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# Heterologous expression of the *Streptococcus pneumoniae yoeB* and *pezT* toxin genes is lethal in *Chlorella vulgaris*☆

Shet Lee Ng [a,b], Jennifer Ann Harikrishna [a,*], Fauziah Abu Bakar [a], Chew Chieng Yeo [b], Thye San Cha [c,*]

[a] Centre for Research in Biotechnology for Agriculture (CEBAR) and Institute for Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia
[b] Biomedical Research Centre, Faculty of Medicine, Universiti Sultan Zainal Abidin, 20400 Kuala Terengganu, Malaysia
[c] Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia

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ABSTRACT

*Chlorella vulgaris* is a eukaryotic microalga with potential for the production of biofuels. However, its thick and rigid cell wall is an impediment to cost-effective, large-scale harvesting of biofuels from these cells. Bacterial toxin-antitoxin (TA) systems, comprising of a stable proteic toxin and its labile cognate antitoxin, have no known homologs in eukaryotic cells. Several bacterial TA toxins have been found to be lethal when expressed in eukaryotes such as yeasts, animal and human cell lines. In this study, the functionality of the $yoeB_{Spn}$ and *pezT* toxin genes from the Gram-positive bacterium *Streptococcus pneumoniae* in *C. vulgaris* was investigated using a two-component inducible expression system. The $yoeB_{Spn}$ and *pezT* toxin genes were each cloned as green fluorescent protein (GFP) fusion constructs and introduced into *C. vulgaris* by *Agrobacterium tumefaciens*-mediated co-transformation with recombinant activator and responder vectors. Following induction for the expression of the toxin-GFP fusion transgenes, GFP fluorescence was observed in the transformed *C. vulgaris* cells which also showed signs of cellular damage and lysis. This is the first report of the lethal expression of bacterial TA toxins in eukaryotic microalgae, which can form the basis of a novel method for harvesting of microalgal cellular contents.



## 1. Introduction

*Chlorella vulgaris* is a unicellular eukaryotic microalga that shares many similar production characteristics with plants such as photosynthesis and carbon dioxide fixation with oxygen production [1]. *C. vulgaris* can multiply by asexual reproduction every 24 h, if grown under optimal conditions [2]. This microalga is widely found in natural waters such as ponds or lakes as well as in wastewater ponds. The cultivation of *C. vulgaris* next to combustion power plants with excess carbon dioxide release enabled the microalgae to absorb the unwanted carbon dioxide or secondary products such as nitrates which the microalgae utilizes during the conversion into potential biofuel or feeds [3]. Under nitrogen starvation, *C. vulgaris* is able to accumulate high lipid content that results in favorable fatty acid profiles for biodiesel production [4]. The production of biohydrogen using *C. vulgaris* was also deemed attractive as it requires only the available solar energy and can be used as gas fuel for electricity generation [5]. The production of bio-energies from microalgae are of major importance as they can eventually replace the use of agricultural crops since microalgae-based bioenergy production can be carried out on large scales with higher fuel yield as compared to the former [6]. Besides that, the cost can be effectively decreased as smaller areas are required [7] and the microalgae can be reused for the bioremediation of waste products [8].

Since microalgae are deemed attractive candidates for the generation of a wide range of bioenergy products such as biofuel, biohydrogen and bioethanol [8], a lot of research has been carried out to improve methods to efficiently harvest the cell contents of microalgae. The most used microalgae-biomass harvesting techniques include centrifugation, filtration and flocculation [9] which can provide high biomass recovery from the culture medium [10]. However, these harvesting techniques have limitations which restrict them from being widely used in the microalgal-biomass harvesting industry: the high gravitational force and shear forces during centrifugation was reported to cause cell structural damage and the energy requirement negatively impacted the $CO_2$ balances in microalgae-based biodiesel production [11] while filtration methods are limited by the differing sizes of the microalgae, which ranged from as small as 1 μM to as large as over 70 μM, leading to high costs for membrane filter replacement used for different microalgae species [11]. Although flocculation can have up to

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* Corresponding authors.
*E-mail addresses:* shetlee@gmail.com (S.L. Ng), jennihari@um.edu.my (J.A. Harikrishna), afung87@gmail.com (F. Abu Bakar), chewchieng@gmail.com (C.C. Yeo), cha_ts@umt.edu.my (T.S. Cha).

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80% flocculation efficiency, it is not feasible to sustain large-scale microalgae-biomass harvesting as production plants require large quantities of flocculants which produce excess cationic flocculent that eventually has to be removed, leading to additional operating costs [12]. Furthermore, microalgae have rigid cell walls which impede the harvesting of cell contents, for example, *C. vulgaris* is protected by a rigid trifluoroacetic cell wall that is composed of glucosamine polymer [13]. Although enzymatic degradation of microalgal cell walls prior to biomass harvesting can address this issue, the feasibility of this pretreatment is still questionable in terms of large-scale microalgae-biomass recovery for bio-energy production [13]. Therefore, the need for new approaches for maximizing the harvesting potential from *C. vulgaris* is desirable. One of the potential alternative approaches is through the genetic manipulation of *C. vulgaris* to introduce genes to induce rapid cell death and cell lysis that will release the valuable microalgal cell contents.

Toxin-antitoxin (TA) systems are nearly ubiquitous genetic elements that are found in prokaryotic genomes and have been implicated in the regulation of bacterial cell death and dormancy [14]. The mechanism of TA action relies heavily on the differential stability of the two components, namely the stable toxin and the labile cognate antitoxin. Under normal growth conditions, the antitoxin is continuously produced to bind to the toxin, thus neutralizing the toxin's lethal effects. When bacterial cells are under stress conditions, endogenous cellular proteases degrade the antitoxin causing the liberation of the toxin from the TA complex. This will result in the toxin acting on its particular cellular targets, often resulting in cell lysis and death [15]. Bacterial TA systems are currently classified into six types (Types I–VI) with Type II systems being the most prevalent and well-characterized [16–18]. In Type II TA systems, the proteic antitoxin prevents the lethal action of the toxin through tight binding with the toxin, usually at the active site of the toxin [16,18,19]. Most type II toxins are endoribonucleases while other toxins disrupt DNA replication by targeting DNA gyrase and helicase [14], and some inhibits the synthesis of the bacterial cell wall [20].

No eukaryotic homologs of bacterial TA systems have been reported but several bacterial TA toxins have been shown to be functionally lethal in eukaryotic cells, leading to several interesting and novel applications [21]. Recently, it was reported that the expression of the *Streptococcus pneumoniae*-encoded $YoeB_{Spn}$ toxin was lethal in the model plant *Arabidopsis thaliana* [22]. A two-component XVE-based expression system comprising of an activator vector and a responder vector [23] was used to enable strict inducibility with 17-$\beta$-estradiol for the cloned $YoeB_{Spn}$ transgene in *A. thaliana* [22]. Until now, there has yet to be any report on the heterologous expression of bacterial TA toxins in eukaryotic microalgae. In this study, the same two-component XVE-based expression system that was used in *A. thaliana* was used to investigate the functionality of the $YoeB_{Spn}$ as well as another *S. pneumoniae*-encoded toxin, PezT [24] in *C. vulgaris*. The functional lethality of the bacterial $YoeB_{Spn}$ and PezT toxins in *C. vulgaris* would thus ultimately pave the way for the development of novel cloning strategies to efficiently harvest valuable cell contents such as biofuels from microalgae.

## 2. Materials and methodology

### 2.1. Agrobacterium tumefaciens and Chlorella vulgaris cultures and conditions

Recombinant *A. tumefaciens* LBA4404 cells were cultured in Luria-Bertani (LB) broth and on LB agar solidified with 1.2% (w/v) bacto-agar, supplemented with the appropriate antibiotics. For *A. tumefaciens* harboring the recombinant pMDC150-derived activator vector, the antibiotics used were rifampicin (50 μg $mL^{-1}$) and spectinomycin (50 μg $mL^{-1}$); whereas for *A. tumefaciens* harboring recombinant pMDC221-derived responder vector, the antibiotics used were rifampicin (50 μg $mL^{-1}$) and ampicillin (100 μg $mL^{-1}$) [23]. Bacterial culture on agar was incubated at 27 °C while those in broth were incubated at 27 °C with shaking at 220 rpm. Both agar and broth cultures of *A. tumefaciens* were incubated in the dark at all times as rifampicin is a light sensitive antibiotic.

*C. vulgaris* UMT-M1 [25] was cultured in Bold's Basal Medium (BBM) broth and on BBM agar [26] solidified with 1.2% w/v bacto-agar. Microalgae culture on agar was incubated at 27 °C while those in broth were incubated at 27 °C with shaking at 220 rpm. Both agar and broth culture of *C. vulgaris* were exposed to continuous photon flux density of 40 μmol $m^{-2}$ $s^{-1}$. For selection, the transformed *C. vulgaris* was cultured on selective BBM supplemented with kanamycin (50 μg $mL^{-1}$) and hygromycin (20 μg $mL^{-1}$). The agar and broth culture of transformed *C. vulgaris* were incubated in the dark for the first 24 h before exposure to light as hygromycin is a light-sensitive antibiotic.

### 2.2. Recombinant plasmids

A two-component 17-$\beta$-estradiol-inducible expression system comprising of the pMDC150 activator vector and the pMDC221 responder vector [23] was used for the expression of the two prokaryotic toxins genes in this study.

A recombinant activator vector pMDC150_35S, containing the CaMV 35S constitutive promoter as previously described [22] was used for the constitutive expression of the chimeric XVE transcriptional activator in the transformed microalgae, while the responder vector pMDC221_yoeBGFP carries the $yoeB_{Spn}$ toxin gene from *S. pneumoniae* as a translational fusion with a *GFP* gene as described by Abu Bakar et al. [22]. The resulting pMDC221_yoeBGFP (Fig. 1B) recombinant responder places the $yoeB_{Spn}$-*GFP* fusion under the control of the XVE-responsive promoter (OlexA-TATA promoter) thus making its expression dependent on the presence of 17-$\beta$-estradiol. A *GFP*-expressing recombinant vector pMDC221_GFP (Fig. 1D) [22] was used a positive control.

The *pezT* toxin gene (GI: 446327505; coordinates 1657560–1658321 of accession no. NZ_AKBW01000001) from *S. pneumoniae* was also cloned as a translational fusion with the *GFP* gene into pMDC221. The *pezT* toxin gene was PCR-amplified as a 762 bp fragment from the pET11a-PezT recombinant plasmid [24] using the primers pezT_F and pezT_R (Table 1). A *Bam*HI (5′-GGATCC) restriction site was included at the 5′-end of the pezT_R reverse primer and the 5′-end of the *GFP* forward primer (GFP_F) (Table 1) to enable the synthesis of a *pezT-GFP* fusion. The *GFP* gene was PCR-amplified as a 732 bp fragment as described in [22]. PCR amplifications were carried out under the following conditions: initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 s; annealing at 57 °C for 30 s; and extension at 72 °C for 3 min; and a final extension at 72 °C for 7 min. Both the *pezT* and *GFP* amplified products were subjected to *Bam*HI digestion for 2 h at 37 °C prior to overnight ligation using T4 DNA ligase (Promega, WI, USA) at 4 °C. The resulting 1497 bp *pezT-GFP* ligated product was then cloned into the Gateway pENTR_D_TOPO cloning vector (Invitrogen, USA) according to the supplier's instructions. The recombinant *pezT-GFP* Gateway entry clone was validated by conventional Sanger dideoxy sequencing prior to the transfer of the *pezT-GFP* fragment into pMDC221 as the Gateway destination vector using the LR clonase reaction (Invitrogen, USA). The constructs obtained were transformed into *Escherichia coli* TOP10 cells and transformants screened by colony PCR using pezT_F and GFP_R primers. The responder recombinant vector was designated pMDC221_pezTGFP (Fig. 1C) after validation by conventional sequencing.

### 2.3. Co-transformation of two-component inducible expression vectors into C. vulgaris

*A. tumefaciens*-mediated transformation of *C. vulgaris* was carried out according to Cha et al. [25] with modifications. A total of $5 \times 10^7$