CHARACTERIZATION OF $F_A/F_B$-CONTAINING PROTEINS PshBI AND PshBII
IN THE PHOTOSYNTHETIC REACTION CENTER OF *Heliobacterium modesticaldum*

A Thesis in
Biochemistry and Molecular Biology
by
Yili Sun

© 2011 Yili Sun

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2011
The thesis of Yili Sun was reviewed and approved* by the following:

John H. Golbeck  
Professor of Biochemistry and Biophysics  
Professor of Chemistry  
Thesis Advisor

Donald A. Bryant  
Ernest C. Pollard Professor in Biotechnology  
Professor of Biochemistry and Molecular Biology

Mark Maroncelli  
Professor of Chemistry

Craig E. Cameron  
Paul Berg Professor of Biochemistry and Molecular Biology  
Associate Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School
ABSTRACT

Similar to all known Type I photosynthetic reaction centers (RCs), the homodimeric RCs found in the *Heliobacteriaceae* utilize three [4Fe-4S] clusters termed $F_X$, $F_A$ and $F_B$ that function as terminal electron acceptors. However, unlike their heterodimeric Photosystem I (PS I) counterpart, in which a single, tightly-bound polypeptide, named PsaC, is responsible for ligating the $F_A$ and $F_B$ clusters, two loosely-bound proteins, termed PshBI and PshBII, function as $F_A/F_B$ harboring subunits in the heliobacterial RC (HbRC). Although the recombinant PshBI and PshBII with His-tags have been proved to be capable of functioning as electron acceptors, untagged PshBI and PshBII are needed to understand their 3-dimensional structures and protein interactions.

In this study, reverse transcription PCR and western blot analysis have been carried out to study the transcription and translation of PshBI and PshBII in *Heliobacterium modesticaldum*. PshBI and PshBII have been over-expressed in *Eschericia coli* with a cleavable thioredoxin tag. After enterokinase cleavage, only one additional alanine remains on the N-termini of the two proteins. Un tagged PshBI and PshBII have been reconstituted with inorganic iron and sulfide. Optical and low temperature X-band EPR studies indicate that untagged PshBI and PshBII harbor two [4Fe-4S] clusters. Room temperature flash-induced charge recombination kinetics and low temperature light-induced EPR spectroscopic studies indicate both untagged PshBI and PshBII are able to accept electron from $F_X$ in HbRC cores. The long lifetime kinetic phase observed in the charge recombination kinetics suggests that PshBI and PshBII might be mobile or semi-mobile subunits. Pull-down assays have been carried out using PshBI-His and PshBII-His holoproteins as bait. Several potential proteins that interact with PshBI and PshBII in *H. modesticaldum* have been captured and identified by mass spectrometry.
TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. vi

LIST OF TABLES ................................................................................................................... vii

ACKNOWLEDGEMENTS .......................................................................................................... viii

Chapter 1 General introduction ........................................................................................... 1

1.1 Photosynthesis .............................................................................................................. 1
1.2 Photosynthetic reaction centers .................................................................................. 2
1.3 Heliobacteria and Heliobacterial reaction centers ...................................................... 2
1.4 PshBI and PshBII in *H. modesticaldum* ................................................................. 3

Chapter 2 Characterization of PshBI and PshBII in *H. modesticaldum* ....................... 6

2.1 Introduction .................................................................................................................... 6
2.2 Materials and methods ............................................................................................... 6
  2.2.1 RNA isolation and reverse transcription-PCR .................................................... 6
  2.2.2 Purification of polyclonal antibodies raised from PshBI-His and PshBII-His .......... 7
  2.2.3 Western blot ......................................................................................................... 8
  2.2.4 Construction of the expression vectors for PshBI and PshBII ............................ 9
  2.2.5 Purification of untagged PshBI and PshBII ......................................................... 10
  2.2.6 Reconstitution with FeS cluster .......................................................................... 11
  2.2.7 Preparation of Heliobacterial reaction centers .................................................. 11
  2.2.8 Time-resolved optical spectroscopy in the near-infrared region ...................... 12
  2.2.9 Low-temperature X-band EPR spectroscopy .................................................... 13
2.3 Results ......................................................................................................................... 14
  2.3.1 RT-PCR revealed that both *pshBI* and *pshBII* are transcribed into mRNA .... 14
  2.3.2 Western blot confirmed the expression of PshBI and PshBII in *H. modesticaldum* ...................................................................................................................................... 14
  2.3.3 Codon usage optimization and the expression constructs ................................. 15
  2.3.4 Purification of the untagged PshBI and PshBII .................................................. 16
  2.3.5 Reconstitution of FeS clusters in PshBI and PshBII .......................................... 17
  2.3.6 Room temperature electron transfer from FeS to FeS/FB .................................. 17
  2.3.7 EPR spectroscopy of HbRC cores with PshBI or PshBII holoprotein .............. 19
2.4 Discussion ..................................................................................................................... 20
  2.4.1 The transcription and translation of PshBI and PshBII ....................................... 20
  2.4.2 Over-expression small proteins like PshBI and PshBII in *E. coli* .................... 20
  2.4.3 Untagged PshBI and PshBII holoproteins contain two [4Fe-4S] clusters ......... 21
  2.4.4 Comparison of PshBI and PshBII in accepting electron from HbRC cores .... 22
2.5 Figure legends ............................................................................................................. 24
Chapter 3 Proteins that may interact with PshBI/PshBII in *H. modesticaldum* ................................ 39

3.1 Introduction ........................................................................................................................ 39
3.2 Materials and methods ....................................................................................................... 39
   3.2.1 Preparation of soluble proteins from *H. modesticaldum* ............................................ 39
   3.2.2 Pull down assay with PshBI-His and PshBII-His ........................................................ 40
   3.2.3 Co-Immunoprecipitation ............................................................................................ 40
3.3 Results ................................................................................................................................. 41
   3.3.1 Potential electron transfer partners of PshBI and PshBII ............................................ 41
   3.3.2 Pull-down assays using apoproteins PshBI-His and PshBII-His as baits ................. 42
   3.3.3 Co-IP using antibodies against PshBI-His and PshBII-His ........................................ 43
3.4 Discussion .......................................................................................................................... 43
   3.4.1 Potential interacting partners of PshBI and PshBII identified from pull-down assays .... 43
   3.4.2 Possible protein-protein interaction pattern utilized by PshBI and PshBII ............... 46
   3.4.3 Future attempts to study the potential partners of PshBI/PshBII ................................. 46
3.5 Conclusion .......................................................................................................................... 47
3.6 Figure legends ..................................................................................................................... 48

References ............................................................................................................................... 55
LIST OF FIGURES

Figure 2.1: Schematic representation of plasmid pET15bPshBI ................................................................. 27
Figure 2.2: RT-PCR results in a 1% agarose gel .......................................................................................... 28
Figure 2.3: Immunoblot analysis of PshBI (A) and PshBII (B) in a *H. modesticaldum* cell lysate. ................................................................................................................................. 29
Figure 2.4: Codon usages of PshBI (A), optimized PshBI (B), PshBII (C) and optimized PshBII (D). ................................................................................................................................. 30
Figure 2.5: Schematic representations of plasmid pET32aPshBI (A), plasmid pET32aPshBII (B), the fusion tags and the enterokinase cleavage site of the fusion proteins (C). .............................................................................. 31
Figure 2.6: SDS-PAGE gel of untagged PshBI (A) and PshBII (B) purification ........................................ 32
Figure 2.7: Absorbance spectra of the untagged PshBI (A) and PshBII (B) after reconstitution with 2-ME, ferrous ammonium sulfate and sodium sulfide. .................................................. 33
Figure 2.8: Low temperature EPR spectra of untagged PshBI (A) and PshBII (B) holoproteins. ................................................................................................................................. 34
Figure 2.9: Charge recombination kinetics of HbRC cores and PshBI followed by flash-induced absorption changes at 798 nm. ................................................................................................. 35
Figure 2.10: Charge recombination kinetics of HbRC cores and PshBII followed by flash-induced absorption changes at 798 nm. ................................................................................................. 36
Figure 2.11: Light-induced EPR difference spectra of HbRC cores reconstituted with untagged (A) PshBI and (B) PshBII holoproteins.............................................................................. 37
Figure 3.1: SDS-PAGE of pull-down assay results using PshBI-His holoprotein as bait. ...... 49
Figure 3.2: SDS-PAGE of pull-down assay results using PshBII-His holoprotein as bait. .... 50
Figure 3.3: SDS-PAGE of pull-down assay results using PshBII-His/PshBII-His holoprotein and apoprotein as bait. .............................................................................................................. 51
Figure 3.4: SDS-PAGE gel picture of Co-IP results....................................................................................... 52
LIST OF TABLES

Table 2.1: Room temperature charge recombination kinetics followed by flash-induced absorption changes at 798 nm. .............................................................. 38

Table 3.1: Proteins pulled-down by PshBI-His holoprotein .......................................................... 53

Table 3.2: Proteins pulled-down by PshBII-His holoprotein .......................................................... 54
ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my supervisor, Dr. John H. Golbeck, who granted me the opportunity to join this amazing group, and who guided and supported me through my graduate studies. I am very grateful to him for providing a free research environment and encouraging me to try my own ideas.

I would like to thank Dr. Donald A. Bryant and Dr. Mark Maroncelli for their time and support as my committee members.

I would like to express my gratitude to all the members of our lab, past and present. Especially thanks to Steve Romberger, Dr. Gaozhong Shen and Dr. Christian Castro, for their scientific advice and technical support for the Heliobacterial project; Dr. Nithya Srinivasan and Junlei Sun, for their instructions and help in the Cyanobacterial project.

I would like to acknowledge my friends and my parents, who always believe in me and encourage me. Finally, I would like to thank my husband Bo Yang Yu for his unwavering love, understanding and support.
Chapter 1

General Introduction

1.1 Photosynthesis

Photosynthesis is the most fundamental biological process in biology. It provides food, fiber and energy to almost all life on the earth, either directly or indirectly. Literally meaning “synthesis with light”, photosynthesis harvests sunlight and converts solar energy into chemical energy to drive cellular processes. The process of photosynthesis takes place within the membranes of plants, photosynthetic bacteria and algae using lipid bilayer membranes to store energy in early stages (I).

Photosynthetic organisms can be divided into oxygenic and anoxygenic types. Oxygenic phototrophs use water as the source of electrons and liberate \( \text{O}_2 \) as a by-product. Anoxygenic phototrophs derive electrons from organic or inorganic molecules, and they do not produce \( \text{O}_2 \). Before the appearance of oxygenic photosynthetic bacteria, anoxygenic phototrophs were dominant. About 2.7 billion years ago, oxygenic photosynthetic cyanobacteria started to appear, and gradually changed the composition of atmosphere in the following 400 million years (I). Since then, advanced forms of life began to emerge. Among the six well-established phototrophic bacterial phyla, Firmicutes, Chloroflexi, Chlorobi, Proteobacteria, Acidobacteria and Cyanobacteria, only Cyanobacteria are oxygenic phototrophs. Furthermore, all eukaryotic phototrophs liberate \( \text{O}_2 \) during photosynthesis.
1.2 Photosynthetic Reaction Centers

In the process of photosynthesis, light energy is harvested and transformed into a stable charge-separated state by membrane bound, protein-pigment complexes called photosynthetic reaction centers (RCs). Only two types of RCs exist on earth (1): Type I RCs use a \([4\text{Fe}-4\text{S}]\) cluster as the final electron acceptor, and Type II RCs use a mobile quinone as the final electron acceptor. The phototrophs in the phyla Firmicutes, Chlorobi (also known as green sulfur bacteria), and Acidobacteria contain Type I RCs, whereas Chloroflexi and Proteobacteria contain Type II RCs. Cyanobacteria and eukaryotic phototrophs contain both Type I and Type II RCs. Each of the two types of RCs can be further divided into two subgroups. Type I RCs can be divided into heterodimeric RCs, such as Photosystem I (PS I), and homodimeric bacterial RCs, including the green sulfur bacterial RC (GbRC) and the heliobacterial RC (HbRC). Type II RCs contain only heterodimeric RCs, and can be divided into the purple bacterial RC, and the heterodimeric Photosystem II (PS II) RC. X-ray crystal structures are available for PS I (2), PS II (3, 4), and the purple bacterial RC (5). However, no high-resolution structural information is available for Type I homodimeric RCs. The Type I homodimeric RC from *Heliobacterium modesticaldum* is the topic of study in this thesis.

1.3 Heliobacteria and Heliobacterial Reaction Centers

The Heliobacteriaceae is a unique family of phototrophic bacteria in the phylum *Firmicutes*. Heliobacteria are the only known group of gram-positive photoheterotrophs (6-8). They are also unique among all phototrophs in that they produce endospores (9). Heliobacteria are strict anaerobes and contain the unusual pigment bacteriochlorophyll (BChl) g, which imparts a characteristic brown color (10, 11). Heliobacteria are obligately heterotrophic and grow only on
certain organic substrates, including pyruvate, lactate, butyrate and acetate (12, 13). They do not contain ribulose 1,5-bis-phosphate or ATP citrate-lyase, two enzymes that are commonly used in carbon fixation (12). All heliobacteria known can fix dinitrogen, and this ability is thought to be the major ecological role of these anoxygenic photoheterotrophs (14). The major habitat for heliobacteria is paddy field soil (15), where they use the organic plant exudates to grow and, in exchange, provide the plants with fixed nitrogen.

HbRCs are homodimeric Type I RCs (16, 17) and are considered to be the evolutionary precursors of the heterodimeric PS I RCs (17). Without any peripheral antenna system, the HbRC consists of two different polypeptides, PshA and PshB. The HbRC core is formed by a homodimer of PshA, an intrinsic membrane protein with a molecular weight of ~68 kDa (17, 18). The PshA homodimer binds 22 to 40 BChl g (16, 19), as well as several carotenoid and quinone molecules (17). More importantly, there are three electron transfer cofactors within PshA: 1) the primary donor P798, a dimer of BChl g⁺; 2) the primary acceptor A₀, an 8₁-OH Chl a₉ monomer² (20); and 3) the [4Fe-4S]¹⁺/²⁺ cluster Fₓ (19, 21, 22). Although the presence of a menaquinone-9 in HbRC has been shown (23), whether it functions as a secondary electron acceptor such as phylloquinone in PS I is controversial (17). The other polypeptide, PshB, which contains the terminal [4Fe-4S]¹⁺/²⁺ clusters Fₐ and Fₜ (24), is found loosely bound to the HbRC core, and can be removed by washing the intact HbRC with 0.1 M to 1 M NaCl (24).

1.4 PshBI and PshBII in H. modesticaldum

Unlike the Fₐ/Fₜ containing protein PsaC, which requires 6.8 M urea for removal from PS I (25, 26), the PshB proteins in Heliobacteria are loosely bound to the HbRC core, and are removed using ionic detergents such as deriphat 160c and sucrose monolaurate (23, 27). PshB in H. modesticaldum was first isolated from n-dodecyl-β-D-maltopyranoside treated membranes, by
washing the intact HbRC complexes with 1.0 M NaCl under anaerobic conditions and performing ultrafiltration using a 30 kDa cutoff membrane (24). Isolated PshB showed a broad absorbance at around 410 nm in the optical spectrum, and a complex set of resonances attributed to $F_A^-/F_B^-$ in the low temperature chemically reduced EPR spectrum (24). Upon addition of isolated PshB to isolated HbRC cores, the flash-induced kinetics shows that a lifetime of 75 ms kinetic phase in the room temperature, as well as light-induced EPR signals of $F_A^-/F_B^-$ at 15 K (17, 24). These results indicate that PshB is the $F_A/F_B$-containing protein in the HbRC.

In 2005, Hatano and colleagues reported the isolation and characterization of two soluble ferredoxins from H. mobilis, FD1 and FD2 (28). The N-terminal amino acid sequence of FD2 was found to be similar as the N-terminal sequence of PshB from H. modesticaldum (29). The gene pshB from H. modesticaldum was cloned using primers designed on the basis of the N- and C-terminal amino acid sequences of FD2 in H. mobilis, and the recombinant PshB with a histidine tag (His-tag) on the N-terminus was over-expressed and characterized (29).

Interestingly, in the upstream region of the pshB gene there exists a second gene, HM1_1462, which also codes for a dicluster ferredoxin (18). The predicted protein shows 62% identity and 69% similarity with PshB in its amino acid sequence. Both proteins contain 54 amino acids, including two CxxCxxCxxxC binding motifs characteristic of two [4Fe-4S] clusters. After reporting this upstream gene, PshB was renamed PshBI, and the protein product of HM1_1462 was named PshBII (30). According to the protein sequences, PshBI has a molecular weight of 5440 Da and an estimated pI of 4.27, whereas PshBII has a molecular weight of 5379 Da and an estimated pI of 3.76. The similarities between these two proteins imply that they share similar biochemical functions. It has been shown that like PshBI, recombinant PshBII is also capable of binding to HbRC cores and accepting electrons from $F_X$ (30).

Both PshBI and PshBII are bacterial dicluster ferredoxins. The three-dimensional structures of many members from this family are known from high resolution X-ray and NMR
studies (17). Homology models of PshBI and PshBII have been constructed using the programs http://Swissmodel.expasy.org, and two templates: 1FDN (Clostridium acidiurici) and 1DUR (Peptococcus asacharolyticus) (31). In both models of PshBI and PshBII, the N- and C-termini of the protein form an antiparallel β-sheet, and the polypeptide backbone wraps around the two [4Fe-4S] clusters, forming two α-helixes (31). Compared to the sequence of PsaC in PS I, which is ~9 kDa in mass, two features are missing in PshBI and PshBII: 1) a region about 14-15 amino acid in the C-terminus, which has been shown to orienting PsaC as it docks to the heterodimeric core of PS I (32); and 2) an 8 amino acid insertion between two [4Fe-4S] cluster binding motifs, which has been shown to interact with ferredoxin (coded by petF in cyanobacteria) and flavodoxin (33). The first missing feature may indicate that the binding mode between HbRC cores and PshBI/PshBII is different from the one employed by PsaC and PsaA/PsaB heterodimer. The second missing feature, the loosely binding mode of PshBI/PshBII and HbRC core, as well as the absence of a petF-like gene in H. modesticaldum, suggest that after accepting electrons, PshBI and PshBII may dissociate from the HbRC core, interact with other target redox protein(s), and return to the HbRC core to complete the electron transfer process.

In this study, experiments have been designed to achieve three goals. The first one is to study the expression of PshBI and PshBII in H. modesticaldum using RT-PCR and western blot analysis. The second goal is to over-express and characterize untagged PshBI and PshBII. The third goal is to search for potential interacting partners of PshBI and PshBII using pull-down assays and co-immunoprecipitation.
Chapter 2

Characterization of PshBI and PshBII in *Heliobacterium modesticaldum*

2.1 Introduction

Previous studies had indicated that both recombinant His-tagged PshBI (PshBI-His) and His-tagged PshBII (PshBII-His) are able to interact with HbRC cores and to accept electrons from $F_X$ (29, 30). However, based on the assays performed with PshBI-His and PshBII-His, different concentrations of PshBI and PshBII are required to achieve the same degree of electron transfer (29, 30). An important detail about these two recombinant proteins is that the expression vectors used in the two studies are different, and hence the sequences of the His-tags are different. In addition, the 21/20-residue His-tag is 40% the size of native PshBI/PshBII. Such a large tag may alter the interaction between PshBI/PshBII and the HbRC. To eliminate these artificial factors and gain a better understanding of PshBI and PshBII in *H. modesticaldum*, the untagged proteins were expressed and studied. In addition, the presence of PshBI and PshBII proteins in *H. modesticaldum* was monitored at the transcriptional and translational levels.

2.2 Materials and Methods

2.2.1 RNA isolation and reverse transcription-PCR

RNA was isolated from late exponential phase *H. modesticalum* cells using the High Pure RNA Isolation Kit from Roche. The protocol from the manufacturer was followed except the cells were lysed via incubation at 30 °C for 10 min in the presence of 200 µg lysozyme and 40 µ
RNase inhibitor before mixing with the lysis-binding buffer. To verify that no DNA was present in the sample, the isolated RNA was tested by PCR using the same primers as in reverse transcription-PCR (RT-PCR). RT-PCR was performed using *C. therm.* Polymerase One-Step RT-PCR System from Roche. The forward and reverse primers for HM1_1461 were, respectively: 5’-AGGGGGTGAACTCATATGGTCTATAAAATC-3’ (Fwd1) and 5’-ACCCAGGGATCTCAATTCTGCTTAACCCTC-3’ (Rvs1). The forward and reverse primers for HM1_1462 were, respectively: 5’-CAGGTGAAGAGACATATGGCTTACAAAATC-3’ (Fwd2) and 5’-GCTTTTGAGGATTCCGATCAGCCAGGAAT-3’ (Rvs2). The 50 µl RT-PCR system contained 10 µl 5× RT-PCR buffer, 2 µl of *C. therm.* Polymerase mixture, 400 µM dNTPs, 7% DMSO, 5 mM DTT solution, 20 U RNase inhibitor, 0.1 µM of each primer, and 50 ng RNA template in RNase-free water.

2.2.2 Purification of polyclonal antibodies raised from PshBI-His and PshBII-His

The expression vectors pET15bPshBI (Figure 2.1) and pET28bFD1 (30) were transformed into Rosetta 2 (DE3) cells to over-express PshBI-His and PshBII-His respectively, which contain the identical His-tag sequence at their N-termini. Proteins were purified using the methods described in ref. (30). Polyclonal antibodies against PshBI-His and PshBII-His were produced in rabbits by Rockland Immunochemicals, Inc. Two rabbits were used for each protein, yielding approximately 100 ml of anti-sera. Cyanogen bromide-activated-Sepharose® 4B (Sigma-Aldrich, C9142) was used to purify antibody against PshBI-His or PshBII-His from the anti-sera. Firstly, 600 µg purified PshBI-His or PshBII-His was bounded to 500 mg CNBr-activated resin following the manufacturer’s protocol. An aliquot of 200 µl antigen-coupled resin was used each time to affinity purify the antibody. Prior to incubating with serum, the resin was washed with elution buffers that will be used for the affinity purification. The resin was firstly
washed with 500 µl of 100 mM glycine, pH 2.5, followed by 100 mM Tris-HCl, pH 8.8, until the pH of the column effluent was neutral; then washed with 500 µl of 100 mM triethylamine, pH 11.5, followed by 10 mM Tris-HCl, pH 7.4, until the pH of the column effluent was neutral. One ml of the crude anti-PshBI-His or anti-PshBII-His serum was diluted with 9 ml of 10 mM Tris-HCl, pH 7.4, and then incubated with the specific antigen-coupled resin at 4 °C overnight with agitation. The resin was washed with 4 ml of 10 mM Tris-HCl, pH 7.4; and the antibody was eluted with 1 ml of 100 mM glycine, pH 2.5. The eluate was collected in an Eppendorf tube containing 100 µl of 1 M Tris-HCl, pH 7.4, and 34 µl of 5 M NaCl. The resin was then washed with 100 mM Tris-HCl, pH 8.8, until the pH of the column effluent was neutral. A basic elution was done with 1 ml of 100 mM triethylamine, pH 11.5; the eluate was collected in a tube containing 100 µl of 1 M Tris-HCl, pH 7.4, and 34 µl of 5 M NaCl. The acidic and basic eluates were analyzed by UV absorbance and Western blot.

### 2.2.3 Western blot

For the SDS-PAGE, all purified protein samples contain 50 ng protein; and the *H. modesticaldum* cell lysate contain 1.43 µg BChlg. The BChlg concentration of the *H. modesticaldum* cells was determined by acetone extraction with the extinction coefficient 76 mM⁻¹ cm⁻¹. The cell pellet was treated with BugBuster® Master Mix (Novagen, 71456-4) to liberate proteins. All samples were incubated with loading buffer (125 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 2% 2-mercaptoethanol) and heated at 65 °C for 10 min before loading into a 15% gel.

Proteins separated by SDS-PAGE were semi-dry transferred from gel onto nitrocellulose membrane using Trans-Blot SD (Bio-Rad). The membrane was blocked by 2% BSA in TBST buffer at room temperature for 1 h before incubated with first antibody at 1:100 dilution at 4 °C.
overnight. The membrane was washed 3 times with TBST buffer, and incubated with HRP-
conjugated secondary antibody (Rockland Immunochemicals for Research, 611-1302) at 1:20000
dilution at room temperature for 1 h. The membrane was again washed with TBST for 3 times
before detected by Pierce ECL western blot substrate (Thermo Scientific, 32106).

2.2.4 Construction of the expression vectors for PshBI and PshBII

Both HM1_1462 (pshBI) and HM1_1461 (pshBII) contain codons that are rarely used in
E. coli. To increase the expression level of the two proteins, codon optimization was carried out.
The gene pshBI was manipulated by PCR to change the rare codons in both N- and C-termini.
The forward and reverse primers were designed as: 5’-
TATATGGATCCGTACAAAAATCACCAGATGCGTGCATGCACCACGCCGTGC
GCGCGGTGC-3’
(FpshIr) and 5’-TTCTAAGATCCGTACAGCCGGGATGATGGCGTCCACCAGG
CGAC-3’
(RpshIr). The template used in PCR was pET15bPshBI. The PCR product was double digested
with Ndel and BamHI, and inserted into the plasmid pET42b (Novagen) digested using same two
enzymes, resulting in pET42bPshBI.

The codon-optimized pshBII was synthesized by Integrated DNA Technologies (IDT).
All the codons in this oligo have been changed into the most frequently used ones in E.coli, and
an Ndel restriction site in N terminus and a BamHI site in C-terminus were designed in it. The
synthesized oligo was double digested by Ndel and BamHI, and then inserted into the backbone
of pET42b digested by the same two enzymes, resulting a pET42bPshBII.

Primers were designed to sub-clone the codon changed DNA fragments from
pET42bPshBI and pET42bPshBII into another plasmid pET32a. The forward and reverse
primers for pshBI are: 5’-TACCATGGCGTGACAAAATACTCACC-3’ (FPshBI) and 5’-
ACAGAAATTCTGATCCGTACAGCTAC-3’ (RPshBI). The forward and reverse primers for
pshBII are: 5’-TACCATGGTGATATAGCGATGCG-3’ (FPshBII) and 5’-TGTACAGAATTCGGATCTTAGCCTCC-3’ (RPshBII). Template pET42bPshBI and pET42bPshBII were used in PCR. NcoI and BamHI double digested PCR products and pET32a were ligated to generate pET32aPshBI and pET32aPshBII. All sequences were determined by the Penn State Nucleic Acid Facility.

2.2.5 Purification of untagged PshBI and PshBII

The pET32aPshBI, or pET32aPshBII, plasmid, was transformed into Rosetta 2 (DE3) competent cells, and plated onto LB plate that contained 100 µg/ml Ampicillin (A100) and 20 µg/ml Chloramphenicol (C20). A single colony was picked from the overnight-incubated plate to inoculated 100 ml A100C20 LB medium, and grown overnight at 37°C. Three 10 ml aliquots of the overnight-grown culture were used to inoculate 3 liters of A100C20 LB medium. Until the OD<sub>600</sub> reached 0.6, IPTG was added to a final concentration of 1 mM. The cells were harvested by centrifugation after a 4-hour growth period. The cell pellet was resuspended in lysis buffer, which contained 50 mM Tris, pH 7.5, 500 mM NaCl, 500 µM PMSF, 10% glycerol, 10 mM 2-ME, and 5 mM imidazole, and lysed using a French Press. The cell lysate was centrifuged at 30,000 rpm for 20 min, and the supernatant was loaded onto a Ni-NTA column. The column was washed with 10 volumes of washing buffer (50 mM Tris, pH7.5, 500 mM NaCl, 10 mM imidazole), and fusion proteins PshBI and PshBII were eluted with 3 volumes of elution buffer (50 mM Tris, pH7.5, 500 mM NaCl, 250 mM imidazole). The purity of the protein was established by SDS-PAGE.

The eluted fusion protein was dialyzed against 50 mM Tris, pH 7.5, and concentrated under argon using an Amicon ultrafiltration cell fitted with a Millipore 3 kDa membrane. The purified fusion protein was then cleaved by recombinant enterokinase (Novagen, 69066-3).
overnight at room temperature. The enterokinase cleavage mixture was loaded onto a Ni column that was equilibrated with 50 mM Tris, pH 7.5, and the untagged PshBI or PshBII protein was collected in the flow-through. The purified, untagged PshBI and PshBII proteins were analyzed by SDS-PAGE with silver stain, and quantified using the Lowry method (Thermo Scientific, 23240).

2.2.6 Reconstitution with FeS clusters

The purified untagged PshBI or PshBII protein was diluted with 50 mM Tris, pH8.3, to a final concentration of 100 µM and a final volume of 25 ml, and then incubated with 500 mM 2-ME. Ferrous ammonium sulfate was added drop wise to a final concentration of 2 mM, and the protein was allowed to incubate for 10 minutes. When the mixture turned brown, sodium sulfide was added drop-wise to a final concentration of 2 mM, and the protein was allowed to incubate at 4°C overnight. The reconstituted protein was washed with the buffer contained 50 mM Tris, pH8.3, 40 mM 2-ME, and 15% glycerol, and concentrated by ultrafiltration with 1 kDa membrane. After reconstitution, the concentration of holoprotein was determined by using a previously published molar extinction coefficient (34). All manipulations were performed anaerobically.

2.2.7 Preparation of Heliobacterial reaction centers

The cells of *H. modesticaldum* were grown anaerobically in PYE medium plus 0.001% resazurin, an oxygen reporter dye. The medium was degassed and incubated in an anaerobic chamber until it turned yellow, indicating it was devoid of oxygen. The medium was then inoculated using Hungate technique (35), and grown under white light illumination at 48°C. The
cells were harvested in their late-exponential phase (about 48 to 72 h) by centrifugation at 7000 rpm, and resuspended in 50 mM MOPS, pH 7.0. Whole cells were lysed by sonication, and the unbroken cells were removed by centrifugation at 10000 rpm. The cell membranes were obtained by ultracentrifugation at 45,000 rpm for 1 h, and resuspended in 50 mM MOPS, pH 7.0, to a BChl g concentration of 500 µM. To solubilize the membranes, 1% n-dodecyl-β-D-maltoside (β-DM) was added to the membrane and incubated for 2 h; insoluble fragments were removed by ultracentrifugation at 45,000 rpm for 30 min. To obtain HbRC cores devoid of PshBI/PshBII, the detergent-solubilized membranes were loaded to a diethylaminoethyl cellulose anion-exchange column equilibrated with 50 mM MOPS, pH 7.0, plus 0.02% β-DM. All manipulations were performed anaerobically.

### 2.2.8 Time-resolved optical spectroscopy in the near-infrared region

The charge recombination kinetics between \( F_X^- \), or \([F_A/F_B]\) and P798\(^+\) were measured by a flash induced absorbance change at room temperature after a saturating laser flash. The spectrophotometer was laboratory built. The sample was probed by a continuous measuring beam at 798 nm isolated from a 400 W tungsten-halogen lamp with a ¼ meter monochromator. A shutter admitted the measuring beam 10 ms prior to the laser flash. The laser flash was produced by an Nd:YAG laser operating in the second harmonic (\( \lambda = 532 \) nm) with a 7 ns pulse duration and an energy of about 2 mJ/cm\(^2\). After passing through the sample, the measuring beam was monitored by a reverse-biased Si photodiode that was shielded from stray light with a 798 nm interference filter. The signal was amplified by a Tektronix AM502 differential amplifier and digitized by a 16-bit NI SCC-68 National Instruments digitizer (Austin, TX) operating at 100,000 samples s\(^{-1}\). The sequence of events for this assay was controlled by a custom Labview software. The samples were prepared anaerobically in dark, and placed in a quartz cuvette with a
10 mm path length. All of the samples contained 9.1 µM HbRC cores (which corresponds to an absorbance of ~1 at 788 nm), 0.01% β-DM and 10 mM ascorbate in 5 mM Tris, pH 8.0. The concentration of untagged PshBI or PshBII holoprotein for each experiment is indicated in the figure legend. The kinetic traces were analyzed using IgorPro (Lake Oswego, OR) by fitting to a multiexponential decay.

### 2.2.9 Low-temperature X-band EPR spectroscopy

Low-temperature EPR spectroscopy was performed using a Bruker ECS-106 X-band spectrometer equipped with an Oxford liquid helium cryostat and temperature controller. The concentration of reconstituted untagged PshBI was 364 µM and the concentration of reconstituted untagged PshBII was 123 µM. The samples were chemically reduced with 33 mM sodium dithionite in 100 mM glycine at pH 10.0. A difference spectrum was constructed by subtracting the as prepared EPR spectrum (no additions) from the chemically reduced EPR spectrum. The sample of HbRC cores and PshBI holoprotein complexes contained 1.14 mM BChlg, 51.8 µM reconstituted PshBI, and 10 mM ascorbate. The sample of HbRC cores and PshBI holoprotein complexes contained 1.73 mM BChlg, 78.5 µM reconstituted PshBI, and 10 mM ascorbate. The samples were prepared anaerobically, frozen in dark, and illuminated using a Millennia™ diode-pumped cw visible laser operated at 2.0 W. A difference spectrum was prepared by subtracting the spectrum of a dark-adapted sample from the spectrum of an illuminated sample.
2.3 Results

2.3.1 RT-PCR revealed that both *pshBI* and *pshBII* are transcribed into mRNA

As mentioned in the introduction, analysis of the two adjacent genes *pshBII* and *pshBI* indicated that their protein products show a high degree of similarity. To address the question of whether both of the two genes are transcribed in *H. modesticaldum*, RT-PCR was carried out. Figure 2.2 shows the RT-PCR products in a 1% agarose gel. Lane 1 shows the RT-PCR product amplified from primer pair Fwd1 and Rvs1. Lane 2 shows the RT-PCR product amplified from primer pair Fwd2 and Rvs2. Lane 3 shows a RT-PCR product using the forward primer of *pshBII* Fwd1 and the reverse primer of *PshBI* Rvs2. These results indicate that both *pshBII* and *pshBI* are transcribed under the prescribed cell growth conditions. The single band in Lane 3 further indicates that both genes are transcribed as a single transcript in *H. modesticaldum*.

2.3.2 Western blot confirmed the expression of PshBI and PshBII in *H. modesticaldum*

To study the expression of PshBI and PshBII in *H. modesticaldum*, western blot analysis was carried out using affinity-purified polyclonal antibodies against PshBI-His (Figure 2.3 A) and PshBII-His (Figure 2.3 B), respectively. The concentration of the purified antibody was calculated from the absorbance on 280 nm using an extinction coefficient 210000 M\(^{-1}\)cm\(^{-1}\). Both of the antibodies were eluted in acid elution buffer with a concentration of 0.1 mg/ml. The acid eluates were used as the first antibodies to detect the presence of PshBI and PshBII in *H.modesticaldum* cell lysate.

In Figure 2.3 A, the band around 5 kDa in lane 1 shows the presence of PshBI in the *H. modesticaldum* cell lysate; compare with lane 2, which contains 50 ng of untagged PshBI. Because the antibody was produced against PshBI-His, 50 ng of PshBI-His was used as a positive
control of the western blot in lane 3. The strong signal at ~7.5 kDa suggests a high affinity of the anti-PshBI-His antibody. The higher molecular mass band represents an impurity during the purification of the PshBI-His protein. Lane 4 contains 50 ng of PshBII untagged protein, which is used to test the specificity of anti-PshBI-His. No signal was detected in lane 4.

In Figure 2.3 B, anti-PshBII-His antibody is shown to detect purified PshBII-His (lane 3), untagged PshBII (lane 2), and PshBII protein in *H. modesticaldum* cell lysate (lane 1). No cross-reaction was found between anti-PshBII-His antibody and PshBII untagged protein (lane 4).

### 2.3.3 Codon usage optimization and the expression constructs

*H. modesticaldum* and *E. coli* have different codon usage preferences. The gene sequences of *pshBI* and *pshBII* reveal that the tRNAs for 10 codons of PshBI and 13 codons of PshBII are poorly expressed in *E. coli* (Figure 2.4 A and C). To express PshBI and PshBII efficiently in *E.coli*, the codon usage of these two proteins was optimized. Figure 2.4, panel A and B show the codon usage of PshBI before and after optimization, respectively. The Y-axis values indicate the frequencies of the codon usage by *E. coli*. Using PCR, most of the rare codons in *pshBII* were changed into more frequently used codons in *E. coli*, leaving only three codons in the middle region of the gene with a usage frequency under 20. Panel C and D respectively show the codon usage of PshBII before and after optimization. In the synthesized gene fragment (Figure 2.4 D), all codons were changed into the most frequently used codons in *E. coli*.

The over-expression of PshBI and PshBII was first attempted with pET42bPshBI and pET42bPshBII, and thereafter with several other pET constructs, including pET3atr, pET11a. However, the expression of wild-type PshBI/PshBII was not detected by SDS-PAGE, even after silver staining. Therefore, the expression vector was changed to pET32a. The plasmid pET32a
adds a cleavable tag in the N-terminus of the fusion protein. The cleavable tag contains a 109 aa Trx-Tag™ thioredoxin protein, His-Tag®, S-Tag™ sequences, and an enterokinase site. The large mass of the fusion tag makes the expression of small proteins much easier, and the enterokinase site leaves only one extra amino acid, alanine, at the N-terminus of the wild-type protein.

The construction of expression vectors for PshBI and PshBII are shown in Figure 2.5, panel A and panel B respectively. The plasmid pET32aPshBI is 6059 bp; and pET32aPshBII is 6053 bp. Panel C shows a zoom-in depiction of the expression constructs, including the cleavable thioredoxin-tag, His-tag, S-tag, enterokinase cleavage site, and the codon-optimized genes. The His-tag in the construct was used in the purification process of the fusion proteins and the enterokinase was used to cleave the fusion tag.

2.3.4 Purification of the untagged PshBI and PshBII

The purification of untagged PshBI and PshBII was achieved in three steps: 1, purification of the fusion protein using a Ni column; 2, cleavage of the fusion tag using enterokinase; 3, removal of the cleaved tag by chromatography over another Ni column. Figure 2.6 A shows the purification of untagged PshBI. Lane 1 shows the pre-stained protein ladder. Lane 2 shows the purified fusion PshBI, which is about 23 kDa in mass. Lane 3 shows the enterokinase cleaved mixture in which the 23 kDa band has disappeared, indicating the fusion protein had been fully digested. Lane 4 and 5 show two fractions of purified untagged PshBI, which is about 5.5 kDa in mass. Figure 2.6 B shows purified untagged PshBII, which is also about 5.5 kDa in mass.
2.3.5 Reconstitution of FeS clusters in PshBI and PshBII

Both purified untagged PshBI and PshBII were reconstituted with FeS clusters using inorganic reagents. The spectra of the PshBI and PshBII apoproteins show no significant absorbance in the visible region, whereas the spectra of the reconstituted holoproteins show a broad absorbance around 400 nm (Figure 2.7 A and B). This feature is characteristic of S → Fe charge transfer bands, indicating that the FeS clusters have been inserted into the untagged proteins. The absorbance at 400 nm was used to measure the concentration of reconstituted PshBI and PshBII using a previously published extinction coefficient 30.3 mM⁻¹cm⁻¹ (34).

Low temperature EPR spectra of the chemical reduced PshBI or PshBII holoproteins show a magnetic interaction spectrum from the F_A⁻ and F_B⁻ resonances. The spectrum of sodium dithionite-reduced PshBI at 15 K shows apparent resonances at g = 2.053, 1.995, 1.975, 1.948, 1.914, 1.887 (Figure 2.8 A); the spectrum of PshBII at 15 K shows apparent resonances at g = 2.046, 2.003, 1.973, 1.927, 1.889 (Figure 2.8 B). Both optical and EPR spectra are characteristic of the presence of two [4Fe-4S]¹⁺/²⁺ clusters in PshBI and PshBII.

2.3.6 Room temperature electron transfer from F_X⁻ to F_A/F_B

To study the ability of exogenously expressed, untagged PshBI and PshBII to interact with HbRC cores, the kinetics of P₇₉₉⁺ charge recombination was measured at room temperature using time-resolved optical spectroscopy. It has been shown that the charge recombination kinetics in freshly isolated HbRC complexes are biphasic, with lifetimes of 15 ms and 75 ms (24). The τ = 15 ms phase originates from the charge recombination between P₇₉₉⁺ and F_X⁻, and the τ = 75 ms phase originates from the charge recombination between P₇₉₉⁺ and [F_A/F_B]⁻ (24, 29). In this study, the isolated HbRC cores show monoexponential kinetics with a lifetime of 15 ms.
(Figure 2.9 A), indicating the complete removal of F_A/F_B protein. When PshBI was added into the HbRC cores in a 1:1 ratio, a slow kinetic phase appeared with $\tau = 79$ ms and an amplitude that accounted for 32.4% of total signal (Figure 2.9 B). At a 5-fold molar excess of PshBI over HbRC cores, the signal amplitude of the $\tau = 71$ ms phase increased to 55.4%, while the amplitude of the $\tau = 10$ ms phase decreased to 28.2%. At the same time, a kinetic phase with longer lifetime ($\tau = 518$ ms) appeared, which accounted for 16.5% of the total signal amplitude (Figure 2.9 C). At a 10-fold molar excess of PshBI over HbRC cores, the kinetics remained same as those with a 5-fold molar excess of PshBI over HbRC cores (Table 2.1).

When PshBII was added into the HbRC cores in a 1:1 ratio, both the $\tau = 60$ ms and $\tau = 213$ ms kinetic phases appeared in addition to a kinetic phase with $\tau = 11$ ms. The signal amplitude of the $[F_A/F_B]^- \text{to } P_{798}^+$ contribution is about 1/4 of the signal amplitude of the $F_X^- \text{to } P_{798}^+$ contribution (Figure 2.10 A). When 5-fold molar excess of PshBII was added to the HbRC cores, the signal amplitude of the $\tau = 247$ ms phase increased to 47.2%, while the ratio of the $[F_A/F_B]^- \text{to } P_{798}^+$ contribution relative the $F_X^- \text{to } P_{798}^+$ contribution increased to 1:1 (Figure 2.10 B). At a 10-fold molar excess of PshBII over HbRC cores, the amplitude of $\tau = 274$ ms phase was 54.9%, while the ratio of the $[F_A/F_B]^- \text{to } P_{798}^+$ contribution relative the $F_X^- \text{to } P_{798}^+$ contribution was 2:1 (Figure 2.10 C). Even with 10-fold molar excess of PshBI or PshBII over HbRC cores, the charge recombination from $F_X^- \text{to } P_{798}^+$ can still be detected, indicating that not all binding sites on the HbRCs were occupied by PshBI or PshBII.

When both PshBI and PshBII were added into the HbRC cores in a 1:1:1 ratio, biphasic kinetics were detected, with lifetimes of 16 ms and 132 ms (Table 2.1). When 5-fold molar excess of PshBI and PshBII were added to the HbRC cores, the signal amplitude of the $\tau = 76$ ms phase increased to 51.5%, while the kinetic phase with a longer lifetime ($\tau = 256$ ms) appeared, which accounted for 23.8% of the total signal amplitude (Table 2.1). At a 10-fold molar excess of PshBI and PshBII over HbRC cores, the signal amplitude of $\tau = 9$ ms phase decreased to 9.6%;
the amplitude of \( \tau = 209 \) ms phase increased to \( 42.3\% \); while the amplitude of the \( \tau = 45 \) ms phase remained \( \sim 50\% \) (Table 2.1). When 20-fold molar excess of PshBI and PshBII were added into the HbRC cores, the amplitude of each kinetic phase remained the same as which obtained when 10-fold molar excess of PshBI and PshBII were combined with HbRC cores (Table 2.1).

### 2.3.7 EPR spectroscopy of HbRC cores with PshBI or PshBII holoprotein

Low temperature EPR spectroscopy was also carried out to monitor the electron transfer from HbRC cores to PshBI and PshBII. When HbRC cores are reconstituted with either PshBI or PshBII holoprotein, both samples show two sets of light-induced signals. One is the isotropic resonance at \( g = 2.003 \) (Figure 2.11 A and B), which is characteristic of \( P_{798}^+ \) (29). The other is a set of rhombic resonances characteristic of the reduced \( F_A \) cluster (29, 30), consistent with the proposal that \( F_A \) cluster is reduced at a lower temperature and \( F_B \) is reduced at a higher temperature (31). The resonances of PshBI after combination with HbRC cores and illumination occur at \( g = 2.068, 1.928, 1.890 \) (Figure 2.11 A), whereas the resonances of PshBII after combination with HbRC cores and illumination occur at \( g = 2.079, 1.928, 1.892 \) (Figure 2.11 B). The g-values as well as the spectra shapes are similar to those found either with genuine PshBI (29), or with His-tagged recombinant PshBI (29) or PshBII (30). It can be concluded that both PshBI and PshBII are able to accept electron from HbRC cores at low temperature.
2.4 Discussion

2.4.1 The transcription and translation of PshBI and PshBII

The family Helionacteriaceae was discovered in 1983 (10). Hence, the study of the Heliobacterial reaction center till today has only about 20 years of history. The anoxic living conditions of Heliobacteria make the research in this field relatively more difficult than the study of cyanobacteria. Unlike the $F_A/F_B$ containing protein PsaC in PS I, there are two $F_A/F_B$ containing proteins in $H. modesticaldum$, PshBI and PshBII, which function as the terminal electron acceptors in HbRC (30). This is the first study of its kind to monitor the transcription and translation of PshBI and PshBII, and to show the presence of these two proteins in $H. modesticaldum$ cells.

RT-PCR results show that $pshBI$ and $pshBII$ are transcribed into a single transcript. Western blot analysis indicates that PshBI and PshBII are both expressed under the same standard conditions. The affinity and specificity of the affinity-purified antibodies were also studied by western blot analysis. Because the identity between PshBI and PshBII is as high as 62%, the specificity of the two antibodies was a concern in generating polyclonal rather than monoclonal antibodies. Surprisingly, no signal was detected with anti-PshBII-His antibody and PshBI untagged protein, or between anti-PshBI-His antibody and PshBII untagged protein. This confirms that the band detected by anti-PshBII-His in the cell lysate is PshBII and not PshBI.

2.4.2 Over-expression small proteins like PshBI and PshBII in $E. coli$

To express His-tag labeled PshBII in $E. coli$, the rare codons near the 5’ and 3’ ends of the gene $pshBII$ were replaced with codons expressed at higher levels in $E. coli$ (30). However, in this study, even with all codons changed into the most frequently used in $E. coli$, the over-
expression of wild type PshBII was not detectable. After trying several different expression vectors, we found that PshBI and PshBII are easier to express with a fusion tag. With a His-tag or a thioredoxin-tag, the expression levels of the fusion proteins were high enough to be visualized by Coomassie-stained SDS-PAGE. The low expression levels of PshBI and PshBII without fusion tags might be because the particular translation initiation sequence is not efficiently utilized in *E. coli* (36). It is also possible that the expressed exogenous proteins PshBI and PshBII are quickly degraded by proteases because of their small molecular masses. Due to its cleavable tag, pET32a was used in constructing expression vectors for PshBI and PshBII. After cleavage with enterokinase, only one additional amino acid, alanine, remained on the N-terminus of PshBI or PshBII. The untagged PshBI and PshBII expressed and purified by this method can be used to determine protein structures by NMR or by X-ray crystallography. This work is being carried out in a separate effort.

### 2.4.3 Untagged PshBI and PshBII holoproteins contain two [4Fe-4S] clusters

Both of PshBI and PshBII untagged proteins have two CxxCxxCxxxC motifs in their amino acid sequences indicating that the proteins are able to bind two [4Fe-4S] clusters. After aerobic purification, both apoproteins were reconstituted with ferrous ammonium sulfate and sodium sulfide in present of 2-ME. The reconstituted holoproteins were examined by optical absorbance spectroscopy and by low temperature EPR spectroscopy. Both reconstituted, untagged PshBI and PshBII proteins showed a brown color and a broad absorbance peak at 400 nm in their absorbance spectra, indicating that their FeS clusters had been inserted. The low temperature EPR spectra of $F_A^-$ and $F_B^-$ in the PshBI and PshBII holoproteins showed a set of resonances similar to that of $F_A^-$ and $F_B^-$ in genuine PshB (29), as well as that of recombinant, His-tagged PshBI (30) and PshBII (29). Therefore, it is confirmed that two [4Fe-4S] clusters
have been successfully reconstituted in untagged PshBI and PshBII. The g values of the resonances are similar to His-tagged PshBI and PshBII, although the features of the spectra obtained with untagged PshBI and PshBII are more pronounced than those in His-tagged PshBI and PshBII. It is possible that the His-tag at the N-termini of the recombinant PshBI and PshBII alters the protein structures in a manner that changes the environment of the \( F_A/F_B \) clusters.

### 2.4.4 Comparison of PshBI and PshBII in accepting electron from HbRC cores

It has been proposed that as a consequence of their high similar amino acid sequences, their masses, and their acidic isoelectric points PshBI and PshBII share similar biochemical functions \((30)\). Indeed, the recombinant His-tagged PshBI and PshBII proteins have been shown to accept electrons from \( F_X \) cluster in HbRC cores by time-resolved optical spectroscopy and by light-induced EPR spectroscopy \((29, 30)\). However, to achieve the same level of electron transfer activity, a 10-fold excess of PshBI-His over PshBII-His is required. To eliminate possible artifacts resulting from different protein tags, untagged PshBI and PshBII were compared using the absorbance at 400 nm to normalize holoprotein concentrations.

Light-induced EPR spectra of HbRC cores combined with same molar ratio of untagged PshBI or PshBII holoprotein indicate that both proteins can bind to the \( P_{798}/F_X \) cores and accept electrons from the \( F_X \) cluster. However, to detect the resonances arising from light-reduced PshBII, the sample of PshBII and HbRC cores needed to be concentrated 1.5 fold compared to that of PshBI and HbRC cores. The light induced resonances of PshBI and \( P_{798} \) at 15 K are visible at \( g = 2.068, 2.003, 1.928 \) and 1.890. The light induced resonances of PshBII and \( P_{798} \) at 15 K are visible at \( g = 2.079, 2.003, 1.928 \) and 1.892. These \( g \) values are consistent with those in genuine PshB, and in recombinant His-tagged PshBI and PshBII \((29, 30)\), confirming that the \( F_A \) clusters in PshBI and PshBII are reduced by electrons from \( F_X \).
In addition to low temperature EPR spectroscopy, charge recombination kinetics of HbRC cores in the presence and absence of PshBI/PshBII were studied at room temperature. In the absence of PshBI or PshBII, the HbRC cores display monoexponential kinetics with a lifetime of 15 ms. As PshBI/PshBII is added to HbRC cores, the signal amplitude of the short lifetime ($\tau_1$) kinetic phase decreases, and the amplitude of the faster ($\tau_2$) lifetime phase increases (Table 2.1). A longer phase with a lifetime of ~500 ms appears when a 5-fold molar excess of PshBI is added to HbRC cores. Similar results were found with PshBII, except that the lifetime of longer phase was ~200 ms and it appeared when PshBII was added in a 1:1 ratio with HbRC cores. This longer phase in the charge recombination kinetics was previously observed in a study of HbRC cores and His-tagged PshBII (30). It was thought to be caused by a diffusion-mediated interaction of PshBII with HbRC cores (30). The longer phases (500 ms for PshBI and 200 ms for PshBII) found in this study can also be explained using the mobile or semi-mobile model of PshBI and PshBII. Recently, this model has also been used to explain the fluorescence modulation in H. modesticaldum (37).

If we compare the ability of the two holoproteins PshBI and PshBII to accept electrons from HbRC cores, it appears that PshBI has a higher affinity for HbRC cores than does PshBII. For the same ratio of $F_A/F_B$ protein over HbRC cores, the signal amplitude fitting for $\tau_2$ phase (from $[F_A/F_B]$ to $P_{798^+}$) is always higher with PshBI than with PshBII (Table 2.1). It has been argued that the presence of PshBI and PshBII may simply serve to increase the titer of $F_A/F_B$ proteins in the cell (30). This reasoning is supported by the fact that HM1_1461, which codes for PshBII, and HM1_1462, which codes for PshBI, are in the same operon and thus likely to be similarly controlled. However, the differences observed between the two proteins in the charge recombination kinetics experiments hint they may have different affinities and therefore may interact preferentially with different redox partners in the cell.
2.5 Figure legends

**Figure 2.1** Schematic representation of plasmid pET15bPshBI.

**Figure 2.2** RT-PCR results in a 1% agarose gel. Lane 1 shows RT-PCR products using primers Fwd1 and Rvs1. Lane 2 shows RT-PCR products using primers Fwd2 and Rvs2. Lane 3 shows RT-PCR products using primers Fwd1 and Rvs2.

**Figure 2.3** Immunoblot analysis of PshBI (A) and PshBII (B) in a *H. modesticaldum* cell lysate. For panel A: lane 1 shows PshBI in a *H. modesticaldum* cell lysate; lane 2 shows purified untagged PshBI apoprotein; lane 3 shows purified PshBI-His apoprotein; lane 4 shows purified untagged PshBII apoprotein. For panel B: lane 1 shows PshBII in a *H. modesticaldum* cell lysate; lane 2 shows purified untagged PshBII apoprotein; lane 3 shows purified PshBII-His apoprotein; lane 4 shows purified untagged PshBI apoprotein. All purified protein samples contain 50 ng protein; the *H. modesticaldum* cell lysate contains 1.43 µg BChlg.

**Figure 2.4** Codon usages of PshBI (A), optimized PshBI (B), PshBII (C) and optimized PshBII (D).

**Figure 2.5** Schematic representations of plasmid pET32aPshBI (A), plasmid pET32aPshBII (B), the fusion tags, and the enterokinase cleavage site of the fusion proteins (C).

**Figure 2.6** SDS-PAGE gel of untagged PshBI (A) and PshBII (B) after purification. For panel A: lane 1 shows the pre-stained protein marker; lane 2 shows the purified fusion protein of PshBI; lane 3 shows the enterokinase cleaved fusion PshBI protein; lane 4 shows the first fraction of
untagged PshBI; lane 5 shows the second fraction of untagged PshBI. For panel B: lane 1 shows the pre-stained protein marker; lane 2 shows untagged PshBII.

**Figure 2.7** Absorbance spectra of the untagged PshBI (A) and PshBII (B) after reconstitution with 2-ME, ferrous ammonium sulfate and sodium sulfide.

**Figure 2.8** Low temperature EPR spectra of untagged PshBI (A) and PshBII (B) holoproteins. The samples were treated with 33 mM sodium dithionite in 100 mM glycine, pH10, to reduce the FeS clusters. The spectrometer conditions were as follows: temperature, 15K; power, 126 mW; microwave frequency, 9.486 GHz; receiver gain 1600; modulation amplitude, 10 G at 100 kHz. Corresponding g values are displayed above the spectrum.

**Figure 2.9** Charge recombination kinetics followed by flash-induced absorption changes at 798 nm in (A) HbRC cores, (B) HbRC cores with untagged PshBI holoprotein in a 1:1 ratio, (C) HbRC cores with untagged PshBI holoprotein in a 1:5 ratio. The residuals for each of the fits are shown in the upper panel of each trace.

**Figure 2.10** Charge recombination kinetics followed by flash-induced absorption changes at 798 nm in (A) HbRC cores with untagged PshBII holoprotein in a 1:1 ratio, (B) HbRC cores with untagged PshBII holoprotein in a 1:5 ratio, (C) HbRC cores with untagged PshBII holoprotein in a 1:10 ratio. The residuals for each of the fits are shown in the upper panel of each trace.

**Figure 2.11** Light-induced EPR difference spectra of HbRC cores reconstituted with untagged (A) PshBI and (B) PshBII holoproteins. The spectrometer conditions were as follows:
temperature, 15 K; power, 100 mW; microwave frequency, 9.486 GHz; receiver gain, 20000; modulation amplitude, 10 G at 100 kHz.
Figure 2.1
Figure 2.2

![ gel electrophoresis image showing bands at 400 bp and 200 bp with lanes labeled 1, 2, 3]
Figure 2.3

A

10 kDa

4.6 kDa

B

10 kDa

4.6 kDa
Figure 2.5

A

B

C

Trx•tag  His•tag  S•tag  Enterokinase
codon optimized HM1_1461 or 1462
Figure 2.6

A

B
Figure 2.7

A

B
Figure 2.8

A

B
Figure 2.9

A

B

C
Figure 2.10

A

B

C
Figure 2.11

A

B

mT

300 320 340 360 380

2.068 2.003 1.928 1.890

2.079 2.003 1.928 1.892
Table 2.1 Room temperature charge recombination kinetics followed by flash-induced absorption changes at 798 nm. The lifetimes $\tau_1$, $\tau_2$ and $\tau_3$ represent three kinetic phases extracted from the multiexponential decay. The numbers in parenthesis are the percentages of the total signal amplitude.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\tau_1$, ms</th>
<th>%</th>
<th>$\tau_2$, ms</th>
<th>%</th>
<th>$\tau_3$, ms</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbRC core</td>
<td>15</td>
<td>(100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PshBI:core=1:1</td>
<td>12</td>
<td>(67.6)</td>
<td>79</td>
<td>(32.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PshBI:core=5:1</td>
<td>10</td>
<td>(28.1)</td>
<td>71</td>
<td>(55.4)</td>
<td>518</td>
<td>(16.5)</td>
</tr>
<tr>
<td>PshBI:core=10:1</td>
<td>16</td>
<td>(22.9)</td>
<td>85</td>
<td>(52.3)</td>
<td>544</td>
<td>(24.8)</td>
</tr>
<tr>
<td>PshBII:core=1:1</td>
<td>11</td>
<td>(63.1)</td>
<td>60</td>
<td>(15.1)</td>
<td>213</td>
<td>(21.8)</td>
</tr>
<tr>
<td>PshBII:core=5:1</td>
<td>8</td>
<td>(26.3)</td>
<td>52</td>
<td>(26.5)</td>
<td>247</td>
<td>(47.2)</td>
</tr>
<tr>
<td>PshBII:core=10:1</td>
<td>6</td>
<td>(14.5)</td>
<td>55</td>
<td>(30.6)</td>
<td>274</td>
<td>(54.9)</td>
</tr>
<tr>
<td>PshBI:PshBII:core=1:1:1</td>
<td>16</td>
<td>(76.5)</td>
<td>132</td>
<td>(23.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PshBI:PshBII:core=5:5:1</td>
<td>10</td>
<td>(25.1)</td>
<td>76</td>
<td>(51.1)</td>
<td>256</td>
<td>(23.8)</td>
</tr>
<tr>
<td>PshBI:PshBII:core=10:10:1</td>
<td>9</td>
<td>(9.6)</td>
<td>45</td>
<td>(48.1)</td>
<td>209</td>
<td>(42.3)</td>
</tr>
<tr>
<td>PshBI:PshBII:core=20:20:1</td>
<td>8</td>
<td>(13.4)</td>
<td>61</td>
<td>(51.4)</td>
<td>234</td>
<td>(35.2)</td>
</tr>
</tbody>
</table>
Proteins that may interact with PshBI/PshBII in *H. modesticaldum*

3.1 Introduction

Unlike their counterpart protein PsaC in PS I, PshBI and PshBII are loosely bound to HbRC cores and therefore might function as mobile electron carriers. In cyanobacterial PS I, the light-driven electron transferred from P700 to F₈ is further transferred to the soluble electron carrier ferredoxin, and finally to the enzyme ferredoxin: NADP⁺-reductase (FNR), which reduces NADP⁺ to NADPH. In *H. modesticaldum*, however, no petF-like gene that would code for a [2Fe-2S] cluster ferredoxin has been found. Therefore, PshBI and PshBII might associate directly with partner redox protein(s). In the following section, a study using pull-down assays and co-immunoprecipitation (co-IP) to search for potential interacting partners of PshBI and PshBII is reported. Several candidates were obtained from pull-down assays and identified by mass spectrometry (MS).

3.2 Materials and Methods

3.2.1 Preparation of soluble proteins from *H. modesticaldum*

The methods described in Chapter 2 were used to grow, harvest, and break *H. modesticaldum* cells, and to prepare HbRC cores. After sonication, the cell lysate was ultracentrifuged at 45,000 rpm for 1 h, and the supernatant containing soluble proteins were saved for pull down assays and Co-IP.
3.2.2 Pull down assay with PshBI-His and PshBII-His

The expression vectors pET15bPshBI (Figure 2.1) and pET28bFD1 (30) were transformed into Rosetta 2 (DE3) competent cells to over-express PshBI-His and PshBII-His respectively. A Ni-NTA resin was used to purify the two proteins. The FeS clusters were reconstituted into the two recombinant proteins by using methods described in Chapter 2.

The pull-down assays were performed anaerobically in darkness. A 100 µg aliquot of PshBI-His holoprotein or PshBII-His holoprotein was incubated with 200 µl Ni-NTA resin at room temperature for 1 h. Unbound proteins were eliminated by 3 ml of washing buffer (5 mM MOPS, pH 7). The immobilized PshBI-His or PshBII-His holoproteins were incubated with 60 mg of soluble proteins from *H. modesticaldum* at room temperature for 1 h. The resin was washed with 3 ml of washing buffer, 1 ml of 5 mM imidazole, 1 ml of 10 mM imidazole, 1 ml of 20 mM imidazole and 1 ml of 30 mM imidazole to eliminate unspecifically-bound proteins. Candidate protein: protein interaction complexes were eluted using 40 µl of 250 mM imidazole and analyzed by SDS-PAGE. A bait control and a prey control were included in the experiment. In the bait control, no soluble proteins from *H. modesticaldum* were added into the system; in the prey control, no bait protein was added into the system. Protein identification was carried out using LC-MS/MS at the Proteomics and Mass Spectrometry Core Facility in University Park, PA.

3.2.3 Co-Immunoprecipitation

To immobilize antibodies against PshBI-His or PshBII-His onto Protein A-Sepharose, 100 µg of the affinity purified polyclonal antibody against PshBI-His or against PshBII-His was incubated with 25 µl of Protein A-Sepharose 4B Fast Flow (Sigma, P-9424) at 4 °C overnight under agitation. A negative control group was performed with 40 µl of the rabbit pre-bleed serum
and 25 µl of Protein A-Sepharose. Following procedures were performed anaerobically. A total of 10 mg of soluble protein from \textit{H. modesticaldum} was pre-cleared with 15 µl Protein A-Sepharose, and then incubated with antibody Protein A-coupled Sepharose beads on ice for 4 h under agitation. After the incubation, the beads were washed three times in 5 mM MOPS, pH 7.0. The protein complexes were eluted with 100 mM glycine, pH 2.5, followed by 2X SDS-PAGE loading buffer (4% SDS, 250 mM Tris, pH 6.8, 20% glycerol, and 4% 2-ME).

### 3.3 Results

#### 3.3.1 Potential electron transfer partners of PshBI and PshBII

Pull-down assays using reconstituted PshBI-His holoprotein and PshBII-His holoprotein as the bait were performed, and several potential proteins were identified. In each experiment, two controls were carried out, a bait control and a prey control. Those proteins that interact with PshBI/PshBII should be found in the experimental groups but not in the control groups. Using PshBI-His as the bait, five protein bands were found in the Coomassie stained-SDS-PAGE gel (Figure 3.1, lane 2 to lane 5). Multiple identical wells were used in SDS-PAGE to increase the sample amount for MS analysis. These five hits show molecular weights from about 25 kDa to 50 kDa. They are labeled as A\textsubscript{1}, B\textsubscript{1}, C\textsubscript{1}, D\textsubscript{1}, and E\textsubscript{1} from higher to lower molecular weight, respectively (Figure 3.1). Several proteins were pulled down by the PshBII-His holoprotein as shown in Figure 3.2, lane 2-5. Five protein bands, namely A\textsubscript{2}, B\textsubscript{2}, C\textsubscript{2}, D\textsubscript{2}, and E\textsubscript{2}, were cut from the gel and analyzed by MS.

Proteins were identified by MS/MS in a search of the \textit{H. modesticaldum} Ice1 genome. Results of the PshBI pull-down assay are listed in Table 3.1. The band of B\textsubscript{1} has not been identified due to the low concentration of the protein recovered from the gel. The proteins with
the highest PLGS score identified in band A, C, and E, as well as the proteins with the highest two scores found in D are shown in Table 3.1. Three proteins out of the five are NADP-dependent enzymes, including riboflavin biosynthesis protein RibD, 3-oxoacyl-(acyl-carrier-protein) reductase, and pyrroline-5-carboxylate reductase. The other two identified are sporulation protein spoIID and uracil phosphoribosyltransferase.

Among the proteins identified from the PshBII pull-down assay, the one with the highest PLGS cores in band A, C, D, E, and the highest two from band B are listed in Table 3.2. Except for the protein found in band C, which is a hypothetical protein with an unknown function, all the other proteins are enzymes involved in primary metabolism, such as glutamate metabolism, riboflavin biosynthesis, reverse TCA cycle, arginine and proline metabolism, and fatty acid biosynthesis. Also, three proteins have been found in both PshBI and PshBII pull-down assays, namely riboflavin biosynthesis protein RibD, 3-oxoacyl-(acyl-carrier-protein) reductase, and pyrroline-5-carboxylate reductase.

### 3.3.2 Pull-down assays using apoproteins PshBI-His and PshBII-His as bait

A group of control pull-down assays using PshBI-His and PshBII-His apoproteins as bait were also carried out. Unexpectedly, the proteins pulled down by PshBI-His and PshBII-His holoproteins (Figure 3.3, lane 5 and 3 respectively) from the soluble protein pool of *H. modesticaldum* were also pulled down by PshBI-His and PshBII-His apoproteins (Figure 3.3, lane 9 and 7 respectively). Without their FeS clusters, PshBI and PshBII are likely unable to fold correctly and most certainly do not function as electron carriers. These results imply that the proteins identified may be able to interact with PshBI/PshBII via some manner in which the presence of FeS clusters is not necessary. Alternatively, the potential proteins shown to interact with PshBI and PshBII in the pull-down assays are simply false positives.
3.3.3 Co-IP using antibodies against PshBI-His and PshBII-His

The polyclonal antibodies against PshBI-His and PshBII-His have been shown effective by Western blots in Chapter 2 to bind to PshBI and PshBII, respectively, in *H. modesticaldum* cell lysates with a respectable affinity and specificity. The two affinity-purified antibodies were used in a Co-IP experiment to search for the proteins that may interact with PshBI and PshBII in *H. modesticaldum*. However, no proteins were found in the experimental groups (Figure 3.4 lane1 and 2) that did not also occur in the pre-bleed control group (Figure 3.4 lane3).

3.4 Discussion

3.4.1 Potential interacting partners of PshBI and PshBII identified from pull-down assays

Using PshBI-His holoprotein and PshBII-His holoprotein as bait, several proteins have been pulled down from the pool of soluble proteins in *H. modesticaldum*. Candidates were identified from each protein band by MS/MS, but only those with PLGS scores higher than 450 are listed in the results section. Three of the candidates obtained were pulled down by both PshBI-His holoprotein and PshBII-His holoprotein: riboflavin biosynthesis protein RibD, 3-oxoaeryl-(acyl-carrier-protein) reductase, and pyrroline-5-carboxylate reductase.

Riboflavin biosynthesis protein RibD is the enzyme catalyzes the second step in riboflavin biosynthesis, from the monophosphorylated pyrimidine derivative, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate, to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinone 5′-phosphate (38, 39). RibD is a bifunctional protein containing an N-terminal deaminase and a C-terminal reductase domain; therefore it can complete the deamination of the 2-amino group and the reduction of the ribose ring at the same time (39). The C-terminal reductase
domain contains a NADPH binding site and the N-terminal deaminase active site contains a tightly bound zinc ion (40).

The enzyme 3-oxoacyl-(acyl-carrier-protein) reductase, also called as beta-ketoacyl-[acyl-carrier protein](ACP) reductase, is an enzyme involved in type II fatty acid biosynthesis (41). This NADPH-dependent enzyme belongs to the short-chain dehydrogenase/reductase superfamily, and catalyzes the conversion from 3-oxoacyl-[acyl-carrier-protein] to (3R)-3-hydroxyacyl-[acyl-carrier-protein] (41).

The enzyme pyrroline-5-carboxylate reductase catalyzes the last step in the biosynthesis of proline from glutamate, the NAD(P)H-dependent (42) reduction of 1-pyrroline-5-carboxylate into L-proline (43).

These three proteins are all NADP dependent enzymes, indicating the interaction could occur through the enzyme ferredoxin-NADP$^+$ oxidoreductase (FNR), even though the interaction between FNR and PshBI/PshBII was not captured under the experimental condition in this study. The activity of FNR has been detected in the cell extracts of *H. modesticaldum* during phototrophic and chemotrophic growth (44), and the source of electron to generate reducing power is considered to be PshBI/PshBII from photosynthesis. Therefore, FNR and PshBI/PshBII may have direct or indirect interactions, leading to detection of NADP$^\pm$ dependent enzymes of physiological relevance.

The other two proteins pulled down by PshBI-His are identified to be sporulation protein spoIID and uracil phosphoribosyltransferase. The sporulation protein spoIID is a membrane-anchored enzyme that degrades peptidoglycan and plays an essential role in engulfment during bacterial endospore formation (45). Uracil phosphoribosyltransferase is the enzyme catalyzes the conversion of uracil to uridine 5'-monophosphate (UMP) utilizing 5'-phosphoribosyl--1-pyrophosphate (PRPP) (46).
The other three proteins identified from the PshBII-His pull-down assay are putative glutamate synthase subunit, putative 2-oxoglutarate oxidoreductase, alpha subunit, and a hypothetical protein (HM1_2460). The putative glutamate synthase (GltS) subunit contains a FMN bind site, a substrate binding site and a [3Fe-4S] cluster binding site, and is very likely to be the GltS FMN binding domain. GltS is a complex iron-sulfur flavoprotein that catalyzes the reductive synthesis of L-glutamate from 2-oxoglutarate and L-glutamine using intramolecular channeling of ammonia (47). The enzyme 2-oxoglutarate oxidoreductase, also called α-ketoglutarate:ferredoxin oxidoreductase (KFOR), catalyzes the synthesis of 2-oxoglutarate from succinyl-CoA and CO₂ (48). It is a ferredoxin dependent enzyme involved in citrate cycle (TCA cycle) and reductive carboxylate cycle.

The pull-down assay is a screen for the potential interaction partners of PshBI and PshBII. It provides information about possible protein-protein interaction in *H. modesticaldum*, but it cannot capture all proteins that may interact with PshBI and PshBII. First, the nature of bacterial dicluster ferredoxins makes most interactions with enzymes transient, rapidly binding and transferring electron(s), followed by dissociation of the oxidized ferredoxins (49). The transient interactions are difficult to detect without using cross-linking methods. Second, although the whole experiment was performed in the dark under anoxic conditions, no reductant was added into the samples. Therefore, the conditions tested may limit the interactions between some proteins and the bait, redox proteins being the best example. Third, proteins used as bait are His-tag labeled PshBI and PshBII. Even though PshBI-His and PshBII-His have been shown to be able to interact HbRC core and accept electrons (29, 30), and the homology models of PshBI and PshBII (31) indicate that the N-terminal His-tag just lengthen one of the antiparallel β-sheets, it is still possible that the His-tag may affect the interaction between PshBI/PshBII and partner proteins.
3.4.2 Possible protein-protein interaction pattern utilized by PshBI and PshBII

The PshBI-His and PshBII-His apoproteins were found to be able to pull down the same candidates from *H. modesticaldum* cells as the holoproteins. This result is unexpected, if only because the presence of the FeS clusters in PshBI and PshBII should be necessary for the three-dimensional structure of these proteins, which should be necessary for protein-protein interactions with other enzymes. The interaction patterns between ferredoxin and ferredoxin-dependent enzymes have been characterized in many studies of oxygenic photosynthetic organisms. The commonly used pattern is a series of ionic bridges formed between negatively charged residues surrounding the FeS cluster of ferredoxin and the positive residues surrounding the co-factors of the partner enzymes (50-52). The fact that both PshBI and PshBII are negatively charged proteins, similar to [2Fe-2S] ferredoxin in oxygenic photosynthetic organisms, indicates that they might interact with their electron acceptor partner(s) via a similar electrostatic mechanism. However, whether ferredoxin apoproteins are able to bind to ferredoxin dependent enzymes is questionable. A recent study using ferredoxin chromatography to screen for potential ferredoxin electron transfer partners in *Synechocystis* sp. PCC 6803 indicates that many putative ferredoxin-binding proteins bind to the ferredoxin affinity column under both oxidizing and reducing conditions (53).

3.4.3 Future attempts to study the potential partners of PshBI/PshBII

This project is still in a preliminary stage: the results of pull-down assays need to be confirmed and no positive results have been obtained in the co-IP experiment. Commercial available crosslink IP kits can be used in co-IP to eliminate contamination of the antibody in the eluate. For both pull-down assays and co-IP, reductants such as sodium dithionite, can be added
to the system to generate reducing conditions so that protein-protein interactions under different redox conditions may be compared. Furthermore, potential PshBI/PshBII electron transfer partners can be cloned, expressed, purified, and the functional interaction of the partner(s) and PshBI/PshBII can be studied by biochemical approaches. To better understand possible protein-protein interactions and intermolecular electron transfers within complexes, 3-D structures of PshBI and PshBII are eagerly needed.

### 3.5 Conclusion

In the pull-down assays, several potential interacting partners of PshBI and PshBII have been captured and identified. Most of these candidates function in primary metabolism, and some of them are NADP-dependent enzymes. However, these potential interacting proteins have also been pulled down by PshBI-His and PshBII-His apoproteins. It is difficult to distinguish whether the proteins obtained in the pull-down assays are potential interacting partners of PshBI/PshBII or just false positives. Unfortunately, no candidates have been found in the co-IP experiments to support the results of the pull-down assays.
3.6 Figure Legends

**Figure 3.1** SDS-PAGE of pull-down assay results using PshBI-His holoprotein as the bait. Lane 1, molecular weight markers; lane 2 to lane 5, proteins eluted from Ni-NTA resin in the experimental group; lane 6, bait control; lane 7, prey control. The proteins pulled-down by PshBI-His holoprotein are labeled as A₁, B₁, C₁, D₁, and E₁.

**Figure 3.2** SDS-PAGE of pull-down assay results using PshBII-His holoprotein as the bait. Lane 1, molecular weight markers; lane 2 to lane 5, proteins eluted from Ni-NTA resin in the experimental group; lane 6, bait control; lane 7, prey control. The proteins pulled-down by PshBII-His holoprotein are labeled as A₂, B₂, C₂, D₂, and E₂.

**Figure 3.3** SDS-PAGE of pull-down assay results using PshBII-His and PshBI-His (holoprotein and apoprotein) as bait. Lane 1, molecular weight markers; lane 2, prey control; lane 3, PshBII-His holoprotein as bait; lane 4, bait control of PshBII-His holoprotein; lane 5, PshBI-His holoprotein as bait; lane 6, bait control of PshBI-His holoprotein; lane 7, PshBII-His apoprotein as bait; lane 8, bait control of PshBII-His apoprotein; lane 9, PshBI-His apoprotein as bait; lane 10, bait control of PshBII-His apoprotein. The proteins identified by MS from the gel pictures in Figure 3.2 and 3.3 are pointed out in this figure.

**Figure 3.4** SDS-PAGE gel picture of Co-IP results using antibody against PshBII (lane 1), antibody against PshBI (lane 2), and the pre-bleed serum as the negative control (lane 3).
Figure 3.1
Figure 3.2
Figure 3.3
Figure 3.4
Table 3.1 Proteins pulled-down by PshBI-His holoprotein

<table>
<thead>
<tr>
<th>Protein accession number and name</th>
<th>Biological pathway</th>
<th>Presumed cofactors(s)</th>
<th>PLGS sores</th>
<th>Band in Fig 3-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>YP_001680403.1 sporulation protein spoIID</td>
<td>engulfment and sporulation</td>
<td>-</td>
<td>540</td>
<td>A₁</td>
</tr>
<tr>
<td>YP_001680708.1 riboflavin biosynthesis protein RibD 3-oxoacyl-(acyl-carrier-protein) reductase</td>
<td>riboflavin biosynthesis</td>
<td>Zinc, NADP</td>
<td>1067</td>
<td>C₁</td>
</tr>
<tr>
<td>YP_001680732.1</td>
<td>fatty acid biosynthesis</td>
<td>NADP</td>
<td>893</td>
<td>D₁</td>
</tr>
<tr>
<td>YP_001680659.1 pyrroline-5-carboxylate reductase</td>
<td>arginine and proline metabolism</td>
<td>NAD(P)</td>
<td>471</td>
<td>D₁</td>
</tr>
<tr>
<td>YP_001679494.1 uracil phosphoribosyltransferase</td>
<td>uracil salvage</td>
<td>-</td>
<td>610</td>
<td>E₁</td>
</tr>
</tbody>
</table>
Table 3.2 Proteins pulled-down by PshBII-His holoprotein

<table>
<thead>
<tr>
<th>Protein accession number and name</th>
<th>Biological pathway</th>
<th>Presumed cofactor(s)</th>
<th>PLGS scores</th>
<th>Band in Fig 3-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>YP_001681170.1</td>
<td>glutamate synthase subunit, putative</td>
<td>FMN, FeS cluster</td>
<td>523</td>
<td>A_2</td>
</tr>
<tr>
<td>YP_001680708.1</td>
<td>riboflavin biosynthesis protein RibD</td>
<td>Zinc, NADP</td>
<td>515</td>
<td>B_2</td>
</tr>
<tr>
<td>YP_001681306.1</td>
<td>2-oxoglutarate oxidoreductase, alpha</td>
<td>FeS clusters</td>
<td>478</td>
<td>B_2</td>
</tr>
<tr>
<td>YP_001681021.1</td>
<td>hypothetical protein HM1 2460</td>
<td></td>
<td>691</td>
<td>C_2</td>
</tr>
<tr>
<td>YP_001680659.1</td>
<td>pyrroline-5-carboxylate reductase</td>
<td>NAD(P)</td>
<td>755</td>
<td>D_2</td>
</tr>
<tr>
<td>YP_001680732.1</td>
<td>3-oxoacyl-(acyl-carrier-protein) reductase</td>
<td></td>
<td>488</td>
<td>E_2</td>
</tr>
</tbody>
</table>
REFERENCES

bacterium Heliobacillus mobilis: structural implications and relations to other photosystems, *Proc Natl Acad Sci U S A* 90, 7124-7128.


