

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.

240 **Acknowledgements**

241 I thank Professor Sarah Gilbert of the Jenner Institute, Oxford and my colleagues, Dr
242 Lindsay Marshall, Professor Yvonne Perrie and Alan Taylor, for critical comments on the
243 manuscript.

244 References

- 245 1. C.M. Kao, R.J. Schneyer, and J.A. Bocchini, Jr. Child and adolescent immunizations:
246 selected review of recent US recommendations and literature. *Curr Opin Pediatr*
247 (2014).
- 248 2. S. Liljeqvist and S. Stahl. Production of recombinant subunit vaccines: protein
249 immunogens, live delivery systems and nucleic acid vaccines. *Journal of*
250 *biotechnology*. 73:1-33 (1999).
- 251 3. R.L. Coffman, A. Sher, and R.A. Seder. Vaccine adjuvants: putting innate immunity
252 to work. *Immunity*. 33:492-503 (2010).
- 253 4. S.A. Plotkin. Vaccines: past, present and future. *Nat Med*. 11:S5-11 (2005).
- 254 5. R.M. Bill. Playing catch-up with *Escherichia coli*: using yeast to increase success
255 rates in recombinant protein production experiments. *Front Microbiol*. 5:85 (2014).
- 256 6. M.D. Spring, J.F. Cummings, C.F. Ockenhouse, S. Dutta, R. Reidler, E. Angov, E.
257 Bergmann-Leitner, V.A. Stewart, S. Bittner, L. Juompan, M.G. Kortepeter, R. Nielsen,
258 U. Krzych, E. Tierney, L.A. Ware, M. Dowler, C.C. Hermsen, R.W. Sauerwein, S.J.
259 de Vlas, O. Ofori-Anyinam, D.E. Lanar, J.L. Williams, K.E. Kester, K. Tucker, M. Shi,
260 E. Malkin, C. Long, C.L. Diggs, L. Soisson, M.C. Dubois, W.R. Ballou, J. Cohen, and
261 D.G. Heppner, Jr. Phase 1/2a study of the malaria vaccine candidate apical
262 membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A.
263 *PLoS One*. 4:e5254 (2009).
- 264 7. A. Roldao, M.C. Mellado, L.R. Castilho, M.J. Carrondo, and P.M. Alves. Virus-like
265 particles in vaccine development. *Expert review of vaccines*. 9:1149-1176 (2010).
- 266 8. S.J. Peacock, D. Limmathurotsakul, Y. Lubell, G.C. Koh, L.J. White, N.P. Day, and
267 R.W. Titball. Melioidosis vaccines: a systematic review and appraisal of the potential
268 to exploit biodefense vaccines for public health purposes. *PLoS Negl Trop Dis*.
269 6:e1488 (2012).
- 270 9. S.N. Cohen, A.C. Chang, H.W. Boyer, and R.B. Helling. Construction of biologically
271 functional bacterial plasmids *in vitro*. *Proceedings of the National Academy of*
272 *Sciences of the United States of America*. 70:3240-3244 (1973).
- 273 10. K. Itakura, T. Hirose, R. Crea, A.D. Riggs, H.L. Heyneker, F. Bolivar, and H.W.
274 Boyer. Expression in *Escherichia coli* of a chemically synthesized gene for the
275 hormone somatostatin. *Science*. 198:1056-1063 (1977).
- 276 11. D.V. Goeddel, D.G. Kleid, F. Bolivar, H.L. Heyneker, D.G. Yansura, R. Crea, T.
277 Hirose, A. Kraszewski, K. Itakura, and A.D. Riggs. Expression in *Escherichia coli* of
278 chemically synthesized genes for human insulin. *Proceedings of the National*
279 *Academy of Sciences of the United States of America*. 76:106-110 (1979).
- 280 12. M. Goodman. Market watch: Sales of biologics to show robust growth through to
281 2013. *Nat Rev Drug Discov*. 8:837 (2009).
- 282 13. N. Ferrer-Miralles, J. Domingo-Espin, J.L. Corchero, E. Vazquez, and A. Villaverde.
283 Microbial factories for recombinant pharmaceuticals. *Microbial cell factories*. 8:17
284 (2009).
- 285 14. J. Zhu. Mammalian cell protein expression for biopharmaceutical production.
286 *Biotechnol Adv*. 30:1158-1170 (2012).
- 287 15. D. Mattanovich, P. Branduardi, L. Dato, B. Gasser, M. Sauer, and D. Porro.
288 Recombinant protein production in yeasts. *Methods Mol Biol*. 824:329-358 (2012).

- 289 16. P. Valenzuela, A. Medina, W.J. Rutter, G. Ammerer, and B.D. Hall. Synthesis and
290 assembly of hepatitis B virus surface antigen particles in yeast. *Nature*. 298:347-350
291 (1982).
- 292 17. L.E. Nigrovic and K.M. Thompson. The Lyme vaccine: a cautionary tale. *Epidemiol
293 Infect.* 135:1-8 (2007).
- 294 18. M.I. Meltzer, D.T. Dennis, and K.A. Orloski. The cost effectiveness of vaccinating
295 against Lyme disease. *Emerg Infect Dis.* 5:321-328 (1999).
- 296 19. A. Soliakov, I.F. Kelly, J.H. Lakey, and A. Watkinson. Anthrax sub-unit vaccine: the
297 structural consequences of binding rPA83 to Alhydrogel(R). *Eur J Pharm Biopharm.*
298 80:25-32 (2012).
- 299 20. S.M. Jones, F. Day, A.J. Stagg, and E.D. Williamson. Protection conferred by a fully
300 recombinant sub-unit vaccine against *Yersinia pestis* in male and female mice of four
301 inbred strains. *Vaccine.* 19:358-366 (2000).
- 302 21. J.L. Holley, M. Elmore, M. Mauchline, N. Minton, and R.W. Titball. Cloning,
303 expression and evaluation of a recombinant sub-unit vaccine against *Clostridium*
304 *botulinum* type F toxin. *Vaccine.* 19:288-297 (2000).
- 305 22. H. Kleanthous, C.K. Lee, and T.P. Monath. Vaccine development against infection
306 with *Helicobacter pylori*. *Br Med Bull.* 54:229-241 (1998).
- 307 23. C. Colonna, R. Dorati, B. Conti, P. Caliceti, and I. Genta. Sub-unit vaccine against *S.*
308 *aureus*-mediated infections: set-up of nano-sized polymeric adjuvant. *Int J Pharm.*
309 452:390-401 (2013).
- 310 24. M.S. Duthie, L.H. Sampaio, R.M. Oliveira, V.S. Raman, J. O'Donnell, H.R. Bailor,
311 G.C. Ireton, A.L. Sousa, M.M. Stefani, and S.G. Reed. Development and pre-clinical
312 assessment of a 73 kD chimeric fusion protein as a defined sub-unit vaccine for
313 leprosy. *Vaccine.* 31:813-819 (2013).
- 314 25. B.C. Mufalo, F. Gentil, D.Y. Bargieri, F.T. Costa, M.M. Rodrigues, and I.S. Soares.
315 *Plasmodium vivax* apical membrane antigen-1: comparative recognition of different
316 domains by antibodies induced during natural human infection. *Microbes Infect.*
317 10:1266-1273 (2008).
- 318 26. Y. Vanloubbeeck, S. Pichyangkul, B. Bayat, K. Yongvanitchit, J.W. Bennett, J.
319 Sattabongkot, K. Schaecher, C.F. Ockenhouse, J. Cohen, A. Yadava, and *P. vivax*
320 vaccine study group. Comparison of the immune responses induced by soluble and
321 particulate *Plasmodium vivax* circumsporozoite vaccine candidates formulated in
322 AS01 in rhesus macaques. *Vaccine.* 31:6216-6224 (2013).
- 323 27. A. Weinrich Olsen, L.A. van Pinxteren, L. Meng Okkels, P. Birk Rasmussen, and P.
324 Andersen. Protection of mice with a tuberculosis subunit vaccine based on a fusion
325 protein of antigen 85b and esat-6. *Infection and immunity.* 69:2773-2778 (2001).
- 326 28. R. Ramirez, R. Falcon, A. Izquierdo, A. Garcia, M. Alvarez, A.B. Perez, Y. Soto, M.
327 Mune, E.M. da Silva, O. Ortega, R. Mohana-Borges, and M.G. Guzman.
328 Recombinant dengue 2 virus NS3 protein conserves structural antigenic and
329 immunological properties relevant for dengue vaccine design. *Virus genes* (2014).
- 330 29. K.O. Jang, J.H. Park, H.H. Lee, D.K. Chung, W. Kim, and I.S. Chung. Expression
331 and immunogenic analysis of recombinant polypeptides derived from capsid protein
332 VP1 for developing subunit vaccine material against hepatitis A virus. *Protein*
333 *expression and purification.* 100C:1-9 (2014).
- 334 30. K. Wu, X. Xue, M. Li, X. Qin, C. Zhang, W. Li, Q. Hao, Z. Wang, Q. Liu, W. Zhang,
335 and Y. Zhang. High level expression, purification and characterization of recombinant
336 CCR5 as a vaccine candidate against HIV. *Protein expression and purification.*
337 89:124-130 (2013).
- 338 31. X. Wen, D. Cao, R.W. Jones, J. Li, S. Szu, and Y. Hoshino. Construction and
339 characterization of human rotavirus recombinant VP8* subunit parenteral vaccine
340 candidates. *Vaccine.* 30:6121-6126 (2012).
- 341 32. N. Dagouassat, V. Robillard, J.F. Haeuw, H. Plotnicky-Gilquin, U.F. Power, N.
342 Corvaia, T. Nguyen, J.Y. Bonnefoy, and A. Beck. A novel bipolar mode of attachment

- 343 to aluminium-containing adjuvants by BBG2Na, a recombinant subunit hRSV
344 vaccine. *Vaccine*. 19:4143-4152 (2001).
- 345 33. J.J. Treanor, D.N. Taylor, L. Tussey, C. Hay, C. Nolan, T. Fitzgerald, G. Liu, U.
346 Kavita, L. Song, I. Dark, and A. Shaw. Safety and immunogenicity of a recombinant
347 hemagglutinin influenza-flagellin fusion vaccine (VAX125) in healthy young adults.
348 *Vaccine*. 28:8268-8274 (2010).
- 349 34. A. Tanomand, S. Farajnia, S. Najar Peerayeh, and J. Majidi. Cloning, expression and
350 characterization of recombinant exotoxin A-flagellin fusion protein as a new vaccine
351 candidate against *Pseudomonas aeruginosa* infections. *Iranian biomedical journal*.
352 17:1-7 (2013).
- 353 35. R.M. Chura-Chambi, E. Nakajima, R.R. de Carvalho, P.A. Miyasato, S.C. Oliveira, L.
354 Morganti, and E.A. Martins. Refolding of the recombinant protein Sm29, a step
355 toward the production of the vaccine candidate against schistosomiasis. *Journal of*
356 *biotechnology*. 168:511-519 (2013).
- 357 36. H.P. Sørensen. Towards universal systems for recombinant gene expression.
358 *Microbial cell factories*. 9:27 (2010).
- 359 37. A.D. Bandaranayake and S.C. Almo. Recent advances in mammalian protein
360 production. *FEBS Letters* (2013).
- 361 38. R. Kunert and E. Casanova. Recent advances in recombinant protein production:
362 BAC-based expression vectors, the bigger the better. *Bioengineered*. 4:258-261
363 (2013).
- 364 39. M. Widmann and P. Christen. Comparison of folding rates of homologous prokaryotic
365 and eukaryotic proteins. *J Biol Chem*. 275:18619-18622 (2000).
- 366 40. D. Null, Jr., B. Pollara, P.H. Dennehy, J. Steichen, P.J. Sanchez, L.B. Givner, D.
367 Carlin, B. Landry, F.H. Top, Jr., and E. Connor. Safety and immunogenicity of
368 palivizumab (Synagis) administered for two seasons. *The Pediatric infectious disease*
369 *journal*. 24:1021-1023 (2005).
- 370 41. L.R. Stanberry, S.L. Spruance, A.L. Cunningham, D.I. Bernstein, A. Mindel, S. Sacks,
371 S. Tying, F.Y. Aoki, M. Slaoui, M. Denis, P. Vandepapeliere, and G. Dubin.
372 Glycoprotein-D-adjuvant vaccine to prevent genital herpes. *N Engl J Med*. 347:1652-
373 1661 (2002).
- 374 42. O.B. Villaflores, C.M. Hsei, C.Y. Teng, Y.J. Chen, J.J. Wey, P.Y. Tsui, R.H. Shyu,
375 K.L. Tung, J.M. Yeh, D.J. Chiao, and T.Y. Wu. Easy expression of the C-terminal
376 heavy chain domain of botulinum neurotoxin serotype A as a vaccine candidate using
377 a bi-cistronic baculovirus system. *Journal of virological methods*. 189:58-64 (2013).
- 378 43. D.E. Arnot, D.R. Cavanagh, E.J. Remarque, A.M. Creasey, M.P. Sowa, W.D.
379 Morgan, A.A. Holder, S. Longacre, and A.W. Thomas. Comparative testing of six
380 antigen-based malaria vaccine candidates directed toward merozoite-stage
381 *Plasmodium falciparum*. *Clin Vaccine Immunol*. 15:1345-1355 (2008).
- 382 44. C.H. Venkateswarlu and V.A. Arankalle. Recombinant glycoprotein based vaccine for
383 Chandipura virus infection. *Vaccine*. 27:2845-2850 (2009).
- 384 45. C.P. McAtee, Y. Zhang, P.O. Yarbough, T.R. Fuerst, K.L. Stone, S. Samander, and
385 K.R. Williams. Purification and characterization of a recombinant hepatitis E protein
386 vaccine candidate by liquid chromatography-mass spectrometry. *Journal of*
387 *chromatography B, Biomedical applications*. 685:91-104 (1996).
- 388 46. Y.P. Shi, S.E. Hasnain, J.B. Sacchi, B.P. Holloway, H. Fujioka, N. Kumar, R.
389 Wohlhueter, S.L. Hoffman, W.E. Collins, and A.A. Lal. Immunogenicity and *in vitro*
390 protective efficacy of a recombinant multistage *Plasmodium falciparum* candidate
391 vaccine. *Proceedings of the National Academy of Sciences of the United States of*
392 *America*. 96:1615-1620 (1999).
- 393 47. Z. Zhou, P. Post, R. Chubet, K. Holtz, C. McPherson, M. Petric, and M. Cox. A
394 recombinant baculovirus-expressed S glycoprotein vaccine elicits high titers of
395 SARS-associated coronavirus (SARS-CoV) neutralizing antibodies in mice. *Vaccine*.
396 24:3624-3631 (2006).

- 397 48. N. Bonafe, J.A. Rininger, R.G. Chubet, H.G. Foellmer, S. Fader, J.F. Anderson, S.L.
398 Bushmich, K. Anthony, M. Ledizet, E. Fikrig, R.A. Koski, and P. Kaplan. A
399 recombinant West Nile virus envelope protein vaccine candidate produced in
400 *Spodoptera frugiperda* expresSF+ cells. *Vaccine*. 27:213-222 (2009).
- 401 49. J.K. Ma, P.M. Drake, and P. Christou. The production of recombinant pharmaceutical
402 proteins in plants. *Nature reviews Genetics*. 4:794-805 (2003).
- 403 50. S.J. Streatfield and J.A. Howard. Plant-based vaccines. *International journal for*
404 *parasitology*. 33:479-493 (2003).
- 405 51. Z. Huang, I. Dry, D. Webster, R. Strugnell, and S. Wesselingh. Plant-derived measles
406 virus hemagglutinin protein induces neutralizing antibodies in mice. *Vaccine*.
407 19:2163-2171 (2001).
- 408 52. K. De Schutter, Y.C. Lin, P. Tiels, A. Van Hecke, S. Glinka, J. Weber-Lehmann, P.
409 Rouze, Y. Van de Peer, and N. Callewaert. Genome sequence of the recombinant
410 protein production host *Pichia pastoris*. *Nat Biotechnol*. 27:561-566 (2009).
- 411 53. A. Goffeau, B.G. Barrell, H. Bussey, R.W. Davis, B. Dujon, H. Feldmann, F. Galibert,
412 J.D. Hoheisel, C. Jacq, M. Johnston, E.J. Louis, H.W. Mewes, Y. Murakami, P.
413 Philippsen, H. Tettelin, and S.G. Oliver. Life with 6000 genes. *Science*. 274:546, 563-
414 547 (1996).
- 415 54. D. Porro, B. Gasser, T. Fossati, M. Maurer, P. Branduardi, M. Sauer, and D.
416 Mattanovich. Production of recombinant proteins and metabolites in yeasts: when are
417 these systems better than bacterial production systems? *Applied microbiology and*
418 *biotechnology*. 89:939-948 (2011).
- 419 55. J.M. Cregg, K.J. Barringer, A.Y. Hessler, and K.R. Madden. *Pichia pastoris* as a host
420 system for transformations. *Mol Cell Biol*. 5:3376-3385 (1985).
- 421 56. F. Habersetzer, T.F. Baumert, and F. Stoll-Keller. GI-5005, a yeast vector vaccine
422 expressing an NS3-core fusion protein for chronic HCV infection. *Current opinion in*
423 *molecular therapeutics*. 11:456-462 (2009).
- 424 57. B. Upadhyaya and R. Manjunath. Baker's yeast expressing the Japanese encephalitis
425 virus envelope protein on its cell surface: induction of an antigen-specific but non-
426 neutralizing antibody response. *Yeast*. 26:383-397 (2009).
- 427 58. D.J. Scott, L. Kummer, D. Tremmel, and A. Pluckthun. Stabilizing membrane proteins
428 through protein engineering. *Curr Opin Chem Biol*. 17:427-435 (2013).
- 429 59. M.W. Traxlmayr and C. Obinger. Directed evolution of proteins for increased stability
430 and expression using yeast display. *Arch Biochem Biophys*. 526:174-180 (2012).
- 431 60. F. Oberg, J. Sjöhamn, M.T. Conner, R.M. Bill, and K. Hedfalk. Improving recombinant
432 eukaryotic membrane protein yields in *Pichia pastoris*: the importance of codon
433 optimization and clone selection. *Mol Membr Biol*. 28:398-411 (2011).
- 434 61. M. Halliday and G.R. Mallucci. Targeting the unfolded protein response in
435 neurodegeneration: A new approach to therapy. *Neuropharmacology*. 76 Pt A:169-
436 174 (2014).
- 437 62. C. Rebnegger, A.B. Graf, M. Valli, M.G. Steiger, B. Gasser, M. Maurer, and D.
438 Mattanovich. In *Pichia pastoris*, growth rate regulates protein synthesis and
439 secretion, mating and stress response. *Biotechnol J* (2013).
- 440 63. O. Spadiut, D. Zalai, C. Dietzsch, and C. Herwig. Quantitative comparison of dynamic
441 physiological feeding profiles for recombinant protein production with *Pichia pastoris*.
442 *Bioprocess Biosyst Eng* (2013).
- 443 64. M. Jazini and C. Herwig. Effects of temperature shifts and oscillations on recombinant
444 protein production expressed in *Escherichia coli*. *Bioprocess Biosyst Eng*. 36:1571-
445 1577 (2013).
- 446 65. N. Bora, Z. Bawa, R.M. Bill, and M.D. Wilks. The implementation of a design of
447 experiments strategy to increase recombinant protein yields in yeast (review).
448 *Methods Mol Biol*. 866:115-127 (2012).
- 449 66. W.J. Holmes, R.A. Darby, M.D. Wilks, R. Smith, and R.M. Bill. Developing a scalable
450 model of recombinant protein yield from *Pichia pastoris*: the influence of culture
451 conditions, biomass and induction regime. *Microbial cell factories*. 8:35 (2009).

- 452 67. C. Dietzsch, O. Spadiut, and C. Herwig. A dynamic method based on the specific
453 substrate uptake rate to set up a feeding strategy for *Pichia pastoris*. *Microbial cell*
454 *factories*. 10:14 (2011).
- 455 68. D. Drew, S. Newstead, Y. Sonoda, H. Kim, G. von Heijne, and S. Iwata. GFP-based
456 optimization scheme for the overexpression and purification of eukaryotic membrane
457 proteins in *Saccharomyces cerevisiae*. *Nat Protoc*. 3:784-798 (2008).
- 458 69. M.P. Ashe and R.M. Bill. Mapping the yeast host cell response to recombinant
459 membrane protein production: relieving the biological bottlenecks. *Biotechnol J*.
460 6:707-714 (2011).
- 461 70. K. De Pourcq, K. De Schutter, and N. Callewaert. Engineering of glycosylation in
462 yeast and other fungi: current state and perspectives. *Applied microbiology and*
463 *biotechnology*. 87:1617-1631 (2010).
- 464 71. S.M. Kitson, W. Mullen, R.J. Cogdell, R.M. Bill, and N.J. Fraser. GPCR production in
465 a novel yeast strain that makes cholesterol-like sterols. *Methods*. 55:287-292 (2011).
- 466 72. M. Guerfal, K. Claes, O. Knittelfelder, R. De Rycke, S.D. Kohlwein, and N.
467 Callewaert. Enhanced membrane protein expression by engineering increased
468 intracellular membrane production. *Microbial cell factories*. 12:122 (2013).
- 469 73. N. Bonander and R.M. Bill. Relieving the first bottleneck in the drug discovery
470 pipeline: using array technologies to rationalize membrane protein production. *Expert*
471 *Rev Proteomics*. 6:501-505 (2009).
- 472 74. N. Bonander, K. Hedfalk, C. Larsson, P. Mostad, C. Chang, L. Gustafsson, and R.M.
473 Bill. Design of improved membrane protein production experiments: quantitation of
474 the host response. *Protein Sci*. 14:1729-1740 (2005).
- 475 75. K. Baumann, N. Adelantado, C. Lang, D. Mattanovich, and P. Ferrer. Protein
476 trafficking, ergosterol biosynthesis and membrane physics impact recombinant
477 protein secretion in *Pichia pastoris*. *Microbial cell factories*. 10:93 (2011).
- 478 76. N. Bonander, R.A. Darby, L. Grgic, N. Bora, J. Wen, S. Brogna, D.R. Poyner, M.A.
479 O'Neill, and R.M. Bill. Altering the ribosomal subunit ratio in yeast maximizes
480 recombinant protein yield. *Microbial cell factories*. 8:10 (2009).
- 481 77. K. Norden, M. Agemark, J.A. Danielson, E. Alexandersson, P. Kjellbom, and U.
482 Johanson. Increasing gene dosage greatly enhances recombinant expression of
483 aquaporins in *Pichia pastoris*. *BMC Biotechnol*. 11:47 (2011).
- 484 78. R.W. Hepler, R. Kelly, T.B. McNeely, H. Fan, M.C. Losada, H.A. George, A. Woods,
485 L.D. Cope, A. Bansal, J.C. Cook, G. Zang, S.L. Cohen, X. Wei, P.M. Keller, E. Leffel,
486 J.G. Joyce, L. Pitt, L.D. Schultz, K.U. Jansen, and M. Kurtz. A recombinant 63-kDa
487 form of *Bacillus anthracis* protective antigen produced in the yeast *Saccharomyces*
488 *cerevisiae* provides protection in rabbit and primate inhalational challenge models of
489 anthrax infection. *Vaccine*. 24:1501-1514 (2006).
- 490 79. M.A. Romanos, A.J. Makoff, N.F. Fairweather, K.M. Beesley, D.E. Slater, F.B.
491 Rayment, M.M. Payne, and J.J. Clare. Expression of tetanus toxin fragment C in
492 yeast: gene synthesis is required to eliminate fortuitous polyadenylation sites in AT-
493 rich DNA. *Nucleic acids research*. 19:1461-1467 (1991).
- 494 80. N.L. Nguyen, J.M. Kim, J.A. Park, S.M. Park, Y.S. Jang, M.S. Yang, and D.H. Kim.
495 Expression and purification of an immunogenic dengue virus epitope using a
496 synthetic consensus sequence of envelope domain III and *Saccharomyces*
497 *cerevisiae*. *Protein expression and purification*. 88:235-242 (2013).
- 498 81. L. Antoniukas, H. Grammel, and U. Reichl. Production of hantavirus Puumala
499 nucleocapsid protein in *Saccharomyces cerevisiae* for vaccine and diagnostics.
500 *Journal of biotechnology*. 124:347-362 (2006).
- 501 82. N. Tomo, T. Goto, and Y. Morikawa. Trans-packaging of human immunodeficiency
502 virus type 1 genome into Gag virus-like particles in *Saccharomyces cerevisiae*.
503 *Microbial cell factories*. 12:28 (2013).
- 504 83. O. Mendoza-Vega, E. Keppi, B. Bouchon, M. Nguyen, and T. Achstetter.
505 Recombinant outer-surface protein A (des-Cys1-OspA) from the Lyme disease

506 spirochete *Borrelia burgdorferi*: high production levels in *Saccharomyces cerevisiae*
507 yeast cultures. *Applied microbiology and biotechnology*. 44:624-628 (1996).

508 84. N.J. Schuldt and A. Amalfitano. Malaria vaccines: focus on adenovirus based vectors.
509 *Vaccine*. 30:5191-5198 (2012).

510 85. M.M. Gozar, V.L. Price, and D.C. Kaslow. *Saccharomyces cerevisiae*-secreted fusion
511 proteins Pfs25 and Pfs28 elicit potent *Plasmodium falciparum* transmission-blocking
512 antibodies in mice. *Infection and immunity*. 66:59-64 (1998).

513 86. B. Rombaut and J.P. Jore. Immunogenic, non-infectious polio subviral particles
514 synthesized in *Saccharomyces cerevisiae*. *The Journal of general virology*. 78 (Pt
515 8):1829-1832 (1997).

516 87. S.R. Klepfer, C. Debouck, J. Uffelman, P. Jacobs, A. Bollen, and E.V. Jones.
517 Characterization of rabies glycoprotein expressed in yeast. *Archives of virology*.
518 128:269-286 (1993).

519 88. W.A. Rodriguez-Limas, K.E. Tyo, J. Nielsen, O.T. Ramirez, and L.A. Palomares.
520 Molecular and process design for rotavirus-like particle production in *Saccharomyces*
521 *cerevisiae*. *Microbial cell factories*. 10:33 (2011).

522 89. L. Tan, H. Wang, X. Tan, J. Zou, and Z. Yao. Yeast expressed foldable quadrivalent
523 Abeta15 elicited strong immune response against Abeta without Abeta-specific T cell
524 response in wild C57BL/6 mice. *Hum Vaccin Immunother*. 8:1090-1098 (2012).

525 90. G.H. Fontanella, K. De Vusser, W. Laroy, L. Daurelio, A.L. Nocito, S. Revelli, and R.
526 Contreras. Immunization with an engineered mutant trans-sialidase highly protects
527 mice from experimental *Trypanosoma cruzi* infection: a vaccine candidate. *Vaccine*.
528 26:2322-2334 (2008).

529 91. G. Batra, C. Gurramkonda, S.K. Nemani, S.K. Jain, S. Swaminathan, and N. Khanna.
530 Optimization of conditions for secretion of dengue virus type 2 envelope domain III
531 using *Pichia pastoris*. *J Biosci Bioeng*. 110:408-414 (2010).

532 92. M. Wang, S. Jiang, X. Liu, and Y. Wang. Expression, purification, and immunogenic
533 characterization of Epstein-Barr virus recombinant EBNA1 protein in *Pichia pastoris*.
534 *Applied microbiology and biotechnology*. 97:6251-6262 (2013).

535 93. M. Wang, S. Jiang, and Y. Wang. Recombinant VP1 protein expressed in *Pichia*
536 *pastoris* induces protective immune responses against EV71 in mice. *Biochem*
537 *Biophys Res Commun*. 430:387-393 (2013).

538 94. A.A. O'Riordan, V.A. Morales, L. Mulligan, N. Faheem, H.J. Windle, and D.P.
539 Kelleher. Alkyl hydroperoxide reductase: a candidate *Helicobacter pylori* vaccine.
540 *Vaccine*. 30:3876-3884 (2012).

541 95. C. Gurramkonda, M. Zahid, S.K. Nemani, A. Adnan, S.K. Gudi, N. Khanna, T.
542 Ebbesen, H. Lunsdorf, C.A. Guzman, and U. Rinas. Purification of hepatitis B surface
543 antigen virus-like particles from recombinant *Pichia pastoris* and *in vivo* analysis of
544 their immunogenic properties. *J Chromatogr B Analyt Technol Biomed Life Sci*.
545 940:104-111 (2013).

546 96. W. Cai, L. Su, Q. Liao, L. Ye, Y. Wu, Z. Wu, and Y. She. Expression, purification and
547 immunogenic characterization of hepatitis C virus recombinant E1E2 protein
548 expressed by *Pichia pastoris* yeast. *Antiviral Res*. 88:80-85 (2010).

549 97. E. Curti, C.A. Seid, E. Hudspeth, L. Center, W. Rezende, J. Pollet, C. Kwityn, M.
550 Hammond, R.K. Matsunami, D.A. Engler, P.J. Hotez, and M. Elena Bottazzi.
551 Optimization and revisions of the production process of the *Necator americanus*
552 glutathione S-transferase 1 (Na-GST-1), the lead hookworm vaccine recombinant
553 protein candidate. *Hum Vaccin Immunother*. 10: (2014).

554 98. W.Z. Jiang, N.Y. Jin, Z.J. Li, L.S. Zhang, H.W. Wang, Y.J. Zhang, and W.Y. Han.
555 Expression and characterization of Gag protein of HIV-1(CN) in *Pichia pastoris*.
556 *Journal of virological methods*. 123:35-40 (2005).

557 99. E.C. Coimbra, F.B. Gomes, J.F. Campos, M. D'Arc, J.C. Carvalho, F.C. Mariz, A.L.
558 Jesus, R.C. Stocco, W. Becak, and A.C. Freitas. Production of L1 protein from
559 different types of HPV in *Pichia pastoris* using an integrative vector. *Braz J Med Biol*
560 *Res*. 44:1209-1214 (2011).

- 561 100. E. Curti, C. Kwityn, B. Zhan, P. Gillespie, J. Brelsford, V. Deumic, J. Plieskatt, W.C.
562 Rezende, E. Tsao, B. Kalampanayil, P.J. Hotez, and M.E. Bottazzi. Expression at a
563 20L scale and purification of the extracellular domain of the *Schistosoma mansoni*
564 TSP-2 recombinant protein: A vaccine candidate for human intestinal
565 schistosomiasis. *Hum Vaccin Immunother.* 9: (2013).
- 566 101. M. Subathra, P. Santhakumar, M.L. Narasu, S.S. Beevi, and S.K. Lal. Evaluation of
567 antibody response in mice against avian influenza A (H5N1) strain neuraminidase
568 expressed in yeast *Pichia pastoris*. *J Biosci.* 39:443-451 (2014).
- 569 102. T.N. Athmaram, A.K. Singh, S. Saraswat, S. Srivastava, P. Misra, M. Kameswara
570 Rao, N. Gopalan, and P.V. Rao. A simple *Pichia pastoris* fermentation and
571 downstream processing strategy for making recombinant pandemic Swine Origin
572 Influenza a virus Hemagglutinin protein. *J Ind Microbiol Biotechnol.* 40:245-255
573 (2013).
- 574 103. W.T. Kwon, W.S. Lee, P.J. Park, T.K. Park, and H. Kang. Protective immunity of
575 *Pichia pastoris*-expressed recombinant envelope protein of Japanese encephalitis
576 virus. *J Microbiol Biotechnol.* 22:1580-1587 (2012).
- 577 104. Y.L. Lau, G. Thiruvengadam, W.W. Lee, and M.Y. Fong. Immunogenic
578 characterization of the chimeric surface antigen 1 and 2 (SAG1/2) of *Toxoplasma*
579 *gondii* expressed in the yeast *Pichia pastoris*. *Parasitol Res.* 109:871-878 (2011).
- 580 105. D. Jacob, C. Ruffie, M. Dubois, C. Combredet, R. Amino, P. Formaglio, O. Gorgette,
581 G. Pehau-Arnaudet, C. Guery, O. Pujalon, J.C. Barale, R. Menard, F. Tangy, and M.
582 Sala. Whole *Pichia pastoris* yeast expressing measles virus nucleoprotein as a
583 production and delivery system to multimerize Plasmodium antigens. *PLoS One.*
584 9:e86658 (2014).
- 585 106. E.C. Vicentin, K.S. Francoso, M.V. Rocha, D. Iourtov, F.L. Dos Santos, F.S.
586 Kubrusly, M.A. Sakauchi, I. Raw, F. Nosten, L. Renia, M.M. Rodrigues, B. Russell,
587 and I.S. Soares. Invasion-inhibitory antibodies elicited by immunization with
588 Plasmodium vivax apical membrane antigen-1 expressed in *Pichia pastoris* yeast.
589 *Infection and immunity.* 82:1296-1307 (2014).
- 590 107. M. Xia, T. Farkas, and X. Jiang. Norovirus capsid protein expressed in yeast forms
591 virus-like particles and stimulates systemic and mucosal immunity in mice following
592 an oral administration of raw yeast extracts. *Journal of medical virology.* 79:74-83
593 (2007).

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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 meningitides</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)
	Tritanrix-HB [®]	Hepatitis B virus,	HBsAg	GlaxoSmithKline	1996 (EU); vaccine

	<i>Corynebacterium diphtheria</i> (causative agent of diphtheria), <i>Clostridium tetani</i> (causative agent of tetanus) and <i>Bordetella pertussis</i> (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, <i>C. diphtheria</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. diphtheria</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 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
<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	(89)
	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	(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)
<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 (Leptospira 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)

Norovirus	Capsid protein (strain VA387, genogroup II.4)	mice 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)

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