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Chapter 25
Halobacterium Expression System for Production of Full-Length *Plasmodium falciparum* Circumsporozoite Protein

Wolf T. Pecher, Jong-Myoung Kim, Priya DasSarma, Ram Karan, Photini Sinnis, and Shiladitya DasSarma

1 Introduction

As an innovative platform for antigen display and vaccine development, we are employing an extremophilic microbe, *Halobacterium* sp. NRC-1, that provides a number of advantages for production of antigenic proteins and vaccines (DasSarma 2007; DasSarma et al. 2013). This member of the archaeal domain has been

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determined to be non-toxic, lipopolysaccharide (LPS) free, and is biocompatible by virtue of its presence as a natural component of salt and salty condiments (http://www.els.net/WileyCDA/ElsArticle/refId-a0000394.html). The genome is completely sequenced and its biology has been studied in detail using genetic, transcriptomic, and proteomic methodologies (Berquist et al. 2006; DasSarma 2004; Ng et al. 1998, 2000). High-level regulated promoters have been used to develop expression vectors for the production of native and foreign proteins (Karan et al. 2013, 2014). Moreover, with *Halobacterium* sp., hypotonic conditions may be used to lyse cells and release expressed proteins in a stable form.

One of the most interesting applications of the *Halobacterium* sp. NRC-1 expression system is the display of antigenic protein arrays on nanoparticles called gas vesicles (GVNPs), that are about 300 nm long, lemon-shaped buoyant organelles used by cells for flotation and are both bioengineerable and highly adjuvanting (DasSarma and Arora 1997; DasSarma et al. 2013; Stuart et al. 2001, 2004). GVNPs are composed only of proteins, one of which (GvpC) can be used for fusion to foreign antigenic proteins for display on the external surface of the nanoparticle. GVNPs have been shown to successfully display SIV, *Chlamydia,* and *Salmonella* antigens, and are processed slowly by macrophages (Childs and Webley 2012; DasSarma and DasSarma 2015; DasSarma et al. 2014; Sremac and Stuart 2008, 2010; Stuart et al. 2001, 2004). For nanoparticles displaying *Salmonella* SopB antigens, immunized mice were found to exhibit reduced bacterial load in organs (DasSarma et al. 2014).

Our recent interest has been to apply the *Halobacterium* sp. NRC-1 expression system for production of the major surface protein of sporozoites, the pre-erythrocytic form of *Plasmodium falciparum* which is transmitted from mosquitoes to humans. Among diseases for which there is a critical need for vaccines, malaria is one of the most widespread, with 200 million infections and over half a million deaths per year, primarily from infection by *P. falciparum* (http://who.int/malaria/publications/world_malaria_report_2013/en/index.html). The circumsporozoite protein, or CSP, is known to be highly immunogenic and is encoded by a single-copy gene unique to *Plasmodium* (Nussenzweig and Nussenzweig 1989). CSP consists of an N-terminal region containing a signal sequence, a central region with multiple tetrapeptide repeats, and a C-terminal cell-adhesion domain that ends with a putative glycosylphosphatidylinositol (GPI) anchor addition sequence (Fig. 25.1) (Coppi et al. 2011; Dame et al. 1984; del Portillo et al. 1987). The number of tetrapeptide repeats may vary from 25 to 49 copies, depending on the source (Bowman et al. 2013). A subunit vaccine, RTS,S, based on *P. falciparum* CSP consisting of most of the C-terminal half of the protein, including 19 copies of the tetrapeptide repeat fused to the hepatitis B surface protein, has been produced in *Pichia pastoris*, tested in different formulations with various immunostimulatory compounds, and shown to provide partial protection. However, protection is limited in infants, and in long term immunity (Campo et al. 2014; Mo and Augustine 2014). The ultimate potential of any pre-erythrocytic vaccine is underscored by reports of sterile immunity obtained with irradiated sporozoites administered intravenously (Seder et al. 2013).

We sought to utilize the *Halobacterium* sp. NRC-1 expression system for production of full-length *P. falciparum* CSP. In order to maximize the production of

parasite protein in *Halobacterium* sp., we designed a codon-optimized gene and cloned it into expression vectors for production of the CSP protein alone or as a fusion to the GVNPs, including the N- and C-terminal regions not present in the mature protein in *Plasmodium*, which may contribute to enhanced immunogenicity or immunostimulation (Kastenmüller et al. 2013; Sedegah et al. 2013). In this report, we provide evidence for the biosynthesis of full-length CSP.

2 Materials and Methods

2.1 *Design and Cloning of Codon-Optimized* P. falciparum *CSP Gene*

To design a codon-optimized *P. falciparum* CSP gene for expression in *Halobacterium* sp. NRC-1, we obtained the CSP gene sequence from the *P. falciparum* strain 3D7 (NCBI reference number XM_001351086.1). The codon usage table for predicted genes in the genome of *Halobacterium* sp. NRC-1 was used to replace rare and infrequent codons (Kennedy et al. 2001). A codon modification (AAC > GGC) was incorporated in the design to add a unique *KasI* restriction site, which resulted in the mutation of an asparagine to glycine residue at amino acid position 201 (Fig. 25.1). The codon-optimized gene, flanked by *AfeI* restriction sites, was synthesized commercially for cloning into *Halobacterium* sp. NRC-1 expression vectors (Life Technologies, Carlsbad, CA, USA).

2.2 *Construction and Culturing of Expression Strains*

The *AfeI* restriction fragment coding the engineered and codon-optimized full-length CSP gene was cloned into the *Halobacterium* expression plasmids, pDRK and pSD, after cleavage of each at the unique *AfeI* sites in these vectors (Karan et al. 2014). The constructed pDRKcsp6 plasmid (Fig. 25.2a) was transformed into *Halobacterium* sp. strain SD109 (DasSarma et al. 1988, 1995), a derivative of wild-type strain NRC-1 deleted for the active *gvp* gene cluster A in pNRC100 and pNRC200, to construct *Halobacterium* strain SD109 (pDRKcsp6). The pSDcsp20 plasmid construct (Fig. 25.2b) was also transformed into SD109 to construct *Halobacterium* strain SD109 (pSDcsp20). The CSP gene region of each plasmid was completely sequenced in both strands using primer walking. *Halobacterium* strains transformed with expression plasmids were grown in CM^+ media with 20 μg/ml mevinolin (Merck, Sharp, and Dohme, Rahway, NJ, USA). Cultures were grown at 42 °C at 220 rpm to stationary phase. Cell lysates and GVNPs were prepared as described previously (DasSarma et al. 1995, 2013).

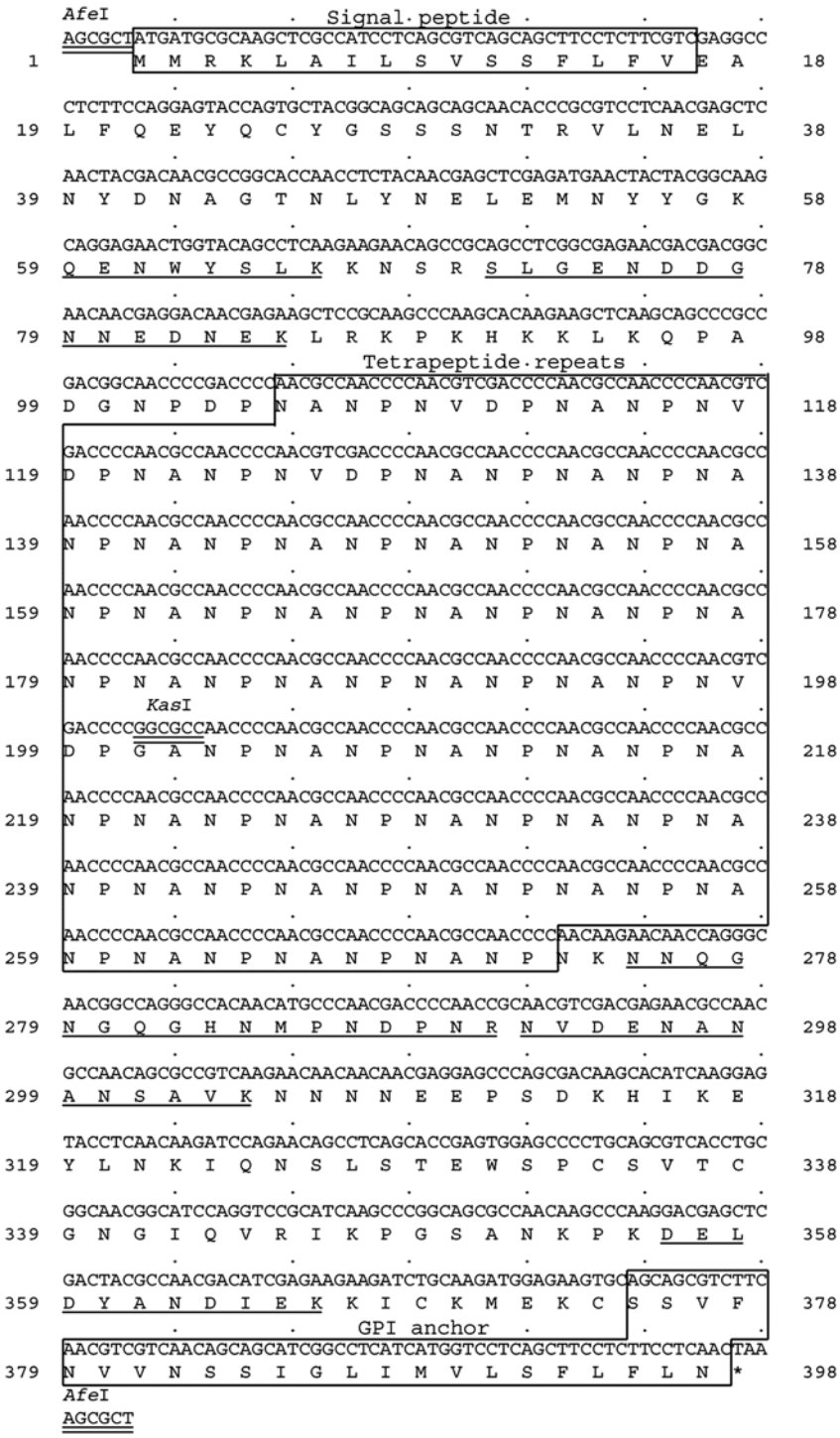

Fig. 25.1 Sequence of the synthetic codon-optimized *P. falciparum* CSP gene for expression in *Halobacterium* sp. and translation product. *AfeI* and *KasI* restriction sites are shown with recognition sequences *double-underlined*. Tryptic peptides of CSP identified by LC-MS/MS analysis are *underlined*. The signal peptide, tetrapeptide repeats, and the putative GPI anchor are boxed

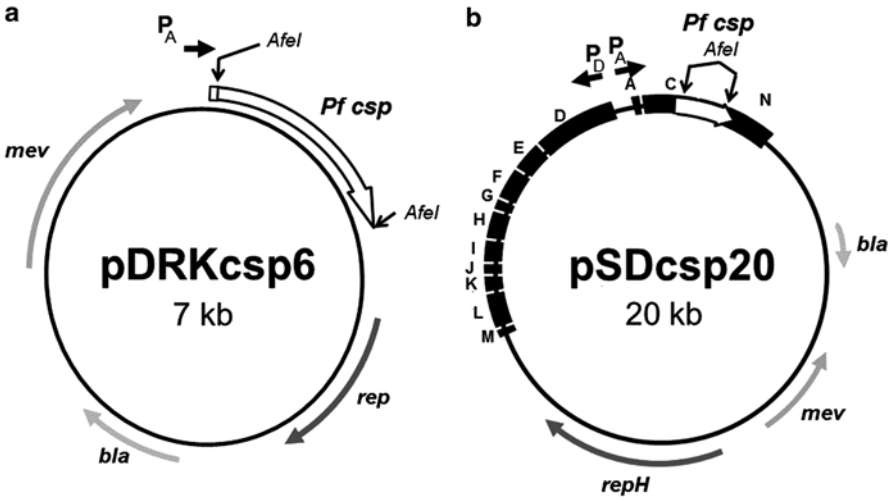

Fig. 25.2 *P. falciparum* CSP expression vectors pDRKcsp6 (**a**) and pSDcsp20 (**b**). The synthetic gene coding *P. falciparum* CSP (labeled *Pf csp*) is shown by *wide white arrows* between *AfeI* restriction sites used for cloning. Location of promoters are indicated by *narrow black arrows*. Locations of selection markers for mevinolin resistance (*mev*) and β-lactamase (*bla*) are shown as *narrow gray arrows*, as are locations of replicase genes (*rep*, *repH*). Location of the hexa-histidine tag is indicated by a small *white box* at the 5′-end of the CSP gene in pDRKcsp6 and locations of *gvpACN* and *gvpDEFGHIJKLM* genes are shown by *black boxes* in pSDcsp20

2.3 Western Blotting Analysis

Western blotting was carried out as previously described (Shukla and DasSarma 2004). Briefly, preparations of cell lysates were electrophoresed on 9 % or 12 % polyacrylamide-SDS gels. Proteins were transferred to 0.45 μm Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Boston, MA, USA). Membranes were blocked with PTM buffer (PBS, 0.1 % Tween 20, 5 % nonfat dry milk), followed by overnight incubation at 4 °C after addition of the CSP-specific monoclonal antibody 2A10 (1:1,000 dilution) (Wirtz et al. 1987). Membranes were washed with PTM buffer, and incubated with rabbit anti-mouse secondary antibodies (1:2,500 dilution) labeled with alkaline phosphatase (Sigma Aldrich, St. Louis, MO, USA). For detection of immunoreactive proteins, membranes were incubated in 1-Step NBT/BCIP substrate per manufacturer's specifications (Thermo Fisher Scientific, Rockford, IL, USA). Bands were quantified by densitometry using Image J (http://imagej.nih.gov/ij/).

2.4 *Proteomic Analysis*

To identify CSP by LC-MS/MS analysis, *Halobacterium* proteins were reduced with DTT, alkylated with iodoacetamide, and subsequently proteolyzed with trypsin (Shevchenko et al. 1996). Digested peptides were desalted by C18 stage-tip and eluted with 0.1 % trifluoroacetic acid in 60 % acetonitrile and resuspended in 0.1 % formic acid for LC–MS/MS analysis. Identification of peptides was performed on a Q-Exactive instrument (Thermo Scientific, Rockford, IL, USA) interfaced with a Proxionnano flow LC system. Peptide sequences were identified from isotopically resolved masses in MS and MS/MS spectra extracted with and without deconvolution using a Thermo Scientific MS2 processor and Xtract software. MS/MS spectra were searched against protein databases using the Sequest search engine interfaced with Proteome Discoverer 1.4 software.

3 Results

3.1 *Construction of* Halobacterium *sp. Expression Strains Containing the Codon-Optimized* P. falciparum *CSP Gene*

Due to the great difference in GC-composition between *P. falciparum* and *Halobacterium* sp., (19 % versus 66 % GC, respectively), we designed a synthetic CSP gene for expression in *Halobacterium* using the codon usage data from the genome of the wild-type NRC-1 strain (Fig. 25.1). The synthetic gene design contained 62.3 % GC, incorporating the full-length CSP coding region from *P. falciparum* strain 3D7, including the N-terminal signal and C-terminal anchor sequence, and was flanked by *AfeI* restriction sites for cloning.

For construction of expression plasmids, the synthetic CSP gene was inserted into plasmid pDRK at an *AfeI* site downstream of the *gvp*A promoter, resulting in plasmid pDRKcsp6 (Fig. 25.2a). The CSP encoded by pDRKcsp6 has an N-terminal 15 amino acid residue extension (MHHHHHHLKRLPRSA) containing a hexa-histidine tag, followed by the 397 amino acid residue full-length CSP (Fig. 25.1). The synthetic CSP gene was also cloned into expression plasmid pSD at the *AfeI* site in *gvp*C for expression of CSP and display on GVNPs in *Halobacterium* (Fig. 25.2b). The resulting pSDcsp20 encodes a fusion protein with 293 amino acids of GvpC followed by the full-length CSP.

3.2 *Expression of Full-Length* P. falciparum *CSP in* Halobacterium *sp.*

The expression plasmids, pDRKcsp6 and pSDcsp20, were transformed into *Halobacterium* sp. strain SD109, a gas vesicle-deficient mutant of the wild-type strain. Cultures were grown to stationary phase, collected by centrifugation, and lysed

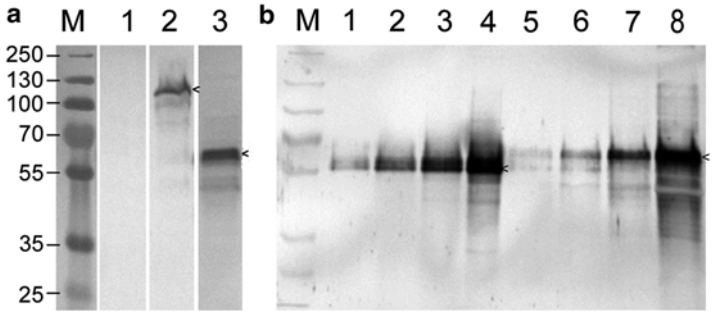

Fig. 25.3 Western blotting analysis of CSP expression strains. *Halobacterium* sp. SD109, SD109 (pSDcsp20), and SD109 (pDRKcsp6) (**a**, lanes 1–3, respectively) and *P. falciparum* sporozoite lysates (**b**, lanes 1–4, 1.9×10^2, 7.5×10^2, 3×10^3, and 1.2×10^4 cells, respectively) and *Halobacterium* sp. SD109 (pDRKcsp6) lysates (**b**, lanes 5–8, 8×10^4, 3×10^5, 1.3×10^6, and 5×10^6 cells, respectively), probed using the CSP monoclonal antibody 2A10. Lanes M contain molecular weight markers, with sizes indicated in kDa. *Arrowheads* indicate the prominent CSP band

using hypotonic conditions. Growth rates of the transformants were comparable to that of the parental strain (data not shown). Cell lysates were fractionated by polyacrylamide gel electrophoresis, transferred, and probed using the CSP monoclonal antibody 2A10. Protein bands corresponding to CSP at the expected positions, 120 kDa for strain SD109 (pSDcsp20) and 60 kDa for strain SD109 (pDRKcsp6), were found (Fig. 25.3a). Proteins of the *Halobacterium* sp. SD109 (pDRKcsp6) were also subjected to trypsin digestion followed by LC-MS/MS. Five unique peptides corresponding to *P. falciparum* CSP were observed, confirming the identity of the expressed protein (Fig. 25.1). The peptides identified covered 64/397 amino acids or 16 % of CSP; it should be noted that the entire tetrapeptide repeat region is devoid of trypsin sites and would not be expected to be detected by this method.

We determined the level of production of CSP in *Halobacterium* sp. by Western blotting analysis using *P. falciparum* sporozoite lysates as standards. Sporozoites were prepared from disrupted salivary glands of *Anopheles stephensi* adult females. Measured numbers of *P. falciparum* sporozoites and *Halobacterium* sp. SD109 (pDRKcsp6) cells were lysed, fractionated by SDS-PAGE, transferred, and probed with the CSP monoclonal antibody 2A10 (Fig. 25.3b). The CSP band in the sporozoites was observed at 55 kDa (lanes 1–4), while the free CSP band in *Halobacterium* was observed at 60 kDa (lanes 5–8), which is larger due to the presence of the signal sequence, the putative GPI anchor, and the hexa-histidine tag. Based on the intensity of these major bands apparent in the Western blot, we determined that 10^3 *P. falciparum* sporozoites produce similar quantities of CSP as 8.3×10^5 *Halobacterium* sp. SD109 (pDRKcsp6) cells. This level of expression represents 3.3 mg of free CSP per liter *Halobacterium* culture. The level of CSP fusion protein for display on GVNPs, observed as a 120 kDa protein, corresponds to about 15 % of this amount or 0.5 mg of CSP per liter of culture (Fig 25.3a and data not shown).

4 Discussion

Using the Archaeal expression host *Halobacterium* sp. NRC-1, we have documented the expression of full-length *P. falciparum* CSP. This extremophilic expression system provides a yield of CSP sufficient for immunological studies and the potential for vaccine development. Additional benefits include biocompatibility, non-toxicity, lack of LPS, and potential for scaling. Another significant advantage is that the cells are easily disrupted by hypotonic conditions, releasing the content without the need for mechanical or enzymatic processes. The *Halobacterium* sp. cells contain large quantities of intracellular GVNPs, which have been shown to be highly adjuvanting, eliciting strong and long-lived immune responses (Childs and Webley 2012; DasSarma and DasSarma 2015; DasSarma et al. 2013, 2014; Sremac and Stuart 2008, 2010; Stuart et al. 2001, 2004). The expression system permits CSP to be produced freely or potentially bound and displayed as an ordered array on the GVNPs. The CSP protein produced in *Halobacterium* is recognized by the 2A10 CSP monoclonal antibody, indicating that its antigenicity is retained in this host. These results may allow the development of multiple formulations for immunological testing and vaccine development in the future.

CSP sequences are available from diverse *Plasmodium* strains and several have been previously expressed in *Escherichia coli*, *P. pastoris*, yeast and other organisms (Dame et al. 1984; Kastenmüller et al. 2013; Kolodny et al. 2001; Plassmeyer et al. 2009; Young et al. 1985). However, in nearly all of these cases, successful expression has employed truncated forms of CSP lacking either the N-terminal signal peptide, the C-terminal putative GPI anchor regions, or both. Early attempts to generate full-length CSP in *E. coli* resulted in the expression of unstable products at low levels (Young et al. 1985). In our case, the entire CSP molecule, either free or fused to GvpC, is produced in *Halobacterium* sp. The presence of significant quantities of the entire CSP molecule offers a potentially attractive alternative to the other expression systems.

In a recent study, near full-length CSP (lacking the signal sequence and putative GPI anchor sequence) was shown to elicit a stronger antibody reaction in mice and non-human primates compared to the RTS,S vaccine that lacks the entire N-terminus of the CSP (Kastenmüller et al. 2013). Adjuvants were shown to differentially mediate effects on antibody and Th1 immunity as well as CD4+ T cell immunity and protection in mice. Haloarchaeal GVNPs may provide a significant boost to immune responses against displayed antigens, as suggested when *Salmonella* SopB antigen-GVNPs successfully reduced bacterial burden upon administration with live attenuated bacteria after challenge (DasSarma et al. 2014). Immunostimulatory effects of SopB-GVNPs were confirmed by significant increases in levels of IFN-γ, IL-2, and IL-9 in immunized mice.

The additional sequences present in full-length CSP produced in *Halobacterium* sp. that are lacking in the mature sporozoite CSP may potentially be either immunogenic or immunostimulatory in ways not observed with the truncated proteins. Consistent with this notion, the signal peptide of CSP contains epitopes recognized

by CD8+ T cells of individuals from malaria endemic areas (Sedegah et al. 2013). Moreover, a combination of cell mediated (i.e. CD4+ Th1, CD8+ T cell) and humoral responses have been shown to be critical to provide protective immunity (Ménard et al. 2013; Schofield et al. 1987). Additional studies are necessary to determine the immunogenic effects of the full-length CSP produced in *Halobacterium* free or displayed on GVNPs.

The biocompatibility of the *Halobacterium* cells and GVNPs represents another significant distinguishing feature of this system (DasSarma et al. 2010). Furthermore, antigens fused to GNVPs were reported to be stable without refrigeration in *Halobacterium* cells for extended periods of time, suggesting that this platform is a valuable alternative for vaccines targeting diseases in developing countries (DasSarma et al. 2010, 2014). Although the ability to partially purify GVNPs by flotation may be valuable, the potential for direct use of CSP-containing cells or cell lysates can also be pursued and may offer advantages for immunogenicity. However, since in some cases, hexa-histidine tagged N-termini could facilitate purification of expressed proteins in other hosts, the inclusion of this affinity tag may be utilized, should purification be desired (Kolodny et al. 2001; Plassmeyer et al. 2009). Finally, the *Halobacterium* sp. CSP expression system may also be modified to express variant proteins, including shorter or truncated forms, should those become needed in future.

Conflict of Interest Wolf T. Pecher, Jong-Myoung Kim, Priya DasSarma, Ram Karan, Photini Sinnis, and Shiladitya DasSarma declare that they have no conflict of interest.

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