UNIVERSIDADE FEDERAL DO PARANÁ

HELOISA BRUNA SOLIGO SANCHUKI

CARACTERIZAÇÃO DA PROTEÍNA DPS DE *Campylobacter jejuni*

CURITIBA

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Dissertação apresentada como requisito parcial à obtenção do grau de Mestre pelo Programa de Pós-Graduação em Ciências - Bioquímica, do Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Luciano Fernandes Huergo
Co-orientador: Dr. Glaucio Valdameri

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TERMO DE APROVAÇÃO

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Curitiba, 17 de março de 2015.

Dedico este trabalho ao meu pai
Joel A. Soligo (*in memoriam*).

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RESUMO

As proteínas Dps (*DNA-binding protein from starved cells*) formam uma subfamília particular de ferritinas. Os membros deste grupo possuem uma estrutura tridimensional comum em forma de dodecâmeros esféricos com uma cavidade central oca. Estas proteínas são capazes de armazenar na sua cavidade central ~500 átomos de ferro por dodecâmero. O centro ferro-oxidase é a assinatura mais marcante deste grupo. Ele está localizado na interface entre as subunidades adjacentes e é composto por aminoácidos altamente conservados, onde ocorre a ligação e oxidação do Fe^{+2} para Fe^{+3}, seguido por sua acomodação no interior da proteína em uma forma não reativa. A proteína Dps de *Campylobacter jejuni* (CjDps) tem sido relatada por suas inúmeras funções, mas principalmente, por proteger o DNA de radicais hidroxila altamente tóxicos e reativos. A fim de facilitar estudos de caracterização *in vitro* da proteína CjDps, foi importante estabelecer um protocolo simples e reprodutível para sua purificação. Neste trabalho, foi demonstrado que a temperatura de desnaturação da proteína CjDps nativa (CjDps wt) é de 94,2°C. Esta característica foi utilizada para auxiliar no processo de purificação, estabelecendo-se um protocolo com um prévio tratamento térmico a 80°C seguido de dois passos cromatográficos. Com objetivo de melhor entender o funcionamento da CjDps, também foi utilizada a proteína CjDps H25G/H37G que possui alterações em resíduos conservados localizados no centro ferro-oxidase. Foi observado que a proteína CjDps H25G/H37G tem menor atividade de oxidação de ferro e menor capacidade de ligar metais em relação à proteína selvagem. A proteína CjDps H25G/H37G não apresentou capacidade de ligar DNA nas condições testadas sugerindo que as histidinas H25 e H37 podem ser importantes para ligação ao DNA.

ABSTRACT

The Dps (DNA-binding protein from starved cells) proteins form a particular subfamily of ferritins. All members of this group share a commum three-dimensional structure assembled forming spherical dodecamers with a hollow central cavity. These proteins are capable of storing ~500 iron atoms per dodecamer. The ferroxidase center (FOC) is the most remarkable signature of these proteins. The FOC is located at the interface between the subunits and is composed by highly conserved aminoacids that bind Fe^{+2} and oxidize to Fe^{+3}, followed by the storage of the latter ion into the central cavity of the dodecamer. The *Campylobacter jejuni* Dps protein is related to various cellular functions, including DNA protection against the highly toxic and reactive hydroxyl radicals. In order to facilitate *in vitro* characterization studies of CjDps, it is important to stablish a simple and reprodutive protocol for its purification. In this study, it was determined that CjDps has a high melting temperature (94.2°C). This thermal stability was used to facilitate the purification process by the introduction of a thermal treatment at 80°C followed by two chromatographic steps. In order to better understand the function of CjDps, we employed a CjDps H25G/H37G variant, where the conserved histidines at the FOC were substituted to glycines. It was observed that the CjDps H25G/H37G variant had iron oxidation and metal binding activities reduced in comparison to the wild-type protein. This protein variant was not able to bind DNA under the conditions tested suggesting that the histidines H25 and H37 might be important for the CjDps DNA-binding activity.

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CjDps – *Campylobacter jejuni* Dps protein

CjDps wt – *Campylobacter jejuni* Dps protein wild-type

DPS - DNA-binding protein from starved cells

EPR – Electron paramagnetic resonance

FOC – Ferroxidase center

GBS – Guillain-Barré syndrome

HP – *Helicobacter pylori*

NAP - Neutrophil-activating protein

ROS – Reactive oxigen species

T_m – Melting temperature

SUMÁRIO

1 INTRODUÇÃO

1.1 *Campylobacter*

O gênero *Campylobacter* (do grego: Kampylos, encurvado; bacter: bactéria) pertence à classe épisilon das protobactérias, ordem Campylobacterales e família Campilobacteraceae. Este gênero é constituído de várias espécies de natureza zoonótica e vem recebendo crescente atenção nos últimos 30 anos devido ao aumento da frequência com que tem sido isolado do homem, animais, alimentos e água (BUTZLER, 2004).

As bactérias pertencentes a este gênero são microrganismos gram-negativos microaerófilos e levam de 72-96 horas para formar colônias a uma temperatura ótima de crescimento de 42°C. Essas bactérias possuem a forma de bacilos curvos, em forma de "S" ou espirais, finos e compridos, cujas dimensões variam de 0,2 a 0,9 µm de largura por 0,5 a 5 µm de comprimento. São móveis através de flagelo único em uma ou ambas extremidades que é responsável pelo seu movimento característico de "serrote ou saca-rolha" (FRANCO, 2002). Em culturas jovens é possível observar a morfologia em "asa de gaivota" enquanto que em culturas de vários dias as bactérias adquirem morfologia esférica ou cocóide. Não são formadores de esporos (FRANCO 2002; VANDAMME; DE LEY, 1991). Uma das características mais marcantes do gênero *Campyobacter* é a microaerofilia. Eles crescem em concentrações de oxigênio de 3-15% sendo que 5% é a concentração ideal. Além disso, eles são capnofílicos, ou seja, requerem CO_2 para o crescimento (FRANCO, 2002). O gênero *Campylobacter* é conhecido por conter espécies que causam gastroenterites (ALLOS, 2001; VÉRON; CHATELAIN, 1973).

Campylobacter spp. são patógenos em humanos, sendo responsáveis por 400-500 milhões de casos de diarréia anualmente (RUIZ-PALACIOS, 2007). Dentre os organismos deste gênero, a espécie melhor estudada é o *C. jejuni*. No ano 2000, seu genoma, de aproximadamente 1.7 Mb, foi completamente sequenciado e observou-se que 94,3% dele codifica para proteínas (PARKHILL et al., 2000). *C. jejuni* não é capaz de usar carboidratos comuns como fonte de carbono, pois além de não possuir transportadores para açúcares como glucose ou lactose, ele não possui diversas enzimas chaves da via glicolítica (VELAYUDHAN; KELLY, 2002). Dentre as fontes de nutrientes primárias deste organismo, encontram-se vários aminoácidos, que podem ser encontrados no intestino do hospedeiro (STAHL; BUTCHER; STINTZI, 2012). Dentre os aminoácidos disponíveis, serina é usada

preferencialmente, seguida de aspartato, asparagina e glutamato, sendo que a preferência pode ser variável entre estirpes (WRIGHT et al., 2009). Estes aminoácidos são biomoléculas comuns no sistema excretor de frangos, o que pode explicar o porque eles são chaves no metabolismo do *C. jejuni*, uma vez que está bactérias é comensal desses animais (PARSONS, 1984). Além de utilizarem aminoácidos como fonte de nutrientes, *C. jejuni* também é capaz de transportar e metabolizar acetato e lactato (THOMAS et al., 2011; WRIGHT et al., 2009).

C. jejuni é o principal causador de infecção em humanos, participando em 92% dos casos de infecção por *Campylobacter spp.* (GILLESPIE et al., 2008). A transmissão para humanos pode ocorrer a partir de várias fontes como água, leite ou alimentos (Figura 1). Dentre estas fontes, tem sido relatado que a ingestão de carne de frango mal cozida é a principal via de transmissão (DEMING et al., 1987; OLSON et al., 2008). As aves abrigam *Campylobacter spp.* no intestino que, por meio de manipulação sem a observação de práticas de higiene necessárias, contamimam a carne.

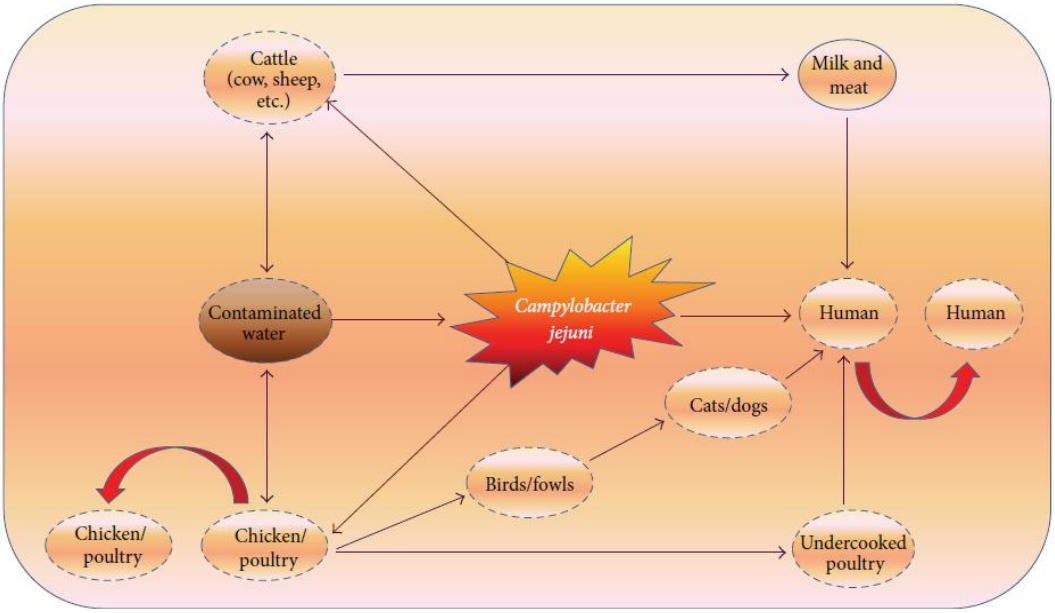

Figura 1. Fontes de transmissão de *C. jejuni*. Frango é o hospedeiro natural de *C. jejuni* onde coloniza a mucosa gastrointestinal do animal. Humanos que bebem água contaminada, consomem carne de frango mal cozida e leite não pasteurizado contaminados são infectados. Fonte: (NYATI; NYATI, 2013). Copyright © 2013 Pubmed

Os fatores de virulência deste organismo incluem motilidade flagelar e quimiotaxia, que o possibilitam o movimento através da camada mucosa que recobre as células intestinais (SZYMANSKI et al., 1995; TAKATA; FUJIMOTO; AMAKO, 1992), adesão e invasão de células hospedeiras (SZYMANSKI et al., 1995), produção de toxinas (WASSENAAR, 1997) entre outros.

Campilobacteriose, como é chamada a infecção por *C. jejuni*, pode ser facilmente causada por baixa dose infecciosa (500 células) (ROBINSON, 1981). Os principais sintomas da doença são diarréia (que pode ser aquosa ou sanguinolenta), febre, cólicas abdominais e dores musculares. A diarréia normalmente se inicia após 24 horas do início dos sintomas. O período de infecção é de 1-7 dias, afetando tanto o intestino delgado quanto o intestino grosso. A doença é comumente autolimitada (COKER et al., 2002).

A complicação mais importante causada por infecção de *C. jejuni* é o desenvolvimento da síndrome neurológica de Guillain-Barré (GBS). GBS é uma doença aguda desmielinizante do sistema nervoso periférico (NYATI; NYATI, 2013). Cerca de 25-40% dos pacientes com GBS estiveram infectados com *C. jejuni* de 1-3 semanas antes de desenvolver a doença (MISHU; BLASER, 1993). As principais características da síndrome são a perda motora ascendente e anormalidades sensoriais (ASBURY; CORNBLATH, 1990).

Devido a infecção por *C. jejuni* ser uma das principais causas de gastroenterites em todo o mundo e poder levar ao desenvolvimento de doenças mais graves, como por exemplo, a GBS, este organismo vendo sendo alvo de muitos estudos.

1.2 FERRO E ESTRESSE OXIDATIVO EM BACTÉRIAS

O ferro é um micronutriente essencial para a maioria dos organismos, sendo cofator em várias reações enzimáticas e essencial para processos como fotossíntese, fixação de nitrogênio, respiração, regulação gênica, transporte de oxigênio, biossíntese de DNA entre outros (ANDREWS; ROBINSON; RODRÍGUEZ-QUIÑONES, 2003). Apesar de participar de todas essas funções, o ferro é um problema para todos os organismos que dele necessitam. Isto porque, embora Fe^{+2} seja solúvel em ambiente fisiológico, na presença de oxigênio ele gera radicais hidroxila que são tóxicos e reativos. Já o ferro na sua forma oxidada Fe^{+3}, é insolúvel em água ou em pH neutro e pode ser reduzido pelo $O_2^{\bullet-}$ formando Fe^{+2} (BARASCH; MORI, 2004).

Bactérias e outros organismos desenvolveram sistemas de regulação precisos do nível de ferro, permitindo que as funções celulares aconteçam, mas sem causar danos através da geração de espécies reativas de oxigênio (ROS) (MASSÉ et al., 2007).

Organismos aeróbicos utilizam oxigênio para respiração e oxidação de nutrientes para obter energia. Como resultado deste consumo, são gerados subprodutos do metabolismo como radicais superóxido ($O_2^{\bullet-}$), peróxido de hidrogênio (H_2O_2) e radicais hidroxila ($OH^{\bullet}$).

Peróxido de hidrogênio e o radical superóxido são pouco reativos fisiologicamente, entretanto, o ferro pode reagir com essas espécies e gerar os altamente tóxicos e reativos radicais hidroxila. Essas espécies reativas de oxigênio têm como alvo macromoléculas biológicas como DNA, RNA, proteínas e lipídios. (CABISCOL; TAMARIT; ROS, 2000). Na forma de Fe^{+2}, o ferro pode reagir com o peróxido de hidrogênio e produzir radicais hidroxila a partir da reação de Fenton. As reações chaves são apresentadas abaixo:

Redução do Ferro: $O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$ (1)

Reação de Fenton: $Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^{\bullet}$ (2)

Reação de Haber-Weiss, (1) + (2) : $O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$

O papel do ferro como protagonista no estresse redox é indicado pelo aumento da sensibilidade a este tipo de estresse após crescimento de bactérias em ambiente rico em ferro (ABDUL-TEHRANI et al., 1999).

De forma a manter o ferro disponível mas tomando o cuidado de evitar os efeitos tóxicos da combinação de Fe^{+2} livre e peróxido de hidrogênio, os organismos estocam ferro dentro de proteínas armazenadoras denominadas ferritinas. O ferro intracelular é sequestrado de maneira reversível dentro dessas proteínas evitando a formação de radicais hidroxilas, e os radicais superóxidos e peróxido de hidrogênio são detoxificados pelas enzimas superóxidos dismutases e catalases respectivamente (CHIANCONE et al., 2004; STORZ; IMLAY, 1999).

1.3 PROTEÍNAS ARMAZENADORAS DE FERRO – FERRITINAS

Levando em consideração que o ferro é essencial para vários processos biológicos é contraditório que ele seja tão indisponível em condições fisiológicas. As ferritinas são peças fundamentais no controle da química entre ferro e oxigênio (LEWIN; MOORE; LE BRUN, 2005). Elas são capazes de sequestrar o ferro intracelular de forma reversível e estoca-lo de forma inerte para impedir reações danosas, e também servem como fonte de ferro quando este se torna escasso no meio intracelular (THEIL, 2007). As ferritinas captam ferro na forma Fe^{+2} mas o depositam na cavidade central como ferro oxidado Fe^{+3}. Portanto, o armazenamento do ferro requer uma etapa de ferro-oxidação que é realizado por sítios específicos dentro das

ferritinas (ANDREWS; ROBINSON; RODRÍGUEZ-QUIÑONES, 2003). O Fe^{+2} é oxidado em sítios que possuem atividade de ferroxidase, cujos aminoácidos ligantes de ferro são altamente conservados evolutivamente (CHIANCONE et al., 2004). Para realizar essa etapa de ferro-oxidação, as ferritinas podem usar oxigênio molecular ou peróxido de hidrogênio (THEIL, 2007)

Há três tipos de ferritinas encontradas em bactérias: as ferritinas típicas (Ftn) que estão presentes também em eucariotos, as bacterioferritinas (Bfr) encontradas somente em bactérias e as proteínas Dps (*DNA-binding protein from starved cells*) encontradas em procariotos (archaea e bactéria) (ANDREWS; ROBINSON; RODRÍGUEZ-QUIÑONES, 2003; VELAYUDHAN et al., 2007). As três sub-famílias de ferritinas podem existir na mesma bactéria e múltiplos genes de ferritinas, bacterioferritinas e Dps são comums.

As ferritinas compartilham muitas características, dentre elas as estruturais. Elas são compostas por 24 (Ftn e Bfr) ou 12 (Dps) subunidades que formam uma proteína esférica com a cavidade central oca onde os átomos de ferro são acomodados (ANDREWS; ROBINSON; RODRÍGUEZ-QUIÑONES, 2003). O centro catalítico onde ocorre a etapa de ferro-oxidação está dentro das subunidades (Ftn e Bfr) (LAWSON et al., 1989, 1991) ou entre elas (Dps) (ILARI et al., 2002).

1.4 PROTEÍNAS Dps

As proteínas Dps formam uma subfamília particular de ferritinas. A primeira proteína Dps foi caracterizada em *Escherichia coli* e verificou-se tratar de uma proteína induzida na fase estacionária ou sob estresse, que liga DNA de forma não específica, o protege de estresse oxidativo e afeta o padrão da expressão gênica (ALMIRÓN et al., 1992). O principal papel da Dps é proteger o DNA contra radicais hidroxila gerados pela ação combinada de Fe^{+2} e H_2O_2 (ZHAO et al., 2002). As proteínas Dps são amplamente distribuídas em procariotos. Elas são caracterizadas por suas subunidades possuirem um núcleo central comum e por regiões N- e C- terminais de comprimentos variados e com diferentes números de cargas positivas. Estas proteínas contém vários aminoácidos conservados que estão localizados perto do centro ferroxidase ou estão envolvidos em importantes interações estruturais (CHIANCONE; CECI, 2010). As proteínas Dps possuem uma arquitetura tridimensional comum e são encontradas como dodecâmeros quase esféricos com uma cavidade central oca. Os monômeros de Dps se dobram formando quatro hélices compactas (Figura1A). Em contraste com as outras

ferritinas, que possuem 24 subunidades, os monômeros de Dps se compactam em conjunto para formar uma estrutura de 12 subunidades (Figura1B). Cada dodecâmero consegue acomodar até ~500 átomos de ferro em sua cavidade, quantidade menor que nos outros tipos de ferritinas (HAIKARAINEN; PAPAGEORGIOU, 2010).

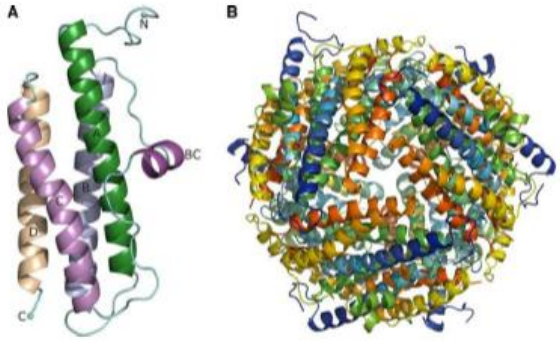

Figura 2 - Representação da estrutura das proteínas Dps de *Escherichia coli*. (A) Um dos monômeros da proteína e (B) dodecâmero visto ao longo do eixo de simetria. Fonte: (HAIKARAINEN; PAPAGEORGIOU, 2010). Copyright © 2010 Springer.

O centro ferroxidase representa a assinatura estrutural mais marcante da família Dps. Ele está localizado na interface entre as subunidades, sendo os aminoácidos que ligam ferro altamente conservados (FRANCESCHINI et al., 2006). As ferritinas em geral usam O_2 como oxidante de ferro com a produção de peróxido de hidrogênio, ao passo que as proteínas Dps tipicamente utilizam H_2O_2, que é aproximadamente 100 vezes mais eficiente que O_2 em carrear a oxidação do ferro (FRANCESCHINI et al., 2006; ZHAO et al., 2002). A remoção concomitante de Fe^{+2} e H_2O_2 pela Dps em uma solução resulta em uma prevenção da reação de Fenton. Como resultado, a formação de radicais hidroxilas altamente tóxicos será significantemente atenuada e, como consequência desse atenuamento, DNA, RNA proteínas e lipídeos (que são alvos biológicos) estarão protegidos dessas espécies altamente reativas de oxigênio (CHIANCONE; CECI, 2010). Esse mecanismo fornece uma explicação molecular para as observações iniciais feitas em *E. coli* (MARTINEZ; KOLTER, 1997), onde ocorre um aumento na síntese de Dps sob condições de estresse oxidativo, enquanto em mutantes com deleção do gene *dps* observa-se uma maior susceptibilidade ao dano oxidativo. THEORET et al., (2011), também demonstraram que mutantes de *C. jejuni* com deleção do gene *dps* tiveram uma diminuição na capacidade de sobrevivência em macrófagos comparado com a estirpe selvagem, fornecendo mais evidências para os achados de Martinez e Kolter (1997).

Cada monômero da proteína Dps possui aproximadamente 17 kDa e sua sequência gênica possui 450 pb. A comparação da sua sequência de aminoácidos revelou que os sítios de ligação de ferro da Dps de *Listeria innocua* proposto por Ilari (ILARI et al., 2000), são conservados também em *C. jejuni* (ISHIKAWA et al., 2003).

A proteína Dps de *C. jejuni* (CjDps) danifica fibras nervosas mielinizadas possivelmente através da ligação com a função sulfatídeo (PIAO et al., 2010). A função sulfatídeo está presente em diferentes tipos celulares, mas principalmente no sistema nervoso, onde são importantes componentes da bainha mielínica correspondendo de 4 a 6% do total dos lipídios mielínicos (ECKHARDT, 2008). A ligação da proteína Dps ao grupamento sulfatídeo da região paranodal (região da célula de Schwann de ambos os lados do nodo de Ranvier dos neurônios) pode contribuir para o desenvolvimento da patologia GBS que possui relação com infecção por *C. jejuni* (PIAO et al., 2010, 2011).

A proteína CjDps compartilha 41% e 24% de identidade com a proteína NAP (*neutrophil-activating protein*) de *Helicobacter pylori* e Dps de *E. coli*, respectivamente. *H. pylori* é um organismo que coloniza as camadas mucosas, bem como a superfície celular do epitélio gástrico, e é o principal agente infeccioso de gastrite crônica (MAHDAVI et al., 2002). A proteína HP-NAP é um dos representantes mais estudados da família Dps. A infecção pelo *H. pylori* está relacionada com a liberação desta proteína após autólise, capaz de induzir uma resposta imune (SATIN et al., 2000). A HP-NAP conduz a resposta inflamatória associada com a infecção e é a única da família com atividade quimiotáxica para neutrófilos, que acarreta em danos ao tecido gástrico (DE BERNARD; D'ELIOS, 2010). Estudos apontam que a imunização oral de ratos com HP-NAP é uma ferramenta promissora para desenvolvimento de vacinas contra infeção por *H. pylori* (SATIN et al., 2000). *H. pylori* é capaz de ligar mucina gástrica, o que o auxilia no processo de colonização (TZOUVELEKIS et al., 1991). Ensaios de ligação de HP-NAP à mucina salivar, utilizada como um sistema modelo para avaliar as mucinas em geral, indicaram que está proteína é capaz de ligar carboidratos sulfatados da mucina, e portanto, desempenhar papel de adesina (NAMAVAR et al., 1998). Esta habilidade de ligar carboidratos seletivamente poderia explicar a diminuição da capacidade de *C. jejuni* mutante do gene *dps* em ligar células epiteliais quando comparada com a estirpe selvagem (THEORET et al., 2011).

Além de protegerem as células contra o estresse oxidativo e estarem envolvidas com adesão e ligação a carboidratos, as proteínas Dps têm um papel fundamental na formação de biofilme. Os biofilmes protegem as bactérias de vários tipos de estresses ambientais,

aumentam sua resistência a antibióticos e fornecem proteção contra mecanismos de defesa do hospedeiro sendo importante para algumas bactérias patogênicas durante o processo de infecção (COSTERTON; STEWART; GREENBERG, 1999). Eles são usualmente definidos como comunidades microbianas estruturadas, contendo uma ou várias espécies, envoltas em uma matriz polimérica (COSTERTON; STEWART; GREENBERG, 1999). A matriz polimérica pode ser uma mistura de carboidratos, proteínas, lipídios e DNA extracelular, além de outras substâncias em menor quantidade (FLEMMING; WINGENDER, 2010). Resultados apontam que a proteína CjDps auxilia na sobrevivência natural de *C. jejuni* na colonização de frangos através da formação de biofilmes. *C. jejuni* com deleção do gene *dps* apresentam diminuição significativa na produção de biofilme comparada com células tipo selvagem (THEORET et al., 2012). Em *Salmonella enterica* foram encontradas 30 proteínas diferencialmente expressas através de análise de proteoma comparando bactérias cultivadas em biofilme ou em solução, sendo que a proteína Dps foi expressa apenas quando as células foram cultivada em biofilme, sugerindo que também neste organismo Dps desempenha um papel neste processo (GIAOURIS et al., 2013).

Estudos recentes mostraram que a proteína Dps de *C. jejuni* tem ainda uma habilidade única de ligar DNA estimulada pela presença de Fe^{+2} ou H_2O_2. Ela é capaz de proteger o DNA de danos oxidativos e degradação enzimática *in vitro*, assim como aumentar a resistência celular a danos causados pelo H_2O_2 *in vivo* (HUERGO et al., 2013). Embora se saiba que a Dps de *C. jejuni* liga DNA estimulada pela presença de H_2O_2, os mecanismos moleculares pelos quais isto acontece ainda são desconhecidos.

2 OBJETIVOS

Objetivo geral

- Purificar e caracterizar a proteína Dps de *Campylobacter jejuni*

Objetivos específicos

- Estabelecer protocolo para purificação da proteína Dps wt;
- Determinar a temperatura de desnaturação da proteína Dps wt;
- Avaliar a atividade de ligação da Dps wt e Dps H25G/H37G ao DNA em diferentes pHs;
- Determinar a atividade de oxidação de íons Fe^{+2} da Dps wt e Dps H25G/H37G *in vitro*;
- Avaliar a ação do peróxido de hidrogênio sob a atividade de oxidação do Fe^{+2};
- Avaliar a capacidade da Dps wt e Dps H25G/H37G ligarem Mn^{+2};

3 CAPÍTULO I

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Purification of the *Campylobacter jejuni* Dps protein assisted by its high melting temperature

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Running title: *C. jejuni* Dps DNA interaction

Abstract

Dps (DNA binding protein from starved cell) form a distinct group within the ferritin superfamily. All Dps members are composed of 12 identical subunits that assemble into a conserved spherical protein shell. Dps oxidize Fe^{2+} in a conserved ferroxidase center located at the interface between monomers, the product of the reaction Fe^{3+}, is then stored inside the protein shell in the form of non-reactive insoluble Fe_2O_3. The *Campylobacter jejuni* Dps (CjDps) has been reported to play a plethora of functions, such as DNA binding and protection, iron storage, survival in response to hydrogen peroxide and sulfatide binding. CjDps is also important during biofilm formation and cecal colonization in poultry. In order to facilitate *in vitro* studies with CjDps it is important to have a simple and reproducible protocol for protein purification. Here we observed that CjDps has an unusual high melting temperature. We exploited this property for protein purification by introducing a thermal treatment step which allowed achieving homogeneity by using only two chromatographic steps. Gel filtration chromatography, circular dichroism, mass spectrometry, DNA-binding and iron oxidation analysis confirmed that the CjDps structure and function were preserved.
Keywords: Dps protein, heat treatment, melting temperature, DNA binding

Introduction

Dps proteins are highly conserved and widely distributed iron storage proteins present in a vast range of prokaryotes. Dps form a distinct group within the ferritin superfamily. The crystallographic structure of various Dps members has been solved; in all cases they are composed of 12 identical subunits (of approximately 150 to 250 residues) that assembled into a conserved spherical protein shell [1–3].

The central core of the Dps dodecamer contains conserved residues that forms the ferroxidase center were Fe^{2+} is oxidized to Fe^{3+} and then stored inside the protein shell as insoluble and non-reactive Fe_2O_3 [4,5]. The Dps ferroxidase center is the most remarkable signature of the Dps family, it is located at the interface between monomers related by 2-fold symmetry [6]. Dps can either use hydrogen peroxide or oxygen to facilitate Fe^{+2} oxidation [7]. The concomitant removal of iron and hydrogen peroxide inhibits Fenton chemistry (H_2O_2 + $Fe^{+2} \rightarrow OH^- + OH^{\cdot} + Fe^{+3}$) thereby relieving the formation of toxic hydroxyl radicals.

Besides iron storage and hydrogen peroxide detoxification, some Dps members perform other cellular functions, such as DNA binding and protection [8,9], resistance to various forms of stress [10–13], carbohydrate binding and cell adhesion [14] and promotion of bacterial survival within biofilms [15,16].

Campylobacter jejuni is one of the most common causes of bacterial gastroenteritis worldwide. Infection by *C. jejuni* results in rapid onset of fever, abdominal cramps and diarrhea [17,18]. The disease is usually self-limiting; nonetheless complications such as reactive arthritis and the neuroparalytic Guillain-Barré syndrome have been associated with *C. jejuni* infections [19]. Multiple functions have been ascribed to the *C. jejuni* Dps (CjDps) protein such as DNA binding and protection; survival in response to hydrogen peroxide; and binding to the galactoceramide sulfatide [20]. *In vivo* studies showed that CjDps is important to biofilm formation and cecal colonization in poultry. Furthermore, preliminary studies demonstrated the potential of using the CjDps protein as a vaccine antigen in chicks [15].

In order to facilitate full functional characterization of CjDps, it is important to have a simple and reproducible protocol for protein purification. Several studies have reported the expression and purification of recombinant CjDps. In all cases, a His-tag has been introduced either at N or C-terminal to facilitate protein purification by affinity chromatography [15,21,22]. As Dps exist as dodecamer complex, the presence of His tags in each of its monomers may alter the overall Dps surface. It has been reported that Dps from different organisms can bind DNA in a pH-depend manner, probably through electrostatic interactions [9,21,23]. The presence of histidines, which are particularly sensitive to protonation at near physiological pH, might influence the overall Dps charge and thus DNA binding. It is therefore imperative to be able to purify this protein without a tag to enable accurate functional characterization. Here, we report a purification protocol for the untagged *C. jejuni* Dps protein.

Material and Methods

Vectors and cloning

The gene coding for *C. jejuni* Dps was removed as a *Nde*I-*Bam*HI fragment from the pLHPETcj1534c plasmid [21] and ligated into the pT7-7 vector previously digested with the same enzymes. The resulting plasmid was named pLHPT71534c and its integrity was confirmed by DNA sequencing.

Protein expression

The CjDps was overexpressed in *E. coli* BL21 (DE3) harboring the pLHPT71534c plasmid. Cells were grown at 37°C in LB medium containing ampicillin (100 μg.ml^{-1}) to an optical density at 600 nm of 0.7. At this point, 0.3 mM of IPTG was added and the cells were incubated at 37°C, 180 rpm for 3 h. Cells were harvested by centrifugation at 6,000 g for 15 min and resuspended in cold buffer 50 mM Tris–HCl pH 8.8 when used Hi-Trap Q FF or buffer 50mM Tris-HCl pH 7.5 for other columns tested. The cell suspension was stored at -80°C until purification.

Binding of CjDps to different matrices

We used five different chromatographic matrices during the initial attempts to purify the untagged CjDps. For 1 ml Hi-Trap Heparin HP (GE Healthcare), 1 ml Hi-Trap DEAE FF (GE Healthcare) and 1 ml Hi-Trap CM FF (GE Healthcare) we used 50 mM Tris-HCl pH 7.5 as buffer during cell lysis, column equilibration and wash. The columns were washed with 10 ml of buffer and the protein was eluted in buffer with increasing KCl concentrations (0 to 1 M) using an Äkta system (GE Healthcare). For 1 ml Hi-Trap Q FF (GE Healthcare), we used 50 mM Tris-HCl pH 8.8 during cell lysis, column equilibration and wash. The column was washed with 10 ml of buffer and the protein was eluted in buffer with increasing KCl concentrations (0 to 1M). For 1ml Hi-Trap Phenyl FF (GE-healthcare), after lysis in 50 mM Tris-HCl pH 7.5, the extract received 30% (w/v) of ammonium sulfate and was kept on ice for 40 min. The extract was centrifuged and loaded to a Hi-Trap Phenyl FF column previously equilibrated with 50mM Tris-HCl pH 7.5 and 1 M ammonium sulfate. The column was washed with 10 ml of Tris-HCl pH 7.5 and 1 M ammonium sulfate; proteins were eluted by linearly decreasing the ammonium sulfate concentrations from 1M to zero. Fractions of 1 ml were collected and analyzed by 15% SDS-PAGE.

Monitoring protein unfolding with Sypro Orange

A real-time PCR device (Applied Biosystems Step One Plus) was used to monitor protein unfolding by the fluorescent dye Sypro Orange as described [24]. 10 μM of CjDps was kept in 50 mM Tris-HCl pH 7.0 containing Sypro Orange diluted to 20x (Invitrogen). Data were collected at 0.3°C intervals from 25°C to 99.9°C. The first derivative curve of

normalized fluorescence intensity was plotted as function of temperature. The inflection point corresponds to the melting temperature (T_m). The result is representative of two independents experiments performed in triplicate.

Investigation of thermal stability by SDS-PAGE

Three different thermal treatments were tested in systems containing 2.8 µM of Dps in 50 mM Tris-HCl pH 7.0 (Fig. 1A). The first set was kept at room temperature, the second was heated at 70°C for 5 min and the third at 80°C for 5 min. After heating, all samples were kept on ice for 10 minutes and subjected to centrifugation for 10 minutes 12,100 g at room temperature. The supernatant of each sample was separated into two aliquots in new microtubes containing SDS-PAGE loading buffer. One aliquot of each set was boiled at 100°C for 7 min to visualize the CjDps monomers, the other aliquot was not heated to visualize the CjDps dodecamer. The samples were analyzed by 15% SDS-PAGE stained with Coomassie blue.

Final CjDps purification protocol

CjDps protein was overexpressed in 1.5 liters of LB medium and harvested by centrifugation. The pellet was stored at -80°C and suspended in 40 ml sonication buffer 50 mM Tris-HCl pH 8.8 at the day of purification. After lysis by sonication on ice bath, the buffer was supplemented with 1 mM PMSF and 20 U ml^{-1} of DNAseI. The extract was clarified by centrifugation at 30,000 g for 30 min at 4°C. The supernatant was kept on water bath at 80°C for 15 min following incubation on ice for 15 min. The soluble fraction was loaded into a 20 ml Hi-Trap Q FF column (GE Healthcare) pre-equilibrated with 40 ml of 50mM Tris-HCl pH 8.8 using an Äkta system (GE Healthcare). The column was washed with 20 ml of buffer and CjDps was eluted by linearly increasing the KCl concentration (from 0 to 1 M). 1 ml fractions were collected and analyzed on 15% SDS-PAGE. The most pure fractions were pooled and loaded into a Sephacryl S200 26/60 column (GE Healthcare) pre-equilibrated with 400 ml buffer containing 50 mM Tris-HCl pH 8.0, 0.1 M KCl and 5% glycerol. 2 ml fractions were collected and analyzed on 15% SDS-PAGE stained with Coomassie blue. CjDps protein was concentrated up to 34 µM using Vivaspin (10 kDa MWCO, Millipore), frozen in liquid nitrogen and stored in aliquots at – 80°C until use.

Protein quantification

Total protein concentration was determined using Bradford reagent (SIGMA) according to the manufacturer's supplied protocol. Bovine serum albumin (BSA) was used as standard.

DNA-binding assay using agarose gel eletrophoresis

Purified CjDps was diluted in 40 mM BisTris pH 6.0 buffer at the following dodecamer concentrations: 0.12, 0.60 or 2.4 µM. 200 ng of super coiled plasmid DNA (pET29a, Novagen) were added to each protein dilution. DNA-binding was allowed to occur for 10 min at room temperature. The reactions were mixed with 40 mM BisTris pH 6.0, 50% glycerol and 0.01% bromophenol blue and loaded into a 0.7% agarose gel prepared in 40mM BisTris pH 6.0 buffer. The gel was subjected to 90V for 20 min at room temperature, DNA was stained with ethidium bromide and visualized using a UVP BioImaging system.

Circular dichroism (CD)

CD analyses of CjDps diluted in 20 mM TrisHCl pH 6.0 to final dodecamer concentration of 2.4 µM were performed on a JASCO J-815 (JAPAN Spectroscopy & Chromatography Technology) spectropolarimeter couple to a temperature controller. The CD spectra were recorded at 25°C in the measure range of 200-300 nm, the baseline was corrected by subtracting the buffer spectrum.

Iron uptake experiment

Purified CjDps was diluted to 0.35 µM (dodecamer concentration) in 500 µL of 50 mM Tris-HCl (pH 6.8). Freshly prepared $FeSO_4$ was added to a final concentration of 500 µM. immediately after the iron addition the absorbance was monitored at 305 nm for 20 minutes under aerobic condition. Control of iron oxidation was performed without the addition of CjDps protein.

Results and discussion

Untagged *C. jejuni* Dps was expressed in *E. coli* and its ability to bind different matrices was evaluated. Giving that Dps binds DNA, we first attempted to use a Hi-Trap Heparin FF column (GE-healthcare), however, Dps did not bind efficiently to this column (Fig. S1A). We next tested Dps interaction with the cation exchanger Hi-Trap CM FF (GE-healthcare), again, Dps failed to bind (Fig. S1B). Attempts to fractionate the cell extract with ammonium sulfate precipitation followed by the hydrophobic interaction Hi-Trap Phenyl FF (GE-healthcare), also resulted in poor Dps recovery (Fig. S2). Finally, we tested two anion exchangers, a weak Hi-Trap DEAE FF and a strong Hi-Trap Q FF column (GE-healthcare). CjDps was retained by both matrices with better binding to Hip-Trap Q FF (Figure S1, panels C and D). Still, the gradient fractions containing CjDps had great number of contaminants (Fig. S1D).

Several lines of evidence indicated that Dps from different organisms are resistant to denaturing conditions keeping its dodecameric assembly even under high concentrations of urea or in the presence of SDS [11,21,25]. Furthermore, incubation of *E. coli* Dps at 75°C for 15 min did not affect the Dps DNA-binding activity [11], suggesting that EcDps retain its native structure even after heat. These data lead us to speculate that CjDps could also be heat resistant.

In order to determine the thermal stability of CjDps, the partially purified CjDps (Fig S1D, fraction 4) had its melting temperature determined using the fluorescent probe Sypro Orange (Invitrogen). Interaction between Sypro Orange and hydrophobic residues that are exposed due to protein unfolding events results in increased fluorescence. The protein melting temperature (T_m) was determined by measuring the fluorescence under a range of temperatures in a Real-time PCR apparatus following previously described methodology [24,26]. CjDps T_m was usually high at of 94.2°C (± 0.4) (Fig S3).

As it is difficult to determine whether the T_m reflects the unfolding of the Dps dodecamer, monomer or both we applied another method to determine the integrity of the CjDps dodecamers. Previous data showed that the His-tagged CjDps dodecamer does not unfold during SDS-PAGE as long as the protein is not heated in the presence of SDS before loading on the gel [21]. Untagged CjDps behaved in a similar manner (Fig 1B, lane 1). It was noted that heating of CjDps at 70°C or 80°C (in buffer without SDS) for 5 min prior to loading onto the gel did not dissociate the CjDps dodecamers (Fig. 1), confirming resistance of CjDps dodecameric structure to heat at up to 80°C.

We exploited thermo stability of CjDps by introducing a thermal treatment step in the purification protocol to improve protein homogeneity; a similar strategy has been successfully applied for the purification of Bacterial PII proteins [24]. The cell extract of *E. coli* expressing CjDps was subjected to 80°C for 15 min and kept on ice for 15 min. After centrifugation, most of the contaminants precipitated while CjDps was retained in the soluble fraction (Fig 2A). By applying only this step, Dps concentration in the soluble fraction was enriched to 48% (Table 1).

To further purify the protein, heat treated CjDps was subjected to a 20 ml Q sepharose FF column (GE-healthcare) (Fig. 2A), followed by gel filtration chromatography (Fig. 2B). CjDps was eluted as a single peak corresponding to a molecular mass of ~150 kDa (Fig.2C) confirming the dodecameric assembly. Furthermore, CjDps purified with or without thermal treatment showed a similar and expected pattern for a folded protein containing only α-helices as determined by circular dichroism analysis (Fig. 3). The helical was estimated to be 91% using the K2D3 software [27] for both preparations. MALDI-TOF analysis of the Dps monomers purified with or without thermal treatment indicated that both samples similar identical molecular masses (17258 ± 12 Da and 17274 ± 12 Da, respectively, Fig. S4) in excellent agreement with the calculated molecular mass (17.2 kDa). Therefore, the thermal step did not alter either the protein secondary structure or the protein molecular mass.

By combining the heat treatment followed by anion exchanger and gel filtration, 95% purity of Dps was achieved (Table 1 and Fig. 4). Hence, the heat treatment enabled extraction of Dps at 95% purity without additional chromatography steps.

The DNA-binding activity of CjDps was assessed using electrophoretic mobility shift assays as previously described [21]. CjDps was able to bind DNA as evidenced by its ability to change the mobility of purified plasmid DNA through agarose gel in a dose dependent manner (Fig. 5). CjDps was also able to catalyze iron oxidation as evidenced by the formation of Fe_2O_3 inside the protein shell by measuring the absorbance at 305 nm (Fig. 6). Therefore, the addition of the heat treatment step facilitated CjDps purification without compromising the protein structure and/or biological activity.

It is well established that ancestral proteins have high melting temperatures as ancient life forms lived in hot environments [28,29]. Hence, it is reasonable to expect that proteins that are wide-spread in nature and highly conserved in structure, such as those belonging to the PII [30] and Dps [4] family, may all have high T_m. We speculate that the heat treatment step introduced in this work may help to assist purification of other Dps members.

Conclusions

The CjDps has an unusually high melting temperature keeping its folding structure even at up to 80^{o}C (Fig. 1). We took advantage of such thermal resistance by introducing a thermal treatment step in the Dps purification protocol, a similar strategy has been successfully applied to purify PII proteins from various organisms [24].

The addition of the heat treatment helped to reach 95% homogeneity by using only two chromatographic steps. Gel filtration chromatography, circular dichroism, mass spectrometry, DNA-binding analysis and iron oxidation activity confirmed that the CjDps structure and function were preserved. The purification protocol described here will facilitate studies of the multifunctional CjDps protein.

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References

[1] R.A. Grant, D.J. Filman, S.E. Finkel, R. Kolter, J.M. Hogle, The crystal structure of Dps, a ferritin homolog that binds and protects DNA, Nat. Struct. Biol. 5 (1998) 294–303.

[2] K. Zeth, S. Offermann, L.-O. Essen, D. Oesterhelt, Iron-oxo clusters biomineralizing on protein surfaces: Structural analysis of Halobacterium salinarum DpsA in its low- and high-iron states, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 13780–13785. doi:10.1073/pnas.0401821101.

[3] G. Zanotti, E. Papinutto, W.G. Dundon, R. Battistutta, M. Seveso, G.D. Giudice, et al., Structure of the Neutrophil-activating Protein from Helicobacter pylori, J. Mol. Biol. 323 (2002) 125–130. doi:10.1016/S0022-2836(02)00879-3.

[4] E. Chiancone, P. Ceci, The multifaceted capacity of Dps proteins to combat bacterial stress conditions: Detoxification of iron and hydrogen peroxide and DNA binding, Biochim. Biophys. Acta BBA - Gen. Subj. 1800 (2010) 798–805. doi:10.1016/j.bbagen.2010.01.013.

[5] K. Zeth, Dps biomineralizing proteins: multifunctional architects of nature, Biochem. J. 445 (2012) 297–311. doi:10.1042/BJ20120514.

[6] B. Ren, G. Tibbelin, T. Kajino, O. Asami, R. Ladenstein, The Multi-layered Structure of Dps with a Novel Di-nuclear Ferroxidase Center, J. Mol. Biol. 329 (2003) 467–477. doi:10.1016/S0022-2836(03)00466-2.

[7] S. Franceschini, P. Ceci, F. Alaleona, E. Chiancone, A. Ilari, Antioxidant Dps protein from the thermophilic cyanobacterium Thermosynechococcus elongatus, FEBS J. 273 (2006) 4913–4928. doi:10.1111/j.1742-4658.2006.05490.x.

[8] M. Almirón, A.J. Link, D. Furlong, R. Kolter, A novel DNA-binding protein with regulatory and protective roles in starved Escherichia coli, Genes Dev. 6 (1992) 2646–2654.

[9] P. Ceci, S. Cellai, E. Falvo, C. Rivetti, G.L. Rossi, E. Chiancone, DNA condensation and self-aggregation of Escherichia coli Dps are coupled phenomena related to the properties of the N-terminus, Nucleic Acids Res. 32 (2004) 5935–5944. doi:10.1093/nar/gkh915.

[10] S.H. Choi, D.J. Baumler, C.W. Kaspar, Contribution of dps to Acid Stress Tolerance and Oxidative Stress Tolerance in Escherichia coli O157:H7, Appl. Environ. Microbiol. 66 (2000) 3911–3916.

[11] K.C. Jeong, K.F. Hung, D.J. Baumler, J.J. Byrd, C.W. Kaspar, Acid stress damage of DNA is prevented by Dps binding in Escherichia coli O157:H7, BMC Microbiol. 8 (2008) 181. doi:10.1186/1471-2180-8-181.

[12] A. Martinez, R. Kolter, Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps, J. Bacteriol. 179 (1997) 5188–5194.

[13] S. Nair, S.E. Finkel, Dps Protects Cells against Multiple Stresses during Stationary Phase, J. Bacteriol. 186 (2004) 4192–4198. doi:10.1128/JB.186.13.4192-4198.2004.

[14] S. Teneberg, H. Miller-Podraza, H.C. Lampert, D.J. Evans, D.G. Evans, D. Danielsson, et al., Carbohydrate Binding Specificity of the Neutrophil-activating Protein of Helicobacter pylori, J. Biol. Chem. 272 (1997) 19067–19071. doi:10.1074/jbc.272.30.19067.

[15] J.R. Theoret, K.K. Cooper, B. Zekarias, K.L. Roland, B.F. Law, R. Curtiss, et al., The Campylobacter jejuni Dps Homologue Is Important for In Vitro Biofilm Formation and Cecal Colonization of Poultry and May Serve as a Protective Antigen for Vaccination, Clin. Vaccine Immunol. CVI. 19 (2012) 1426–1431. doi:10.1128/CVI.00151-12.

[16] B. Pang, W. Hong, N.D. Kock, W.E. Swords, Dps promotes survival of nontypeable Haemophilus influenzae in biofilm communities in vitro and resistance to clearance in vivo, Front. Cell. Infect. Microbiol. 2 (2012). doi:10.3389/fcimb.2012.00058.

[17] B.M. Allos, Campylobacter jejuni Infections: update on emerging issues and trends, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 32 (2001) 1201–1206. doi:10.1086/319760.

[18] M.J. Blaser, Epidemiologic and Clinical Features of *Campylobacter jejuni* Infections, J. Infect. Dis. 176 (1997) S103–S105. doi:10.1086/513780.

[19] A.O. Coker, R.D. Isokpehi, B.N. Thomas, K.O. Amisu, C.L. Obi, Human Campylobacteriosis in Developing Countries1, Emerg. Infect. Dis. 8 (2002) 237–243. doi:10.3201/eid0803.010233.

[20] H. Piao, M. Minohara, N. Kawamura, W. Li, Y. Mizunoe, F. Umehara, et al., Induction of paranodal myelin detachment and sodium channel loss in vivo by Campylobacter jejuni DNA-binding protein from starved cells (C-Dps) in myelinated nerve fibers, J. Neurol. Sci. 288 (2010) 54–62. doi:10.1016/j.jns.2009.10.007.

[21] L.F. Huergo, H. Rahman, A. Ibrahimovic, C.J. Day, V. Korolik, Campylobacter jejuni Dps Protein Binds DNA in the Presence of Iron or Hydrogen Peroxide, J. Bacteriol. 195 (2013) 1970–1978. doi:10.1128/JB.00059-13.

[22] H. Piao, M. Minohara, N. Kawamura, W. Li, T. Matsushita, R. Yamasaki, et al., Tissue Binding Patterns and In Vitro Effects of Campylobacter jejuni DNA-Binding Protein from Starved Cells, Neurochem. Res. 36 (2011) 58–66. doi:10.1007/s11064-010-0263-7.

[23] P. Ceci, L. Mangiarotti, C. Rivetti, E. Chiancone, The neutrophil-activating Dps protein of Helicobacter pylori, HP-NAP, adopts a mechanism different from Escherichia coli Dps to bind and condense DNA, Nucleic Acids Res. 35 (2007) 2247–2256. doi:10.1093/nar/gkm077.

[24] V.R. Moure, G. Razzera, L.M. Araújo, M.A.S. Oliveira, E.C.M. Gerhardt, M. Müller-Santos, et al., Heat stability of Proteobacterial PII protein facilitate purification using a single chromatography step, Protein Expr. Purif. 81 (2012) 83–88. doi:10.1016/j.pep.2011.09.008.

[25] M.D. Hitchings, P. Townsend, E. Pohl, P.D. Facey, D.H. Jones, P.J. Dyson, et al., A tale of tails: deciphering the contribution of terminal tails to the biochemical properties of two Dps proteins from Streptomyces coelicolor, Cell. Mol. Life Sci. (2014) 1–16. doi:10.1007/s00018-014-1658-4.

[26] F.H. Niesen, H. Berglund, M. Vedadi, The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability, Nat. Protoc. 2 (2007) 2212–2221. doi:10.1038/nprot.2007.321.

[27] C. Louis-Jeune, M.A. Andrade-Navarro, C. Perez-Iratxeta, Prediction of protein secondary structure from circular dichroism using theoretically derived spectra, Proteins Struct. Funct. Bioinforma. 80 (2012) 374–381. doi:10.1002/prot.23188.

[28] E.A. Gaucher, S. Govindarajan, O.K. Ganesh, Palaeotemperature trend for Precambrian life inferred from resurrected proteins, Nature. 451 (2008) 704–707. doi:10.1038/nature06510.

[29] S. Akanuma, Y. Nakajima, S. Yokobori, M. Kimura, N. Nemoto, T. Mase, et al., Experimental evidence for the thermophilicity of ancestral life, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 11067–11072. doi:10.1073/pnas.1308215110.

[30] L.F. Huergo, G. Chandra, M. Merrick, PII signal transduction proteins: nitrogen regulation and beyond, FEMS Microbiol. Rev. 37 (2013) 251–283. doi:10.1111/j.1574-6976.2012.00351.x.

Table and Figures

Table 1. Purification of the CjDps protein

Purification step	Protein (mg/mL)	[b] Total protein (mg)	[c] Purity (%)	Protein yield (mg)	[a] Yield (%)
Soluble fraction	8	308	29	90	100
Soluble fraction*	4	144	48	69	77
QFF	2	32	73	24	34
Sephacryl S200	1	14	95	13	54

*Soluble fraction after thermal treatment at 80°C. [a]Yield% was calculated as the ratio of the amount of purified protein achieved and the amount present in the previous step. [b]Total protein was measured by Bradford. [c]Purity was determined by densitometric analyses of samples loaded on 15% SDS-PAGE and stained with Comassis Blue.

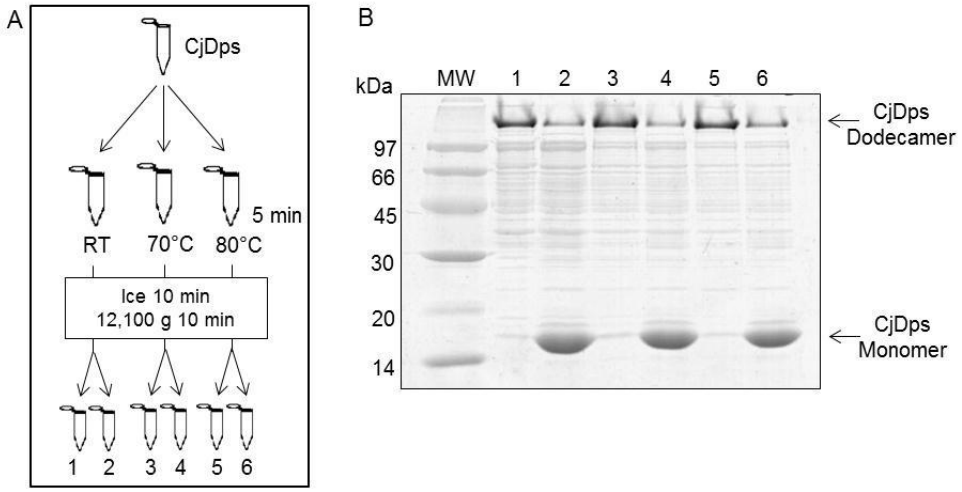

Figure 1. Investigation the thermal stability the CjDps protein. (A) Schematic view of the thermal stability assay. (B) 15% SDS-PAGE Comassie Blue stained showing the CjDps monomeric and dodecameric form. Three different thermal treatments were tested. Lanes 1 and 2 were kept at room temperature for 5 min, lanes 3 and 4 were heated at 70°C for 5 min and lanes 5 and 6 were heated at 80°C for 5 min (see panel A). After thermal treatment, samples were kept on ice for 10 minutes, centrifuged and separated into two aliquots in tubes containing SDS-PAGE loading buffer. One aliquot was boiled at 100°C for 7 min to visualize CjDps monomer (lanes 2, 4 and 6) and the other aliquot was not heated to visualize CjDps dodecamer (lanes 1, 3 and 5). Each lane contains 2.8 µM CjDps.

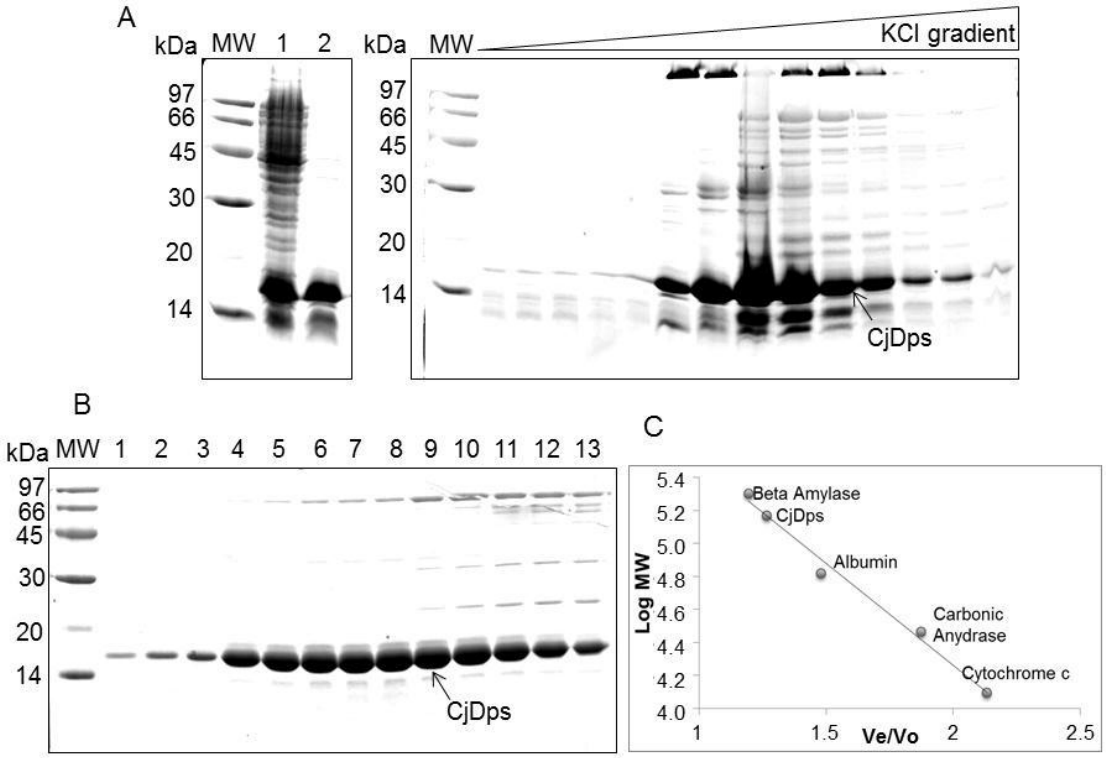

Figure 2. Purification of the *Campylobacter jeuni* Dps protein. (A) Protein purification using the thermal treatment at 80°C for 15 min and Hi-Trap Q FF. Lane 1 contains soluble fraction after lysis and lane 2 contains soluble fraction after thermal treatment. The protein was eluted with KCl linear gradient. The samples were visualized on 15% SDS-PAGE gel Comassie Blue stained. (B) Purification using gel filtration. The fractions with higher purity in Hi-Trap Q FF purification were pooled and loaded on Sephacryl S200 26/60. The samples were visualized on 15% SDS-PAGE gel Comassie Blue stained. (C) Gel filtration on Sephacryl S200 26/20 column was calibrated with a range of molecular mass standards. The log of MW vs the ratio between the elution volume (Ve) and the void volume (Vo) are indicated in the graphic. CjDps was eluted as homogeneous peak corresponding to a molecular weight of ~150 kDa.

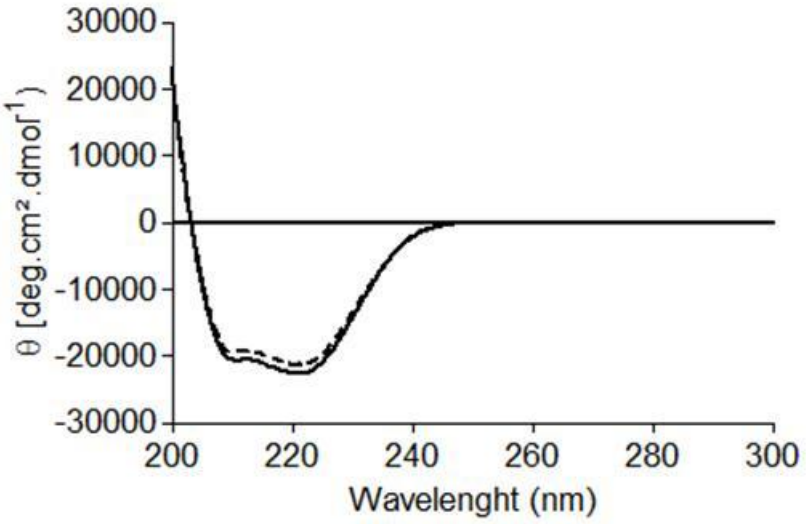

Figure 3. Comparison of the CD spectrum of CjDps purified with or without the thermal treatment. The CD spectrum shows two minima of similar magnitude at 222 nm and 208 nm. This spectrum profile is typical of proteins that contain alpha-helix. Dashed line, CjDps without thermal treatment; solid line, CjDps purified with thermal treatment.

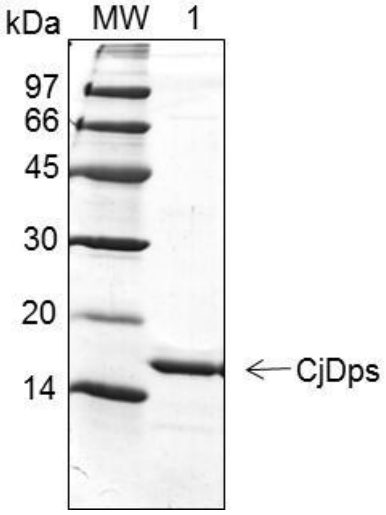

Figure 4. Final result of the purification of CjDps. Lane 1 contains one microgram of the purified CjDps. MW corresponds to molecular weight markers. The samples loaded on a 15% SDS-PAGE, the was Comassie Blue stained.

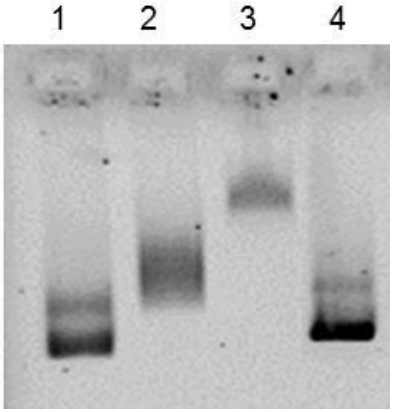

Figure 5. DNA-binding activity of CjDps. DNA-binding activity of CjDps purified with thermal treatment was evaluated by the capacity to retard the migration of DNA in 0.7% agarose gel electrophoresis. Each lane contains 200 ng circular DNA (pET29a) and 40 mM BisTris buffer pH 6.0. Lane 1, 2 and 3 contain, respectively, 0.12, 0.60 and 2.4 µM CjDps purified with thermal treatment. Lane 4 is a control and contains only DNA.

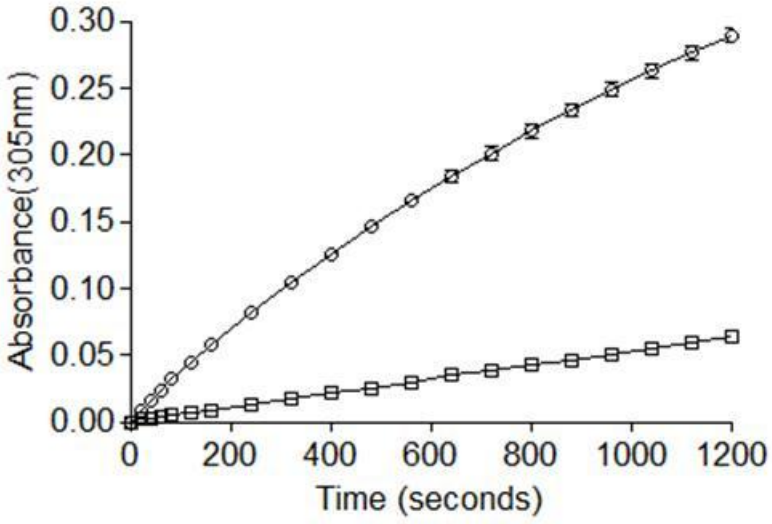

Figure 6. Spectrophotometric kinetic curve of Fe^{+2} oxidation by CjDps. Reactions were performed under aerobic conditions in 50 mM Tris-HCl (pH 6.8) at room temperature. The formation of iron core was monitored by measure absorbance at 305 nm. Reactions started by addition of 500 µM $FeSO_4$. Circles: experiment in the presence of 0.35 µM CjDps. Squares: control experiment without CjDps showing non-catalyzed iron oxidation.

Supplementary Material

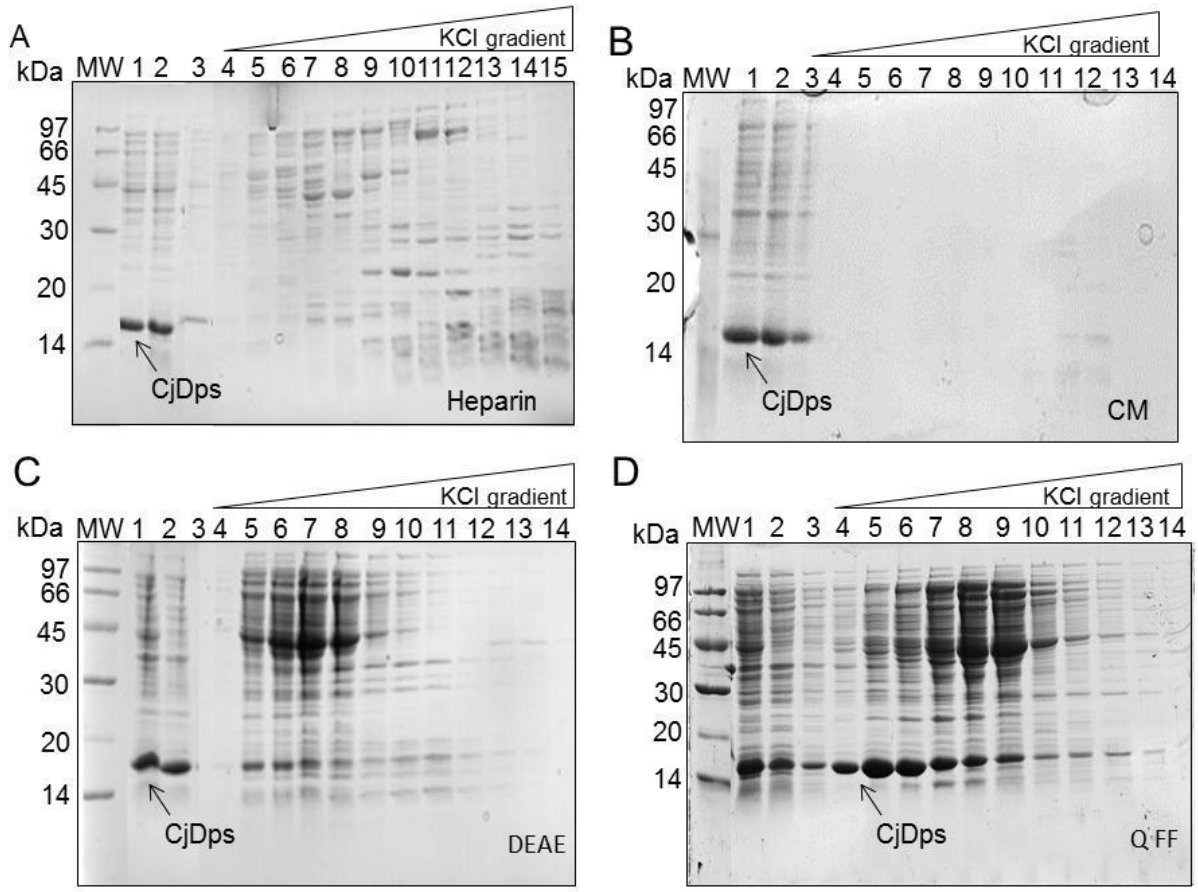

Figure S1. Binding of CjDps to different matrices. (A) Protein purification profile using 1 ml Heparin column. (B) Protein purification profile using 1 ml DEAE column. (C) Protein purification profile using 1 ml DEAE column. (D) Protein purification profile using 1ml Q FF column. MW, molecular weight marker. Lanes 1, soluble fractions after cell disruption. Lanes 2, flow through fractions after column injection. Lanes 3, washed fractions. Panel A: Lanes 4-15, eluted fractions with KCl gradient. Panels B, C and D: Lanes 4-14, eluted fractions with KCl gradient. All fractions were analyzed by 15% SDS-PAGE Comassie blue stained.

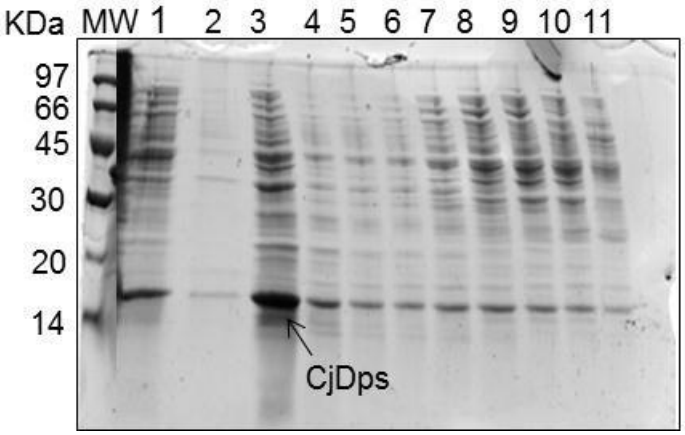

Figure S2. Binding of Cjdps to a 1 mL Hi-Trap Phenyl FF column. The cell extract was precipitated with 30% of ammonium sulfate (w/v), the soluble fraction was loaded onto a Hi-Trap Phenyl FF column. MW, molecular weight marker. Lane 1, soluble fraction after ammonium sulfate. Lane 2, flow through fraction. Lane 3, washed fraction. Lane 4-11, eluted fractions with linear decreasing gradient of ammonium sulfate. All fractions were analyzed by 15% SDS-PAGE Comassie blue stained.

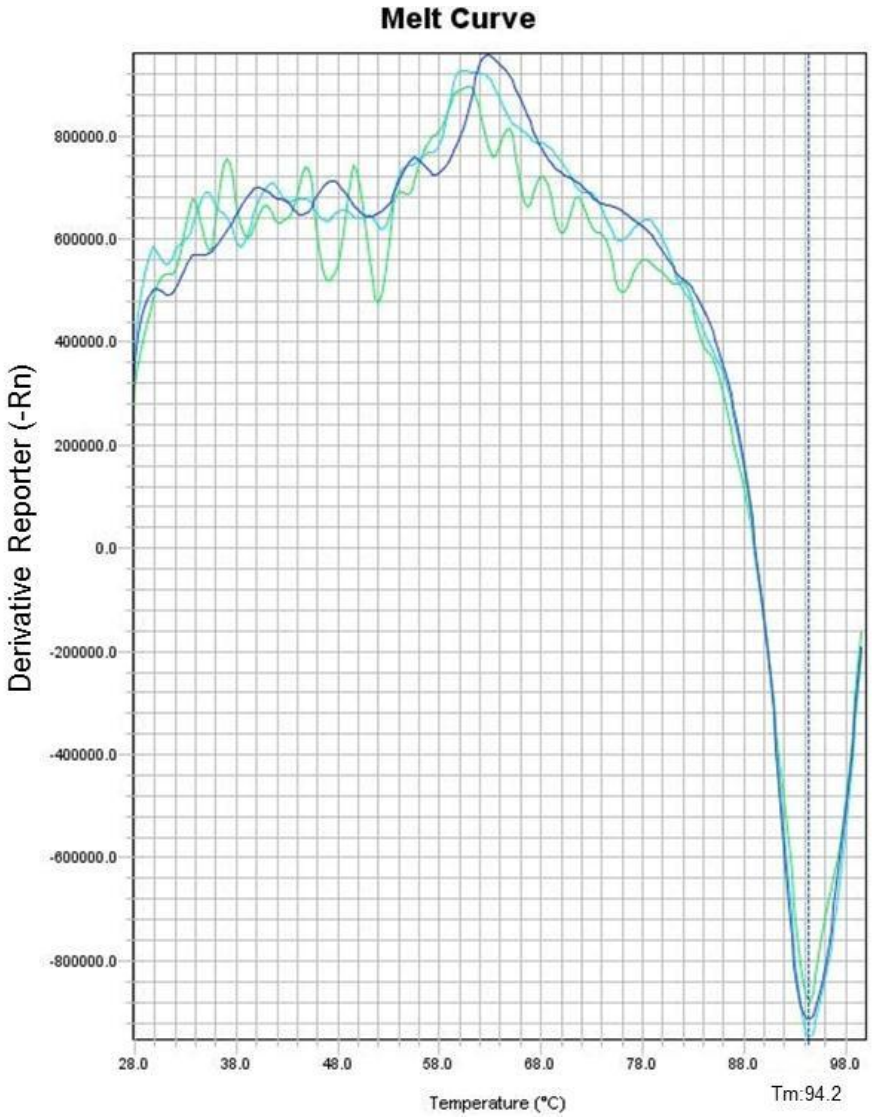

Figure S3. Melt profiles of CjDps using Sypro-Orange. The maximum of derivatives curves determines the melting value. The melting temperature estimated for CjDps protein is 94.2°C ± 0.4. Data were recorded at 0.3ºC intervals from 25 to 99.9ºC. The data is representative of two independent experiments performed in triplicate (technical replicates are indicated by the green, light blue and dark blue lines).

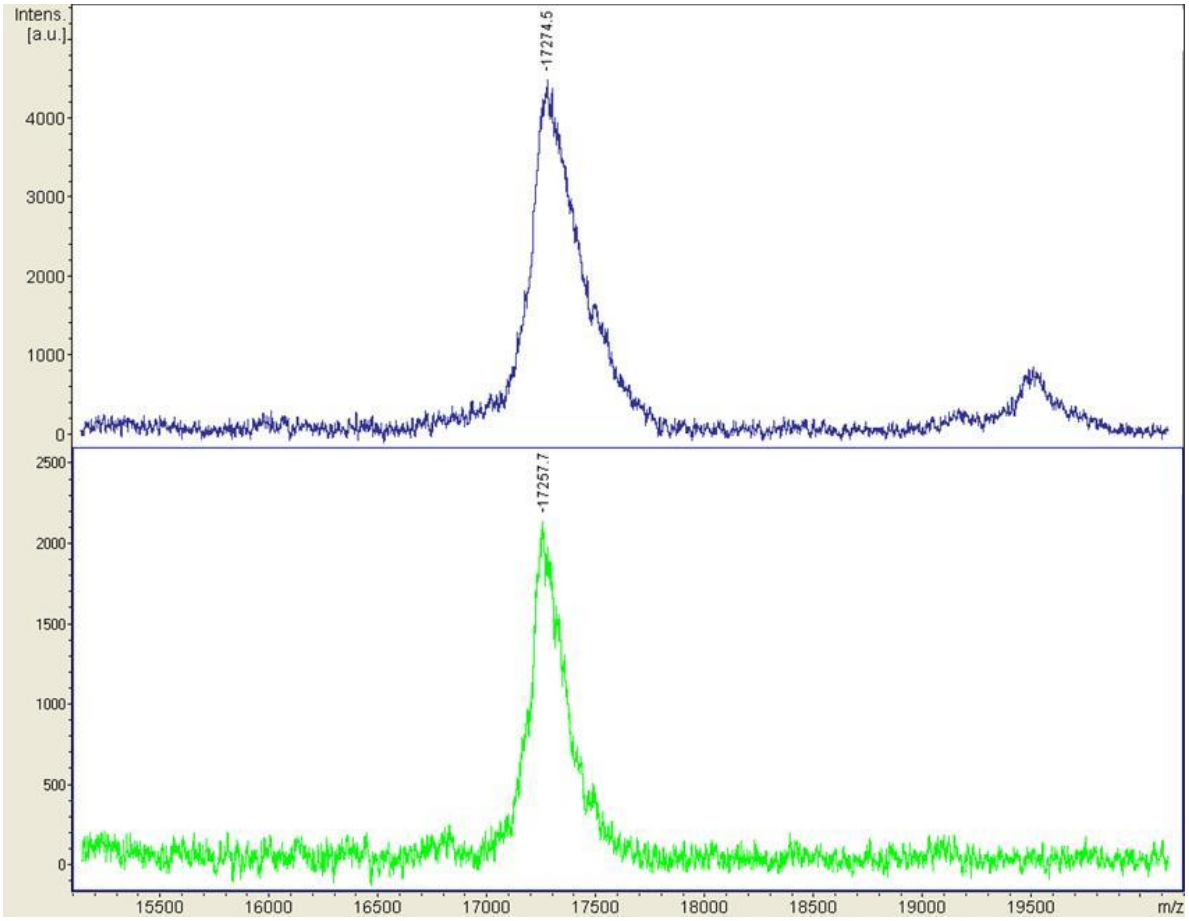

Figure S4. MALDI-TOF mass spectrometry analysis of CjDps purified with (blue) or without (green) the thermal treatment. Purified CjDps was mixed at 1:10 ratio with a saturated solution of the MALDI matrix HCCA dissolved in acetonitrile 50% (v/v) and trifluoracetic acid 2.5% (v/v). Samples were placed on a MALDI target plate and allowed to dry. Mass spectra were acquired using a MALDI-TOF autoflex (Bruker Daltonics) operating in a linear positive mode. The calculated mass for the CjDps monomer is 17.2 KDa. The error of this analysis is ± 12 Da. Hence, the mass of both samples are similar.

4 CAPÍTULO II

Manuscrito ainda não submetido.

Conserved histidine residues at the ferroxidase center of the *Campylobacter jejuni* Dps protein are required for full iron oxidation and DNA binding activities

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Running title: *C. jejuni* Dps DNA interaction, ferroxidase center

Abstract

Iron is an essential micronutrient for a vast range of microorganism participating in a variety of important physiological processes. However, under physiological conditions, iron could be potentially toxic generating hydroxyl radical through Fenton reaction. Dps (DNA-binding protein from starved cells) is a subfamily of ferritins and can store approximately 500 iron atoms per dodecamer. The ferroxidase center is a signature of this protein family and is composed by highly conserved residues. In this study, we analyzed the role of two conserved histidine residues (H25 and H37) located at the ferroxidase center of the *Campylobacter jejuni* Dps protein. The *C. jejuni* H25G/H37G variant showed reduced iron binding and ferroxidase activities in comparison to the wild-type Dps (CjDps wt). We also noted that both CjDps wt and CjDps H25G/H37G were able to bind manganese atoms. The ability of CjDps to interact with DNA was analyzed by electrophoresis DNA shift assays. While CjDps wt was able to shift DNA migration, the H25G/H37G variant failed to do so. These results support that the H25 and H37 residues at the ferroxidase center of *C. jejuni* Dps are not completely required for metal binding and oxidation; nonetheless, they might play a role in DNA binding.

Keywords: Dps protein, ferroxidase center, DNA binding

Introduction

Campylobacter jejuni is the most frequent cause of human gastroenteritis worldwide. After ingestion of contaminated food or water, an incubation period of 24-72 h is followed by symptoms as fever, abdominal cramps, diarrhea and headache [1–3]. Infection by this organism is the most common antecedent to Guillain-Barré syndrome that causes acute demyelinating of the peripheral nervous systems [4]. Pathogenic organisms such as *C. jejuni* need to developed strategies to counteract the various environments stress found during colonization, such extreme pHs and low availability of iron [5].

Iron is an essential micronutrient for life, acting as cofactor for many enzymes and participating in important process such as photosynthesis, gene regulation and nitrogen fixation [6]. However, under physiological conditions, iron is potentially toxic because it can react with H_2O_2 generating highly toxic hydroxyl radical through Fenton reaction that may damage DNA, fatty acids and proteins [7,8]. Hence, aerobic organisms developed a variety of strategies to store iron in non-reactive forms.

Dps proteins are a subfamily of ferritins composed by 12 identical subunits that assembled into a spherical protein shell (Fig. 1A) which is able to store approximately 500 iron atoms. Each Dps subunit is formed by a compact four-helix bundle with molecular mass of ~20 kDa. The ferroxidase center (FOC) located at the two fold interface between subunits is the most remarkable signature of Dps [9]. The FOC is conserved among the members of this family including *C. jejuni* Dps, in which the conserved aminoacids residues are His 25, Trp 26, His 37, Asp 52 and Glu 56 [10,11] (Fig. 1B and 1C). It is well accepted that the Dps FOC contains two metal binding sites with different affinity for Fe^{2+} ions [12]. Subsequently to Fe^{2+} binding, Fe^{2+} is oxidized to Fe^{3+} using H_2O_2 or O_2 as oxidant. The Fe^{3+} ions are then stored inside the protein cavity in a non-reactive iron-oxide-form Fe_2O_3 [13]. Most Dps members described to date preferentially use H_2O_2 over O_2 [14]. In *Escherichia coli* Dps the rate reaction increased 100-fold with H_2O_2 [15]. In addition to Fe^{2+}, some Dps members are able to bind other metal ions [16,17]. The *Streptococcus suis* Dps can bind Cu^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+} to the FOC as determined by isothermal titration calorimetry (ITC) and X-ray crystallography [18]. Furthermore, *Kineococcus radiotolerans* Dps can form a Mn-Fe heteronuclear metal cluster at the FOC, however, only iron but not manganese can be stored inside the protein shell [19].

Dps proteins are widely distributed in prokaryotes [20], being required for survival during stressful conditions such as: nutrient starvation, thermal stress, oxidative conditions and inside biofilm [21–26]. The archetypical function of Dps seems to be DNA protection against hydroxyl radicals that are produced when Fe^{2+} and H_2O_2 are combined [15]. DNA protection occurs indirectly via iron oxidation and storage, furthermore, some Dps members can physically protect DNA through the formation of Dps-DNA complexes that are independent of the DNA sequence [12].

Some non-conserved Dps structural motifs have been implicated in DNA binding activity such as: a lysine rich N-terminus in *E. coli* Dps [27]; a positive charged C-terminus in *Mycobacterium smegmatis* [28]; or a positively charged protein surface in *H. pylori* [29]. The *C. jejuni* Dps is able to bind DNA, and the DNA binding activity is apparently stimulated by Fe^{2+} or H_2O_2 [30]. In this study, we analyzed how the integrity of the FOC affected the ferroxidase and DNA binding activities of *C. jejuni* Dps.

Material and Methods

Site-direct mutagenesis and cloning

A synthetic condon-optimized gene encoding *C. jejuni* Dps carrying the H25G and H37G substitutions was purchased from Integrated DNA Technologies (IDT) and cloned into pIDTsmartKm. The synthetic gene was subcloned into the pET29a expression vector using the *Nde*I-*Bam*HI restriction sites to generating the pLHPETcjH25G-H37G plasmid. The insert of the final construct was verified by DNA sequencing.

Expression and purification of CjDps wt and CjDps H25G/H37G

E. coli BL21 (λDE3) carrying the expression plasmid of interest (Table 1) were cultivated on 800 mL LB at 37°C until $O.D._{600\ nm}$ of 0.7 and, after that, 0.3 mM of IPTG was added. After 3 h, cells were harvested by centrifugation at 6,000 g for 15 min. Cells were resuspended in 40 mM Tris-HCl pH 8.8 and lysated by sonication followed by centrifugation at 30,000 g for 30 min at 4°C. The supernatant was collected and the protein of interest was purified using a thermal treatment and two chromatographic steps as previously describe by Sanchuki *et al* (2015, in press)[31].

Protein quantification

Total protein concentration was determined using Bradford reagent (SIGMA) according to the manufacturer's supplied protocol. Bovine serum albumin (BSA) was used as standard.

Mass spectrometry analysis

Mass spectrometry analysis was performed as describe [32]. Proteins bands excised from Comassie-stained SDS-PAGE gels were subjected to in gel digestion with trypsin. The trypsinized mixture was mixed at 1:1 ratio (v/v) with saturated solution of the MALDI matrix α-cyano-4-hydroxycinnamic acid (HCCA) dissolved in acetonitrile 50% (v/v) and trifluoracetic acid 2.5% (v/v). Samples were spotted onto the MALDI target and allowed to dry. Mass spectra were acquired using a MALDI-TOF/TOF Autoflex II spectrometer (Bruker

Daltonics, Bremen, Germany). Database search was performed using a Mascot 2.3 server. Mass list was searched against a database of *C. jejuni*.

Staining for iron-binding proteins

Staining for iron-binding proteins was performed as previously describe [33]. 100 µg of CjDps were incubated with freshly prepared 1 mM $FeSO_4$ solution for 1 hour at room temperature. Subsequently, sample buffer (10% glycerol, 0.01% bromophenol blue, 62.5 mM Tris-HCl pH 6.8) was added to the protein samples and the mixtures were loaded on 10% native polyacrimamide gel electrophoresis for 3 hours. The gel was stained for 10 min (in the dark) with potassium ferricyanide and then sunk into a freshly prepared solution of 10% methanol (v/v) and 10% trichloroacetic acid (w/v). When iron is present, a blue band is visualized. After iron staining, the same gel was stained with Comassie brilliant blue. BSA and commercial horse spleen ferritin were used as negative and positive controls, respectively.

Atomic Absorption Spectroscopy

The iron content of CjDps protein was determined by atomic absorption spectroscopy. These assays were performed by Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco following the official Mexican regulation NOM-117-SSA1-1994.

Iron uptake experiment

Purified CjDps preparation were diluted to 0.35 µM (dodecamer concentration) in 500 µL of 50 mM Tris-HCl (pH 6.8). Iron incorporation experiments were performed using freshly prepared $FeSO_4$ at final concentration of 500 µM. Iron oxidation was monitored spectrophotometrically at 305 nm for 20 min under aerobic condition. As control, reactions were performed without the addition of the CjDps protein. Experiments were performed in presence of H_2O_2 100 µM when indicated.

Tryptophan fluorescence quenching

Experiments were performed at 25°C using a JASCO J-815 equipment (JAPAN Spectroscopy & Chromatography Technology). Proteins were diluted to 1.9 µM (dodecamer

concentration) in 50 mM BisTris pH 7. Tryptophan residues were excited at 295 nm and the emission spectrum was recorded from 310 to 370 nm. The maximal emission at 340 nm was used to fit the metal binding curves. The experimental data for iron and manganese binding was fitted to the one-site specific binding model. Each experiment was performed at least twice in triplicates. Data was analyzed using GraphPad Prism 5.0.

EPR spectroscopy

The Electron Paramagnetic Resonance experiments were carried out using a Bruker Elexsys E500 spectrometer equipped with a Bruker ER4123D dielectric resonator. Proteins were diluted in 40 mM TrisHCl pH 8, 0.1 M KCl. Sample were sealed in glass capillaries and analyzed at room temperature. A 1200 G field sweep was set centered at 3488 G, with microwave frequency stabilized at 9,758977 GHz. 1024 points were recorded for each spectrum, with a sweep time of 671 s, time constant of 40,96 ms under 0,6248 mW of microwave power. The modulation frequency employed was 100 kHz and the modulation amplitude was 9,62 G. The Mn^{2+} spectra were baseline corrected and the absorption was integrated to render the number of paramagnetic centers. The spectral intensities were adjusted to internal isolated Cr^{3+} doped magnesium oxide standard sample.

DNA-binding assay using agarose gel electrophoresis

DNA binding was assessed in gel shift assays using 300 ng of super coiled plasmid DNA (pET28a). Dps was incubated with DNA for 10 min at room temperature in Tris-HCl or BisTris buffer over the pH range of 6 to 8 as indicated in each experiment. Electrophoresis was carried out on 0.7% agarose gel for 20 min at 90 V using as running buffer the very same buffer applied during the incubation of the Dps-DNA mixture. When indicated, DNA-binding assay was performed in the presence of H_2O_2. DNA was stained with ethidium bromide and visualized using a UVP BioImaging system.

Results and Discussion

Previous data showed that CjDps is able to bind DNA and that this DNA binding activity is stimulated by Fe^{2+} [30]. In order to verify if the conserved residues at the Dps FOC

participate in iron oxidation and/or could play a role in DNA binding, a H25G/H37G variant of CjDps was obtained and confirmed both by DNA sequencing (data not shown) and by MALDI-TOF analysis of the trypsin digested protein preparations (Fig. S1). We opted for these substitutions as previous data using a *E. coli* Dps H51G/H63G variant showed that these substitutions did not affect the Dps secondary structure, albeit completely abrogating Fe^{2+} binding and ferroxidase activity [34]. In addition, the crystallographic structure of *Listeria innocua* Dps H31G/H43G, showed that these substitutions did not affect the protein quaternary, tertiary or secondary structure [35]. As expected, the CjDps H25G/H37G variant kept its dodecameric structure as accessed by gel filtration chromatography (data not shown).

Effects of the H25G/H37G substitution in the iron oxidation activity

In order to evaluate the ability of CjDps and its H25G/H37G variant to store iron, we firstly performed native gel electrophoresis followed by iron staining with potassium ferricyanide. This technique is specific for non-heme iron proteins, resulting in blue bands when potassium ferricyanide reacts with iron bound to proteins [33,36]. When CjDps was pre-incubated with ferrous sulfate before electrophoresis a blue band was readily observed in CjDps wt, while a faint blue band was visualized in the CjDps H25G/H37G (Fig. 2A). This result suggests that both proteins were able to accumulated iron in their inner cavity with the latter having less ability to do so.

The same analysis was performed without pre-incubation of the proteins in the presence of iron before electrophoresis, in this case, iron staining was negative (Fig. 2A), supporting that neither CjDps wt nor the H25G/H37G carries significant amount of pre-bound iron during purification. Indeed, atomic absorption spectroscopy confirmed that both proteins had no significant pre-bound iron (less than 1 atom per dodecamer).

In order to further investigate the role of the H25 and H37 residues on iron oxidation, we performed an iron uptake experiment where the formation of the iron core was monitored at 305 nm. The results are in agreement with the in gel iron staining analysis. Both CjDps wt and CjDps H25G/H37G were able to form the iron core; however, the CjDps H25G/H37G variant had a slower activity (Fig. 3). These results support that H25 and H37 residues are important but not completely required to CjDps ferroxidase activity.

The formation of the iron core was faster in the presence of hydrogen peroxide, supporting that H_2O_2 is preferred as oxidant over O_2 (Figure 3). This data is in agreement with

results reported for other Dps members [12,15]. However, it is in stark contrast with the results obtained using a N-terminal 10x his tagged form of CjDps where H_2O_2 did not stimulated the formation of iron core [30]. We suspect that these contrasting results were caused by the presence of the His tag in the previous analysis (see below).

Metal binding analysis

The quenching of the intrinsic fluorescence of tryptophan has been widely used to assess metal binding to the FOC of various Dps members [35,37,38]. Comparison of the apo and iron bound structures of *H. pylori* Dps (Nap protein) suggests that iron binding at the FOC causes a structural change switching the position of Trp26 residue by ~180^o along with the Cβ-Cγ bound [17,39]. This change in tryptophan hydrophobic surroundings could be responsible for the observed fluorescent quenching by Fe^{2+}.

The addition of increasing concentrations of Fe^{2+} proportionally quenched the intrinsic tryptophan fluorescence of CjDps wt (Fig. 4A). Strikingly, a very similar profile was observed for the CjDps H25G/H37G (Fig. 4A). It worth mentioning that these experiments were performed in the presence of oxygen and, therefore, do not allow the distinctions between the effects caused by the binding of Fe^{2+} to the FOC from the effects caused by the accumulation of Fe^{3+} inside the protein cage. Hence, these quenching curves cannot be directly interpreted as binding curves.

It has been reported that Mn^{2+} can bind to the FOC of Dps [19], which is expected as Fe^{2+} and Mn^{2+} can assume similar coordination geometries on proteins crystal structures [18,40]. We evaluated the binding of manganese to CjDps by measuring the intrinsic tryptophan fluorescence (Fig. 4B). Addition of Mn^{2+} increased the tryptophan fluorescence of CjDps wt in concentration-dependent manner and the calculated Kd was 6.9 ± 1.2 μM (Fig. 4B). Surprisingly, the CjDps H25G/H37G variant showed opposite fluorescence response to the Mn^{2+} addition. Mn^{2+} quenched the tryptophan fluorescence of CjDps H25G/H37G variant also in a concentration-dependent manner with a calculated Kd of 12.2 ± 1.0 μM (Fig. 4B).

The quenching of tryptophan fluorescence is affected by the mobility and by the interaction of the side chain with its surrounding [41,42]. We speculate that Mn^{2+} binding is somehow differently affecting the CjDps H25G/H37G conformation, exposing tryptophan residues and consequently, quenching fluorescence. Given that all the tryptophan residues in

CjDps are close to the FOC (Fig. S3), we hypothesize that manganese is binding to or near the FOC even in the H25G/H37G variant.

Electron paramagnetic resonance (EPR) spectroscopy was employed to confirm the binding of Mn^{2+} to Dps. The concentration of free Mn^{2+} solution decreased with increasing concentrations of CjDps wt indicating the formation of protein-bound manganese species (Fig. 5). A similar, although less pronounced, effect was observed using the H25/H37G variant (Fig. 5). The EPR analysis confirmed that both wt and H25/H37G CjDps can bind Mn^{+2}.

DNA-binding analysis

We next tested the ability of CjDps to interact with DNA by measuring a shift in DNA migration during agarose gel electrophoresis. It worth mentioning that in a previous study we used the very same assay to determine the DNA-binding activity of an engineered N-terminal 10x His tagged CjDps [30]. However, we noted that the untagged CjDps used in the present study showed different responses. For instance, while the DNA binding activity of His tagged Dps was stimulated by H_2O_2 [30] that of untagged CjDps was not (Fig. S2). Histidine residues can be oxidized in the presence of H_2O_2 [43], this could explain why the 10x His tagged Dps (which contains 120 extra histidine residues per dodecamer) reacted differently to H_2O_2 during both DNA-binding (Fig. S2) and iron oxidation (Fig. 3) assays.

We compared the ability of untagged CjDps wt and CjDps H25G/H37G to interact with DNA using DNA shift (Fig. 6). When a DNA-protein complex is formed it is possible to visualize a shift in DNA migration compared with free DNA. When CjDps wt was incubated with circular plasmid DNA we observed a Dps concentration-dependent retardation on the DNA migration. However, when CjDps H25G/H37G was incubated with DNA there were no changes in DNA migration (Fig. 6, lanes 5, 6 and 7). The Dps DNA-binding activity was performed in a pH range of 6 to 8 and the same response was visualized (Fig.6 A, B and C). This data suggests that the H25 and H37 residues may somehow be required for full DNA binding activity.

Conclusions

In this study, we found that CjDps was able to bind iron and manganese and that iron oxidation was enhanced when hydrogen peroxide was used as oxidant. The residues H25 and

H37 are not completely required to Dps ferroxidase activity and these residues could be related with the inability of the CjDps H25G/H37G binds DNA under the conditions tested.

Acknowledgments

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References

[1] S.F. Altekruse, N.J. Stern, P.I. Fields, D.L. Swerdlow, Campylobacter jejuni--an emerging foodborne pathogen., Emerg. Infect. Dis. 5 (1999) 28–35.

[2] B.M. Allos, Campylobacter jejuni Infections: update on emerging issues and trends, Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 32 (2001) 1201–1206. doi:10.1086/319760.

[3] M.J. Blaser, Epidemiologic and Clinical Features of *Campylobacter jejuni* Infections, J. Infect. Dis. 176 (1997) S103–S105. doi:10.1086/513780.

[4] K.K. Nyati, R. Nyati, Role of Campylobacter jejuni Infection in the Pathogenesis of Guillain-Barré Syndrome: An Update, BioMed Res. Int. 2013 (2013). doi:10.1155/2013/852195.

[5] C.M. Litwin, S.B. Calderwood, Role of iron in regulation of virulence genes., Clin. Microbiol. Rev. 6 (1993) 137–149. doi:10.1128/CMR.6.2.137.

[6] M. Ilbert, V. Bonnefoy, Insight into the evolution of the iron oxidation pathways, Biochim. Biophys. Acta BBA - Bioenerg. 1827 (2013) 161–175. doi:10.1016/j.bbabio.2012.10.001.

[7] D. Touati, Iron and oxidative stress in bacteria, Arch. Biochem. Biophys. 373 (2000) 1–6. doi:10.1006/abbi.1999.1518.

[8] S.C. Andrews, A.K. Robinson, F. Rodríguez-Quiñones, Bacterial iron homeostasis, FEMS Microbiol. Rev. 27 (2003) 215–237.

[9] T. Haikarainen, A.C. Papageorgiou, Dps-like proteins: structural and functional insights into a versatile protein family, Cell. Mol. Life Sci. CMLS. 67 (2010) 341–351. doi:10.1007/s00018-009-0168-2.

[10] A. Ilari, S. Stefanini, E. Chiancone, D. Tsernoglou, The dodecameric ferritin from Listeria innocua contains a novel intersubunit iron-binding site, Nat. Struct. Biol. 7 (2000) 38–43. doi:10.1038/71236.

[11] T. Ishikawa, Y. Mizunoe, S. Kawabata, A. Takade, M. Harada, S.N. Wai, et al., The Iron-Binding Protein Dps Confers Hydrogen Peroxide Stress Resistance to Campylobacter jejuni, J. Bacteriol. 185 (2003) 1010–1017. doi:10.1128/JB.185.3.1010-1017.2003.

[12] S. Franceschini, P. Ceci, F. Alaleona, E. Chiancone, A. Ilari, Antioxidant Dps protein from the thermophilic cyanobacterium Thermosynechococcus elongatus, FEBS J. 273 (2006) 4913–4928. doi:10.1111/j.1742-4658.2006.05490.x.

[13] F. Bou-Abdallah, The iron redox and hydrolysis chemistry of the ferritins, Biochim. Biophys. Acta BBA - Gen. Subj. 1800 (2010) 719–731. doi:10.1016/j.bbagen.2010.03.021.

[14] E. Chiancone, P. Ceci, The multifaceted capacity of Dps proteins to combat bacterial stress conditions: Detoxification of iron and hydrogen peroxide and DNA binding, Biochim. Biophys. Acta. 1800 (2010) 798–805. doi:10.1016/j.bbagen.2010.01.013.

[15] G. Zhao, P. Ceci, A. Ilari, L. Giangiacomo, T.M. Laue, E. Chiancone, et al., Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of Escherichia coli, J. Biol. Chem. 277 (2002) 27689–27696. doi:10.1074/jbc.M202094200.

[16] H. Havukainen, S. Haataja, A. Kauko, A.T. Pulliainen, A. Salminen, T. Haikarainen, et al., Structural basis of the zinc- and terbium-mediated inhibition of ferroxidase activity in Dps ferritin-like proteins, Protein Sci. Publ. Protein Soc. 17 (2008) 1513–1521. doi:10.1110/ps.036236.108.

[17] H. Yokoyama, S. Fujii, Structures and Metal-Binding Properties of Helicobacter pylori Neutrophil-Activating Protein with a Di-Nuclear Ferroxidase Center, Biomolecules. 4 (2014) 600–615. doi:10.3390/biom4030600.

[18] T. Haikarainen, A. Thanassoulas, P. Stavros, G. Nounesis, S. Haataja, A.C. Papageorgiou, Structural and thermodynamic characterization of metal ion binding in Streptococcus suis Dpr, J. Mol. Biol. 405 (2011) 448–460. doi:10.1016/j.jmb.2010.10.058.

[19] M. Ardini, A. Fiorillo, M. Fittipaldi, S. Stefanini, D. Gatteschi, A. Ilari, et al., Kineococcus radiotolerans Dps forms a heteronuclear Mn–Fe ferroxidase center that may explain the Mn-dependent protection against oxidative stress, Biochim. Biophys. Acta BBA - Gen. Subj. 1830 (2013) 3745–3755. doi:10.1016/j.bbagen.2013.02.003.

[20] E. Chiancone, P. Ceci, The multifaceted capacity of Dps proteins to combat bacterial stress conditions: Detoxification of iron and hydrogen peroxide and DNA binding, Biochim. Biophys. Acta BBA - Gen. Subj. 1800 (2010) 798–805. doi:10.1016/j.bbagen.2010.01.013.

[21] S. Nair, S.E. Finkel, Dps Protects Cells against Multiple Stresses during Stationary Phase, J. Bacteriol. 186 (2004) 4192–4198. doi:10.1128/JB.186.13.4192-4198.2004.

[22] B. Pang, W. Hong, N.D. Kock, W.E. Swords, Dps promotes survival of nontypeable Haemophilus influenzae in biofilm communities in vitro and resistance to clearance in vivo, Front. Cell. Infect. Microbiol. 2 (2012). doi:10.3389/fcimb.2012.00058.

[23] J.R. Theoret, K.K. Cooper, R.D. Glock, L.A. Joens, A Campylobacter jejuni Dps Homolog Has a Role in Intracellular Survival and in the Development of Campylobacterosis in Neonate Piglets, Foodborne Pathog. Dis. 8 (2011) 1263–1268. doi:10.1089/fpd.2011.0892.

[24] M. Almirón, A.J. Link, D. Furlong, R. Kolter, A novel DNA-binding protein with regulatory and protective roles in starved Escherichia coli., Genes Dev. 6 (1992) 2646–2654. doi:10.1101/gad.6.12b.2646.

[25] A. Martinez, R. Kolter, Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps, J. Bacteriol. 179 (1997) 5188–5194.

[26] W.G. Dundon, H. Nishioka, A. Polenghi, E. Papinutto, G. Zanotti, P. Montemurro, et al., The neutrophil-activating protein of Helicobacter pylori, Int. J. Med. Microbiol. IJMM. 291 (2002) 545–550.

[27] P. Ceci, S. Cellai, E. Falvo, C. Rivetti, G.L. Rossi, E. Chiancone, DNA condensation and self-aggregation of Escherichia coli Dps are coupled phenomena related to the properties of the N-terminus, Nucleic Acids Res. 32 (2004) 5935–5944. doi:10.1093/nar/gkh915.

[28] S. Roy, R. Saraswathi, S. Gupta, K. Sekar, D. Chatterji, M. Vijayan, Role of N and C-terminal Tails in DNA Binding and Assembly in Dps: Structural Studies of Mycobacterium smegmatis Dps Deletion Mutants, J. Mol. Biol. 370 (2007) 752–767. doi:10.1016/j.jmb.2007.05.004.

[29] P. Ceci, L. Mangiarotti, C. Rivetti, E. Chiancone, The neutrophil-activating Dps protein of Helicobacter pylori, HP-NAP, adopts a mechanism different from Escherichia coli Dps to bind and condense DNA, Nucleic Acids Res. 35 (2007) 2247–2256. doi:10.1093/nar/gkm077.

[30] L.F. Huergo, H. Rahman, A. Ibrahimovic, C.J. Day, V. Korolik, Campylobacter jejuni Dps Protein Binds DNA in the Presence of Iron or Hydrogen Peroxide, J. Bacteriol. 195 (2013) 1970–1978. doi:10.1128/JB.00059-13.

[31] H.B.S. Sanchuki, G. Valdameri, V.R. Moure, M.A. Oliveia, F.O. Pedrosa, E.M. Souza, et al., Purification of the Campylobacter jejuni Dps protein assisted by its high melting temperature, Protein Expr. Purif. (n.d.). doi:10.1016/j.pep.2014.12.011.

[32] L.F. Huergo, L. Noindorf, C. Gimenes, R.S.P. Lemgruber, D.F. Cordellini, L.J. Falarz, et al., Proteomic analysis of Herbaspirillum seropedicae reveals ammonium-induced AmtB-dependent membrane sequestration of PII proteins, FEMS Microbiol. Lett. 308 (2010) 40–47. doi:10.1111/j.1574-6968.2010.01986.x.

[33] L.M. Leong, B.H. Tan, K.K. Ho, A specific stain for the detection of nonheme iron proteins in polyacrylamide gels, Anal. Biochem. 207 (1992) 317–320.

[34] A.R. Arnold, J.K. Barton, DNA protection by the bacterial ferritin Dps via DNA charge transport, J. Am. Chem. Soc. 135 (2013) 15726–15729. doi:10.1021/ja408760w.

[35] A. Ilari, M.C. Latella, P. Ceci, F. Ribacchi, M. Su, L. Giangiacomo, et al., The unusual intersubunit ferroxidase center of Listeria innocua Dps is required for hydrogen peroxide detoxification but not for iron uptake. A study with site-specific mutants, Biochemistry (Mosc.). 44 (2005) 5579–5587. doi:10.1021/bi050005e.

[36] D.M. Lawson, A. Treffry, P.J. Artymiuk, P.M. Harrison, S.J. Yewdall, A. Luzzago, et al., Identification of the ferroxidase centre in ferritin, FEBS Lett. 254 (1989) 207–210.

[37] X. Wei, H. Mingjia, L. Xiufeng, G. Yang, W. Qingyu, Identification and biochemical properties of Dps (starvation-induced DNA binding protein) from cyanobacterium Anabaena sp. PCC 7120, IUBMB Life. 59 (2007) 675–681. doi:10.1080/15216540701606926.

[38] M. Su, S. Cavallo, S. Stefanini, E. Chiancone, N.D. Chasteen, The so-called Listeria innocua ferritin is a Dps protein. Iron incorporation, detoxification, and DNA protection properties, Biochemistry (Mosc.). 44 (2005) 5572–5578. doi:10.1021/bi0472705.

[39] O. Tsuruta, H. Yokoyama, S. Fujii, A new crystal lattice structure of Helicobacter pylori neutrophil-activating protein (HP-NAP), Acta Crystallogr. Sect. F. 68 (2012) 134–140. doi:10.1107/S1744309111052675.

[40] H. Zheng, M. Chruszcz, P. Lasota, L. Lebioda, W. Minor, Data mining of metal ion environments present in protein structures, J. Inorg. Biochem. 102 (2008) 1765–1776. doi:10.1016/j.jinorgbio.2008.05.006.

[41] A.B.T. Ghisaidoobe, S.J. Chung, Intrinsic Tryptophan Fluorescence in the Detection and Analysis of Proteins: A Focus on Förster Resonance Energy Transfer Techniques, Int. J. Mol. Sci. 15 (2014) 22518–22538. doi:10.3390/ijms151222518.

[42] J.T. Vivian, P.R. Callis, Mechanisms of Tryptophan Fluorescence Shifts in Proteins, Biophys. J. 80 (2001) 2093–2109. doi:10.1016/S0006-3495(01)76183-8.

[43] J.-W. Lee, J.D. Helmann, The PerR transcription factor senses H_2O_2 by metal-catalysed histidine oxidation, Nature. 440 (2006) 363–367. doi:10.1038/nature04537.

Table and figures

Table 1. Strain and plasmids used in this study

Strain or plasmid	Genotype	Reference
Strain		
BL21(ʎDE3)	Expresses T7 RNA polymerase	Agillent
Plasmids		
pET28a	Expression Vector/ T7 promoter	Novagen
pLHPT71534c	Amp^r (pT7-7) expresses *C. jejuni* Dps wt	Sanchuki *et al* (in press, 2015)
pLHPETcjH25G-H37G	Km^r (pET29a) express *C. jejuni* Dps H25G/H37G	This work

Figures

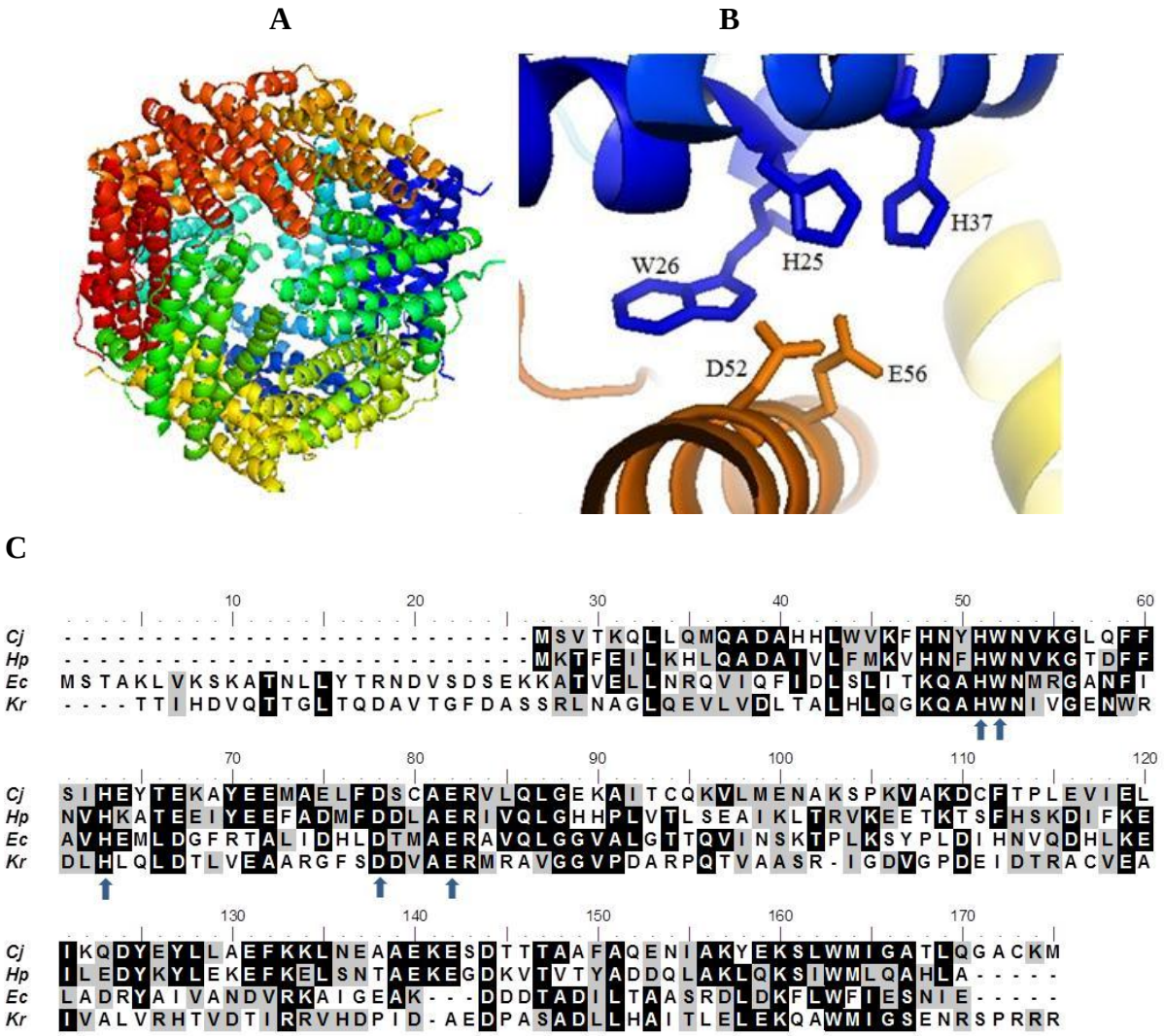

Figure 1. Structure of *C. jejuni* Dps proteins and conserved residues at ferroxidase center. (A) Structure of *C. jejuni* Dps protein dodecamer (PDB 3KWO) (B) Location of the conserved residues at FOC (PDB 3KWO) (C) CulstalW alignament of *C. jejuni* Dps (Cj) with Dps from *Helicobacter pylori* (Hp), *Escherichia coli* (Ec) and *Kineococcus radiotolerans* (Kr). Conserved residues at ferroxidase center are indicated by arrows. Figures were generated using Pymol.

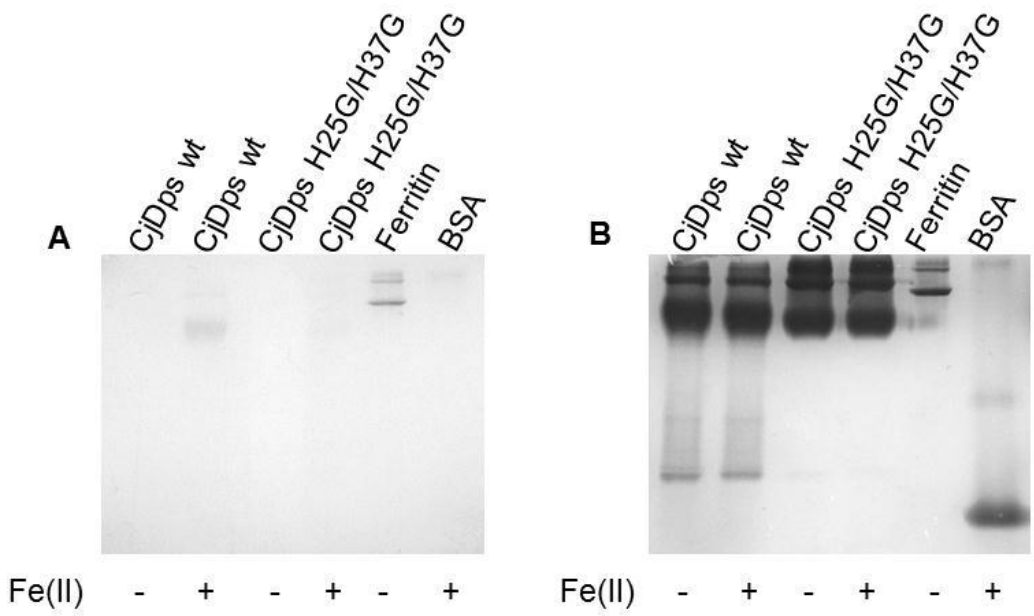

Figure 2. Native polyacrylamide gel electrophoresis analysis of CjDps. (A) Potassium ferricyanide staining for protein iron bound. (B) Coomassie brilliant blue staining for total proteins. A and B represents the same gel first stained with potassium ferricyanide and later with Comassie. Proteins were loaded on sequence: 100 µg Dps, 10 µg commercial Ferritin and 20 µg BSA. When indicated (+), the proteins were pre-incubated with 1 mM $FeSO_4$ for 1 hour.

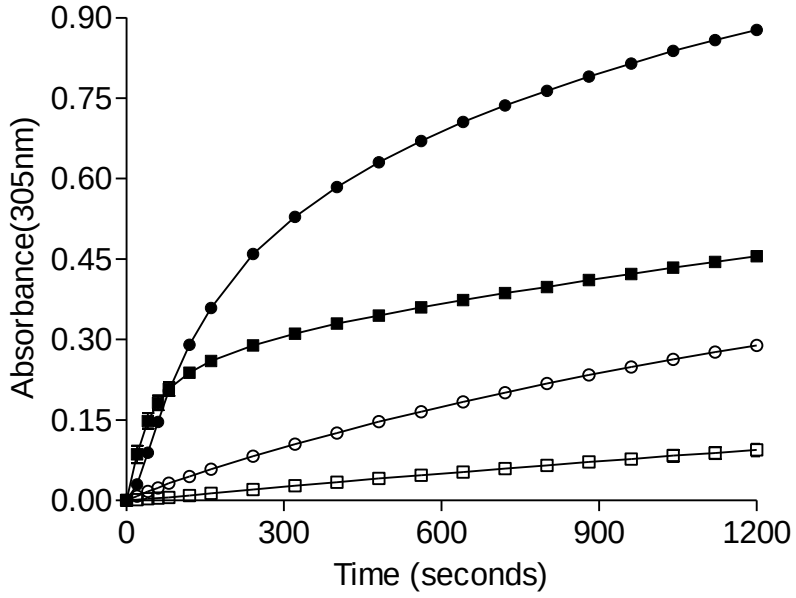

Figure 3. Comparison of the ferroxidation activity between CjDps wt and CjDps H25G/H37G. Spectrophotometric kinetic curve of Fe^{+2} oxidation by CjDps. Reactions were incubated aerobically in 50 mM Tris-HCl pH 6.8 at room temperature and were initiated by the addition of 500 µM $FeSO_4$. The formation of the iron core was monitored by measuring absorbance at 305 nm. White squares, 0.35 µM CjDps H25H/H37G; white circles, 0.35 µM CjDps wt; black squares, 0.35 µM CjDps H25H/H37G + 100 µM H_2O_2; black circles, CjDps wt + 100 µM H_2O_2.

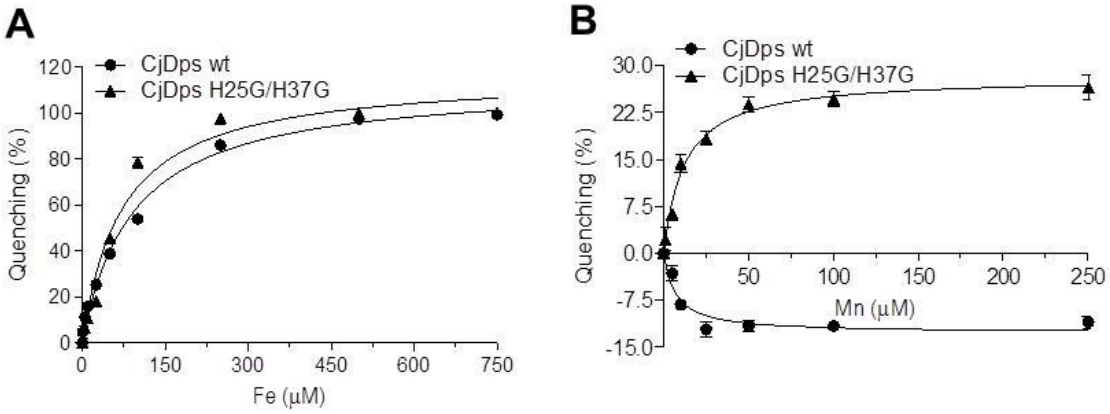

Figure 4. Interaction between Dps and metal accessed by fluorescence quenching. (A) Fluorescence quenching upon increasing Fe^{+2} concentrations to CjDps wt and CjDps H25G/H37G. (B) Fluorescence quenching upon increasing Mn^{+2} concentrations to CjDps wt and CjDps H25G/H37G Conditions were as follows: λ_{ex} = 295nm, λ_{em} = 340nm, 2.0 μM CjDps wt or H25G/H37G variant, 40 mM BisTris, pH 7.0, 25°C. The curves obtained were fitted by the Prism progam, according to One Site – Specific binding model. Fluorescence data presented as the mean (± SD) of two independent experiments.

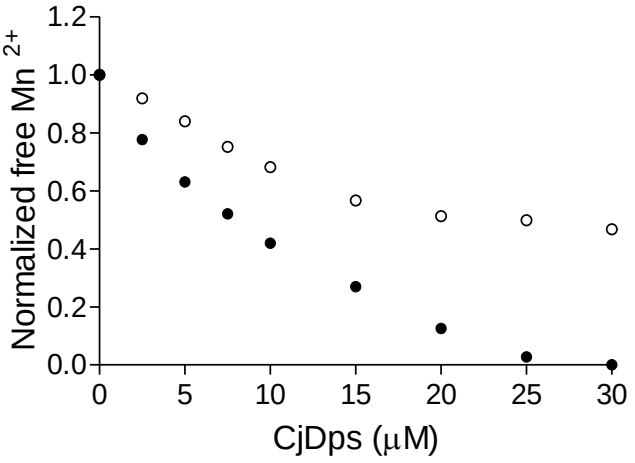

Figure 5. Interaction between CjDps and Mn^{2+} accessed by EPR spectroscopy. Equivalents of free Mn^{2+} versus concentration of CjDps. The signal of free manganese decrease according to addition of increasing CjDps concentration. Black circles, CjDps wt; white circles, CjDps H25G/H37G.

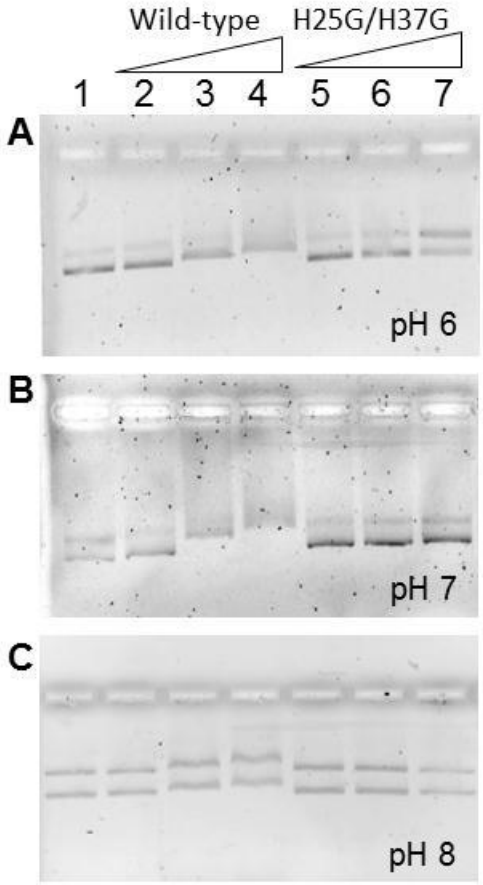

Figure 6. DNA binding activity of CjDps wt and CjDps H25G/H37G t different pHs. DNA binding activity was evaluated by the capacity to retard DNA migration in 0.7 % agarose gel electrophoresis. All reactions mixtures contained 300 ng circular DNA (pET28a). Lanes: 1- DNA, 2- DNA + 0.5 µg CjDps wt, 3 - DNA + 2.5 µg CjDps wt, 4 - DNA + 5.0 µg CjDps wt, 5 - DNA + 0.5 µg CjDps H25G/H37G, 6 - DNA + 2.5 µg CjDps H25G/H37G, 7 - DNA + 5.0 µg CjDps H25G/H37G. Reactions were incubated for 10 minutes at room temperature. Gel retardation increases with increasing protein concentration. Panels A, B and C were performed at different pHs as indicated.

Supplementary material

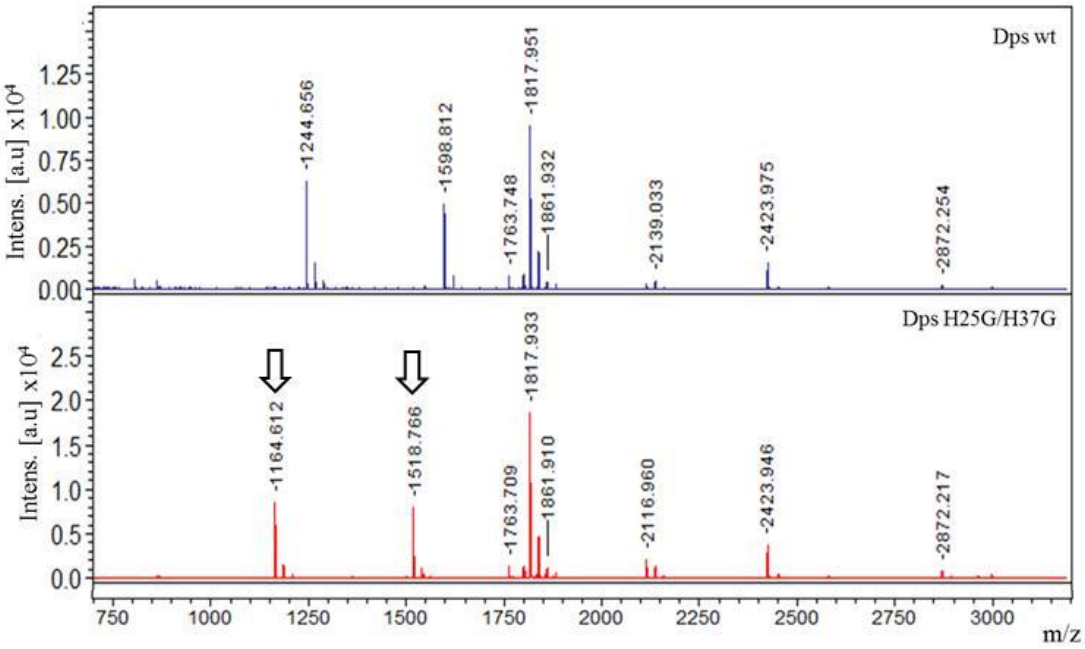

Figure S1. MALDI-TOF mass spectrometry analysis comparing the CjDps wt (blue) and CjDps H25G/H37G (red). Proteins bands excised from Comassie-stained SDS-PAGE gels were subjected to in-gel digestion with trypsin. The trypsinized mixture was mixed at 1:1 ratio (v/v) with a saturated solution of the MALDI matrix HCCA dissolved in acetonitrile 50% (v/v) and trifluoracetic acid 2.5% (v/v). Samples were spotted onto the MALDI target and allowed to dry. The peptides contained the modified histidine (arrows) showed decrease of 80 Da corresponding the change of Histidine to Glycine (21-29 FHNYHWNVK 1244.656/1164.612 Da; 30-42 GLQFFSIHEYTEK 1598.812/1518.766 Da).

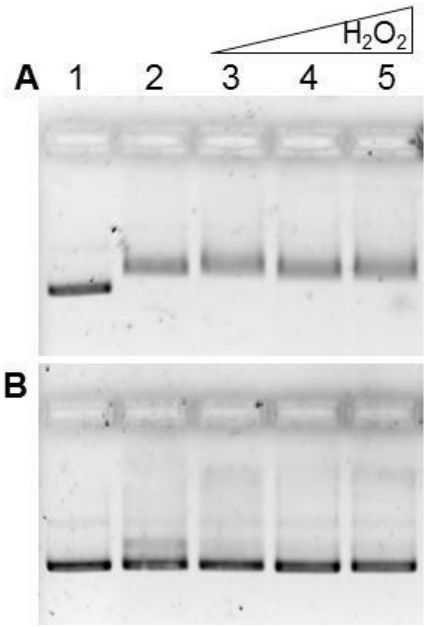

Figure S2. DNA binding activity of CjDps wt and CjDps H25G/H37G in the presence of H_2O_2. DNA binding activity was evaluated by the capacity to retard DNA migration in 0.7 % agarose gel electrophoresis. All reactions mixtures contained 300 ng circular DNA (pET28a). Panel (A) CjDps wild-type and panel (B) CjDps H25G/H37G. Lanes: 1- DNA, 2- DNA + 10 µg CjDps, 3 - DNA + 10 µg CjDps + 1 mM H_2O_2, 4 - DNA + 10 µg CjDps, + 5 mM H_2O_2, 5 - DNA + 10 µg CjDps + 10 mM H_2O_2 . Reactions were incubated for 10 minutes at room temperature.

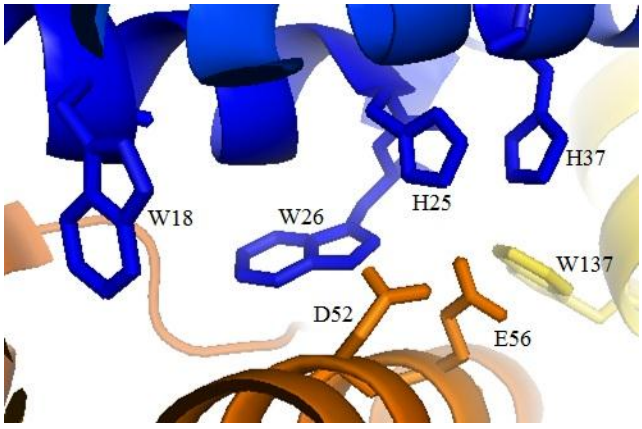

Figure S3. Location of the tryptophan residues at CjDps protein. All the tryptophan residues present in CjDps protein are localized close to the FOC. Figure was generated using Pymol.

5 CONCLUSÕES

1. O protocolo estabelecido para a purificação da proteína CjDps utilizando aquecimento a 80°C seguido de duas etapas cromatográficas é eficiente;
2. A temperatura de desnaturação da proteína CjDps wt é 94,2°C sendo portanto mais alta que a temperatura usada no processo de purificação;
3. As proteínas CjDps wt e CjDps H25G/H37G foram capazes de formar núcleo de ferro, embora CjDps H25G/H37G tenha esta capacidade diminuída;
4. A presença de peróxido de hidrogênio estimula a formação do núcleo de ferro das proteínas CjDps;
5. Ensaios de fluorescência do triptofano e espectroscopia mostraram que as proteínas CjDps são capazes de ligar manganês além de ferro;
6. Nas condições testadas, a proteína CjDps wt é capaz de ligar DNA enquanto que a proteína CjDps H25G/H37G não possui esta capacidade.

REFERÊNCIAS BIBLIOGRÁFICAS

ABDUL-TEHRANI, H.; HUDSON, A.J.; CHANG, Y.S.; TIMMS, A.R.; HAWKINS, C.; WILLIAMS, J.M.; HARRISON, P.M.; GUEST, J.R.; ANDREWS, S.C. Ferritin Mutants of Escherichia coli Are Iron Deficient and Growth Impaired, and fur Mutants are Iron Deficient. **Journal of Bacteriology**, v. 181, p. 1415–1428, 1999.

ALLOS, B. M. Campylobacter jejuni Infections: update on emerging issues and trends. **Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America**, v. 32, p. 1201–1206, 2001.

ALMIRÓN, M.; LINK, A.J.; FURLONG, D.; KOLTER, R. A novel DNA-binding protein with regulatory and protective roles in starved Escherichia coli. **Genes & Development**, v. 6, p. 2646–2654, 1992.

ANDREWS, S. C.; ROBINSON, A. K.; RODRÍGUEZ-QUIÑONES, F. Bacterial iron homeostasis. **FEMS microbiology reviews**, v. 27, p. 215–237, 2003.

ASBURY, A. K.; CORNBLATH, D. R. Assessment of current diagnostic criteria for Guillain-Barré syndrome. **Annals of Neurology**, v. 27, p. S21–S24, 1990.

BARASCH, J.; MORI, K. Cell biology: iron thievery. **Nature**, v. 432, p. 811–813, 2004.

BUTZLER, J. P. Campylobacter, from obscurity to celebrity. **Clinical Microbiology and Infection**, v. 10, p. 868–876, 2004.

CABISCOL, E.; TAMARIT, J.; ROS, J. Oxidative stress in bacteria and protein damage by reactive oxygen species. **International Microbiology: The Official Journal of the Spanish Society for Microbiology**, v. 3, p. 3–8, 2000.

CHIANCONE, E.; CECI, P.; ILARI, A.; RIBACCHI, F.; STEFANINI, S. Iron and proteins for iron storage and detoxification. **Biometals: An International Journal on the Role of Metal Ions in Biology, Biochemistry, and Medicine**, v. 17, p. 197–202, 2004.

CHIANCONE, E.; CECI, P. Role of Dps (DNA-binding proteins from starved cells) aggregation on DNA. **Frontiers in Bioscience (Landmark Edition)**, v. 15, p. 122–131, 2010.

COKER, A. O.; ISOKPEHI, R. D.; THOMAS, B. N.; AMISU, K. O.; OBI, C.L. Human Campylobacteriosis in Developing Countries1. **Emerging Infectious Diseases**, v. 8, p. 237–243, 2002.

COSTERTON, J. W.; STEWART, P. S.; GREENBERG, E. P. Bacterial biofilms: a common cause of persistent infections. **Science (New York, N.Y.)**, v. 284, p. 1318–1322, 1999.

DE BERNARD, M.; D'ELIOS, M. M. The immune modulating activity of the Helicobacter pylori HP-NAP: Friend or foe? **Toxicon: Official Journal of the International Society on Toxinology**, v. 56, p. 1186–1192, 2010.

DEMING, M. S.; TAUXE, R. V.; BLAKE, P.A.; DIXON, S. E.; FOWLER, B. S.; JONES, T. S.; LOCKAMY, E. A.; PATTON, C. M.; SIKES, R. O. Campylobacter enteritis at a university: transmission from eating chicken and from cats. **American Journal of Epidemiology**, v. 126, p. 526–534, 1987.

ECKHARDT, M. The role and metabolism of sulfatide in the nervous system. **Molecular Neurobiology**, v. 37, p. 93–103, 2008.

FLEMMING, H. C.; WINGENDER, J. The biofilm matrix. **Nature Reviews Microbiology**, v. 8, p. 623–633, 2010.

FRANCESCHINI, S.; CECI, P.; ALALEONA, F.; CHIANCONE, E.; ILARI, A. Antioxidant Dps protein from the thermophilic cyanobacterium Thermosynechococcus elongatus. **FEBS Journal**, v. 273, p. 4913–4928, 2006.

FRANCO, B. D. G. M., L., M. **Microbiologia dos alimentos**. 2. ed. São Paulo: Atheneu, 2002.

GIAOURIS, E.; SAMOILIS, G.; CHORIANOPOULOS, N.; ERCOLINI, D.; NYCHAS, G. J. Differential protein expression patterns between planktonic and biofilm cells of Salmonella enterica serovar Enteritidis PT4 on stainless steel surface. **International Journal of Food Microbiology**, v. 162, p. 105–113, 2013.

GILLESPIE, I. A.; O'BRIEN, S. J.; PENMAN, C.; TOMPKINS, D.; COWDEN, J.; HUMPHREY, T. J. Demographic determinants for Campylobacter infection in England and Wales: implications for future epidemiological studies. **Epidemiology and Infection**, v. 136, p. 1717–1725, 2008.

HAIKARAINEN, T.; PAPAGEORGIOU, A. C. Dps-like proteins: structural and functional insights into a versatile protein family. **Cellular and molecular life sciences: CMLS**, v. 67, p. 341–351, 2010.

HUERGO, L. F.; RAHMAN,H.; IBRAHIMOVIC, A.; DAY, C. J; KOROLIK, V. Campylobacter jejuni Dps Protein Binds DNA in the Presence of Iron or Hydrogen Peroxide. **Journal of Bacteriology**, v. 195, p. 1970–1978, 2013.

ILARI, A.; STEFANINI, S.; CHIANCONE, E.; TSERNOGLOU, D. The dodecameric ferritin from Listeria innocua contains a novel intersubunit iron-binding site. **Nature Structural Biology**, v. 7, p. 38–43, 2000.

ILARI, A. CECI, P.; FERRARI, D.; ROSSI, G. L.; CHIANCONE, E. Iron Incorporation into Escherichia coli Dps Gives Rise to a Ferritin-like Microcrystalline Core. **Journal of Biological Chemistry**, v. 277, p. 37619–37623, 2002.

ISHIKAWA, T.; MIZUNOE, Y.; KAWABATA, S.; TAKADE, A.; HARADA, M.; WAY, S. N.; YOSHIDA, S. The Iron-Binding Protein Dps Confers Hydrogen Peroxide Stress Resistance to Campylobacter jejuni. **Journal of Bacteriology**, v. 185, p. 1010–1017, 2003.

LAWSON, D. M.; TREFFRY, A.; ARTYMIUK, P. J.; HARRISON, P. M.; YEWDALL, S. J.; LUZZAGO, A.; CESARENI, G.; LEVI, S.; AROSIO, P. Identification of the ferroxidase centre in ferritin. **FEBS letters**, v. 254, p. 207–210, 1989.

LAWSON, D. M.; ARTYMIUK, P. J.; YEWDALL, S.; SMITH, J. M. A.; LIVINGSTONE, J. C.; TREFFRY, A.; LUZZAGO, A.; LEVI, S.; AROSIO, P.; CESARENI, G.; THOMAS, C. D.; SHAW, W. V.; HARRISON, P. M. Solving the structure of human H ferritin by genetically engineering intermolecular crystal contacts. **Nature**, v. 349, p. 541–544, 1991.

LEWIN, A.; MOORE, G. R.; LE BRUN, N. E. Formation of protein-coated iron minerals. **Dalton Transactions (Cambridge, England: 2003)**, p. 3597–3610, 2005.

MAHDAVI, J.; SONDÉN, B.; HURTIG, M.; OLFAT, F. O.; FORSBERG, L.; ROCHE, N.; ANGSTROM, J.; LARSSON, T.; TENEBERG, S.; KARLSSON, K. A.; ALTRAJA, S.; WADSTRÖM, T.; KERSULYTE, D.; BERG, D. E.; DUBOIS, A.; PETERSON, C.; MAGNUSSON, K. E.; NORBERG, T.; LINDH, F.; LUNDSKOG, B. B.; ARNGVIST, .A.; HAMMARSTRÖM, L.; BORÉN, T. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. **Science**, v. 297, p. 573–578, 2002.

MARTINEZ, A.; KOLTER, R. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. **Journal of Bacteriology**, v. 179, p. 5188–5194, 1997.

MASSÉ, E.; SALVAIL, H.; DESNOYERS, G.; ARGUIN, M. Small RNAs controlling iron metabolism. **Current Opinion in Microbiology**, v. 10, p. 140–145, 2007.

MISHU, B.; BLASER, M. J. Role of Infection Due to Campylobacter jejuni in the Initiation of Guillain-Barre Syndrome. **Clinical Infectious Diseases**, v. 17, p. 104–108, 1993.

NAMAVAR, F.; SPARRIUS, M.; VEERMAN, E. C. I.; APEELMELK, B. J.; VANDENBROUCKE-GRAUS, C. M. J.E. Neutrophil-Activating Protein Mediates Adhesion of Helicobacter pylori to Sulfated Carbohydrates on High-Molecular-Weight Salivary Mucin. **Infection and Immunity**, v. 66, p. 444–447, 1998.

NYATI, K. K.; NYATI, R. Role of Campylobacter jejuni Infection in the Pathogenesis of Guillain-Barré Syndrome: An Update. **BioMed Research International**, v. 2013, 2013.

OLSON, C. K. et al. Epidemiology of Campylobacter jejuni infections in industrialized nations. Ed.3, p. 163–189, 2008.

PARKHILL, J.; WREN, B. W.; MUNGALL, K.; KETLEY, J. M.; CHURCHER, C.; BASHAM, D.; CHILLINGWORTH, T.; DAVIES, R. M.; FELTWELL, T.; HOLROYD, S.; JAGELS, K.; KARLYSHEV, A. V.; MOULE, S.; PALLEN, M. J.; PEEN, C. W.; QUAIL, M. A.; RAJANDREAM, M. W.; RUTHERFORD, K. M.; van VLIET, A. H.; WHITEHEAD, S.; BARREL, B. G. The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. **Nature**, v. 403, p. 665–668, 2000.

PARSONS, C. M. Influence of caecectomy and source of dietary fibre or starch on excretion of endogenous amino acids by laying hens. **British Journal of Nutrition**, v. 51, p. 541–548, 1984.

PIAO, H.; MINOHARA, M.; KAWAMURA, N.; LI, W.; MIZUNOE, Y.; UMEHARA, F.; GOTO, Y.; KUSUNOKI, S.; MATSUSHITA, T.; IKENAKA, K.; MAEJIMA, T.; NABEKURA, J.; YAMASAKI, R.; KIRA, J. Induction of paranodal myelin detachment and sodium channel loss in vivo by Campylobacter jejuni DNA-binding protein from starved cells (C-Dps) in myelinated nerve fibers. **Journal of the Neurological Sciences**, v. 288, p. 54–62, 2010.

PIAO, H.; MINOHARA, M.; KAWAMURA, N.; LI, W.; MATSUSHITA, T.; YAMASAKI R.; MIZUNOE, Y.; KIRA, J. Tissue Binding Patterns and In Vitro Effects of Campylobacter jejuni DNA-Binding Protein from Starved Cells. **Neurochemical Research**, v. 36, p. 58–66, 2011.

ROBINSON, D. A. Infective dose of Campylobacter jejuni in milk. **British Medical Journal (Clinical research ed.)**, v. 282, p. 1584, 1981.

RUIZ-PALACIOS, G. M. The health burden of Campylobacter infection and the impact of antimicrobial resistance: playing chicken. **Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America**, v. 44, p. 701–703, 2007.

SATIN, B.; Del GIUDICE, G.; Della BIANCA, V.; DUSI, S.; LAUDANNA, C.; TONELLO, F.; KELLEHER, D.; RAPPUOLI, R.; MONTECUCCO, C.; ROSSI, F. The Neutrophil-Activating Protein (Hp-Nap) of Helicobacter pylori Is a Protective Antigen and a Major Virulence Factor. **The Journal of Experimental Medicine**, v. 191, p. 1467–1476, 2000.

STAHL, M.; BUTCHER, J.; STINTZI, A. Nutrient acquisition and metabolism by Campylobacter jejuni. **Frontiers in Cellular and Infection Microbiology**, v. 2, p. 5, 2012.

STORZ, G.; IMLAY, J. A. Oxidative stress. **Current Opinion in Microbiology**, v. 2, p. 188–194, abr. 1999.

SZYMANSKI, C. M.; KING, M.; HAARDT, M.; ARMSTRONG, G. D. Campylobacter jejuni motility and invasion of Caco-2 cells. **Infection and Immunity**, v. 63, p. 4295–4300, 1995.

TAKATA, T.; FUJIMOTO, S.; AMAKO, K. Isolation of nonchemotactic mutants of Campylobacter jejuni and their colonization of the mouse intestinal tract. **Infection and Immunity**, v. 60, p. 3596–3600, 1992.

THEIL, E. C. Coordinating responses to iron and oxygen stress with DNA and mRNA promoters: the ferritin story. **Biometals: An International Journal on the Role of Metal Ions in Biology, Biochemistry, and Medicine**, v. 20, p. 513–521, 2007.

THEORET, J. R.; COOPER, K. K.; GLOCK, R. D.; JOENS, L. A. A Campylobacter jejuni Dps Homolog Has a Role in Intracellular Survival and in the Development of Campylobacterosis in Neonate Piglets. **Foodborne Pathogens and Disease**, v. 8, p. 1263–1268, 2011.

THEORET, J. R.; COOPER, K. K.; ZEKARIAS, B.; ROLAND, K. L.; LAW, B. F.; CURTISS, R.; JOENS, L. A. The Campylobacter jejuni Dps Homologue Is Important

for In Vitro Biofilm Formation and Cecal Colonization of Poultry and May Serve as a Protective Antigen for Vaccination. **Clinical and Vaccine Immunology : CVI**, v. 19, p. 1426–1431, 2012.

THOMAS, M. T.; SHEPERD, M.; POOLE, R. K.; van VLIET, A. H. M.; KELLY, D. J.; PEARSON, B. M. Two respiratory enzyme systems in Campylobacter jejuni NCTC 11168 contribute to growth on L-lactate. **Environmental Microbiology**, v. 13, p. 48–61, 2011.

TZOUVELEKIS, L. S.; MENTIS, A. F.; MAKRIS, A. M. SPILIADIS, C.; BLACKWELL, C.; WEIR, D. M. In vitro binding of Helicobacter pylori to human gastric mucin. **Infection and Immunity**, v. 59, p. 4252–4254, 1991.

VANDAMME, P.; DE LEY, J. Proposal for a new family, Campylobacteraceae. **Int. J. Syst. Bacteriol.,** v. 41, p. 451-455, 1991.

VELAYUDHAN, J.; CASTOR, M.; RICHARDSON, A.; MAIN-HESTER, K. L.; FANG, F. C. The role of ferritins in the physiology of Salmonella enterica sv. Typhimurium: a unique role for ferritin B in iron-sulphur cluster repair and virulence. **Molecular Microbiology**, v. 63, p. 1495–1507, 2007.

VELAYUDHAN, J.; KELLY, D. J. Analysis of gluconeogenic and anaplerotic enzymes in Campylobacter jejuni: an essential role for phosphoenolpyruvate carboxykinase. **Microbiology**, v. 148, p. 685–694, 2002.

VÉRON, M.; CHATELAIN, R. Taxonomic Study of the Genus Campylobacter Sebald and Véron and Designation of the Neotype Strain for the Type Species, Campylobacter fetus (Smith and Taylor) Sebald and Véron. **International Journal of Systematic Bacteriology**, v. 23, p. 122–134, 1973.

WASSENAAR, T. M. Toxin production by Campylobacter spp. **Clinical Microbiology Reviews**, v. 10, p. 466–476, 1997.

WRIGHT, J. A.; GRANT, A. J.; HURD, D.; HARRISON, M.; GUCCIONE, E. J.; KELLY, D. J.; MASKELL, D. J. Metabolite and transcriptome analysis of Campylobacter jejuni in vitro growth reveals a stationary-phase physiological switch. **Microbiology**, v. 155, p. 80–94, 2009.

ZHAO, G.; CECI, P.; ILARI, A.; GIANGIACOMO, L.; LAUE, T. M.; CHIANCONE, E.; CHASTEEN, N. D. Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of Escherichia coli. **The Journal of Biological Chemistry**, v. 277, p. 27689–27696, 2002.