Review

Production of recombinant proteins from protozoan parasites

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Although the past decade has witnessed sequencing from an increasing number of parasites, modern high-throughput DNA sequencing technologies have the potential to generate complete genome sequences at even higher rates. Along with the discovery of genes that might constitute potential targets for chemotherapy or vaccination, the need for novel protein expression platforms has become a pressing matter. In addition to reviewing the advantages and limitations of the currently available and emerging expression systems, we discuss novel approaches that could overcome current limitations, including the 'pseudoparasite' concept, an expression platform in which the choice of the surrogate organism is based on its phylogenetic affinity to the target parasite, while taking advantage of the whole engineered organism as a vaccination adjuvant.

Proteins from protozoan parasites as targets for diagnosis and intervention

The availability of genome sequences of several parasites of medical importance has led to exponential progress in our understanding of their biology and enabled identification of potential targets for intervention [1]. Furthermore, continued advances in genome sequencing technologies holds great promise for accomplishment of similar goals for virtually any parasite of interest [2]. When compared to other pathogens of human and veterinary importance, such as viruses and bacteria, large gaps exist in our knowledge of the virulence and pathogenesis mechanisms of protozoan parasites, and methodologies for eradication or management of parasitic diseases through vaccination or treatment are still in the distant future. Characterization of genes of interest for potential intervention identified by mining these genomes has been hindered because of the lack of suitable protein expression systems. A clear example is *Plasmodium falciparum*, the etiological agent of malaria, for which the lack of an effective vaccine and the rapid emergence of drug-resistant strains have made most intervention attempts extremely challenging [3]. Therefore, the development of innovative and efficient systems for the expression of recombinant proteins from protozoan parasites has become an urgent public health matter.

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Applications of recombinant proteins from protistan parasites

Recombinant proteins from parasites are required for numerous applications.

(i) *Development of diagnostic tools*: A rapid and accurate diagnosis of the etiological agent is the key for effective management of diseases caused by protozoan parasites, while avoiding the unnecessary use of therapeutic agents that might result in the selection of drug-resistant strains. The development of diagnostic tools requires substantial amounts of either the parasite at particular life cycle stages or selected proteins expressed at those stages [4,5]. For selected proteins, the recombinant proteins should accurately represent the native equivalents; otherwise, the field performance of the diagnostic test could be compromised [6]. Currently, the only US Federal Drug Administration (FDA) approved kit uses HRP-2 and aldolase antigens (BinaxNow, Inverness Medical, Princeton, NJ, USA) [4]. Diagnostics for trypanosomiasis, babesiosis and leishmaniasis also rely on recombinant antigens [7–9].

(ii) *Immunogens for vaccination*: Vaccination is the most desirable prophylactic method for any infectious

Glossary

Codon harmonization (synonymous translational attenuation): during the translation in the ribosomes, low frequency codons can cause translational pausing, favoring the correct folding of the proteins. Codon harmonization consists of matching the usage frequencies, particularly the low usage frequencies, of the native codons from the gene within the native organism as near as possible to codons recognized as low usage frequency codons by the surrogate host. This ensures that the positional codon frequency of low/intermediate and high usage codons remains unaltered in the surrogate host, allowing the needed halts in the translational processes to match that of the organism of interest (natural host). Differences in synonymous codon usage between the natural host and surrogate cells can lead to low protein expression and the formation of insoluble aggregates.

Codon optimization: most of the 20 genetically determined amino acids are encoded by multiple codons, which each species use at a distinct frequency. Protein synthesis in protozoan parasites relies on codons that are rarely employed by the surrogate organism in a heterologous expression system. Codon optimization consists of matching the frequencies at which different codons are used in the natural host (donor) and the surrogate organism.

Heterologous protein expression system: a system in which production of the protein of interest is carried out in an organism different from the organism source of the target protein.

Homologous protein expression system: a system in which production of the protein of interest takes place in the natural host under a specific (inducible or not) promoter, often different from the native promoter.

Inclusion bodies: insoluble aggregates of denatured/unfolded protein produced in the cytoplasm or nucleus of bacteria.

Surrogate system: organism or cellular components used to study a particular mechanism or express a protein naturally produced by a different organism.

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Table 1. Systems used for producing vaccine candidates against protozoan parasites[a]

Organism	Disease	Candidate[b]	System
Plasmodium	Malaria	ABRA	*Escherichia coli*
		MSP1	*Escherichia coli*
		AMA-1	*Escherichia coli*
		MSP1(42)	*Escherichia coli*
		LSA-1	*Escherichia coli*
		RTSS/AS02A	*Saccharomyces cerevisiae*
		Pfs25	*Pichia pastoris*
		Pvs25	*Saccharomyces cerevisiae*
		CSP	*Saccharomyces cerevisiae*
Toxoplasma	Toxoplasmosis	SAG1, -2	*Salmonella*
		OP2	Plasmid DNA
Cryptosporidium	Cryptosporidiosis	P23, CP15	Plasmid DNA
Theileria	East Coast fever	p67	Baculovirus
Neospora	–	NcSAG1, -2	*Escherichia coli*
		NcGRA7, NcsHSP33	Plasmid DNA
Babesia	Babebiosis	12D3, 11C5	*Escherichia coli*
		Bd37	*Escherichia coli*
Eimeria	–	EtMIC2	Plasmid DNA

[a]This is not a comprehensive list. Not all of these proteins are mentioned in the main text.
[b]ABRA, acidic basic repeat antigen; AMA, apical membrane antigen; EtMIC2, *Eimeria tenella* microneme protein 2; GRA, tachyzoite-dense granules; HSP, heat-shock protein; Pfs25, *P. falciparum* surface 25; Pvs25, *P. vivax* surface 25.

disease. The use of proteins isolated directly from parasites is advantageous over recombinant proteins in that all structural and immunogenic characteristics that are native to the organism are displayed in the vaccine. However, their availability at the required purity standard and quantity has been the main limiting factor. In addition, polymorphism of the protein of interest can result in vaccines with poor reproducibility. Most subunit vaccines rely on the industrial production of the recombinant antigen of choice (Table 1). Furthermore, as compared to whole-organism vaccines, subunit vaccines have low inherent safety risks associated with their manufacture processes [10].

(iii) *Structure–function analysis of proteins from parasites*: Novel approaches for effectively and specifically targeting essential parasite enzymes are currently being developed [11]. However, the rigorous proof of function requires biochemical, biophysical and functional characterization of the protein of interest. Protozoan parasites represent the ultimate challenge in this regard as most of them are obligate intracellular parasites for which no *in vitro* culture methods have been developed to date. Nevertheless, homology modeling and molecular-docking experiments have enabled the design of specific inhibitory drugs that can be subsequently tested experimentally as potential therapeutic agents [12].

(iv) *Screening and profiling of candidate drugs*: The development of therapeutics is a viable alternative to mitigate the effects of a disease, which is particularly useful where a vaccine is not available, or, in those cases where the vaccine is not sufficiently effective, as a complement to the vaccine. Along these lines, great efforts have been invested in studies leading to the production of drugs against apicomplexan parasites, based on interference with metabolic pathways associated with the apicoplast [13]. In some cases, cultured parasites provide enough material for drug screening (e.g. using *Leishmania tarentolae* 20×10^{11} cells, 50 mg of tubulin at 10–30 mg ml^{-1} is enough for screening approximately 1600 compounds) [14]; however, given the availability of extensive combinatorial chemical libraries, current drug-discovery screens require large amounts of proteins (i.e. PfMetAP1b 222 nM for 175,000 compounds arrayed in 384-well plates) [15], a fact that also underscores the urgent need of suitable expression systems.

Production of recombinant proteins from parasites: challenges and limitations

(i) *Scale of production*: Although improved technologies have reduced the amount of protein required for extensive screenings, the application of high-throughput methodologies still requires relatively high protein quantities (i.e. 5–25 mg ml^{-1} for screening 500–1000 crystallization conditions; 10 nM pfDHOD for screening a 208,000 compound library) [16,17], virtually unattainable from parasites isolated from the host. By contrast, recombinant proteins can be produced in relatively large amounts. In some cases, however, the industrial-scale production of recombinant proteins necessary for a selected application can require a complex infrastructure. For example, production of the *P. falciparum* liver stage antigen 1 (LSA-1) for the pre-erythrocyte stage protein-based vaccine, requires 300 l of broth to produce 8.0 kg of *Escherichia coli* paste, which yields 8 mg LSA per gram of paste (<0.005 endotoxin units per 50 μg of protein) [18].

(ii) *Immunogenicity of the recombinant product*: Immunogenicity is of crucial importance when evaluating expression systems for production of recombinant vaccine antigens. Immunogenicity is inherent to the protein and is based mostly on structural features that determine its solubility, degradability and

interactions with multiple components of the innate and adaptive immune responses. For example, differences in the expression constructs for production of proteins evaluated as malaria vaccine candidates resulted in different immunogenicity, even when rabbits were immunized with equimolar amounts of each recombinant protein [19]. A standard method to enhance immunogenicity of the recombinant protein is to conjugate it to a carrier, usually a highly glycosylated protein or polysaccharide, although the outcomes are sometimes unexpected [20]. The *P. falciparum* apical membrane antigen 1 conjugated to bacterial ExoProteinA resulted in a 1000-fold higher antibody titer, as compared to the threefold increase in Pfs25 conjugated to the same carrier [20]. To date, the most promising vaccine against malaria uses the circumsporozoite protein (CSP) region and a T-cell immunogenic epitope fused to the hepatitis B antigen, and formulated with AS02A [10]. Alternatively, multiple protective epitopes can be combined in a single recombinant immunogen [21].

(iii) *Post-translational modifications*: Recombinant proteins should reflect their authentic counterparts as faithfully as possible, including their post-translational modifications. Among these, glycosylation can be critical, not only for immunogenicity in that it can enhance or conceal immunogenic epitopes but also to modulate biological activity. For example, the hyperglycosylated recombinant *Toxoplasma* surface antigen (SAG2) produced in yeast reacted strongly with pooled anti-*Toxoplasma* human serum, pooled anti-*Toxoplasma* mouse serum and a SAG2-specific monoclonal antibody [22]. *Toxoplasma* tachyzoites treated with tunicamycin, which inhibits *N*-glycosylation, were mostly incapable of invading new host cells, thus revealing its relevance for virulence and possibly for immunogenicity [23]. By contrast, the *Trypanosoma brucei* recombinant transferrin receptors expressed in insect cells in the presence of tunicamycin bind ligand effectively [24]. Other post-translational modifications typical from eukaryotes that have been described in apicomplexan proteins and might be required for production of recombinant proteins include the removal of N-terminal methionine and acylation [15,25,26].

Surrogate systems for expression of proteins from protozoan parasites

The selection of heterologous expression systems for proteins from protozoan parasites has been determined, at least in part, by the specific objective(s) pursued, production cost and availability of the required facilities. To date, most efforts have been directed to the production of *Plasmodium* recombinant proteins (reviewed in Ref. [27]). A search of the PDB (Protein Data Bank) database (www.pdb.org, May 2009) yielded a limited number of matches to protozoan protein sequences for which the structures have been resolved (Figure 1a). Although an increase in the number of structures resolved during the past 8 years is evident, the pattern illustrates the limited success attained in the available heterologous expression systems. In the following section, we focus on the systems that have been tested (successfully and unsuccessfully) for expression of parasite proteins (Table 2), those that are currently under development and potentially useful innovative platforms.

Prokaryotic systems

The *E. coli* expression system is the most commonly used for industrial production of recombinant proteins for pharmaceutical applications (reviewed in Ref. [28]) (Figure 1b). Prokaryotic organisms grow rapidly and at high densities in relatively inexpensive growth media, are easy to transform and can produce high quantities of soluble recombinant product; in addition, multiple cloning vectors and host strains are available. Limitations of prokaryotic expression systems include the absence of post-transcriptional modifications, amino acid substitutions or modifications, heterogeneous products, contamination with endotoxin and accumulation of the recombinant product as inclusion bodies. For proteins from protozoan parasites, the overall success rate for obtaining soluble, immunogenic recombinant products in prokaryotic systems remains low [29–32] (Figure 1c). For example, from 303 *Plasmodium* genes, only 7% of the recombinant proteins induced antibody titers [31]. A high-throughput study targeting apicomplexan parasites resulted in 30.2% purified soluble recombinant proteins, with only 3.8% of their structures resolved [29]. Similarly, from a total of 1000 open reading frames (ORFs) from *Plasmodium* tested, <7% were expressed as soluble proteins [30]. Current approaches to improve expression in prokaryotic systems include the production of temperature-induced recombinant protein [33] and the use of thermal melt and/or thermal shift assays for identifying ligands that stabilize recombinant proteins [34]. However, *Plasmodium* proteins of large size or with high p*I* remain difficult to produce in bacteria, and no plastid-targeted recombinant proteins have yet been expressed as soluble products [30]. This has been attributed to the codon bias derived from the high frequency at which a particular codon is used preferentially by the *Plasmodium* AT-rich genome. Although the impact of codon bias on heterologous protein expression has been questioned by a recent large-scale study [30], alternative strategies have been developed to overcome this problem (reviewed in Ref. [35]). These include plasmids or host cells containing tRNA that recognize rare codons [36] and the use of harmonized codon usage frequencies [18]. As it becomes increasingly affordable, gene synthesis represents a viable option for codon usage harmonization, even for large-scale initiatives [37]. Recently, focus has been placed on the identification of sequence features that can modulate protein expression in heterologous systems. For example, the recombinant protein yield in *E. coli* was strongly dependent on the codons used for only a subset of amino acids, predominantly favoring codons read by tRNAs that are most highly charged during amino acid starvation, instead of codons that are most abundant for the highly expressed *E. coli* proteins [38]. In addition to codon bias, features that can be unique to any given protein determine success in

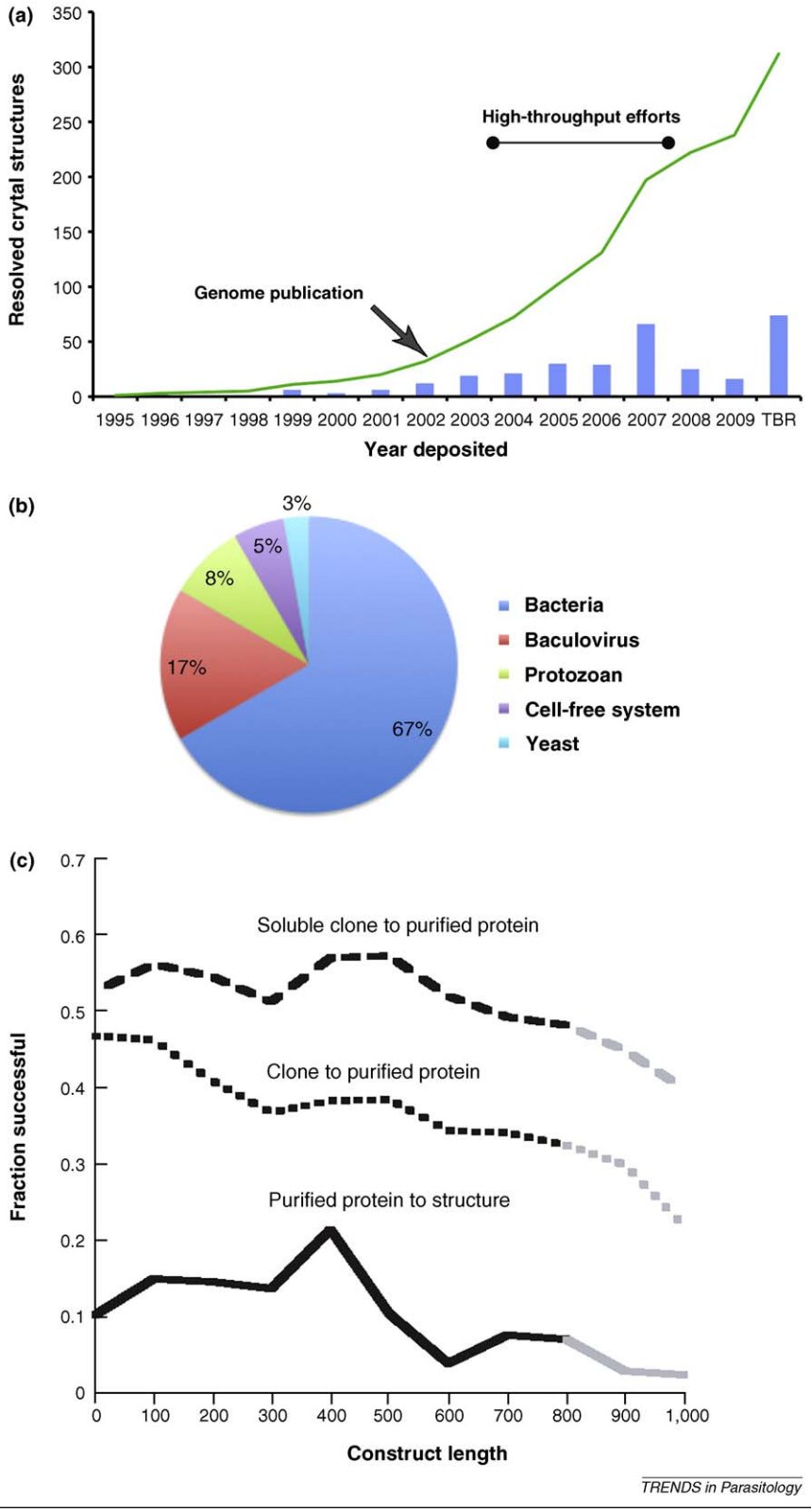

Figure 1. **(a)** Resolved crystal structures of *Plasmodium* genes. PDB database (www.pdb.org, May 2009) was searched for *Plasmodium* protein resolved structures and plotted against the year deposited (bars: number of structures deposited in a given year; line: cumulative structure deposited numbers). After the sequence of the genome, there were two high-throughput efforts that resulted in a limited increase in the number of structures resolved. Overall, recombinant protein production remains a bottleneck for many scientific endeavors. TBR: to be released. **(b)** Heterologous protein expression systems for parasites. A PubMed search of recombinant proteins from parasites in the past 7 years reveals that prokaryotic systems are still the workhorse, followed by insect cells/baculovirus. Interestingly, protozoan organisms are used more often than yeast. Cell-free systems are just starting to take off. **(c)** Solubility as a function of construct length; Ref. [32] is the original source. The overall success rate for soluble recombinant proteins remains very low, particularly for protein targets with lengths >800 amino acids [32]. Dotted line, fraction of cloned targets resulting in successful large-scale purifications. Dashed line, fraction of soluble clones at a 1-ml scale resulted in pure protein at large scale. Solid line, fraction of purified proteins resulting in successful crystal structure determinations.

Table 2. Performance comparison of most common heterologous expression systems

System	Advantages	Disadvantages
Prokaryotic	• Rapid growth at high densities	• Absence of post-transcriptional modifications
	• Inexpensive growth media	• Amino acid substitutions or modifications
	• Suitable for large-scale production	• Heterogeneous products
	• Well known genetics	• Contamination with endotoxin
	• Easy to transform	• Biased codon usage
	• Multiple cloning vectors, purification tags and host strains	• Low level of secretion
	• Abundant literature (troubleshooting)	• Failure to fold in the native active state
	• Multiple commercial brands	• Accumulation as insoluble inclusion bodies
	• High protein yields	• Lack of complexity for functional organelle targeting
Eukaryotic		
Yeast	• Inexpensive	• Glycosylation patterns different from those in the native parasite protein
	• Suitable for large scale production	• Hyperglycosylation
	• Multiple cloning vectors, purification tags and host strains	• Low yield derived from codon bias, depletion of precursors and energy, proteolysis
	• Secretion into the culture medium	• Unfolded proteins shooting down the secretory pathway
	• Trafficking through the secretory pathway favors disulfide bonds formation and protein folding	• Truncated proteins
	• Easy genetic manipulation	
	• Post-transcriptional modifications	
	• Endotoxin-free	
	• Abundant literature (troubleshooting)	
	• Several commercial brands	
	• High protein yields	
Mammalian cell lines	• Post-transcriptional modifications (glycosylation, phosphorylation and the addition of fatty acid chains)	• Labor intensive and expensive
	• Transient or stable transfection	• Increased biosafety (BL2) when using some virus for gene delivery
	• Abundant literature (troubleshooting)	• Slowly growth
		• Limited large-scale production
		• Low protein yield
		• Contamination with mammalian pathogens
Baculovirus expression vector system	• Basic cell culture equipment	• Labor intensive
	• Minimal safety precautions	• Time consuming (up to 3 weeks to obtain enough viruses)
	• Large inserts (up to 15 Kb)	• Expensive methodology
	• Post-transcriptional modifications	• No easy amenable for automation
	• Abundant literature (troubleshooting)	• Suitable only for batch production
		• Glycosylation patterns different from those in the native parasite protein (lack of multiantennary glycans)
D. discoideum	• Easy culture at high densities	• Still under development
	• Easy genetic manipulation	• No commercially available
	• Multiple cloning vectors	• Preferential codon usage
	• Eukaryotic organelle complexity for functional studies	• No FDA approved SOPs
	• AT rich genome	
	• Abundant literature (model organism)	
T. thermophila	• Post-transcriptional modifications	• Still under development
	• AT rich genome	• Not commercially available
	• GPI anchored proteins	• No FDA approved SOPs
Cell-free systems	• Free of translation inhibitors	• Absence of post-transcriptional modifications
	• Non-biased codon usage	• Expensive methodology
	• Non-specialized training	• Low yield
	• Simple equipment required	• Not amenable for automation and parallelization
	• Particularly suitable for integral membrane proteins	• No FDA approved SOPs

Abbreviations: FDA, Federal Drug Administration; SOPs, Standard Operating Procedures.

expression yields and correct folding in prokaryotic systems. For example, cryptopain-1 from *Cryptosporidium* does not require its pro-domain for proper folding when expressed in *E. coli* [39]. In spite of the above-mentioned limitations, a minor subset of protozoan proteins have been produced in prokaryotic systems as soluble, active and immunogenic recombinant products, without any major difficulties. These include the *Entamoeba hystolica* cysteine proteinase EhCP1 [40], the recombinant dihydrofolate reductase-thymidylate synthase (DHFR-TS) from *Babesia gibsoni* [41] and the dominant circumsporozoite CD8 T-cell epitope from *Plasmodium berghei* [42].

Eukaryotic systems

The main advantage of these systems derives from their closer phylogenetic relationship with the parasites of interest, presumably sharing to some extent with the parasites of interest post-translational modifications, such as

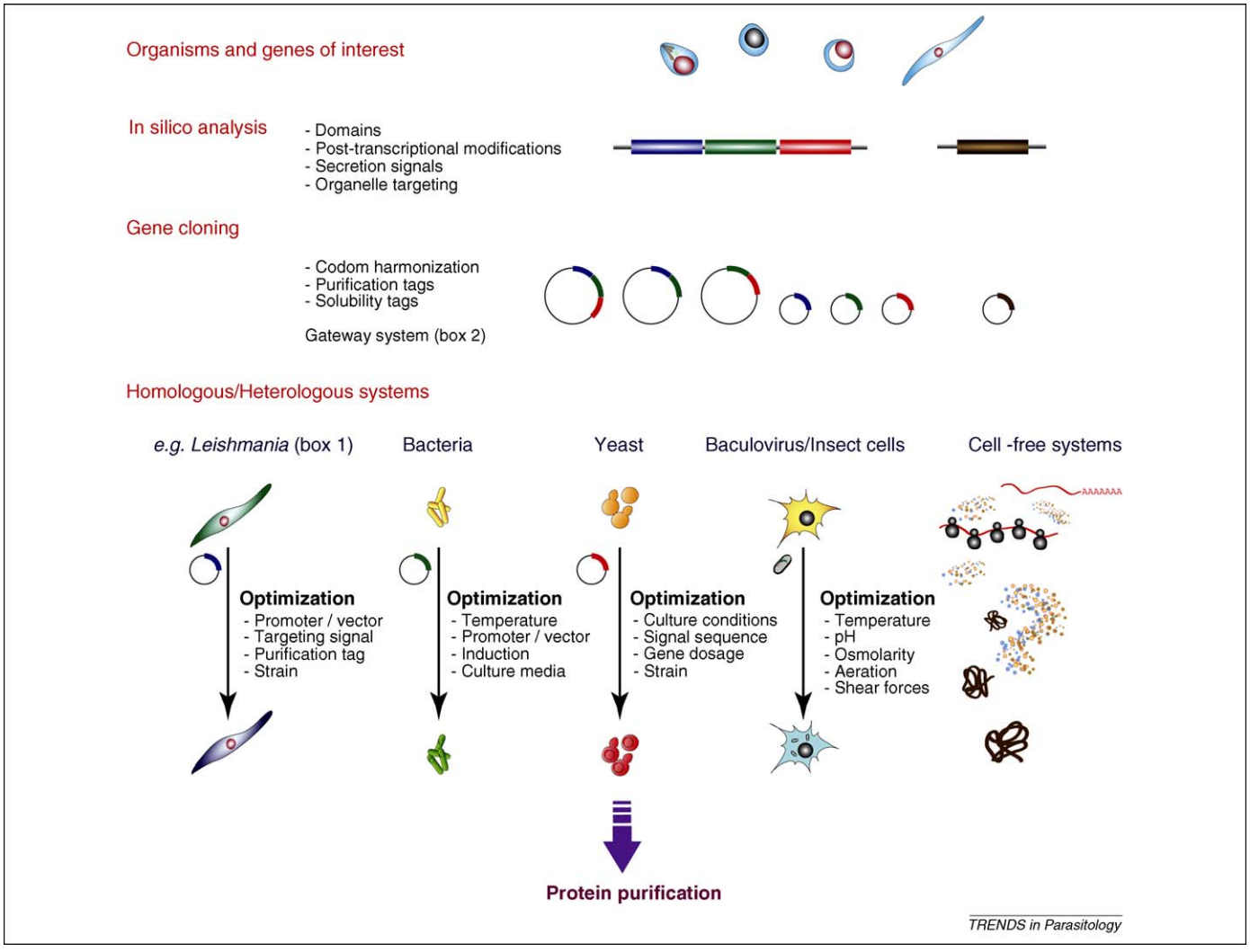

Figure 2. Most frequently used systems for the production of recombinant protein from parasites. After selection of the gene of interest, an extensive *in silico* analysis should corroborate the presence of protein domains, secretion signals, organellar targeting and post-translational modifications. These characteristics and the use of the recombinant protein will help guide the selection of the cloning vector and expression system. The gene of interest can be directly cloned, often under a strong promoter, into a suitable vector to express in a homologous system or in a surrogate system, ideally, the closest related species that can be grown in large quantities and is amenable for genetic manipulation. When no *in vitro* culture is available or the scale needed precludes the use of the homologous system, the bacterial system is often the first choice. General guidelines include removing predicted membrane-spanning regions, avoiding disrupting predicted secondary structural elements, respecting the boundaries of globular domains, if known; and avoiding the inclusion of low-complexity regions or hydrophobic residues at the N- and/or C-termini. When post-translational modifications in the recombinant protein are needed for function or antigenicity or the bacterial systems are not performing as expected, eukaryotic systems are available either using a surrogate organism or cell-free system. In either case, and particularly for systems that have been in the market for long time, there is a suite of clones, strains, culture conditions and tags that can be tested during the optimization process.

glycosylation, acylation, ability to form disulfide linkages, interaction with eukaryotic chaperones and proteolytic processing, together with subcellular compartmentalization, secretion mechanisms that avoid accumulation and low toxicity [43] (Figure 2). The most frequently used eukaryotic expressions systems include:

(i) *Yeast*: *Pichia pastoris* and *Saccharomyces cerevisiae* are usually the first choice expression systems owing to their suitability for large-scale fermentors and their high protein yields. Their main disadvantage resides in the *N*- and *O*-linked glycosylation patterns of the recombinant proteins, which, in some cases, might be qualitatively and quantitatively different from those in the native parasite protein, resulting in inactive products. Nevertheless, PfCP-2.9 and Pvs25 are two good examples of recombinant proteins produced in *Pichia* [27]. Further improvements on the expression of *Plasmodium* recombinant proteins in *S. cerevisiae* are based on the identification of *Plasmodium* protein–protein interactions using a high-throughput version of the yeast two-hybrid assay [44].

(ii) *Mammalian cell lines*: Although sharing numerous features with yeasts, the use of mammalian cells to produce recombinant proteins from parasites has been hindered by the labor-intensive and expensive methodology required for establishing stable recombinant cells. Thus, the use of mammalian cell lines has been limited to the identification and characterization of *Plasmodium* surface ligands involved in host–parasite interactions, binding assays, expression of membrane transporters and testing of DNA-based vaccines [27].

(iii) *Baculovirus-mediated expression systems in insect cells*: As a transient expression system, the virus-mediated synthesis of heterologous proteins in insect cells and larvae (reviewed in Ref. [45]) can yield milligram quantities of recombinant protein, with some required post-transcriptional modifications. Insect cell culture is simpler and requires less equipment than the culture of mammalian cells; it can be easily upgraded to bioreactor scale, and several genes of interest can be expressed simultaneously for the production of multiprotein complexes. Drawbacks include the time-consuming generation of recombinant baculovirus, their inability to synthesize branched multiantennary glycans and their lethal effects on the host cells, which makes the system suitable only for batch production. The full-length *Crithidia fasciculata* mitochondrial topoisomerase II, a promising target for anti-trypanosomatid drugs, was successfully expressed in the Bac-to-Bac baculovirus expression system [46]. Viruses represent one of the most frequently used systems for developing vaccines and have proved suitable both as delivery systems and as adjuvants. These include recombinant poxvirus FP9 and a modified vaccinia virus Ankara [47,48], the malaria protein B in yellow fever 17D virus [49], the *Plasmodium* merozoite surface protein (MSP)-1(42) in *Bombyx mori* nuclear polyhedrosis virus-silkworm [50] and the malaria MSP-1 in a replication-defective virus [51]. *Toxoplasma* surface antigens expressed in replication-deficient recombinant adenoviruses induce a protective immune response in mice [52]. Furthermore, the recombinant MVA rhoptry protein 2 vaccinia virus was developed as a vaccine candidate for toxoplasmosis [53].

(iv) *Tetrahymena thermophila*: Based on its high cell densities with short generation times and suitability for culture in bioreactors, the ciliate *T. thermophila* was proposed around a decade ago as an alternative heterologous expression system [54]. Since then, it has been used successfully to express the I-antigen of the parasitic ciliate of fish *Ichthyophthirius multifiliis* [55] and the glycosylphosphatidylinositol (GPI)-anchored full-length *Plasmodium* CSP [56]. This constitutes the first report of the recognition of targeting and GPI anchoring signals in a heterologous expression host. This successful initiative led to further development of *Tetrahymena* as an expression system [57], although limited progress has been reported since then. Publication of the *Tetrahymena* genome should renew such interest by identifying secretion and organellar signals for secretion and targeting, regulatory elements for constructing transfection vectors, among others, in spite of a potential limitation of this system concerning the expression of plastid-derived genes, because no compelling evidence for its presence was found in the genome [58].

(v) *Dictyostelium discoideum*: This slime mold is recognized as a useful model organism for studies ranging from ameboid motility to multicellular morphogenesis [59]. Because of its multiple advantages, which include axenic *in vitro* culture in synthetic media, *Dictyostelium* has been recently proposed as an alternative expression system for eukaryotic proteins (reviewed in Ref. [60]). As an alternative subunit vaccine against malaria, the *Plasmodium* recombinant CSP anchored to the surface of *Dictyostelium*, elicited antibodies against two different regions of the target protein [61]. In this case, replacement of the CSP–C-terminal segment by the *Dictyostelium* contact site A glycosyl-phosphatidylinositol anchor signal sequence was essential for surface display [61,62]. After these early successful studies [61,62], no further attempts have been reported and, as for *Tetrahymena*, the availability of the genome [63] should facilitate the exploration of this system for the production of recombinant proteins from parasites.

(vi) *Homologous and heterologous parasite expression systems*: As indicated above, proteins directly isolated from parasites are advantageous over recombinant proteins produced in bacteria because they faithfully display all desirable characteristics, such as immunogenicity and biological activity. Thus, the enhanced production of proteins of interest in the particular parasite, either by overexpression or by increasing the parasite numbers by culture scale-up, would represent the approach of choice. Recently, a chemically defined medium has been developed for continuous growth of the *P. falciparum* intraerythrocytic stage [64], which should facilitate the development of a homologous system for production of *Plasmodium* recombinant proteins. For parasites that cannot be cultured *in vitro*, alternative animal models can be used to overcome this limitation, at least in part. For example, rats have been proposed to substitute ruminants for enhancing propagation of *Cryptosporidium* spp. [65].

Protozoan parasites have also been used successfully as surrogate systems for expression of proteins of other parasites (Figure 3), mostly for functional studies, and developed into commercial heterologous expression systems (Box 1). *Neospora caninum* can express genes of the phylogenetically related *T. gondii* [66], whereas engineered *Toxoplasma* ts-4 mutants can express the *Leishmania* antigen kinetoplastid membrane protein-11 and elicit a specific immune response in BALB/c mice [67]. *Trichomonas foetus* can express functional *Trichomonas vaginalis* AP65 adhesin [68]. *Plasmodium vivax* chloroquine-resistance

Box 1. Commercial eukaryotic protein expression systems

The protozoan *Leishmania tarentolae*, which was isolated from the Moorish gecko *Tarentola mauritanica* [82], has been developed into a eukaryotic protein expression system (LEXSY, Jena Bioscience Jena, Germany). Its eukaryotic gene expression machinery, which includes full glycosylation and disulfide bond formation, represents a potential advantage over other expression systems. In addition, the system offers a suite of inducible or constitutive vectors and the choice to target the protein to intracellular compartments or for secretion. Moreover, the strains expressing the protein of interest are stable, easy to culture and amenable of production at fermentation scale. The system is relatively new in the market and its thorough implementation should, in the future, reveal to what extent it can contribute to resolve the current limitations in the production of recombinant protein from protozoan parasites.

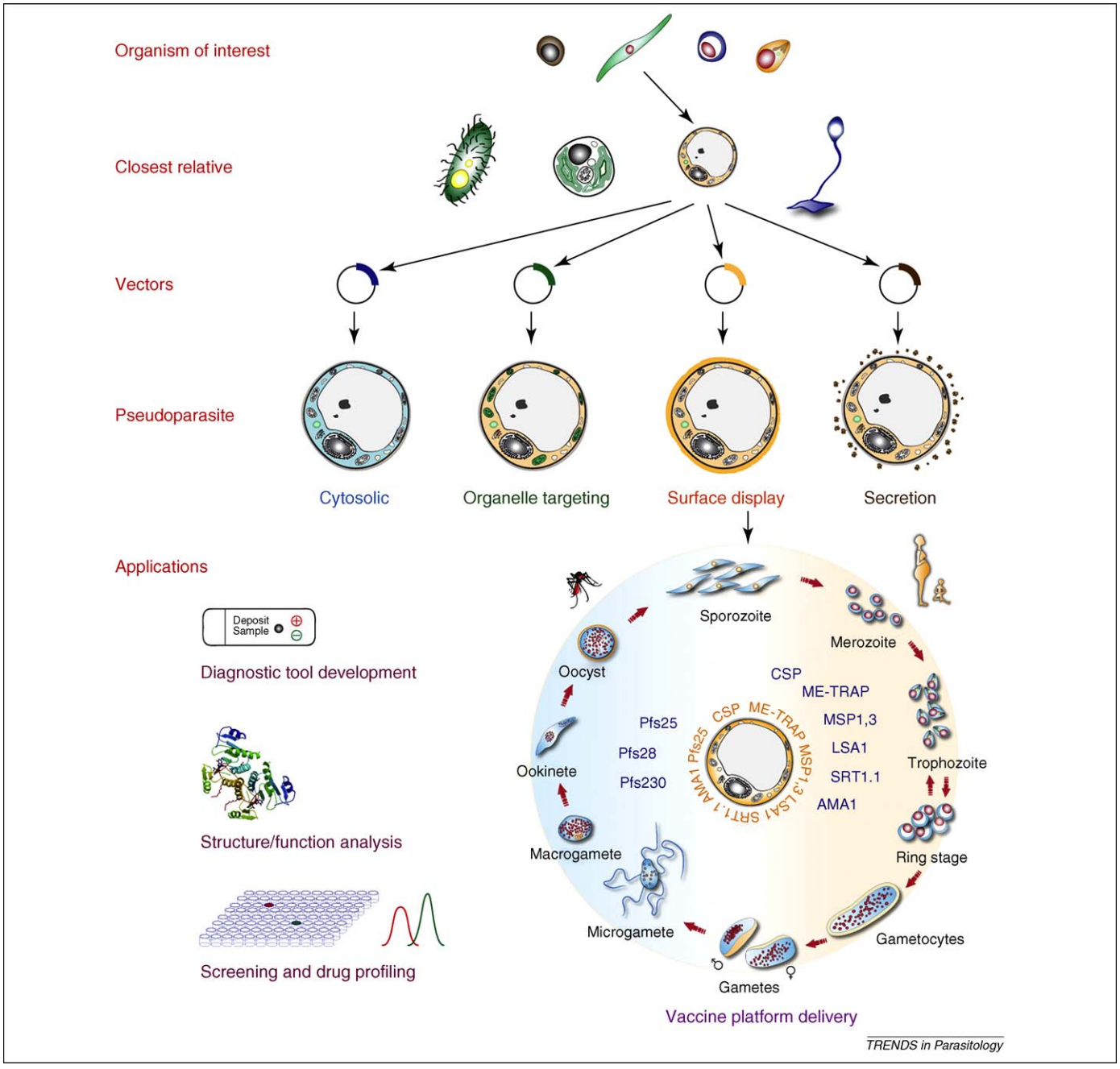

Figure 3. The 'pseudoparasite' concept. The evolutionary relationships between organisms provide us with a 'magnifying glass' to select those with shared structures, mechanisms and perhaps shared processes required for protein function. Therefore, 'phylogenetically tailored' heterologous expression systems might lead to the development of novel alternative platforms for expressing recombinant proteins from parasites. The first step would involve the selection of the closest relative to our organism of interest that shares the targeted structure/pathway/gene. Ideally, this organism would be amenable for genetic manipulation and for large-scale culture in axenic conditions and in a cell-free defined medium. In the second step, the cloning vector (cytosolic, organelle targeting, surface, secretion) would be selected depending on the predicted native targeting, and the potential applications of the recombinant protein, ranging from diagnostic tool development to screening and drug profiling. Taking this phylogenetic relationship further, the engineering of a pseudoparasite might be carried out in an organism that expresses genes that are homologous to those in the parasite of interest. These will enable the correct folding and post-translational modifications that take place in the target organism and that are necessary to elicit a specific, effective immune response, thus, providing a multi-antigen, up-scalable vaccine delivery platform. This approach would enable the identification and selection of the best combination of multiple immunogens to be expressed in their immunologically relevant conformation by the pseudoparasite, while taking advantage of the whole engineered organism as an adjuvant. Hence, the pseudoparasite displaying the selected parasite antigens can be directly tested for the ability to induce an effective immune response in the absence of adjuvants. Gateway systems and alike can also be adopted to incorporate any gene of the organism of interest to the pseudoparasite platform. The example depicted focuses on multiple *Plasmodium* stages (intra-erythrocytic stage omitted from the diagram) from which candidate genes selected for a malaria vaccine are integrated into the pseudoparasite. Other genes of interest identified in future studies could be incorporated to develop a platform expressing as many genes as necessary to provide an effective vaccine.

transmembrane protein was expressed in both *P. falciparum* and *Dictyostelium* for chloroquine drug tests [69]. Similarly, *Trypanosoma cruzi*, the etiological agent of Chagas disease, has been used to screen for drugs against *Crithidia fasciculata* ornithine decarboxylase [70].

Cell-free protein synthesis systems

The wheat-germ cell-free expression system was used to produce a functional PfDHFR-TS, known to have resisted multiple attempts in other expression systems [71]. Its application to malaria vaccine candidates [72] required no

prior optimization or harmonization of the *P. falciparum* AT-biased codon usage and yielded soluble, highly immunogenic proteins. A small-scale format expression analysis of 124 *P. falciparum* genes resulted in the production of 93 recombinant proteins, suggesting that this system could represent a suitable alternative for proteins from genes with codon bias and when post-translational modifications are not required.

The 'next door neighbor' as the optimal expression system

Phylogeny and systematics are powerful tools to establish evolutionary relationships between organisms, and they can contribute significantly to select species that could exhibit the highest potential to produce heterologous recombinant proteins. Similar structures and mechanisms present in distinct organisms might underscore unknown shared processes required for production of functional proteins [73]. Therefore, the identification of close relatives to the parasites of interest might lead to the development of novel alternative platforms for expressing recombinant proteins from protozoan parasites. *Perkinsus marinus* is a protozoan parasite of mollusks within the dinoflagellate lineage, still close to the divergence point from the apicomplexans [74]. In addition to the multiple morphological features that *Perkinsus* shares with the Apicomplexa, the availability of cell-free, fully defined media formulations for its culture, the 16–24 h doubling time reaching densities up to 10^8 cells/ml, and the ease of culture scale-up [75,76], suggest that *Perkinsus* might constitute an optimal expression system for producing recombinant proteins from apicomplexan parasites that meet the necessary qualitative and quantitative requirements. Furthermore, *Perkinsus* is non-pathogenic to humans and available resources include a fully sequenced genome and transfection methodology [77].

The alga *Chromera velia* has been dubbed a 'bridge over troublesome plastids' by providing a strong case for a single endosymbiotic origin of the dinoflagellates and apicomplexan plastids derived from a red algal endosymbiont [78,79]. Because *Chromera* can also be easily grown in culture, it has been deemed to have considerable potential for the successful expression of apicomplexan plastid-encoded proteins, as well as for high-throughput screening for drugs targeting the plastid [79]. Nevertheless, considerable characterization and optimization of the system will be required before it can become useful for these and other applications. On the basis of the extensive body of knowledge on the use of bioengineered algae for industrial applications [80], however, it could be anticipated that its potential might be realized in the near future.

Concluding remarks and future directions

The biological roles of most annotated genes of protozoan parasites remain unknown and the availability of their products, which is key to addressing questions about their structure–function relationships, remains hindered by the lack of optimal expression platforms for producing the recombinant proteins that would facilitate structural studies. From the above discussion, it becomes evident that the available systems are far from optimal. The development of novel expression systems (Box 1) together with the continued optimization of the currently available systems (Boxes 2 and 3) should contribute to rapidly increase the number of functional immunogenic recombinant proteins from protozoan parasites. Greater understanding of the cell biology of parasites and their close relatives by the comprehensive use of the existing resources will be key to advancing the field and enable the development of 'phylogenetically tailored' heterologous expression systems. Hence, strategies for producing parasite recombinant proteins should be designed on an individual basis and gradually developed to higher levels of complexity

Box 2. Gateway recombination strategy

The actual strategies for production of recombinant proteins include the screening of multiple host systems that require building multiple constructs with different purification tags. To reduce the pathway defined as 'linear', build an expression clone for a particular host, if the recombinant protein is not suitable for the application, go back and prepare another vector for the same or a new host [83], Gateway (Invitrogen Carlsbad, CA, USA) (for a review on this method see Ref. [84]) developed a method that relies on multistep recombinational cloning eliminating the need for the traditional enzymatic restriction and DNA ligase [85]. This system can be applied to 'low-throughput' to test a single protein of interest in different hosts or 'high-throughput' to test a large number of proteins in the same heterologous system and under the same conditions, in particular if a ORF library is available with each ORF as an individual clone, and the target vector has all the elements necessary for driving the expression and purifying the recombinant protein. This system has been applied successfully to *P. falciparum* single exon genes, with an 84% cloning efficiency and a 100% success rate when subcloned into three expression vectors [31]. The same approach was used to identify novel malaria antigens for DNA vaccines [86] and it is being adopted for cloning and expressing genes in *Entamoeba histolytica* [87]. Similarly, this system can be quickly adopted for any parasite of interest to build a battery of expression vectors designed specifically to express and produce recombinant proteins in any parasite specific heterologous system developed [88].

Box 3. Source of the currently available expression systems

For a review of widely used prokaryotic and eukaryotic systems, see Ref. [28]. Invitrogen commercializes the *Pichia* Expression Kit and Bac-to-Bac baculovirus expression system (www.invitrogen.com). *Flash*BAC™ (Oxford Expression Technologies, Oxford, UK) offers the potential for automation and parallelization. With the same objective, EMD BioSciences Inc. (Gibbstown, NJ, USA) and Invitrogen (USA) have introduced the InsertDirect™ and HEK 293Free-Style™ systems, respectively. The protein expression system using the protozoan *Leishmania tarentolae* LEXSY has been recently commercialized by Jena Bioscience (www.jenabioscience.com). The wheat germ cell-free protein expression system (WREPO®) was developed by Cell Free Sciences (www.cfsciences.com). Non-commercial systems under development or with potential for became alternative systems for the expression of recombinant proteins are available from the laboratories where it has been developed. *T. thermophila* is the most advanced [54]. In the case of *Dictyostelium*, which has recently been proposed as an alternative expression system for eukaryotic proteins [60], it is available at the *Dictyostelium* consortium (http://www.nih.gov/science/models/d_discoideum/). The *P. marinus* system is under development at the corresponding author laboratory. The alga *Chromera* has also considerable potential for the successful expression of apicomplexan proteins [79]. Most of these organisms are available at ATCC (www.ATCC.org).

(Figure 2). 'Systems biotechnology' is still in its early stages, however, and novel disciplines (such as evolutionary engineering and inverse metabolic engineering) illustrate the emergence of innovative concepts (reviewed in Ref. [81]) that could spearhead progress in addressing parasitic diseases. In this context, the parasite *P. marinus* could be engineered as a suitable surrogate for production of apicomplexan proteins. Thus, designing a non-pathogenic, albeit phylogenetically closely related 'pseudoparasite' (Figure 3), which expresses multiple parasite genes with the correct folding and post-translational modifications, while taking advantage of the whole engineered organism as an adjuvant, might elicit a specific effective immune response and thus constitute a promising alternative to the currently available platforms for vaccine design and delivery.

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