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Heat-stable oral alga-based vaccine protects mice from *Staphylococcus aureus* infection

Imke A.J. Dreesen [a], Ghislaine Charpin-El Hamri [b], Martin Fussenegger [a,*]

[a] *Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, CH-4058 Basel, Switzerland*
[b] *Université de Lyon, 43 Boulevard du 11 Novembre 1918, F-69622 Villeurbanne, France*

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ABSTRACT

While 15 million deaths per year are caused by communicable pathogens worldwide, health care authorities emphasize the considerable impact of poverty on the incidence of infectious diseases. The emergence of antigen-expressing plant tissues (e.g. rice, tomato, potato) has indicated the potential of land plants for low-cost vaccines in oral immunization programs. In this study, we engineered the chloroplasts of the unicellular green alga *Chlamydomonas reinhardtii* for the stable expression of the D2 fibronectin-binding domain of *Staphylococcus aureus* fused with the cholera toxin B subunit (CTB), under the control of *rbcL* UTRs. Analysis of sera and faeces of mice, fed for 5 weeks with transgenic algae grown in confined Wave Bioreactor^{TM}, revealed the induction of specific mucosal and systemic immune responses. Algae-based vaccination significantly reduced the pathogen load in the spleen and the intestine of treated mice and protected 80% of them against lethal doses of *S. aureus*. Importantly, the alga vaccine was stable for more than 1.5 years at room temperature. These results indicate that *C. reinhardtii* may play an important role in molecular pharming, as it combines the beneficial features of land plant vaccines, while offering unmatched ease of growth compared to other members of the plant kingdom.

1. Introduction

Although current vaccines are reliable in preventing most infectious diseases, one fifth of all deaths worldwide are caused by communicable pathogens. This is explained mainly by the high correlation between the incidence of disease and levels of local development. Today, infectious diseases account for about 50% of the deaths in low-income countries and for 1% of all deaths in developed countries (http://www.who.int/topics/infectious_diseases). This imbalance is evidence of the widening gap between the wherewithal of national and international authorities, who are responsible for promoting global immunization programs, and the actual costs of large-scale vaccination campaigns. Therefore, the World Health Organization, the National Institutes of Health, the UNESCO and several other agencies have stressed the need for a new generation of low-cost vaccines to promote vaccination programs in the poorest regions of the world (Arntzen et al., 2005; Jodar et al., 2001). They especially emphasized the need for (i) heat-stable vaccines to avoid the considerable expense in maintaining the cold-chain during production and distribution and (ii) needle-free formulations to eliminate the risk of opportunistic contaminations as well as the need for qualified personnel (Giudice and Campbell, 2006; Jodar et al., 2001).

During the past 15 years, molecular pharming has revealed exciting developments in vaccine research. Through the molecular engineering of plant cells, plant biotechnology has become a cost-effective alternative to microbial and animal cell factories for the production of clinically useful proteins (Gitzinger et al., 2009; Hellwig et al., 2004; Ma et al., 2003). In addition to in-depth research, aimed at increasing already high expression rates, plants can undergo crucial post-translational modifications (Gomord and Faye, 2004; Shimoda et al., 2007). The industrial production of complex proteins, such as functional immunoglobulins (plantibodies), is relatively inexpensive (Ma et al., 1995; Mayfield et al., 2003). Years ago, oral delivery of antigen-containing plant tissues proved effective in eliciting specific immune responses *in vivo* (Haq et al., 1995; Nochi et al., 2007). As well as decreasing costs and increasing ease of administering vaccines without needles, it is unnecessary for plant antigens to undergo costly purification and downstream processing. The rigid plant cell wall protects the antigen from rapid degradation in the acidic environment of the stomach. As a result, plant tissues make effective carriers of antigens (Streatfield, 2005). This is a major advantage because the bioproduction of pharmaceuticals in plants considerably reduces the risk of contamination with mammalian pathogens, intrinsic endotoxins or toxic by-products. This means that approved good manufacturing practices (GMP)

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* Corresponding author at: ETH Institute of Chemical and Bio-Engineering, ETH Hoenggerberg, HCI F115, Wolfgang-Pauli-Strasse 10, 8093 Zurich, Switzerland. Tel.: +41 61 387 31 69; fax: +41 61 387 39 88.
E-mail address: fussenegger@bsse.ethz.ch (M. Fussenegger).

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can be implemented throughout the production pipeline (Doran, 2000). Furthermore, in contrast to current syringe-injected vaccines, oral administration of plant tissues elicits systemic as well as mucosal immune responses (Brennan et al., 1999; Czerkinsky et al., 1999). Owing to the fact that mucosa represents the most important avenue of entry for human pathogenic bacteria and viruses, mucosal immune effectors are crucial in the first line of defense. The induction of this specific two-level immunity has, therefore, become an ideal strategy in fighting emerging and re-emerging infectious diseases (Neutra and Kozlowski, 2006).

The photosynthetic unicellular alga *Chlamydomonas reinhardtii*, a member of the plant kingdom, combines the beneficial features of classical terrestrial plant systems and is grown easily in controlled and confined bioreactors (Franklin and Mayfield, 2005; Walker et al., 2005). Compared with land plants, *C. reinhardtii* grows at a much faster rate, doubling its cell number in about 8 h in a 12 h light/12 h dark regime. Through the molecular engineering of chloroplasts, relatively high amounts of recombinant proteins accumulate in the closed, protective organelle that can account for about 60% of the cell volume (Franklin and Mayfield, 2005). Provided that *C. reinhardtii* is generally regarded as safe (GRAS), this organism is the ideal candidate for a novel oral vaccine formulation. Also, the antigen can be concentrated and stabilized for long-term storage by lyophilization and it is straightforward to package the GMP-compliant free-dried algae powder in standard gelatine capsules which addresses issues such as dosing and taste (Arntzen et al., 2005). To provide proof-of-concept, we engineered *C. reinhardtii* chloroplasts for the stable production of the *Staphylococcus aureus* fibronectin-binding domain D2, fused to the cholera toxin B subunit (CTB) mucosal adjuvant (Rappuoli et al., 1999). The large-scale production of alga biomass was performed in a Wave Bioreactor^{TM}, and mice were subjected to a 5-week oral immunization program with lyophilized algae. The protection from *S. aureus* infection, conferred by the algae, was determined by quantifying specific mucosal (fecal IgA) and systemic (serum IgG) immune responses and by *in vivo* assessment of bacterial dissemination in key organs such as the intestine and spleen after sub-lethal infection. The survival rate of mice fed with transgenic algae was also monitored after exposure to lethal doses of *S. aureus*.

2. Materials and methods

2.1. Expression vector design

The expression vectors p463 (5′*rbcL*-*aadA*-3′*rbcL*; *rbcL*, untranslated region [UTR] derived from the ribulose-1,5-bisphosphatcarboxylase large subunit; *aadA*, spectinomycin adenylyltransferase) and p464 (5′*atpA*-*aadA*-3′*rbcL*; *atpA*, UTR derived from the ATP synthase α), conferring constitutive resistance to spectinomycin (*aadA*) in *C. reinhardtii*, were obtained from the *Chlamydomonas* Stock Center (Duke University, Durham, NC, USA). pID25 (5′*rbcL*-*aadA*-3′*rbcL*—5′*rbcL*-*CTB*-*D2*-3′*rbcL*), constitutively expressing *aadA* and CTB-D2 (cholera toxin B subunit [CTB] fused to the *S. aureus*-specific epitope D2), which had been codon-optimized for maximum expression in *C. reinhardtii* chloroplasts (Geneart, Regensburg, Germany), was constructed following a two-step procedure: (i) CTB-D2 was excised from pID32 (Geneart) using NcoI/PstI and cloned into the corresponding sites (NcoI/PstI) of p463 to result in pID22 (5′*rbcL*–*CTB*-*D2*–3′*rbcL*). (ii) 5′*rbcL*–*CTB*-*D2*–3′*rbcL* was PCR-amplified from pID22 using oligonucleotides OID25 (forward, 5′-gtgatccgcggccgcatgggtttataggtattttgagacc-3′) and OID26 (reverse, 5′-gtgatccgagctcgtatgttactatttcttttattacttataaaatataatac-3′), restricted with NotI/SacI and inserted into the corresponding sites (NotI/SacI) of p463. pID26, which is isogenic to pID25 but harbors 5′*atpA* instead of 5′*rbcL* in front of CTB-D2, was constructed by (i) excising CTB-D2 from pID32 using NcoI/PstI and cloning it into the corresponding sites (NcoI/PstI) of p464, thus replacing the *aadA* gene flanked by the 5′ *atpA* and 3′ *rbcL* UTRs and resulting in pID24 (5′*atpA*-*CTB*-*D2*-3′*rbcL*). (ii) The resulting expression cassette 5′*atpA*-*CTB*-*D2*-3′*rbcL* was excised from pID24 by ClaI/SpeI, blunted and inserted clockwise into NotI-digested and blunted p463 to result in pID26 (5′*rbcL*-*aadA*-3′*rbcL*—5′*atpA*-*CTB*-*D2*-3′*rbcL*). For construction of pID31 (P_{T7}-D2-His$_6$; P_{T7}, phage T7 promoter; His$_6$, hexahistidine tag), the *S. aureus* D2 epitope was PCR-amplified from pID32 using OID50 (forward; 5′-cggaattcccaccatgcatatgggtcaaaataatggtaaccag-3′) and OID51 (reverse; 5′-gctctaggatccaagcttttaatggtgatggtgatgatgtgaggaagaatcaatatcaataatgttaccac-3′), restricted with HindIII/NdeI and inserted into the corresponding sites (HindIII/NdeI) of pWW312 (Weber et al., 2005).

2.2. C. reinhardtii strains

The wild-type *C. reinhardtii* strain CC-125 (mt+) was obtained from the *Chlamydomonas* Stock Center (Duke University, Durham, NC, USA). The *C. reinhardtii* clone CR-463/4 was generated by stably transforming CC-125 (see below) with p463 (5′*rbcL*-*aadA*-3′*rbcL*) followed by clonal selection using 100 µg/ml spectinomycin. The stable CTB-D2-expressing *C. reinhardtii* strains CR-25/1-6 and CR-26/1-6 were generated by stably transforming CC-125 with pID25 or pID26, respectively, followed by clonal selection using 100 µg/ml spectinomycin.

2.3. Stable transformation of C. reinhardtii

Algae were grown at 25 °C in 500 ml Erlenmeyer flasks containing 100 ml Tris–acetate–phosphate medium (TAP; Harris, 1989) while shaking at 100 rpm in an orbital shaking plant incubator (ISF-1-W; Kuehner Shaker, Birsfelden, Switzerland) set to 12 h light (4000 lux)/12 h dark cycles. The algae were collected from 50 ml of a mid-log-phase culture (8×10^6 cells/ml) and harvested by centrifugation at 4000 × *g* for 5 min at room temperature. The algae pellet was resuspended in 4 ml TAP medium and poured on a TAP agar plate (30 ml TAP medium, 4% (wt/v) agar) for subsequent transformation. Transformation was carried out using a PDS-1000/He particle bombardment device (Bio-Rad, Hercules, CA, USA; Kindle et al., 1991) and the transgenic algae were selected on TAP agar plates containing 100 µg/ml spectinomycin (Sigma, St. Louis, MO, USA, cat. no. S4014). Homoplasmic *C. reinhardtii* lines were generated by sequentially streaking the clones ten times on spectinomycin-selective TAP agar plates.

2.4. Small- and large-scale cultivation of C. reinhardtii

For small-scale cultivation of up to 1 l, the algae were grown in TAP medium-containing Erlenmeyer flasks incubated at 25 °C and 100 rpm in an orbital shaking plant incubator set to 12 h (4000 lux)/12 h light/dark cycles. For the large-scale production of alga biomass, *C. reinhardtii* was grown to early-log-phase (3×10^6 cells/ml) and 1 l of culture was transferred to a 20 l Cellbag^{TM} containing 13 l TAP medium supplemented with 100 mg/l spectinomycin and incubated for 7 days in a Wave Bioreactor^{TM} adjusted to the following settings: 25 °C (cooling was provided by a Multitemp II system [Pharmacia, Uppsala, Sweden] set to 6 °C and placed under the Cellbag^{TM}), rocking rate 30 min^{-1}, rocking angle 6 degrees and an aeration rate of 450 ml/min.

The algae were harvested by centrifugation (3000 × g, 4 °C, 15 min), resuspended in 10 ml TAP medium-containing 3% methanol, frozen to −80 °C in an isopropanol bath and lyophilized overnight in a Lyovac GT3 (Leybold-Heraeus, Orsay, France).