

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

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Abstract

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

Recombinant protein sub-unit vaccines

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

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

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

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

57 Recombinant gene expression technology was developed 41 years ago in *E. coli* (9), leading
58 to the recombinant synthesis of the human hormones, somatostatin in 1977 (10) and insulin
59 in 1979 (11). Today, the production of a wide range of recombinant biopharmaceuticals,
60 including recombinant hormones, antibodies and vaccines, is a multi-billion dollar global
61 business (12). More than 150 biopharmaceuticals have been approved by the FDA to date
62 (13, 14), with approximately 20% of these recombinant proteins being produced in yeasts
63 (the vast majority in *S. cerevisiae*), 30% in *E. coli* and 50% in mammalian cell-lines and
64 hybridomas (5, 13, 15). Table 1 summarizes data for recombinant protein sub-unit vaccines
65 that are either currently licensed for human use in the EU or the US or have previously been
66 licensed but are now withdrawn. In contrast to the picture for biopharmaceuticals as a whole,
67 it is notable that the majority of commercial vaccines use antigens that have been
68 synthesized in microbes; 14 out of the 16 vaccines in Table 1 contain an antigen synthesized
69 in either *E. coli* or *S. cerevisiae* although only two distinct antigens are actually synthesized
70 in *S. cerevisiae* and two in *E. coli*. Recombinant hepatitis B surface antigen (HBsAg)
71 synthesized in *S. cerevisiae* has been used in 11 different formulations (Table 1); the first of
72 these was reported in 1982 (16) and was subsequently licensed in 1986 by the FDA for use
73 in humans (2). Due to a lack of demand in the EU, GlaxoSmithKline Biologicals withdrew
74 Tritanrix-HB® in 2009, while Aventis Pasteur MSD withdrew Primavax® in 2000 and
75 Procomvax® in 2009, all of which contain recombinant HBsAg as part of multivalent vaccine
76 formulations. A second antigen synthesized in *S. cerevisiae* is comprised of the
77 major capsid protein, L1, from four human papillomavirus types (6, 11, 16 and 18) to
78 generate the human papillomavirus vaccine, Gardasil®. In both cases these *S. cerevisiae*-
79 derived antigens form VLPs. An alternative VLP vaccine, Cervarix®, is formulated using
80 recombinant major capsid protein, L1, from two human papillomavirus types (16 and 18) that

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

83 In 1998, a vaccine against Lyme disease, Lymerix®, was approved by the FDA. Lymerix®
84 incorporates recombinant surface lipoprotein, OspA, from *Borrelia burgdorferi* that is
85 synthesized in *E. coli* (Table 1); however, the vaccine was withdrawn by the manufacturer in
86 2002 due to a lack of demand in the US that followed extensive media coverage and
87 ongoing litigation concerning adverse side effects (17). This was despite initial studies
88 indicating that the Lymerix® vaccine was a cost-effective public health intervention for people
89 at high risk of Lyme disease; its withdrawal precluded the design of more conclusive studies
90 (18). Bexsero® was licensed in the EU in 2013 to protect against meningococcal meningitis
91 and septicemia caused by meningococcal serogroup B (Table 1). It contains three antigens
92 synthesized in *E. coli* in addition to an outer membrane vesicle from meningococcal strain
93 MZ98/254.

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

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

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

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

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

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

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

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

161 Insect cells have also been used for both commercial vaccine production (Cervarix® and
162 Flublok®, Table 1) and in the synthesis of recombinant protein antigens for new vaccine
163 development. For example, the receptor-binding domain of neurotoxin A (rBoNT/A-HC-6h)
164 from *Clostridium botulinum* was synthesized in insect cells; purified rBoNT/A-HC-6h gave
165 mice full protection against botulinum A toxin with a dose as low as 0.2µg (42). Merozoite
166 surface protein 1 from *P. falciparum* (MSP-1, comprising 43 amino-terminal residues) was
167 also synthesized in insect cells and demonstrated to be immunogenic in rabbits (43). Further
168 examples include development of sub-unit vaccines, some incorporating glycoproteins that
169 would not be possible to synthesize in *E. coli*: these include sub-unit vaccines against
170 chandipura virus (44), hepatitis E virus (45), malaria (specifically disease caused by *P.*
171 *falciparum*) (46), severe acute respiratory syndrome (SARS) virus (47) and West Nile virus
172 (48).

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

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

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

203 **Designing improved recombinant antigen synthesis experiments**

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

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

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

230 **Conclusions**

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

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592 an oral administration of raw yeast extracts. *Journal of medical virology.* 79:74-83
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595

596 **Table 1. Recombinant protein sub-unit vaccines approved for human use.** Sub-unit vaccines containing a recombinant protein antigen
 597 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
 598 which they were approved. Data were retrieved on 28th May 2014 from the FDA
 599 (<http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093830.htm>), EMA
 600 (http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/medicines/medicines_landing_page.jsp&mid=) and the United Kingdom
 601 Department of Health (<https://www.gov.uk/government/collections/immunisation-against-infectious-disease-the-green-book>) websites. While 14
 602 out of 16 vaccine formulations contain antigens synthesized in microbes, only two distinct antigens are synthesized in *Saccharomyces*
 603 *cerevisiae* and a further two in *Escherichia coli*. Insect cells are used to synthesize two additional distinct antigens.
 604

Recombinant host	Vaccine name	Protection conferred against	Recombinant antigen	Manufacturer	Date approved
<i>Escherichia coli</i>	Lymerix®	<i>Borrelia burgdorferi</i> (causative agent of Lyme disease in US)	OspA lipoprotein	GlaxoSmithKline Biologicals	1998 (US); vaccine withdrawn by GlaxoSmithKline Biologicals in 2002
	Bexsero®	<i>Neisseria meningitidis</i> (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)
<i>Saccharomyces cerevisiae</i>	Recombivax-HB®	Hepatitis B virus	Hepatitis B surface antigen (HBsAg)	Merck & Co Inc	1986 (US)
	Comvax®	Hepatitis B virus and <i>Haemophilus influenzae</i> type B; causative agent of pneumonia or meningitis, especially in young children	HBsAg	Merck & Co Inc	1996 (US)
	Titanrix-HB®	Hepatitis B virus,	HBsAg	GlaxoSmithKline	1996 (EU); vaccine

	<i>Corynebacterium diphtheriae</i> (causative agent of diphtheria), <i>Clostridium tetani</i> (causative agent of tetanus) and <i>Bordetella pertussis</i> (causative agent of whooping cough)		Biologics	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, <i>C. diphtheriae</i> and <i>C. tetani</i>	HBsAg	Sanofi Pasteur MSD	1998 (EU); vaccine withdrawn by Sanofi Pasteur MSD in 2000
Procomvax®	Hepatitis B virus and <i>H. influenzae</i> 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, <i>C. diphtheriae</i> , <i>C. tetani</i> , <i>B. pertussis</i> 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	2006 (US)
Insect cells	Cervarix®	Human papillomavirus	Major capsid protein, L1, for human papillomavirus types 16 and 18	Sanofi Pasteur MSD	2006 (EU)
	Flublok®	Influenza virus	Full-length hemagglutinin (influenza virus A strains, H1N1 and H3N2, and one influenza virus B strain)	GlaxoSmithKline Biologicals	2007 (EU); 2009 (US)
				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
<i>Saccharomyces cerevisiae</i>	Anthrax (<i>Bacillus anthracis</i>)	Protective antigen component of the anthrax toxin complex, PA63	Protection against infection was demonstrated in rabbits and non-human primates	(78)
	Tetanus (<i>Clostridium tetani</i>)	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 (<i>Borrelia burgdorferi</i>)	N-terminally truncated form of outer-surface protein A (des-Cys1-OspA)	Improved yields over synthesis in <i>E. coli</i>	(83)
	Malaria (<i>Plasmodium spp</i>)	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 VLPs are each estimated to contain, on average, 100 polypeptides	
Malaria (<i>Plasmodium falciparum</i>)	Sexual-stage surface antigens synthesized as a Pfs25-28 fusion protein	Pfs25-28 elicits potent <i>P. falciparum</i> transmission-blocking antibodies in mice.	(85)
Malaria (<i>Plasmodium vivax</i>)	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)
<i>Pichia pastoris</i>	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
	Chagas' disease (<i>Trypanosoma cruzi</i>)	Trans-sialidase containing the catalytic domain without the immunodominant SAPA (Shed Acute Phase Antigen) repeats	The recombinant sub-unit vaccine was protective in mice
	Dengue virus	Dengue virus type 2 envelope domain III (sEDIII-2)	Demonstration of synthesis of recombinant antigen

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)
<i>Helicobacter pylori</i> 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 (<i>Necator americanus</i>)	<i>N. americanus</i> 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 (<i>Schistosoma</i>	9 kDa recombinant protein corresponding to the extracellular domain of a unique <i>S. mansoni</i> tetraspanin	Development of 20L scale production was demonstrated	(100)

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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 (<i>Leptospira</i> spp)	Leptospiral immunoglobulin-like (Lig) protein LigANI and the immunodominant lipoprotein LipL32	Recombinant proteins produced in <i>E. coli</i> have demonstrated variable results. LigANI and LipL32 from <i>P. pastoris</i> retained the antigenic characteristics of the native proteins	(104)
Malaria (<i>Plasmodium berghei</i>)	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 <i>P. pastoris</i> expressing N-CS RNPs provided significant reduction of parasitemia after intradermal challenge with a high dose of parasites	(105)
Malaria (<i>Plasmodium falciparum</i>)	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 (<i>Plasmodium vivax</i>)	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 (<i>Toxoplasma gondii</i>)	Chimeric surface antigen 1 and 2 (SAG1/2)	Vaccinated mice were significantly protected against lethal challenge with live <i>T. gondii</i>	(104)