Recombinant protein sub-unit vaccine synthesis in microbes: a role for yeast?

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Abstract

Recombinant protein sub-unit vaccines are formulated using protein antigens that have been synthesized in heterologous host cells. Several host cells are available for this purpose, ranging from *Escherichia coli* to mammalian cell-lines. This article highlights the benefits of using yeast as the recombinant host. The yeast species, *Saccharomyces cerevisiae* and *Pichia pastoris*, have been used to optimize the functional yields of potential antigens for the development of sub-unit vaccines against a wide range of diseases caused by bacteria and viruses. *S. cerevisiae* has also been used in the manufacture of eleven approved vaccines against hepatitis B virus and one against human papillomavirus; in both cases the recombinant protein forms highly-immunogenic virus-like particles. Advances in our understanding of how a yeast cell responds to the metabolic load of producing recombinant proteins will allow us to identify host strains that have improved yield properties and enable the synthesis of more challenging antigens that cannot be produced in other systems. Yeasts therefore have the potential to become important host organisms for the production of recombinant antigens that can be used in the manufacture of sub-unit vaccines or in new vaccine development.

Recombinant protein sub-unit vaccines

Most vaccines are based on formulations comprising either live, attenuated- or killed, inactivated-bacteria or viruses (1). Live, attenuated vaccines typically induce strong, long-lasting immunity; however, while they are attenuated in their pathogenicity, concerns remain

about reversion to virulent wild-type strains that might cause disease in immunocompromised individuals (2). In contrast, killed, inactivated vaccines are non-infectious (2), but are less effective in inducing protective immunity often requiring an adjuvant to stimulate antibody responses and effector T cell functions (3). The increasingly stringent demands of regulatory authorities such as the United States Food and Drug Administration (FDA), the European Medicines Agency (EMA) and the World Health Organization (WHO) require new vaccine compositions to be precisely specified. This makes developments using whole cell vaccines particularly challenging because they contain undefined molecules that originate from the source bacterium or the host cell used to produce the virus.

In the last thirty years, there has been a trend towards developing sub-unit vaccine formulations that contain defined antigenic components together with a potent adjuvant (2). The antigen may be a polysaccharide, a nucleic acid or a protein. In the latter case, which is the focus of this article, the protein itself may be (i) a purified protein from the disease-causing pathogen, (ii) a synthetic peptide or (iii) a recombinant protein that has been synthesized in one of many possible heterologous host cells ranging from *Escherichia coli* to mammalian cells (4). This ensures that the antigen has a well-defined composition, that there is effectively no risk of pathogenicity in its use and that antigen synthesis and purification can be scaled up in a cost-effective manner (5). Unfortunately, while many recombinant proteins exhibit immunogenicity in mice, they are not necessarily potent antigens in humans (even when administered with an effective adjuvant), as seen in the case of apical membrane antigen-1 (AMA-1), which is a leading blood-stage malaria vaccine antigen (6). However, some recombinant proteins form virus-like particles (VLP), which are multi-protein structures that mimic the organization and conformation of native viruses but without a viral genome (7). VLPs have been found to be more stable and considerably more immunogenic than purified protein antigens (7). Notably, the two currently-licensed

recombinant antigens manufactured in yeast are VLPs (Table 1).This review examines the role that yeast cells can play in further vaccine development.

Recombinant gene expression technology was developed 41 years ago in *E. coli* (9), leading to the recombinant synthesis of the human hormones, somatostatin in 1977 (10) and insulin in 1979 (11). Today, the production of a wide range of recombinant biopharmaceuticals, including recombinant hormones, antibodies and vaccines, is a multi-billion dollar global business (12). More than 150 biopharmaceuticals have been approved by the FDA to date (13, 14), with approximately 20% of these recombinant proteins being produced in yeasts (the vast majority in *S. cerevisiae*), 30% in *E. coli* and 50% in mammalian cell-lines and hybridomas (5, 13, 15). Table 1 summarizes data for recombinant protein sub-unit vaccines that are either currently licensed for human use in the EU or the US or have previously been licensed but are now withdrawn. In contrast to the picture for biopharmaceuticals as a whole, it is notable that the majority of commercial vaccines use antigens that have been synthesized in microbes; 14 out of the 16 vaccines in Table 1 contain an antigen synthesized in either *E. coli* or *S. cerevisiae* although only two distinct antigens are actually synthesized in *S. cerevisiae* and two in *E. coli*. Recombinant hepatitis B surface antigen (HBsAg) synthesized in *S. cerevisiae* has been used in 11 different formulations (Table 1); the first of these was reported in 1982 (16) and was subsequently licensed in 1986 by the FDA for use in humans (2). Due to a lack of demand in the EU, GlaxoSmithKline Biologicals withdrew Tritanrix-HB® in 2009, while Aventis Pasteur MSD withdrew Primavax® in 2000 and Procomvax® in 2009, all of which contain recombinant HBsAg as part of multivalent vaccine formulations. A second antigen synthesized in *S. cerevisiae* is comprised of the major capsid protein, L1, from four human papillomavirus types (6, 11, 16 and 18) to generate the human papillomavirus vaccine, Gardasil®. In both cases these *S. cerevisiae*-derived antigens form VLPs. An alternative VLP vaccine, Cervarix®, is formulated using recombinant major capsid protein, L1, from two human papillomavirus types (16 and 18) that

have been synthesized in insect cells; insect cells are also used in the manufacture of a second vaccine, Flublok® (Table 1).

In 1998, a vaccine against Lyme disease, Lymerix®, was approved by the FDA. Lymerix® incorporates recombinant surface lipoprotein, OspA, from *Borrelia burgdorferi* that is synthesized in *E. coli* (Table 1); however, the vaccine was withdrawn by the manufacturer in 2002 due to a lack of demand in the US that followed extensive media coverage and ongoing litigation concerning adverse side effects (17). This was despite initial studies indicating that the Lymerix® vaccine was a cost-effective public health intervention for people at high risk of Lyme disease; its withdrawal precluded the design of more conclusive studies (18). Bexsero® was licensed in the EU in 2013 to protect against meningococcal meningitis and septicemia caused by meningococcal serogroup B (Table 1). It contains three antigens synthesized in *E. coli* in addition to an outer membrane vesicle from meningococcal strain MZ98/254.

Recombinant antigen synthesis is possible in a range of host cells: the importance of *Escherichia coli*

E. coli is typically the first choice of host cell for producing recombinant proteins in industrial and academic laboratories; it is a familiar laboratory organism, quick and inexpensive to culture and has the potential to generate high product yields (5). Unsurprisingly, it is therefore widely used in both commercial (for the manufacture of approximately 30% of protein biopharmaceuticals; (13, 15)) and research (in the synthesis of >70% of proteins (5)) laboratories. This situation is reflected by data in the published literature on recombinant antigen synthesis, which suggests a wide range of protein antigens have been produced in *E. coli* for use in the development of new recombinant protein sub-unit vaccines: on 8th October 2014, the PubMed Central database was searched for entries in any field containing "recombinant" and "vaccine" together with the name of the host cell; this returned 3,256 articles for "coli", 266 articles for "pastoris", 288 articles for "cerevisiae", 890 articles for "baculovirus" and 398 articles for CHO (or 107 articles for "Chinese hamster ovary"). While

this type of analysis can only be indicative, *E. coli* does appear to have an important role in research into recombinant antigens, in line with its wider use in recombinant protein production.

Reports using *E. coli* as the host cell most often describe initial characterization of the recombinant antigen and demonstration of immunogenicity in mice, as illustrated by the following examples. Recombinant protective antigen (rPA83) has been characterized as a successful adjuvant-bound sub-unit vaccine against *Bacillus anthracis*, the causative agent of anthrax (19). Recombinant fraction 1 (Caf1) and V (LcrV) antigens induce protection against *Yersinia pestis* infection (the causative agent of bubonic and pneumonic plagues) one year post-vaccination (20). Flaccid paralytic disease or botulism is caused by neurotoxin F from *Clostridium botulinum*; the receptor-binding domain of neurotoxin F was synthesized as a fusion with or without maltose binding protein in *E. coli* and the purified protein protected mice against challenge with *C. botulinum* neurotoxin F ten months after vaccination (21). *Helicobacter pylori* infection causes stomach and duodenal ulcers in humans; the recombinant urease sub-units, UreA and UreB, induced an immunoprotective response in mice (22). Hospital-acquired infection such as pneumonia and sepsis are typically caused by *Staphylococcus aureus*. Data from studies in mice have suggested the potential to develop a protein sub-unit vaccine based on recombinant collagen binding bacterial adhesin fragment (CNA19) (23). A proof-of-principle leprosy vaccine development scheme recently demonstrated efficacy in mice using a 73f fusion protein (coded by aligning the individual gene sequences for ML2028, ML2346 and ML2044 from *Mycobacterium leprae* as a single product) (24). Two approaches to developing malaria vaccines (specifically disease caused by *Plasmodium vivax*) have examined recombinant domain II of AMA-1, which was demonstrated to be immunogenic in mice (25) and a soluble antigen called VMP001 based on the circumsporozoite protein, which was immunogenic in rhesus monkeys (26). A recombinant sub-unit vaccine formulated using a fusion protein between Ag85B and ESAT-6 was shown to be highly protective against *Mycobacterium tuberculosis*

(the causative agent of tuberculosis) in mice (27). Further examples include development of sub-unit vaccines to protect against dengue virus (28), hepatitis A virus (29), human immunodeficiency virus (30), human rotavirus (31), human respiratory syncytial virus (32), H1N1 influenza virus (33), *Pseudomonas aeruginosa* infection (34) and schistosomiasis (35). All these examples use *E. coli* as the recombinant host and illustrate the importance of this prokaryotic microbe as a tool in vaccine development.

The use of eukaryotic hosts in recombinant protein sub-unit vaccine development: an emerging role for yeasts

While *E. coli* has many benefits as a cell factory, producing a recombinant protein in a prokaryotic host cell can often result in inclusion body formation and/or low specific yields of a product lacking post-translational modification (36). This may be one reason for a general decline in the more recent use of *E. coli* as a host cell and the consequent emergence of several eukaryotic options (5).

In principle, the use of mammalian cell-lines should overcome challenges associated with synthesizing eukaryotic proteins in prokaryotes, especially with recent advances in stable recombinant gene expression (37, 38). This is because rates of protein synthesis and folding are almost an order of magnitude faster in prokaryotes than they are in eukaryotes (39), eukaryotic codons are often inefficiently expressed in prokaryotes and authentic eukaryotic post-translational modifications cannot yet be achieved in *E. coli* (36). In support of this, Synagis®, which is used for passive immunization of infants to protect against respiratory syncytial virus, is formulated using a humanized monoclonal antibody ($IgG1_{1K}$; directed against an epitope of the viral F protein) synthesized in mouse myeloma cells (40). In clinical trials, a herpes simplex virus (HSV) vaccine, containing a truncated form of recombinant HSV-2 glycoprotein D from HSV-2 strain G that had been synthesized in Chinese hamster ovary cells, had efficacy in some women dependent on their serologic status, but no efficacy in men (41).

Insect cells have also been used for both commercial vaccine production (Cervarix® and Flublok®, Table 1) and in the synthesis of recombinant protein antigens for new vaccine development. For example, the receptor-binding domain of neurotoxin A (rBoNT/A-HC-6h) from *Clostridium botulinum* was synthesized in insect cells; purified rBoNT/A-HC-6h gave mice full protection against botulinum A toxin with a dose as low as 0.2μg (42). Merozoite surface protein 1 from *P. falciparum* (MSP-1, comprising 43 amino-terminal residues) was also synthesized in insect cells and demonstrated to be immunogenic in rabbits (43). Further examples include development of sub-unit vaccines, some incorporating glycoproteins that would not be possible to synthesize in *E. coli*: these include sub-unit vaccines against chandipura virus (44), hepatitis E virus (45), malaria (specifically disease caused by *P. falciparum)* (46), severe acute respiratory syndrome (SARS) virus (47) and West Nile virus (48).

Plant cells have also been explored as recombinant hosts, with the added possibility of developing edible vaccines. The cholera toxin B subunit, immunoglobulins, α-interferon, VP1 protein from foot-and-mouth disease virus and glycoprotein S from transmissible gastroenteritis virus have all been expressed in transgenic plants or by means of plant viruses (49, 50). Transgenic tobacco plants (*Nicotiana tabacum*) have also been used to synthesize a measles virus hemagglutinin (H) protein that was demonstrated to be immunogenic in mice (51).

Eukaryotic microbes, especially *S. cerevisiae* and *Pichia pastoris* offer many of the benefits of higher eukaryotic host cells, whilst retaining the advantages of being microbial. Despite their propensity to hyper-glycosylate recombinant proteins (5), these two yeasts have many advantages: a wealth of molecular and genetic resources are available for both species (52, 53), growth rates are an order of magnitude higher than mammalian cell-lines and they are cheap to culture (54). As discussed above, *S. cerevisiae* is already used in the manufacture of 12 out of the 16 approved vaccines shown in Table 1; these vaccines are considered safe and efficacious because they are noninfectious and highly immunogenic.

Table 2 shows examples from the literature suggesting that these advantages are becoming more widely known in academic research laboratories both for *S. cerevisiae* and *P. pastoris*; the latter yeast is a relative new-comer, having been first developed as a recombinant host system in 1985 (55). The PubMed Central database was searched for entries containing "sub-unit" and "vaccine" in any field, which returned 189 articles. This was augmented with searches for entries in any field containing "recombinant" and "vaccine" with the name of the host cell; this returned 266 articles for "pastoris" and 288 entries for "cerevisiae". The articles were examined manually to identify the target disease, the recombinant antigen and the recombinant host cell. Many veterinary vaccines are in development, but only data for potential human recombinant sub-unit vaccines are shown. For *S. cerevisiae*, several vaccine candidates are based on inactivated whole yeast cells (56) or involve displaying the antigen on the surface of a yeast cell (57), but these are not included in Table 2; only studies using recombinant protein antigens are listed. What is immediately noticeable is the large proportion of very recent studies that have been published using yeast: for *S. cerevisiae*, 5 out of 12 and for *P. pastoris* 17 out of 21 reports were published between 2010 and 2014.

Designing improved recombinant antigen synthesis experiments

In designing any new recombinant protein production strategy, optimization of the gene sequence should be considered so it is more likely to be stably expressed in the chosen recombinant host cell; there is an extensive literature on engineering stabilized proteins (58, 59), while recent insights suggest that codon optimization (60) might aid functional expression (61). In addition, optimizing culture conditions and induction protocols is essential to increase recombinant protein yields; this has been demonstrated in cultures of both *P. pastoris* (62, 63) and *E. coli* (64). Successful implementation of a "Design of Experiments" approach to bioprocess optimization (65) enables the simultaneous investigation of multiple parameters and their interactions on the functional yield of a recombinant protein. In *P. pastoris* cultures, this approach was shown to increase the yield per cell by matching the induction feed profile to the cellular metabolism (66). In a separate study, pulsing *P. pastoris*

cells with an inducer (methanol) revealed the potential benefit of stress in increasing productivity (67). These advances are all easily applicable to recombinant antigen synthesis.

S. cerevisiae is particularly amenable to studying the mechanistic basis of high-yielding recombinant protein production experiments using the tools of systems and synthetic biology (68). As stated in a recent review (5), identifying or engineering yeast strains with improved yield characteristics may either be targeted towards one particular pathway or may take a more global approach (69). Examples of the targeted approach include “humanizing” the yeast glycosylation (70) and sterol (71) pathways and modifying membrane phospholipid synthesis to proliferate intracellular membranes (72). Studies taking a more global approach in both *S. cerevisiae* (73, 74) and *P. pastoris* (62, 75) have identified the importance of the unfolded protein response (the cellular stress response activated by the accumulation of unfolded or misfolded protein) and reduced translational activity in high yielding cultures. Such insights, which are not yet possible in higher eukaryotic systems, have been used to select specific yeast strains that can substantially improve recombinant protein yields compared to wild-type cells (76, 77).

Conclusions

E. coli is often the first host cell to be considered in the synthesis of a new recombinant protein, although the commercial production of approved sub-unit vaccines relies on *S. cerevisiae* and insect cells as well as *E. coli* (Table 1). Table 2 illustrates the use of yeast as a research tool in vaccine development. This is particularly notable for *P. pastoris*, which has become a popular host very recently. Using both prokaryotic and eukaryotic microbes makes practical sense, since working with bacteria and yeast require similar techniques, equipment and approaches. Yeasts should therefore be considered alongside *E. coli* in developing a strategy to produce recombinant protein sub-unit vaccines, especially those based on VLPs, or as a tool to screen novel antigens in new vaccine development.

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Table 1. Recombinant protein sub-unit vaccines approved for human use. Sub-unit vaccines containing a recombinant protein antigen that have been approved for human use in the United States of America (US) or the European Union (EU) are listed in order of the date on which they were approved. Data were retrieved on 28th May 2014 from the FDA (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093830.htm), EMA (http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/medicines/medicines_landing_page.jsp&mid=) and the United Kingdom Department of Health (https://www.gov.uk/government/collections/immunisation-against-infectious-disease-the-green-book) websites. While 14 out of 16 vaccine formulations contain antigens synthesized in microbes, only two distinct antigens are synthesized in *Saccharomyces cerevisiae* and a further two in *Escherichia coli*. Insect cells are used to synthesize two additional distinct antigens.

Recombinant host	Vaccine name	Protection conferred against	Recombinant antigen	Manufacturer	Date approved
Escherichia coli	Lymerix®	*Borrelia burgdorferi* (causative agent of Lyme disease in US)	OspA lipoprotein	GlaxoSmithKline Biologicals	1998 (US); vaccine withdrawn by GlaxoSmithKline Biologicals in 2002
	Bexsero®	*Neisseria meningitides* (causative agent of meningococcal meningitis and septicemia)	Factor H binding protein (fHbp), Neisserial adhesin A (NadA), Neisseria heparin binding antigen (NHBA) and Porin A (PorA) from meningococcal strain NZ 98/254	Novartis	2013 (EU)
Saccharomyces cerevisiae	Recombivax-HB®	Hepatitis B virus	Hepatitis B surface antigen (HBsAg)	Merck & Co Inc	1986 (US)
	Comvax®	Hepatitis B virus and *Haemophilus influenzae* type B; causative agent of pneumonia or meningitis, especially in young children	HBsAg	Merck & Co Inc	1996 (US)
	Tritanrix-HB®	Hepatitis B virus,	HBsAg	GlaxoSmithKline	1996 (EU); vaccine

	Corynebacterium diphtheria (causative agent of diptheria), *Clostridium tetani* (causative agent of tetanus) and *Bordetella pertussis* (causative agent of whooping cough)		Biologicals	withdrawn by GlaxoSmithKline Biologicals in 2009
Twinrix®	Hepatitis A and B viruses	HBsAg	GlaxoSmithKline Biologicals	1996 (EU; adult vaccine); 1997 (EU; paediatric vaccine)
Engerix-B®	Hepatitis B virus	HBsAg	GlaxoSmithKline Biologicals	1998 (US)
Primavax®	Hepatitis B virus, *C. diphtheria* and *C. tetani*	HBsAg	Sanofi Pasteur MSD	1998 (EU); vaccine withdrawn by Sanofi Pasteur MSD in 2000
Procomvax®	Hepatitis B virus and *H. influenzae* type B	HBsAg	Sanofi Pasteur MSD	1999 (EU); vaccine withdrawn by Sanofi Pasteur MSD in 2009
HBvaxPRO®	Hepatitis B virus	HBsAg	Sanofi Pasteur MSD	2001 (EU)
Pediarix®	Hepatitis B virus, *C. diphtheria*, *C. tetani*, *B. pertussis* and poliovirus	HBsAg	GlaxoSmithKline Biologicals	2002 (US)
Ambirix®	Hepatitis A and B viruses	HBsAg	GlaxoSmithKline Biologicals	2002 (EU)
Fendrix®	Hepatitis B virus	HBsAg	GlaxoSmithKline	2005 (EU)

				Biologicals	
	Gardasil	Human papillomavirus	Major capsid protein, L1, for human papillomavirus types 6, 11, 16 and 18	Merck & Co Inc Sanofi Pasteur MSD	2006 (US) 2006 (EU)
Insect cells	Cervarix®	Human papillomavirus	Major capsid protein, L1, for human papillomavirus types 16 and 18	GlaxoSmithKline Biologicals	2007 (EU); 2009 (US)
	Flublok®	Influenza virus	Full-length hemagglutinin (influenza virus A strains, H1N1 and H3N2, and one influenza virus B strain)	Protein Sciences Corporation	2013 (US)

605 **Table 2. Examples of recombinant protein antigens synthesized in yeast for use in developing human sub-unit vaccines.** Antigens
606 synthesized in *S. cerevisiae* or *P. pastoris* are listed alphabetically by the relevant disease. The PubMed Central database was searched for
607 entries containing "sub-unit" and "vaccine" in any field, which returned 189 articles. This was augmented with searches for entries in any field
608 containing "recombinant" and "vaccine" with the name of the host cell; this returned 266 articles for "pastoris" and 288 entries for "cerevisiae".
609 These articles were examined manually to identify the target disease, the antigen and the recombinant host cell. Many veterinary vaccines are
610 in development, but only data for potential human recombinant sub-unit vaccines are shown.

Recombinant host	Disease (causative organism)	Antigen	Outcome	Reference
Saccharomyces cerevisiae	Anthrax (*Bacillus anthracis*)	Protective antigen component of the anthrax toxin complex, PA63	Protection against infection was demonstrated in rabbits and non-human primates	(78)
	Tetanus (*Clostridium tetani*)	Tetanus toxin fragment C	Protection against infection was demonstrated in mice	(79)
	Dengue virus	Dengue envelope domain III (scEDIII) from all four serotypes	Immunogenicity was demonstrated in mice	(80)
	Hantavirus	Hantavirus N protein	Mid-scale (5L) production demonstrated	(81)
	Human immunodeficiency virus type 1	Gag protein	Spheroplasts released Gag virus-like particles extracellularly	(82)
	Lyme disease (*Borrelia burgdorferi*)	N-terminally truncated form of outer-surface protein A (des-Cys1-OspA)	Improved yields over synthesis in *E. coli*	(83)
	Malaria (*Plasmodium spp)*	RTS,S that consists of sequences of the circumsporozoite protein and the hepatitis B surface antigen (HBsAg). RTS and S spontaneously assemble into mixed	Vaccine is in phase 3 clinical trials; it induced protection in 56% of vaccinees	(84)

		polymeric particulate structures. These VLPsare each estimated to contain, on average, 100 polypeptides		
	Malaria (*Plasmodium falciparum*)	Sexual-stage surface antigens synthesized as a Pfs25-28 fusion protein	Pfs25-28 elicits potent *P. falciparum* transmission-blocking antibodies in mice.	(85)
	Malaria (*Plasmodium vivax*)	A particulate antigen called CSV-S,S based on the circumsporozoite (CSV) protein. It comprises CSV-S (a fusion protein between a soluble form of CSV and HBsAg) and free HBsAg co-expressed in yeast and self-assembled into mixed VLPs	The particulate antigen was immunogenic in rhesus monkeys	(26)
	Poliovirus	P1, the precursor for the structural proteins, and 3CD, the viral protease	VLPs could be isolated	(86)
	Rabies virus	Rabies virus surface glycoprotein	Protective following intramuscular injection in guinea pigs	(87)
	Rotavirus	Structural proteins VP2, VP6 and VP7	Production of triple-layered rotavirus VLP demonstrated	(88)
Pichia pastoris	Alzheimer's disease	Recombinant 4 × Aβ15, four tandem repeats of amyloid β(1-15) interlinked by spacers	Proposed as an alternative to previous human clinical trials of vaccination that were halted due to brain inflammation	(89)
	Chagas' disease (*Trypanosoma cruzi*)	Trans-sialidase containing the catalytic domain without the immunodominant SAPA (Shed Acute Phase Antigen) repeats	The recombinant sub-unit vaccine was protective in mice	(90)
	Dengue virus	Dengue virus type 2 envelope domain III (sEDIII-2)	Demonstration of synthesis of recombinant antigen	(91)

Epstein-Barr virus	EBNA1, the viral protein expressed in all EBV-associated malignancies; truncated EBNA1 (E1ΔGA, codons 390-641) was expressed as a secretory protein with an N-terminal polyhistidine tag	Recombinant E1ΔGA was demonstrated to be immunogenic in mice	(92)
Hand, foot and mouth disease (human enterovirus 71)	VP1, one of the major immunogenic capsid proteins of human enterovirus 71	Recombinant VP1 protein was immunogenic in mice	(93)
Helicobacter pylori infection	Alkyl hydroperoxide reductase (AhpC)	Protection against infection was demonstrated in mice	(94)
Hepatitis B virus	Hepatitis B surface antigen (HBsAg)	Production and purification of VLPs that have potential as a superior vaccine to Engerix-B®	(95)
Hepatitis C virus	E1E2 protein, which consists of E1 residues 187-346 and E2 residues 381-699	E1E2 protein was immunogenic in rabbits	(96)
Human hookworm (*Necator americanus*)	*N. americanus* glutathione S-transferase (Na-GST-1)	Scale-up of production was demonstrated for initial phase 1 clinical testing	(97)
Human immunodeficiency virus type 1	Gag protein	Gag protein was immunogenic in mice	(98)
Human papillomavirus	Major capsid protein, L1, for human papillomavirus type 16	Recombinant protein was produced	(99)
Human schistosomiasis (*Schistosoma*	9 kDa recombinant protein corresponding to the extracellular domain of a unique *S. mansoni* tetraspanin	Development of 20L scale production was demonstrated	(100)

mansoni)			
Influenza virus A (avian origin)	Recombinant neuraminidase (rNA) antigen	The recombinant antigen induced an immunoprotective response in mice	(101)
Influenza virus A (pandemic swine origin)	H1N1 hemagglutinin (HA) protein	Recombinant production of endotoxin-free H1N1 HA was demonstrated	(102)
Japanese encephalitis virus	Viral envelope protein (E)	Immunogenicity and protective efficacy were demonstrated in mice.	(103)
Leptospirosis (Leptospira spp)	Leptospiral immunoglobulin-like (Lig) protein LigANI and the immunodominant lipoprotein LipL32	Recombinant proteins produced in *E. coli* have demonstrated variable results. LigANI and LipL32 from *P. pastoris* retained the antigenic characteristics of the native proteins	(104)
Malaria (*Plasmodium berghei*)	Circumsporozoite protein (CS) multimerized by fusion to the measles virus nucleoprotein (N) known to auto-assemble in yeast in large-size ribonucleoprotein rods (RNPs)	Subcutaneous immunization of mice with heat-inactivated whole *P. pastoris* expressing N-CS RNPs provided significant reduction of parasitemia after intradermal challenge with a high dose of parasites	(105)
Malaria (*Plasmodium falciparum*)	Merozoite surface protein 1 (MSP-1), comprising 43 N-terminal MSP-1 residues (the 19 residue MSP-1 signal sequence, which is removed by processing in baculovirus, plus 24 residues from N-terminal block 1) and the adjacent 16 amino acid residues, and other variants	Immunogenicity was demonstrated in rabbits	(43)
Malaria (*Plasmodium vivax*)	Apical membrane antigen-1 AMA-1	Secreted recombinant forms of AMA-1 were demonstrated to be immunogenic in	(106)

		mice	
Norovirus	Capsid protein (strain VA387, genogroup II.4)	Oral administration of yeast extracts without an adjuvant stimulated an appropriate immune response in mice	(107)
Toxoplasmosis (*Toxoplasma gondii*)	Chimeric surface antigen 1 and 2 (SAG1/2)	Vaccinated mice were significantly protected against lethal challenge with live *T. gondii*	(104)

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