An Oral Delivery System for Controlling White Spot Syndrome Virus Infection in Shrimp Using Transgenic Microalgae

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ABSTRACT

White spot disease (WSD) is a longstanding and serious viral disease of various shrimp species that has caused high mortality rates for many decades. Currently, there is no practical method to control this disease. Therefore, we have explored the development of a novel vaccine-based method to control this disease using transgenic algae. During infection by white spot syndrome virus (WSSV), the interaction between viral envelope proteins and cell surface protein receptors on target cells is the key step of viral entry and replication. Hence, transgenic lines of the green microalga *Chlamydomonas reinhardtii* harboring a WSSV VP28 viral envelope protein were created as an oral delivery system for vaccinating shrimp. Two type of transplastomic lines containing wild-type and codon optimized gene sequences for VP28 were evaluated for recombinant protein levels. Only the codon optimized line gave rise to detectable VP28 in western blot analysis, which demonstrated that optimization for chloroplast codon bias improved the efficiency of expression and that the gene design produced a favorable RNA secondary structure with suitable free energy for translation. In addition, bile salt and acid tolerance tests demonstrated this transgenic *Chlamydomonas* can tolerate mildly acidic (pH 4.0) conditions and 0.30% bile salts. These features indicated that algal cells are suitable for delivering viral antigen through a shrimp's digestive system. In WSSV infection experiments, the highest survival rate (87%) was recorded in shrimps fed with the codon optimized VP28 line mixed into their feed indicating that this line could be employed in the control of WSSV spread in shrimp populations. This algal strategy offers an alternative new, efficient, fast and less labor-intensive method for the control of other diseases in aquatic animals through oral delivery.

Keywords: White spot syndrome virus; oral delivery system; *Chlamydomonas reinhardtii*; chloroplast genetic engineering; transgenic algae

HIGHLIGHT

- An oral delivery system to control WSSV disease in shrimp has been developed based on an edible microalga engineered to synthesize a viral antigen in the chloroplast.
- A codon optimized synthetic gene encoding VP28 protein from WSSV was successfully integrated into the chloroplast genome of *C. reinhardtii.*
- Oral administering of the transgenic alga effectively raised the survival rate of shrimp exposed to WSSV compared to a control group.
- The cell wall deficient *C. reinhardtii* strain used is suited as a shrimp supplement as it can persist for 80 minutes in conditions of mild acid (pH 4.0) and 0.15% bile salts similar to that found in the shrimp digestive system.

INTRODUCTION

White spot disease (WSD) is an infectious disease of decapod crustaceans such as shrimp, lobster and crayfish and is caused by white spot syndrome virus (WSSV) [1]. The virus particularly affects farmed shrimp such as *Litopenaeus vannamei* (whiteleg shrimp) and *Penaeus monodon* (Asian tiger shrimp) and is a global problem for the shrimp industry because practical WSD prevention and control methods during cultivation are poorly developed. Most of the methods rely on the application of different effective biomolecules such as purified protein antigens or antibodies, DNA or RNA vaccines, or natural products [2]. However, a practical method for administration of these biomolecules is required that does not adversely impact shrimp health through handling of individuals; is able to be administered to shrimp of all growth stages; is suitable for treating large numbers of animals, and is time-saving and does not require skilled personnel. Thus, from those requirements, the oral administration of the biomolecule as a supplement in the shrimp feed is an attractive alternative to direct delivery.

VP28, an envelope protein of WSSV, is recognized as a key target for controlling WSD since the protein plays a central role in the infection process through its interaction with PmRab7, a shrimp cell surface protein [3]. Various control methods using VP28 have been evaluated, such as DNA vaccines [4, 5], RNA interference and double-stranded RNA [6, 7, 8] and use of recombinant VP28 (rVP28) [9 - 13]. However, most of these methods employ injection into shrimp as the delivery system, which is not practical or cost-effective. Therefore, various oral delivery systems have been developed and have shown promising disease control. For example, using attenuated bacteria carrying VP28 DNA [14], baculovirus with VP28 expressed on the surface [15], biomaterials such as chitosan as a DNA-nanoparticle complex [16] and double-stranded RNA or protein vaccines synthesized in transgenic alga [7, 17, 18]. However, the major concern of an oral administration method is compatibility of the delivery system and the gastrointestinal tract of the recipient animal. Therefore, a practical oral delivery system should allow the bioactive molecule to persist in the acidic and bile salt environments and then be released at a suitable position in the gastrointestinal tract.

The unicellular microalga, *Chlamydomonas reinhardtii* offers significant potential as a light-driven cell factory for synthesis of recombinant proteins such as subunit vaccines [19 - 22]. Several aspects in particular are attractive for the development of oral vaccines. Firstly, transgenes can be stably integrated into the chloroplast genome at specific loci as a result of efficient homologous recombination, and high-level expression of the transgenes can be achieved [23]. Secondly, the availability of both walled and cell-wall deficient *C. reinhardtii* strains allows the tailoring of the breakdown characteristics of the algal cell in the shrimp gastrointestinal tract. In this study, VP28 was produced in the chloroplast of a cell-wall deficient strain of *C. reinhardtii.* The transgenic

line was shown to be stable under conditions that mimic that in the shrimp gut, and when included in the feed conferred protection from WSD. This feeding strategy therefore offers a more convenient and economic protocol for shrimp immunostimulant/vaccination than other methods such as injection, and the use of the algal platform could be exploited to control other diseases in economically important aquatic animals.

MATERIALS AND METHODS

1. Algal strains and growth conditions

Chlamydomonas reinhardtii strains were maintained on tris-acetate phosphate (TAP) medium with 2% agar at 25°C under continuous white light (40-50 $\mu E.m^{-2}.s^{-1}$) as described in [24]. The TN72 strain that carries both a nuclear cell wall-deficient mutation (*cw15*) and a chloroplast mutation (*psbH*::*aadA*) preventing photosynthesis was used as the recipient strain for chloroplast transformation [25]. Selection for rescue of *psbH* function was on high salt minimum (HSM) medium [24] with a constant light intensity of 40-50 $\mu E.m^{-2}.s^{-1}$. For protein work and shrimp feed preparation, algal cells were grown under similar constant light conditions but in shake flasks containing liquid TAP medium, shaking at 120 rpm.

2. Codon optimization

The sequence of WSSV envelope protein VP28 was obtained from GenBank (accession number: DQ681069.1). The DNA sequence encoding WSSV including a C-terminal polyhistidine tag was optimized to be compatible with *C. reinhardtii*'s chloroplast codon usage (http://www.kazusa.or.jp) by using Codon Usage Optimizer (CUO) beta 0.92 program (http://www.ucl.ac.uk/algae/Genetic_engineering_tools). Multiple sequence alignment and percent similarity were predicted using GeneDoc (Ver. 2.7.000) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Codon bias was verified by computing the expected codon adaptation index (e-CAI). Secondary structure of the optimized VP28 mRNA was predicted using mfold (http://mfold.rna.albany.edu/). The codon optimized DNA for *vp28* (termed "*vp28-op*") was synthesized by Integrated DNA Technology (IDT, USA) and included a unique *Sap*I and *Sph*I site upstream and downstream of the coding region, respectively.

3. Plasmid construction

To construct a plasmid carrying the wild type version of *vp28* the gene was amplified by PCR from pET-*vp28* by using primers CrVP28F: 5'-GCTCTTCAATGGATCTTTCTTTCACTCTTTCGGTC-3' and CrVP28R: 5'-GCATGCCTAGTGATGGTGATGGTGATGCTC, which contained *Sap*I and *Sph*I (underlined) sites. Both *vp28* and *vp28-op* were then cloned into the chloroplast expression vector pASapI [25] at corresponding restriction sites and transformed into *E. coli* (DH5α). Transformed cells were screened by PCR using specific primers for pASapI; pASapF: 5'-CAAGTGATCTTACCACTCAC-3' and pASapR: 5'-CAAACTTCACATGCAGCAGC-3'. The nucleotide sequence of *vp28 and vp28-op* in the two plasmids was

checked by DNA sequencing (Macrogen, Korea) and the plasmids designated as pASapI-*vp28* and pASapI-*vp28op*, respectively.

4. Transformation of Chlamydomonas reinhardtii

Chloroplast expression vectors, pASapI-*vp28op*, pASapI-*vp28*, and empty pASapI, were introduced to chloroplast of *C. reinhardtii* by glass bead transformation as described previously in Economou *et. al*. [26]. A 300 µl of mid-log phase (about 2×10^6 cells.mL^{-1}) TN72 were resuspended with HSM broth and transferred to a sterile tube containing 300 mg glass beads of diameter 425-600 µm (Sigma-Aldrich, U.S.). Five microgram of plasmid was added and vigorously agitated for 15 sec. Warmed 0.5% Bacto^{TM} Agar (Becton Dickinson, U.S.) was added, quickly poured onto an HSM agar plate and incubated at 23 ºC in dim light (1-5 µE.m^{-2}.s^{-1}) overnight, then moved to a higher light (40–50 µE.m^{-2}.s^{-1}) for 2-4 weeks. Putative algal transformant colonies were restreaked onto fresh HSM agar plates, cultured under light intensity of 40–50 µE.m^{-2}.s^{-1} at 23°C and subcultured for at least three rounds to obtain homoplasmic lines. Subsequently, the obtained phototrophic transformants were verified for transgene integration by PCR using a set of three primers: trnE2-F (5′-GTCATTGCGAAAATACTGGTGC-3′), psbH-R (5′-ACGTCCACAGGCGTCGTAAGC-3′) and atpA-R (5′-GATGACGTTTCTATGAGTTGGG-3′) as illustrated in Fig 1. Furthermore, the loss of the *aad*A gene and restoration of *psbH* were confirmed by a 'spot test' growth assay on TAP agar containing 100 µg.mL^{-1} spectinomycin and HSM agar, respectively, under white light for 7 days.

5. Recombinant protein expression, purification and western blot analysis

Transgenic algal cells (100 ml) (designated as TNVP28-op, TNVP28 and TN72-emp; *C. reinhardtii* transformed with pASapI-vp28op, pASapI-vp28, and empty pASapI vector, respectively), were collected and lysed in 50 ml denaturing extraction buffer (20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 8 M urea, 5 mM imidazole and 0.5% Tween-20). For VP28 expression in *E. coli*, strain BL21 (DE3) harboring pET-*vp28* was induced with 3 mM IPTG and employed as a positive control for western blot analysis. All versions of the VP28 protein were purified using Ni-NTA sepharose 6 Fast Flow (GE Healthcare) under denaturing condition and VP28 eluted by using 100, 250, and 500 mM imidazole. Protein concentration was determined by Bradford assay (Bio-Rad) using BSA as a standard protein.

Proteins separated by 12% SDS-PAGE were transferred to PVDF membrane (Pall Corporation, USA). Recombinant protein was detected using rabbit polyclonal anti-VP28 (diluted 1:10,000 with TBST) as the

primary antibody (produced in S. Unajak lab). The secondary antibody was anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP, Cell signaling, USA) and detection used SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, UK). The signal was recorded by Chemiluminescence detector (ImagQuant LAS500, GE Health care, USA).

6. Acid and bile salt tolerance assays

For the acid tolerance test, mid-log phase TNVP28-op cells were resuspended in a sterile TAP medium with pH ranging from of 2 - 8 (adjusted with 5 M HCl). All samples were incubated at 26ºC for 1, 3 and 5 h then subjected to microscope inspection. For the bile salt tolerance test, mid-log phase of TNVP28-op cells were added to bile salt (Himedia) with a final concentration of 0.15%, 0.3%, 0.6%, and 1.2% (v/v) for 30, 60 and 90 min, followed by inspection under the microscope.

7. Feed preparation

Recombinant *C. reinhardtii* (TNVP28-op, TNVP28 and TN72-emp) were cultured in TAP medium with a 40-50 $\mu E.m^{-2}.s^{-1}$ light and 120 rpm shaking until late log phase and centrifuged at 1,500 ×g at 4 °C for 20 min to collect the algal cells. Feed supplemented with the three types of transgenic algae were prepared. Shrimp feed (Sunshine, Thailand) was ground into fine particles prior to mixing with algal samples. Two hundred grams of ground shrimp feed was mixed with an algal pellet of approximately 14×10^{10} cells. The mixtures were the extruded through a 5 ml sterile plastic syringe to form a compacted rod of shrimp feed. The feeds were baked at 50 °C for 16 h and then broken into smaller pieces. Feeds were stored at 4 °C until used.

8. Oral administration and WSSV challenge

Penaeus vannamei (at 6 - 7 g) free from WSSV and yellow head virus were divided into five groups (15 shrimps per group and conducted in triplicate) and fed with commercial feed (Sunshine, Thailand) containing recombinant algae (TNVP28-op, TNVP28 and TN72-emp). Control groups (with and without WSSV challenge) were fed with normal feed. Each group was fed twice daily. At 10 days post-stimulation, all groups (except the negative control) were challenged by a cohabitation system with a WSSV-injected moribund shrimp. The mortality rates of the experimental shrimps were recorded daily. This experiment on animals complied with the ethical standards set by the National Research Council of Thailand (NRCT). Statistical analysis of mortality

relationship was performed with one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test using the SPSS 11.5 program at the significant consideration level of 0.05.

RESULTS

1. *Codon optimization of the gene for WSSV VP28*

Chloroplast genes of *C. reinhardtii* display a strong bias towards AT-rich codons, and several studies have shown that optimization of codon usage in a foreign gene to match this bias can significantly improve the efficiency of translation of the gene in the algal chloroplast [27]. Hence, the VP28 gene was codon-optimized (designated as *vp28-op*) according to *C. reinhardtii* chloroplast codon usage showing the expected codon adaptation index (eCAI) of 0.987 compared to the CAI of the native VP28 which was 0.642. The secondary structure analysis of *vp28-op* mRNA revealed a free energy for mRNA folding of -137.30 kcal.mol^{-1} (Fig 1). A nucleotide sequence comparison of native *vp28* and synthetic *vp28-op* showed a 76.42 % similarity without any change in amino acid sequence (Fig 2).

2. Production of C. reinhardtii transformants harboring WSSV vp28-op or vp28

Synthetic *vp28-op* and *vp28* were cloned into the pASapI vector such that the genes were under the control of the endogenous *atpA* promoter/5' UTR element, and the 3' UTR from *rbcL* [25] to create plasmids pASapI-vp28op and pASapI-vp28. These plasmids, together with the empty pASapI vector, were used to transformed the plastome of the algal recipient TN72. Five colonies were obtained from pASapI-vp28op, six from pASapI-vp28 and eight from pASapI-empty and the putative transformant lines were designated as TNVP28-op, TNVP28, and TN72EMP, respectively (data not shown). After three rounds of restreaking under phototrophic growth conditions, the homoplasmicity of the polyploid plastome in the transformants was verified by PCR analysis. Gel electrophoresis of the PCR products showed only a 1.2 kb band in all transformant lines (Fig 3). This band arises from the transformed copies of the plastome whereas a 1.0 kb and would arise from untransformed copies. The absence of any detectable 1.0 kb in the PCR analysis indicates that homoplasmy was successfully obtained in all lines.

Additionally, homologous recombination between sequences on the pASapI plasmids and the TN72 plastome results in the replacement of the *aadA* gene conferring spectinomycin with a functional copy of the *psbH* gene, allowing photosynthetic growth on minimal medium [25], while the loss of *aadA* gene results in the creation

of transformant lines free from an antibiotic-resistance marker (Fig 1). None of the TNVP28-op (PO1-5) and TNVP28 (CV1-6) transformants could survive in TAP containing 100 μg/ml spectinomycin, unlike the untransformed TN72 recipient (Fig 4), further supporting the conclusion that the transgenes had successfully integrated into the plastome at the specific position downstream of *psbH* and had replaced the *aadA* marker.

3. Determination of protein expression in transgenic Chlamydomonas harboring vp28op or vp28

To determine whether WSSV VP28 is produced in the transgenic algae, western blot analysis was carried out using a polyclonal rabbit anti-VP28 antibody. The expected size of the protein is ~28 kDa and a distinct band of this size was detected in an *E. coli* extract expressing *vp28* (Fig 5, lane P) and in two transformant lines (OP1 and OP2) containing the codon-optimised *vp28*. In addition, the antibodies also detect an endogenous protein of ~35 kDa in the algal extracts and this is seen in all extracts including that from the negative control transformant line (CP) and the untransformed strain (TN72). Interestingly, the 28 kDa VP28 band is not detected in the OP line carrying the wild-type (non-optimised) version of *vp28*, highlighting the benefit of codon optimization for improved synthesis of recombinant protein in the *C. reinhardtii* chloroplast. His-tag affinity purification of VP28 from OP1 further confirmed that the 28 kDa band detected by the antibodies is the His-tagged VP28 as a subsequent western blot (Fig 6) showed detection of this band in the eluted fractions (E1 – E5) with the 35 kDa band eliminated in the flow-through.

4. ***Acid and bile salt tolerance assay***

The efficiency of an oral delivery system is influenced by the effectiveness of digestibility and absorption in the animal's digestive tract. The tolerance of the algal cell to the physical environment of the shrimp gut was determined by assessing the integrity of the cells to changes to acidic conditions and bile salt concentration. The transgenic line TNVP28-op, which carries the cell-wall deficient mutation, was incubated in a range of pH from 2 to 8 and in various concentrations of bile salt; 0.15%, 0.3%, 0.6%, and 1.2% (v/v), respectively. At pH 7 or in 0.15% bile salts as control conditions during the analysis, the algal cells showed round bright green coloration and remained intact until the end of the exposure period. In the unfavorable environment of low pH or high bile salt concentration, the algal cells showed discoloration and extensive cell lysis (Fig. 7 and Fig 8). The lowest concentration of bile salts and pH that the algal cells remained intact and demonstrated green in color are 0.3% bile salts and pH 5.0.

*5. **Efficiency of transgenic algae on controlling white spot syndrome***

To determine the effectiveness of the transgenic *C. reinhardtii* harboring VP28 on controlling WSSV infection, Pacific white shrimp were fed with feed mixed with the transgenic line TNVP28-op for 14 days. WSSV-infected shrimp were then added to the aquarium tank allowing co-habitation with the algae-fed shrimp. Shrimp mortality, caused by WSSV infection, started on day 4 of co-habitation with the infected animals. In all the control treatments including shrimp fed the commercial feed without the algal supplement, and those given feed containing the non-expressing algal lines TNVP28 and TN72-emp gradually died within 7 days with a near 0% survival rate. However, shrimp fed with TNVP28-op showed the lowest mortality with a relative survival of 87%, as compared to a 100% survival for shrimp in an uninfected negative control group (Fig. 9).

DISCUSSION

VP28, a dominant structural envelope protein of WSSV, is recognized as an effective biomolecule which can activate shrimp immunity and elicit an anti-viral response [28-32]. VP28 plays as a key role in WSSV infection by specifically binding to host cell receptors such as Rab7 and facilitating viral entry, which further allows viral propagation [33]. Thus, a rationale protection strategy against WSSV infection is dependent on interfering with the interaction between VP28 and the shrimp cell receptor to reduce viral entry and also later on activating a shrimp immunity against the invading pathogen. Consequently, different biological forms of VP28 such as DNA vaccine [4, 5, 14] interfering RNA targeting *vp28* expression [34, 35] and recombinant VP28 protein [19, 36] were used and demonstrated promising controlling WSSV infectivity. However, most of these methods rely on injection into individual juvenile animals of the active biomolecule, and this is not considered practical in commercial shrimp production. Moreover, preparation of VP28 either as recombinant protein, or as dsRNA or DNA vaccine are costly and time consuming which is considered a further limitation in the preparation and utilization of these agents in aquaculture.

An oral system for delivery of recombinant VP28 protein to shrimp as part of the feeding regime would allow the introduction of this immunostimulant to many animals in a technically simple manner without causing stress or possible injury to the shrimp. Various kinds of biological materials have been employed to encapsulate VP28 such as biodegradable materials (chitosan) [16, 19], bacteria; *Bacillus subtilis* [37] *Escherichia coli* [38] and the single cell alga *Dunaliella salina* [9]. The advantages of encapsulation in expression hosts such as algae or bacteria are that the host not only provides a low-cost platform for synthesis of the recombinant protein, but protects the VP28 in the harsh environment of the shrimp gut until it reaches to the target cells in the gastrointestinal tract. Therefore, one of the most important considerations of a VP28 delivery system is optimizing the release of VP28 at the most suitable site and time after it passes through the shrimp digestive system so that efficient WSSV control can be attained. In this study, oral administration was carried out using a cell-wall deficient microalga expressing VP28 in the chloroplast. The results of the feeding and viral challenge trials show a marked improvement in survival when the shrimp are fed algae containing VP28, indicating that a sufficient amount of the administered protein is surviving digestion and reaching the target cells in the shrimp gut. This promising result suggests that oral administration of transgenic algae is a viable approach to managing viral infection.

Several studies have shown that efficient production of a recombinant protein in the *C. reinhardtii* chloroplast requires codon optimization of the transgene [18, 39, 40]. In this study, VP28 was successfully

expressed in the algal chloroplast after codon optimization whereas the wild-type (non-optimised) gene from WSSV was poorly expressed such that no VP28 was detectable by western analysis. This improvement might have resulted from a rational nucleotide optimization based on five criterions which are 1) codon usage which resembles that used in alga's chloroplast; 2) an expected codon adaptation index (eCAI) close to 1.0; 3) a suitable codon bias with A and T nucleotides at the third position rather than G and C [40, 41]; 4) an acceptable folding free energy ($\Delta G = -137.30$ kcal.mol^{-1}) [42], and 5) a stable mRNA secondary structure which could play a critical role in the regulation of transcription [43] and translation [44]. In this optimization, the complexity of mRNA secondary structures found in genes with low GC content could increase the folding free energy resulting in the reduction of mRNA stability [45]. Thus, unstable mRNA secondary structure could impair the expression by impairing the binding of ribosome and other trans-acting factors during translational initiation [42].

The ability of algal cells to survive in the shrimp digestive system prior to release of VP28 in the lower tract is one of main features required for an efficient oral delivery system. For shrimp, the duration of feed passing through their digestive system is about 80 min [47]. Our results demonstrated that after 80 min of incubation, algal cells could resist mild acid (pH 5.0) and bile salts (0.15%). Additionally, in the digestive tract, it is not only physiological conditions that affect the release of active contents from algae, but digestive enzymes will also result in cell lysis. The release of sufficient VP28 from the cells to stimulate shrimp immunity therefore reflects a balance between the tolerance of the cells to the conditions in the gut and their active breakdown as part of the normal digestive process.

The shrimp fed with the TNVP28-op strain had a significantly lower mortality rate than the control group and the equivalent TNVP28 strain that had undetectable levels of VP28 (i.e. ~ 13% mortality after seven days compared with ~100% mortality). These results indicate a clear protection from WSSV from the VP28 in the TNVP28-op alga, and that other components in *C. reinhardtii* dried biomass itself are not responsible for the observed protection against WSSV. Furthermore, the delivery system plays a pivotal role in mediating the protection. Several types of oral VP28 delivery system have been developed using for example *B. subtilis* spores [48], the marine alga *Dunaliella salina* [9] and the cyanobacterium *Anabaena* sp. [49] and different efficacies in controlling WSD were observed. Reported protective effects of crayfish fed with VP28 expressed in *D. salina* (59% survival rate), *B. subtilis* spore (37.9 – 44.8% survival rate) [48] and *Anabaena* sp. (68% survival rate) [49] are lower than those reported using chloroplast engineered *Chlamydomonas* containing either double stranded RNA (84.1%) [7] or recombinant VP28 (87%) [this study]. Even though it is not possible to directly

compare the values obtained in the different studies, the findings do suggest that *C. reinhardtii* represents a good host for expression and oral delivery. Furthermore, *C. reinhardtii* is considered a harmless feed additive with toxicology studies allowing its classification as a GRAS (Generally Recognised As Safe) organism [50].

In conclusion, transgenic microalga appears to be a good vehicle to deliver immunostimulants in controlling WSD in shrimp through oral administration, and the transgenic *C. reinhardtii* expressing VP28 could be employed to prevent WSSV infection in white shrimp. This delivery system could also offer a new method for preventing other viral diseases in aquatic animals, and represent an environmentally friendly and sustainable system for vaccine production given that the algae are grown by photosynthesis with simple inputs of sunlight, CO_2 and basic nutrients.

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CONFLICT OF INTEREST

No conflict of interest declared.

REFERENCES

1. Pradeep, B., Rai, P., Mohan, S.A., Shekhar, M.S., Karunasagar, I., 2012. Biology, host range, pathogenesis and diagnosis of white spot syndrome virus. Indian J Virol. 23 (2), 161–74.

2. Feng, S., Wang, C., Hu, S., Wu, Q., Li, A., 2017. Recent progress in the development of white spot syndrome virus vaccines for protecting shrimp against viral infection. Arch Virol. 162 (10), 2923–36.

3. Sritunyalucksana, K., Wannapapho, W., Lo, C. F., Flegel, T. W., 2006. PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. J. Virol. 80, 10734-10742.

4. Rout, N., Kumar, S., Jaganmohan, S., Murugan, V., 2007. DNA vaccines encoding viral envelope proteins confer protective immunity against WSSV in black tiger shrimp. Vaccine. 25, 2778-2786.

5. Li, X., Liu, Q.-hui, Huang, L.H., 2010. Effect of VP28 DNA vaccine on white spot syndrome virus in *Litopenaeus vannamei.* Aquaculture International. 18, 1035-1044.

6. Fei, Z., Zhang, X., 2011. The antiviral vp28-siRNA expressed in bacteria protects shrimp against white spot syndrome virus (WSSV). Aquaculture. 319, 311-314.

7. Charoonnart P., Worakajit N., Zedler J.A.Z., Meetam M., Robinson C., Saksmerprome V., 2019. Generation of microalga *Chlamydomonas reinhardtii* expressing shrimp antiviral dsRNA without supplementation of antibiotics. Sci Rep. 9(1). https://doi.org/10.1038/s41598-019-39539-x.

8. Taju, G., Madan, N., Abdul Majeed, S., Kumar, T.R., Thamizhvanan, S., Otta, S.K., Sahul Hameed, A.S., 2015. Immune responses of whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931), to bacterially expressed dsRNA specific to VP28 gene of white spot syndrome virus. J. Fish. Dis. 38, 451-465.

9. Feng, S., Zhao, L., Gu, H., Li, Q., Shi, K., Guo, S., Zhang, N., 2014. Preparation of transgenic *Dunaliella salina* for immunization against white spot syndrome virus in crayfish. Arch. Virol. 159, 519-525.

10. Ha, Y.M., Soo-Jung, G., Thi-Hoai, N., Ra, C.H., Kim, K.H., Nam, Y.K., Kim, S.K., 2008. Vaccination of shrimp (*Penaeus chinensis*) against white spot syndrome virus (WSSV). J. Microbiol. Biotechnol. 18, 964-967.

11. Thomas, A., Sudheer, N.S., Viswanathan, K., Kiron, V., Bright Singh, I.S., Narayanan, R.B., 2014. Immunogenicity and protective efficacy of a major White Spot Syndrome Virus (WSSV) envelope protein VP24 expressed in *Escherichia coli* against WSSV. J. Invertebr. Pathol. 123, 17-24.

12. Valdez, A., Yepiz-Plascencia, G., Ricca, E., Olmos, J., 2014. First *Litopenaeus vannamei* WSSV 100% oral vaccination protection using CotC: Vp26 fusion protein displayed on *Bacillus subtilis* spores surface. J. Appl. Microbiol. 117, 347-357.

13. Solís-Lucero, G., Manoutcharian, K., Hernández-López, J., Ascencio, F., 2016. Injected phage-displayed-VP28 vaccine reduces shrimp *Litopenaeus vannamei* mortality by white spot syndrome virus infection. Fish Shellfish Immunol. 55, 401-406.

14. Ning, J.F., Zhu, W., Xu, J.P., Zheng, C.Y., Meng, X.L., 2009. Oral delivery of DNA vaccine encoding VP28 against white spot syndrome virus in crayfish by attenuated *Salmonella typhimurium*. Vaccine. 27, 1127-1135.

15. Syed, M.S., Kwang, J., 2011. Oral vaccination of baculovirus-expressed VP28 displays enhanced protection against White Spot Syndrome Virus in *Penaeus monodon.* PLoS One. 6, e26427. https://doi.org/10.1371/journal.pone.0026428.

16. Rajeshkumar, S., Venkatesan, C., Sarathi, M., Sarathbabu, V., Thomas, J., Anver Basha, K., Sahul Hameed, A.S., 2009. Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV). Fish Shellfish Immunol. 26. https://scite.ai/reports/10.1016/j.fsi.2009.01.003.

17. Somchai P, Jitrakorn S, Thitamadee S, Meetam M, Saksmerprome V., 2016. Use of microalgae *Chlamydomonas reinhardtii* for production of double-stranded RNA against shrimp virus. Aquac Reports. 3, 178–183.

18. Feng, S., Feng, W., Zhao, L., Gu, H., Li, Q., Shi, K., 2014. Preparation of transgenic *Dunaliella salina* for immunization against white spot syndrome virus in crayfish. Arch Virol. 159 (3), 519–25.

19. Dyo, Y.M., Purton, S., 2018. The algal chloroplast as a synthetic biology platform for production of therapeutic proteins. Microbiol (United Kingdom). 164 (2), 113–21.

20. Scaife, M.A., Nguyen GTDT, Rico, J., Lambert, D., Helliwell, K.E., Smith, A.G., 2015. Establishing *Chlamydomonas reinhardtii* as an industrial biotechnology host. Plant J. 82 (3), 532-546.

21. Charoonnart, P., Purton, S., Saksmerprome, V., 2018. Applications of microalgal biotechnology for disease control in aquaculture. Biology (Basel). 7(2). https://doi.org/10.3390/biology7020024

22. Schroda, M., 2019. Good News for Nuclear Transgene Expression in Chlamydomonas. Cells. 8 (12). https://doi.org/10.3390/cells8121534.

23. Taunt, H.N., Stoffels, L., Purton, S., 2018. Green biologics: The algal chloroplast as a platform for making biopharmaceuticals. Bioengineered. 9 (1), 48-54.

24. Harris, E.H., Stern, D.B., Witman, G.B., 2009. The *Chlamydomonas* Sourcebook; Durham, North Carolina, USA; London: Academic Press. Vol. 3, pp 2000.

25. Wannathong, T., Waterhouse, J.C., Young, R.E.B., Economou, C.K., Purton, S., 2016. New tools for chloroplast genetic engineering allow the synthesis of human growth hormone in the green alga *Chlamydomonas reinhardtii*. Appl Microbiol Biotechnol. 100 (12), 5467–77.

26. Economou, C., Wannathong, T., Szaub, J., Purton, S., 2014. A simple, low-cost method for chloroplast transformation of the green alga *Chlamydomonas reinhardtii*. Methods Mol Biol. 1132, 401–11.

27. Purton, S., Szaub, J.B., Wannathong, T., Young, R., Economou, C.K., 2013. Genetic engineering of algal chloroplasts: Progress and prospects. Russ J Plant Physiol. 60 (4), 491–9.

28. Witteveldt, J., Vlak, J.M., van Hulten, M.C., 2004. Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. Fish Shellfish Immunol. 16, 571-579.

29. Fu, L.L., Li, W.F., Du, H.H., Dai, W., Xu, Z.R., 2008. Oral vaccination with envelope protein VP28 against white spot syndrome virus in *Procambarus clarkii* using *Bacillus subtilis* as delivery vehicles. Lett. Appl. Microbiol. 46, 581-586.

30. Sarathi, M., Simon, M.C., Venkatesan, C., Hameed, A.S., 2008. Oral administration of bacterially expressed VP28dsRNA to protect *Penaeus monodon* from white spot syndrome virus. Mar. Biotechnol. (NY). 10, 242-249.

31. Witteveldt, J., Vlak, J.M., van Hulten, M.C., 2006. Increased tolerance of *Litopenaeus vannamei* to white spot syndrome virus (WSSV) infection after oral application of the viral envelope protein VP28. Dis. Aquat. Organ. 70, 167-170.

32. Witteveldt, J., Cifuentes, C.C., Vlak, J.M., van Hulten, M.C.W., 2004. Protection of *Penaeus monodon* against White Spot Syndrome Virus by Oral Vaccination. J. Virol. 78, 2057-2061.

33. Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J., Hu, L., 2004. Vp28 of shrimp white spot syndrome virus is involved in the attachment and penetration into shrimp cells. J. Biochem. Mol. Biol. 37, 726-734.

34. Akhila, D.S., Mani, M.K., Rai, P., Condon, K., Owens, L., Karunasagar, I., 2015. Antisense RNA mediated protection from white spot syndrome virus (WSSV) infection in Pacific white shrimp *Litopenaeus vannamei.* Aquaculture. 435, 306-309.

35. Nilsen, P., Karlsen, M., Sritunyalucksana, K., Thitamadee, S., 2017. White spot syndrome virus VP28 specific double-stranded RNA provides protection through a highly focused siRNA population. Sci. Rep. 7 (1), 1028. https://doi.org/10.1038/s41598-017-01181-w

36. Taengchaiyaphum, S., Nakayama, H., Srisala, J., Khiev, R., Aldama-Cano, D.J., Thitamadee, S., Sritunyalucksana, K., 2017. Vaccination with multimeric recombinant VP28 induces high protection against white spot syndrome virus in shrimp. Dev. Comp. Immunol. 6, 56-64.

37. Fu, L.L., Wanga, Y., Wu, Z.C., Li, W.F., 2011. In vivo assessment for oral delivery of *Bacillus subtilis* harboring a viral protein (VP28) against white spot syndrome virus in *Litopenaeus vannamei.* Aquaculture. 322-323, 33-38.

38. Jha, R.K., Xu, Z.R., Shen, J., Bai, S.J., Sun, J.Y., Li, W.F., 2006. The efficacy of recombinant vaccines against white spot syndrome virus in *Procambarus clarkii.* Immunol. Lett. 105, 68-76.

39. Franklin, S., Ngo, B., Efuet, E., Mayfield, S.P., 2002. Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. Plant. J. 30, 733-744.

40. Surzycki, R., Greenham, K., Kitayama, K., Dibal, F., Wagner, R., Rochaix, J.D., 2009. Factors effecting expression of vaccines in microalgae. Biologicals. 37 (3), 133–8.

41. Franklin, S.E., Mayfield, S.P., 2004. Prospects for molecular farming in the green alga *Chlamydomonas*. Curr. Opin. Plant. Biol. 7, 159-165.

42. Kozak, M., 1986. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. U S A. 83, 2850-2854.

43. Weik, M., Modrof, J., Klenk, H.D., Becker, S., Mühlberger, E., 2002. Ebola virus VP30-mediated transcription is regulated by RNA secondary structure formation. J Virol. 76, 8532-8539.

44. Schmittgen, T.D., Danenberg, K.D., Horikoshi, T., Lenz, H.J., Danenberg, P.V., 1994. Effect of 5-fluoro- and 5-bromouracil substitution on the translation of human thymidylate synthase mRNA. J. Biol. Chem. 269, 16269-16275.

45. Jia, M., Li, Y., 2005. The relationship among gene expression, folding free energy and codon usage bias in *Escherichia coli.* FEBS. Lett. 579, 5333-5337.

46. Gangl, D., Zedler, J.A., Włodarczyk, A., Jensen, P.E., Purton, S., Robinson, C., 2015. Expression and membrane-targeting of an active plant cytochrome P450 in the chloroplast of the green alga *Chlamydomonas reinhardtii.* Phytochemistry. 110, 22-28.

47. Beseres, J.J, Lawrence, A.L., Feller, R.J., 2006. Practical equivalence of laboratory and field measurements of gut passage time in two penaeid shrimp species. Mar. Ecol. Prog. Ser. 309, 221-231.

48. Ning, D., Leng, X., Li, Q., Xu, W., 2011. Surface-displayed VP28 on *Bacillus subtilis* spores induce protection against white spot syndrome virus in crayfish by oral administration. J. Appl. Microbiol. 111, 1327-1336.

49. Jia, X.H., Zhang, C.L., Shi, D.J., Zhuang, M.M., Wang, X., Jia, R., Zhang, Z.Y., Huang, J., Sun, Y.H., Qian, W.Y., 2015. Oral administration of *Anabaena*-expressed VP28 for both drug and food against white spot syndrome virus in shrimp. J. Appl. Phycol. 28, 1001-1009.

50. *Chlamydomonas* in the Laboratory. In: The *Chlamydomonas* Sourcebook 3-Vol set. Elsevier Inc.; 2009. p. 241–302

LIST OF FIGURES

Figure 1 The pASapI expression vector contains the *atpA* promoter/5'UTR, a multiple cloning site for coding sequence of the gene of interest (GOI) insertion, the *rbcL* 3'UTR and the essential photosynthesis gene *psbH* which serves as a selectable marker. *C. reinhardtii* recipient strain TN72 is non-photosynthetic because *psbH* has been disrupted using the *aadA* gene that confers spectinomycin resistance. Following transformation, two homologous recombination events result in the replacement of *psbH::aadA* with a functional *psbH* and with the expression cassette harboring the GOI.

Figure 2 Nucleotide sequence alignment of the original *vp28* sequence (top sequence) and the codon optimized *vp28* (*vp28-op*, bottom sequence). The black shading indicates identical nucleotides. The numbers on the right indicated the position of nucleotides.

Figure 3 PCR confirmation of homoplasmy of *vp28-op* and *vp28* transformant lines. A three-primer PCR strategy (see Fig 1) was used to confirm the correct integration of the expression cassette into the chloroplast genome of the phototrophic transformants. Heteroplasmic transformants harboring both transformed and untransformed copies of the genome would be expected to produce both a 1.2 kb and a 1.0 kb PCR product. The absence of any detectable 1.0 kb band suggests that all transformant lines are homoplasmic. M = DNA ladder of size markers, Lane 1-5: transformants with *vp28-op*, Lane 6-11= transformants with *vp28*.

Figure 4 Growth on solid media to test for spectinomycin resistance in the different transformant lines. Cultures of: (A) TNVP28-op (OP1-5), (B) TNVP28 (CV1-6) and (C) TNVP28-op (OP), TNVP28 (CV), TN72-emp (CP) and the TN72 recipient strain were grown on TAP medium (left) and TAP containing 100 µg/ml spectinomycin (right). After 1 week, no significant growth of the transformant line is seen on TAP medium containing spectinomycin. Only the untransformed TN72 could grow on the selective medium.

Figure 5 Western blot analysis of total lysate from selected *C. reinhardtii* transformant lines using a polyclonal anti-VP28 antibody. A positive control (lane P) gives a distinct band at ~28kDa and a similar sized band is seen in the recombinant VP28-op extracts (OP1 and OP2). A non-specific band at ~35 kDa is seen in all algal samples including TN72 recipient

and probably represents an endogenous protein that cross-reacts with the antibody (black arrow). (Lane M = protein marker; OP1 and OP2 = TNVP28-op transformants, CV = TNVP28, CP = TN-empty, TN72 = TN72 recipient, P = lysate from *E. coli* expressing recombinant VP28 as a positive control).

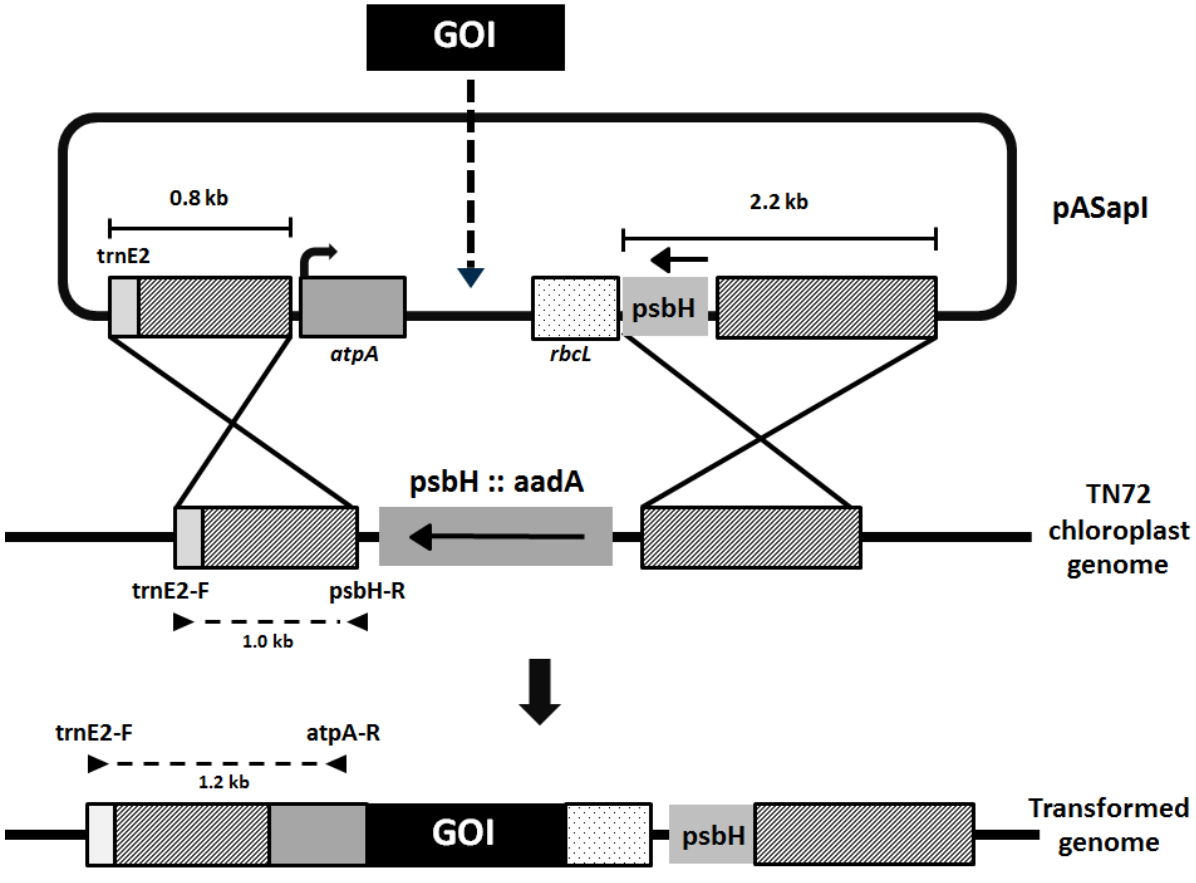
Figure 6 Western blot analysis of the His-tagged VP28 from *C. reinhardtii* transformant (TNVP28-op) affinity purified using a Ni–NTA column. The 28 kDa protein is detected by the anti-VP28 antibody in all eluted fractions. (Lane M, protein marker; lane FT = flow-through protein fraction that did not bind to the column containing the endogenous 35 kDa protein; lane W = last wash fraction; lane E1 – E2 = first eluted fraction with 100 and 250 mM imidazole; lane E3 – E5 = eluted fraction with 500 mM imidazole; lane C = extracted proteins from *E. coli* expressing VP28 as a positive control).

Figure 7 *C. reinhardtii* cells incubated in TAP medium at different pH's for 1, 3 and 5 hours to examine the acid tolerance.

Figure 8 *C. reinhardtii* cells incubating in different bile salt concentrations for 30, 60 and 90 min to examine the bile salt tolerance.

Figure 9 Cumulative mortality rates of shrimp against WSSV from the groups orally immunostimulated with *C. reinhardtii* strain TNVP28-op, TNVP28 and TN72-emp. As a positive control, shrimps were fed only commercial feed. Shrimps were challenged with WSSV 10 days' post-immunization and cumulative mortalities were recorded 8 days after challenge. A negative control group were not challenged with the WSSV

FIGURE 1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pASapI
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TN72
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genome
trnE2-F
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GOI
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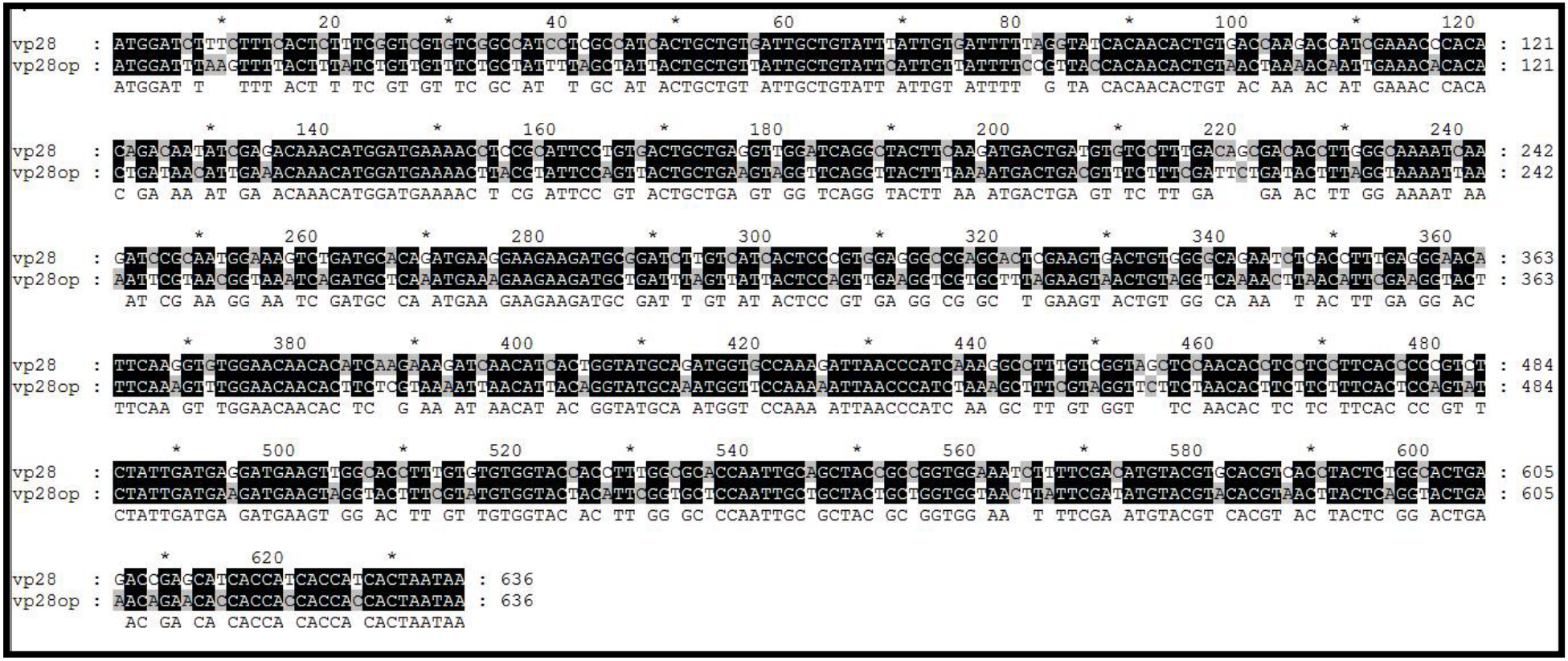
Transformed
genome

FIGURE 2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C GA AA AT GA ACAAACATGGATGAAAAC T CG ATTCC GT ACTGCTGA GT GG TCAGG TACTT AA ATGACTGA GT TC TT GA GA AC TT GG AAAAT AA
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vp28 : GACCGAGCATCACCATCACCATCACTAATAA : 636
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AC GA CA CACCA CACCA CACTAATAA

65 FIGURE 3

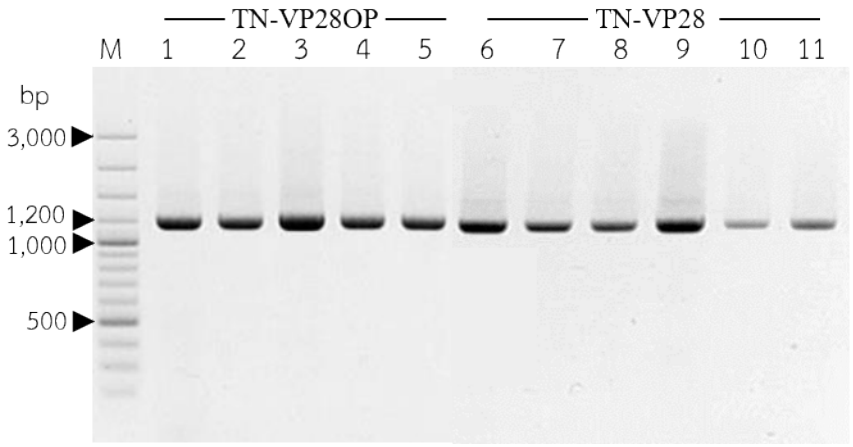

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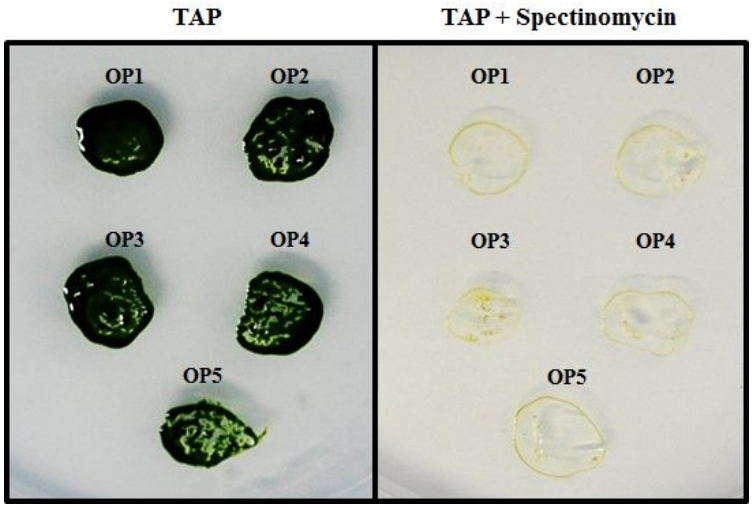
FIGURE 4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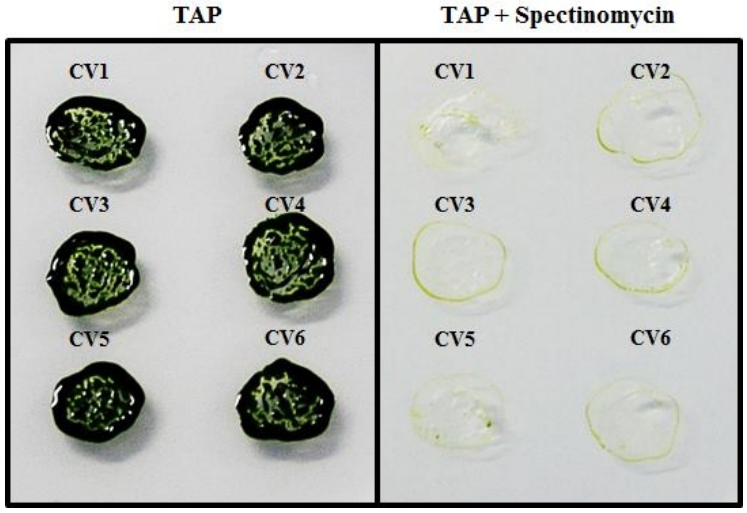
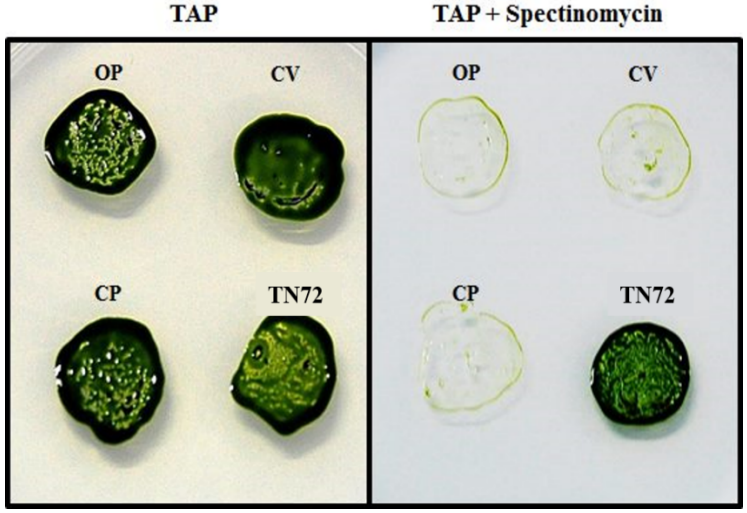
TAP + Spectinomycin
OP1
OP2
OP3
OP4
OP5
OP1
OP2
OP3
OP4
OP5

(A)


TAP
TAP + Spectinomycin
CV1
CV2
CV3
CV4
CV5
CV6
CV1
CV2
CV3
CV4
CV5
CV6

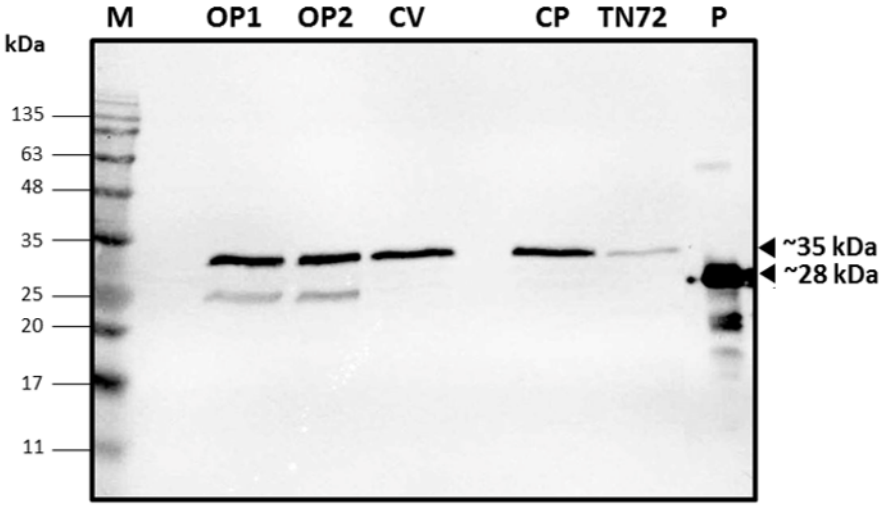
(B)


TAP
TAP + Spectinomycin
OP
CV
CP
TN72
OP
CV
CP
TN72

(C)

74 FIGURE 5

75



76

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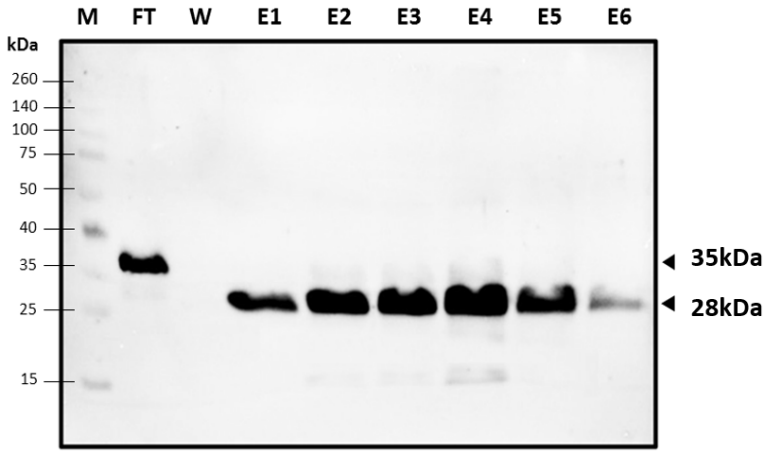
79 FIGURE 6

80



81

FIGURE 7

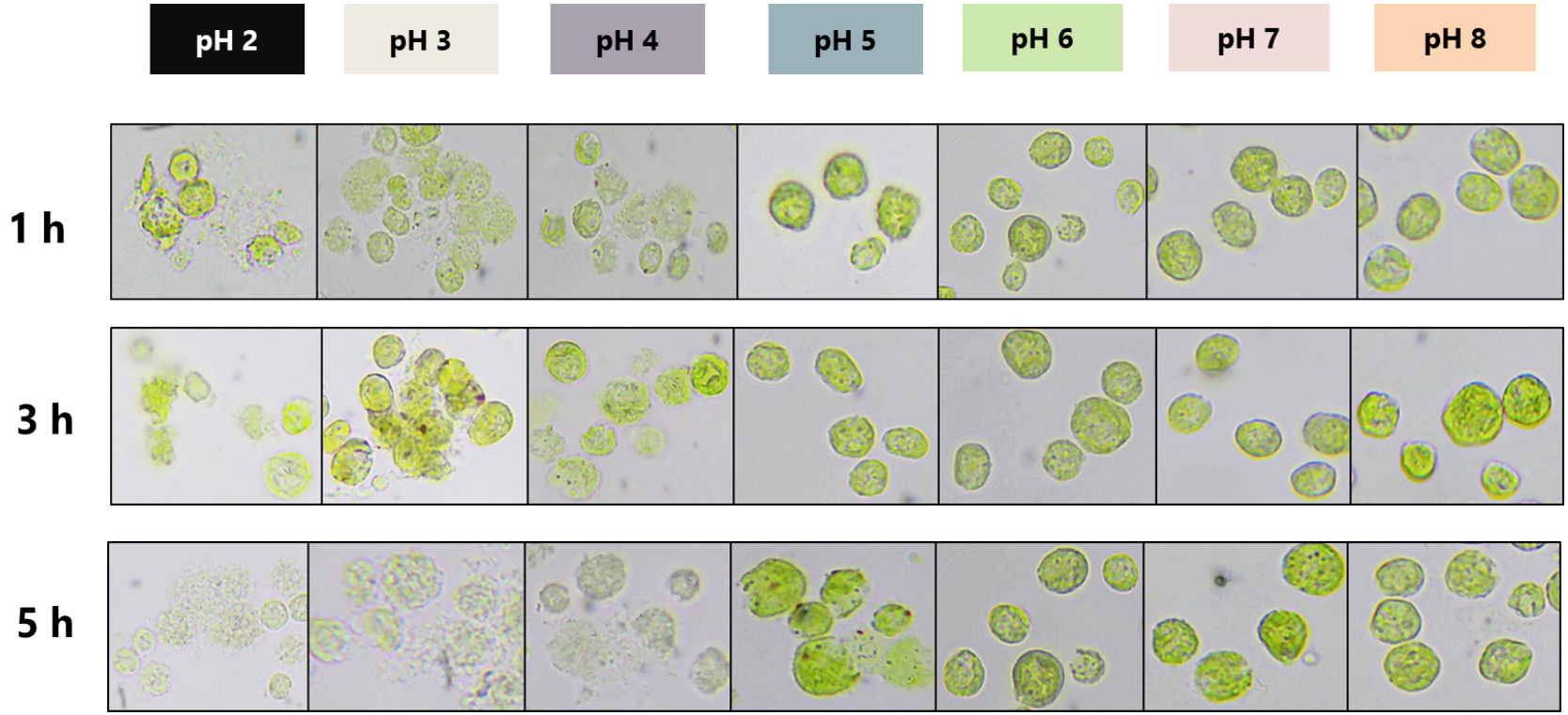


FIGURE 8

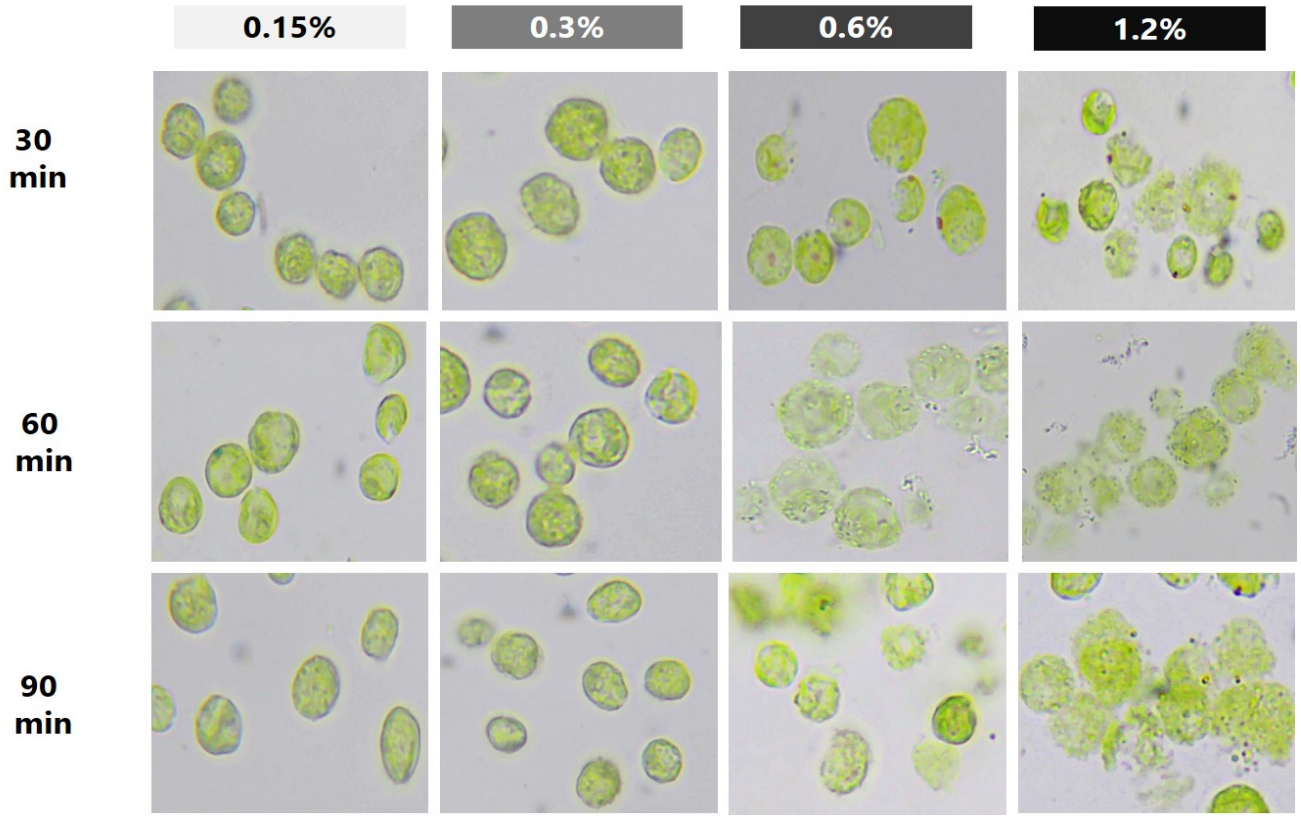
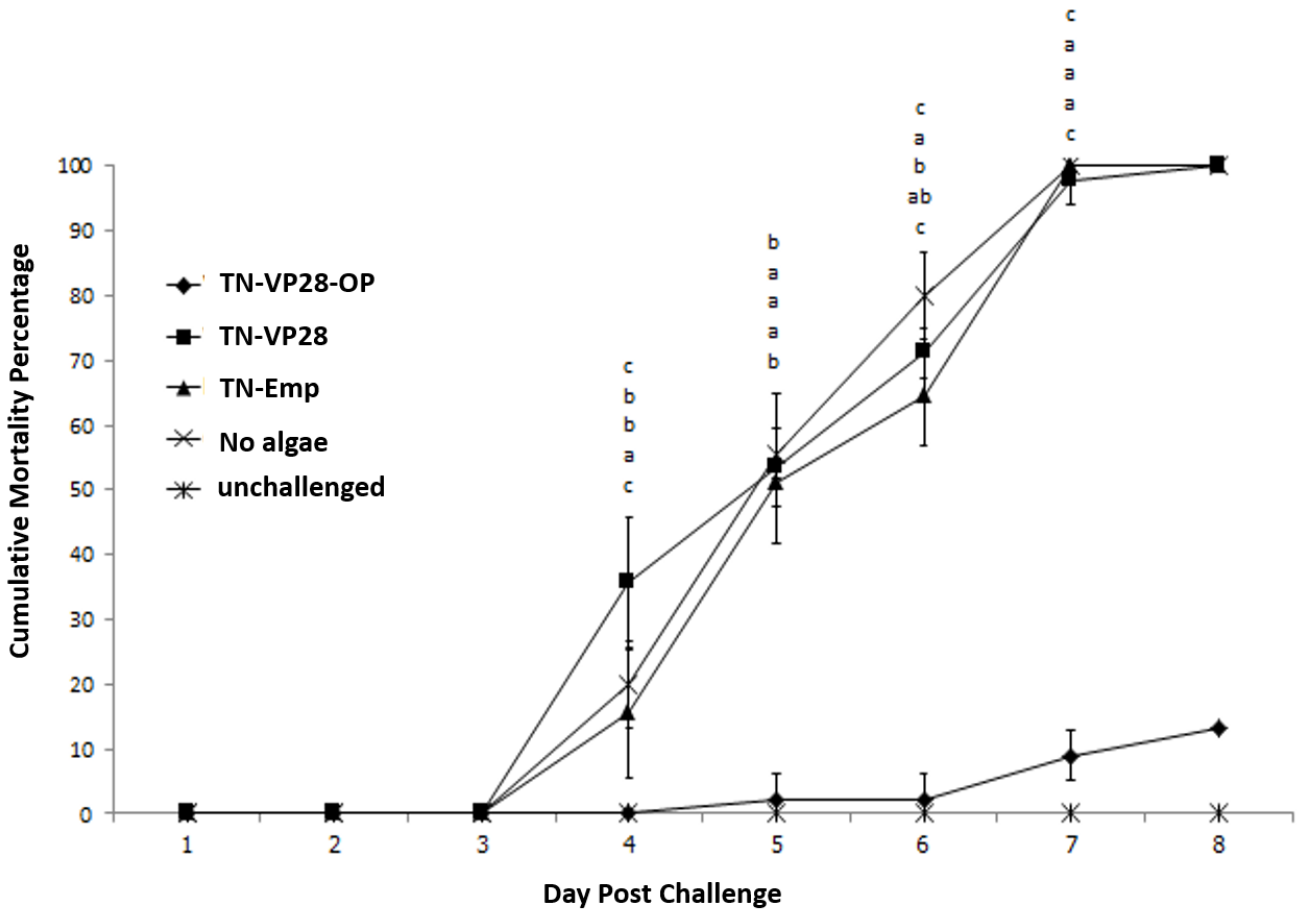


FIGURE 9


Cumulative Mortality Percentage
Day Post Challenge
TN-VP28-OP
TN-VP28
TN-Emp
No algae
unchallenged
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7
8
c b b a c
b a a a b
c a b ab c
c a a a c