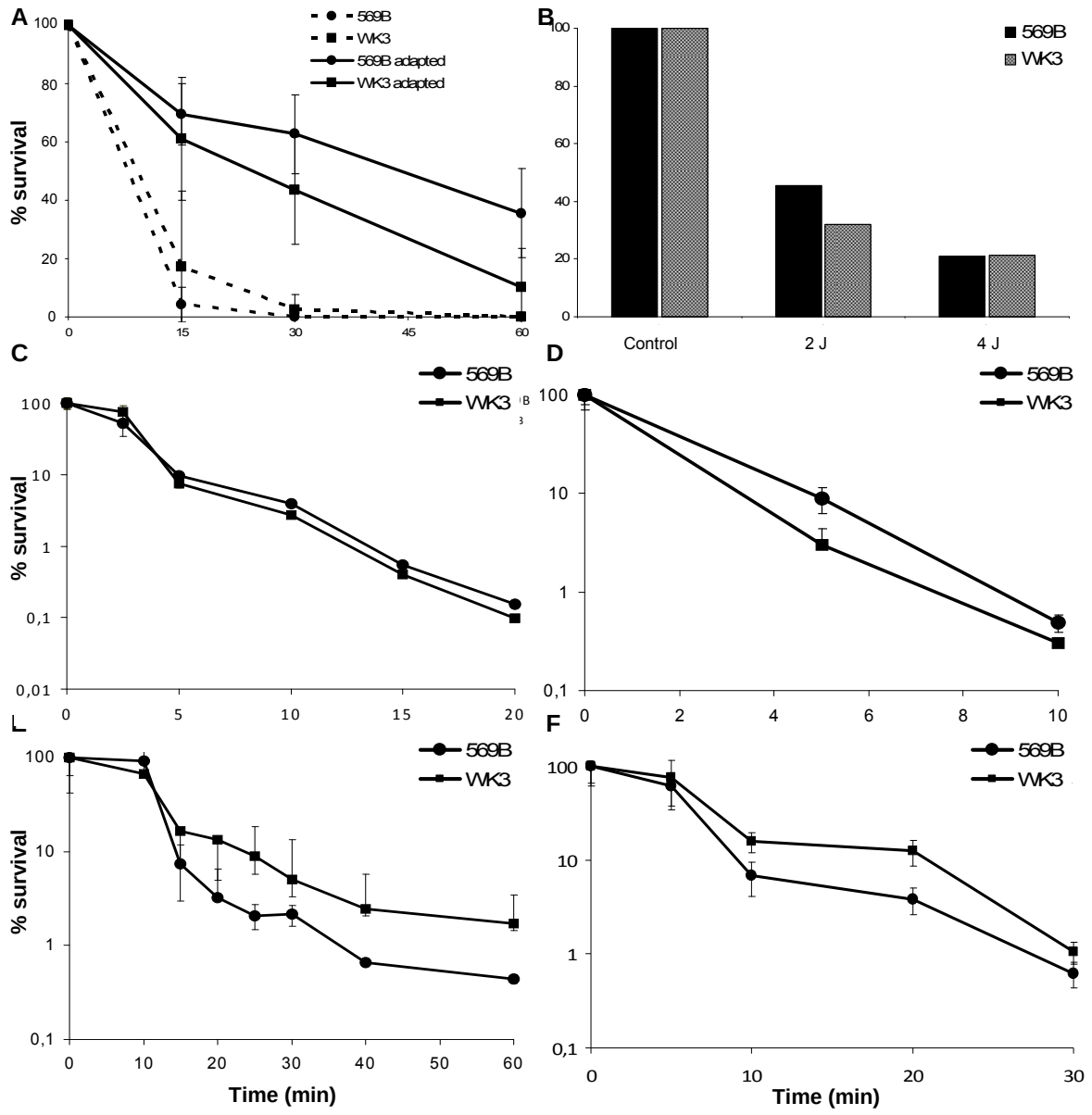

Fig. 4: Stress survival assays. 569BSR and WK3 in LB were subjected to (A) acid shock at pH 4.5 (cells were previously adapted in pH 5.7 or not), (B) 2 or 4J ultraviolet irradiation, (C) 1mM H_2O_2 for oxidative stress, (D) 8% ethanol, (E) 42°C for termic shock and (F) 1mM NaCl for osmotic stress. Data represent the mean and standard deviation of 6 independent measures.

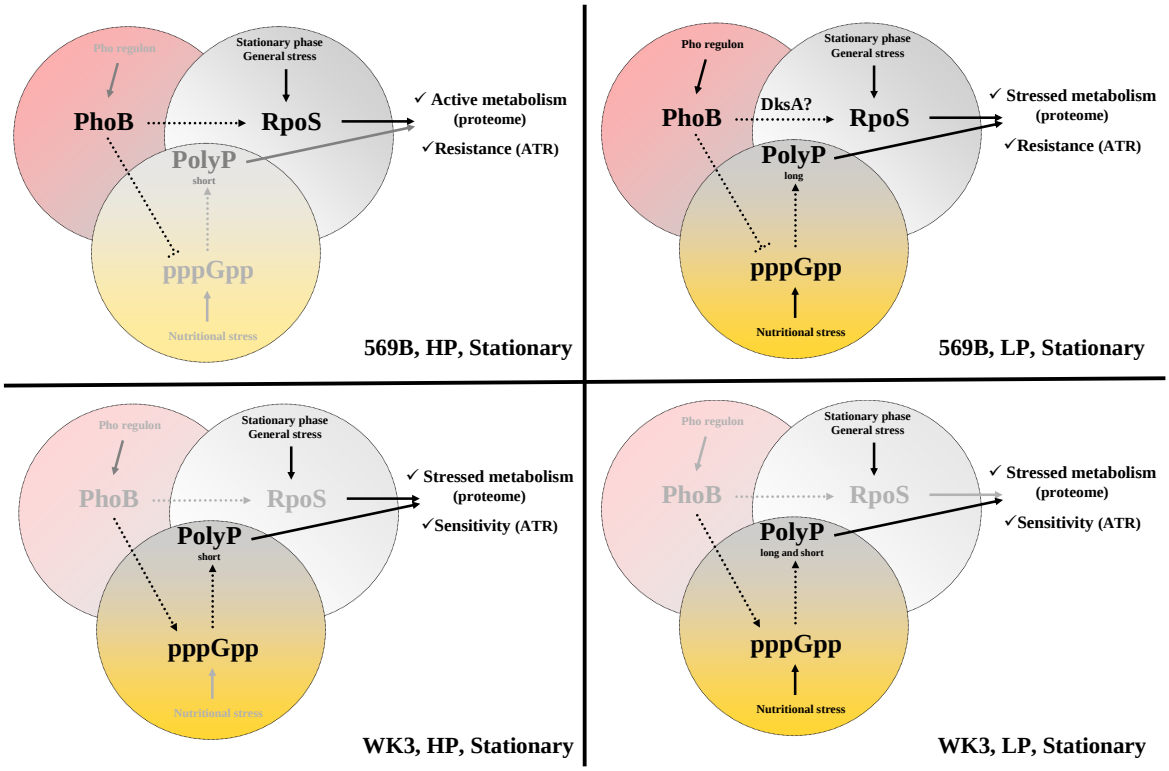

Fig. 5: Scheme illustrating the interactions among the expression of Pho regulon members (red circle), RpoS (stationary phase induced / several stresses responses; gray circle), PolyP biosynthesis and the stringent response (nutrients limitation) major factor pppGpp (yellow circle). Dashed lines represent the interactions, based on the results of this study that could be either direct or indirect. Elements represented in black were detected under the specified condition and the ones in gray were absent. Adapted from (231).

Table 1: Number of spots detected in comparative protein expression analysis of 569BSR and WK3 at stationary phase (16 hours) under TGHP.

Fraction	569B		WK3	
	total	up-regulated	total	up-regulated
Whole lysate	431	60	440	67
Periplasmic	157	15	174	19
Membrane-enriched	190	33	154	15
Total	778	108	768	101

Table 2: Differentially expressed proteins in *V. cholerae* 569BSR. PM: number of peptide matches. Cov: % of protein sequence coverage by identified peptides.

Spot	Locus	Protein	Score	MW	pI	PM	Cov
Adaptation to atypical conditions							
35	VC0018	16 kDa heat shock protein A	28	17	5,2	2	17
Amino acid transport and metabolism							
22	VC1507	phospho-2-dehydro-3-deoxyheptonate aldolase, phe-sensitive	25	43	5,7	1	5
M16	VC1134	histidinol-phosphate aminotransferase	86	39	5,3	6	12
M17	VC1293	aspartate aminotransferase	112	45	5,4	9	11
Cell surface							
43	VC1318	outer membrane protein OmpV	88	28	5,2	3	20
45	VC1318	outer membrane protein OmpV	178	28	5,2	3	30
46	VC2213	outer membrane protein OmpA	18	34	5,1	1	4
47	VC2213	outer membrane protein OmpA	111	34	5,1	5	32
48	VC2213	outer membrane protein OmpA	120	34	5,1	4	22
49	VC2213	outer membrane protein OmpA	88	34	5,1	4	28
50	VC2213	outer membrane protein OmpA	84	34	5,1	4	25
51	VC2213	outer membrane protein OmpA	83	34	5,1	1	5
57	VC2213	outer membrane protein OmpA	60	34	5,1	2	13
21	VC1854	outer membrane protein OmpT	28	40	5	1	25
Energy production and conversion							
M22	VC0550	oxaloacetate decarboxylase alpha subunit	66	62	6,9	8	12
52	VC0477	phosphoglycerate kinase	93	42	5	2	10
53	VC0477	phosphoglycerate kinase	61	42	5	1	5
56	VC0478	fructose-bisphosphate aldolase, class II	72	39		10	39
16	VC1098	acetate kinase	60	43	5,6	2	8
15	VC2086	2-oxoglutarate dehydrogenase, E2 component	111	44		4	16
M26	VC2646	phosphoenolpyruvate carboxylase	74	100	5,9	10	9
Hypothetical							
44	VC1503	conserved hypothetical protein	17	33	9	1	6
38	VC2512	conserved hypothetical protein	83	14	5,2	2	25
Protein fate: folding and stabilization							
30	VC0566	protease DO	33	48	5,7	1	6
Protein synthesis							
19	VC0631	tyrosyl-tRNA synthetase	66	44	5,7	2	8
Transport and binding proteins							
31	VC2210	vibriobactin utilization protein ViuB	62	31	5,3	3	23
32	VC2210	vibriobactin utilization protein ViuB	88	31	5,3	3	23
PP5	VC1302	amino acid ABC transporter, periplasmic amino acid-binding protein	124	37	5,2	10	23

Table 3: Differentially expressed proteins in *V. cholerae* WK3. PM: number of peptide matches. Cov: % of protein sequence coverage by identified peptides.

Spot	Locus	Protein	Score	MW	pI	PM	Cov
Adaptation to atypical conditions							
54	VC0018	16 kDa heat shock protein A	44	17	5,3	3	30
4	VC0985	heat shock protein HtpG	36	72	5,3	2	4
58	VCA0184	cold shock DNA-binding domain protein	21	8	6,5	1	29
Amino acid transport and metabolism							
6	VC1704	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	17	85	5,4	1	2
8	VC1704	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	52	85	5,4	2	5
63	VC2685	5,10-methylenetetrahydrofolate reductase	46	33	6	2	12
Cell surface							
PP1	VC1854	outer membrane protein OmpT	64	40	5	7	20
52	VC1318	outer membrane protein OmpV	13	28	5,2	1	10
42	VC2213	outer membrane protein OmpA	184	34	5,1	4	25
43	VC2213	outer membrane protein OmpA	228	34	5,1	5	29
44	VC2213	outer membrane protein OmpA	78	34	5,1	3	27
DNA metabolism							
3	VC0647	polyribonucleotide nucleotidyltransferase	37	31	4,9	2	23
9	VC0210	ribonuclease PH	19	27	5,3	1	4
31	VC2571	DNA-directed RNA polymerase, alpha subunit	50	37	4,9	2	11
32	VC2571	DNA-directed RNA polymerase, alpha subunit	53	37	4,9	2	10
Energy production and conversion							
2	VC2033	alcohol dehydrogenase/acetaldehyde dehydrogenase	17	97	6,1	1	2
Hypothetical							
46	VCA0006	conserved hypothetical protein	204	27	4,8	2	20
48	VCA0006	conserved hypothetical protein	126	27	4,8	2	20
49	VCA0006	conserved hypothetical protein	122	27	4,8	2	20
M6	VC1507	phospho-2-dehydro-3-deoxyheptonate aldolase, phe-sensitive	73	43	5,7	10	26
Lipopolysaccharide metabolism							
59	VC0258	RfbT protein	33	26	5,4	2	15
Protein fate: folding and stabilization							
60	VC2675	protease HslVU, subunit HslV	100	19	6,3	1	10
50	VC0354	peptidyl-prolyl cis-trans isomerase, FKBP-type	27	28	4,9	1	6
17	VC2674	protease HslVU, ATPase subunit HslU	26	50	5,4	1	4
Protein synthesis							
12	VC0321	elongation factor Tu	103	43	5,1	4	12
19	VC0321	elongation factor Tu	76	43	5,1	3	13
1	VC2342	elongation factor G	48	77	5	3	7
5	VC2342	elongation factor G	21	77	5	3	7
7	VC2342	elongation factor G	25	77	5	2	5
Transport and binding proteins							
M12	VC1929	C4-dicarboxylate-binding periplasmic protein	66	37	5,4	4	5
62	VC0430	TRAP-type uncharacterized transport system, periplasmic component	28	34	5,9	1	4
64	VC2210	vibriobactin utilization protein ViuB	59	31	5,3	10	15

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