# Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*

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**The ciliated protozoan, *Tetrahymena thermophila*, offers an attractive medium for the expression of heterologous proteins and could prove particularly useful for the display of foreign proteins on the cell surface. Although progress has been made in transformation of *Tetrahymena* with heterologous DNA, methods that permit reliable expression of foreign genes have been lacking. Using a mutant strain of *T. thermophila* carrying a negatively selectable allele of a β-tubulin gene, we have been able to direct foreign genes to this locus by homologous recombination. Transformed cell lines producing foreign proteins were readily identified and, in at least one case, targeting of proteins to the plasma membrane was accomplished.**

Keywords: *Tetrahymena,* parasite antigen, -tubulin, paclitaxel, vaccine



Expression of eukaryotic genes in heterologous systems has become increasingly important both for the production of pharmaceuticals and as a basic research tool. Systems currently in use, such as yeast and cultured insect and mammalian cells, vary with respect to yield, cost-effectiveness, and biological activity of resulting products, and no universal host for the expression of foreign genes exists[1]. We are particularly interested in expression of heterologous proteins at the cell surface and are investigating the free-living ciliate, *Tetrahymena thermophila*, as a potential host in this regard. *T. thermophila* is nonpathogenic and can be grown in large volume cultures using a variety of inexpensive media including skimmed milk powder[2]. Generation time is short (1.5–3 h) and cells attain remarkably high density given their size (approximately 50 μm in length). Indeed, fermentation in perfused bioreactors has permitted growth to concentrations as high as 2.2 10[7] cells/ml, equivalent to a dry weight of 48 g/L (ref. 3). *Tetrahymena* has the ability to secrete proteins into the growth medium, and mutant strains defective in the release of hydrolytic enzymes are available[4–8]. Finally, a large part of the cell metabolism is devoted to the production of abundant surface membrane proteins known as immobilization antigens (i-antigens), whose expression is tightly regulated by environmental conditions[9].

By and large, the use of *Tetrahymena* as a system for the expression of foreign proteins has awaited development of suitable cloning vectors for high-level expression and stable maintenance of transgenes. The approach commonly used to transform *Tetrahymena* is based on high copy number vectors containing a replication origin derived from the rDNA minichromosome[10–13]. The level of expression of a transgene subcloned into rDNA vectors is highly variable due to frequent integration of vector molecules into the native rDNA[11,14–16]. Furthermore, no methods are currently available to prevent the loss of transgenes that lack a selectable phenotype. We report here an approach that permits the isolation of stable recombinants for genes that are not inherently selectable, and at the same time places the transgene under control of a highly active promoter. We further show that the resulting gene products can be targeted to the plasma membrane where they are readily detected.

## Results

**Targeted expression of the neomycin resistance gene by disruption of a β-tubulin gene of *T. thermophila.*** *T. thermophila* expresses two major β-tubulin genes, *BTU1* and *BTU2*, which encode identical β-tubulin proteins[17]. A substitution of lysine 350 by methionine in the *BTU1* gene *(btu-1-1K350M)* confers increased resistance to several microtubule-depolymerizing drugs (oryzalin, colchicine, vinblastine), and increased sensitivity to a microtubule-stabilizing agent, paclitaxel[18]. The phenotype of the *btu1-1K350M* allele is expressed in the presence of wild-type copies of the second β-tubulin gene, *BTU2*. Previous studies have shown that cells carrying the *btu1-1K350M* allele can be transformed to paclitaxel resistance by gene replacement of *btu1-1K350M* with a wild-type *BTU1* gene fragment[18]. These results led to the prediction that any loss-of-function mutation of *btu1-1K350M* in the presence of wild-type *BTU2* gene should confer paclitaxel resistance. Consequently, it should be possible to target any exogenous gene to the *BTU1* locus by homologous recombination and selection with paclitaxel. To test this idea, we used a replacement vector that contained the *BTU1* derivative, *btu1-2::neo1*, which substitutes the coding region of a prokaryotic gene, *neo1*, for that of *BTU1*. The *neo1* gene confers resistance to paromomycin in *T. thermophila*[14].

*Tetrahymena*, like most ciliate species, has two distinct nuclei, the germinal micronucleus and somatic macronucleus (MAC). In this study, we used a transformation method that targets genes into the MAC of vegetative cells using biolistic bombardment[19]. Plasmid DNA containing a *btu1-2::neo1* fragment was introduced into the paclitaxel-sensitive strain of *T. thermophila,* and cells were selected for resistance to either paclitaxel (30 μM) or paromomycin (120 μg/ml). As shown in Table 1, transformants were readily obtained following selection with either drug. Random clones that had been initially selected in paclitaxel were all found to be cross-resistant to paromomycin (n=40), and nearly all transformants originally selected with paromomycin, were also resistant to 30 M paclitaxel (89%, n=71). Thus, acquisition of the transformed phenotype (in most, if not all, cases) resulted from disruption of the host *btu1-1K350M* gene, and negative paclitaxel selection based on *BTU1* gene loss-of-function was nearly as effective as a positive selection based on paromomycin resistance conferred by the transgene. In several

transformant cell lines we replaced all copies of the endogenous macronuclear *BTU1* genes by the *neo1* derivative by phenotypic assortment as previously described[18]. Stable transformants produced the *neo1* gene product, neomycin phosphotransferase II (NPT-II), at the level of 50 ± 5 pg/μg (n=3) of total cell protein (equivalent of 0.05 mg/L of culture at standard density of $10^6$ cells/ml) (Fig. 1A).

**Expression of an *Ichthyophthirius multifiliis* surface antigen protein in *T. thermophila*.** Next, we attempted to transform *Tetrahymena* with a eukaryotic gene that expressed a protein product that was not inherently selectable. We chose a gene that encodes a 48 kDa surface antigen of the parasitic ciliate *Ichthyophthirius multifiliis*[20]. The coding region of the gene (*IAG48[G1];* GenBank accession #AF140273*)* was subcloned between the flanking sequences of *BTU1*, and the resulting *btu1-4::IAG48[G1]* fragment was introduced into *Tetrahymena* as described above. As shown in Table 1, paclitaxel-resistant transformants were obtained with a frequency comparable to that seen with the control *btu1-2::neo1* gene construct.

Western blot analysis (Fig. 2) using polyclonal antiserum against affinity-purified i-antigens of *Ichthyophthirius* demonstrated that the *I. multifiliis* gene product was being expressed in *IAG48[G1]* transformants. A protein with an apparent molecular weight of 50 kDa was detected in all transformants carrying the *IAG48[G1]* gene, but not in a control cell line transformed with *neo1* (Fig. 2). The size of the detected protein was in close agreement with the molecular weight of the corresponding surface antigen of *Ichthyophthirius*[20]. An additional band of about 100 kDa seen in both *btu1-4::IAG48[G1]* and control extracts most likely represents an endogenous *Tetrahymena* protein that shares immunologic determinants with the parasite antigen. We subsequently obtained a number of transformant cell lines in which all macronuclear copies of the *BTU1* gene were replaced by the *btu1-4::IAG48[G1]* fragment as described above for the *neo1* gene replacement. We performed a quantitative analysis of the level of expression of the i-antigen in a number of stable transformants and found that they expressed the i-antigen at the level of 269 ± 28 pg/μg (n=4) of total cell protein, which is equivalent to 0.269 mg/L of culture at the standard density of $10^6$ cells/ml (Fig. 1B).

**Protein targeting to the plasma membrane.** To determine the location of transgene protein in *Tetrahymena*, transformed cell lines were fixed, reacted with antibodies against the 48 kDa antigen, and analyzed by indirect immunofluorescence (without permeabilization) using confocal microscopy. Strong surface labeling of oral and somatic cilia was observed, along with a weaker pattern of fluorescence (in the form of longitudinal rows) associated with the cell cortex (Fig. 3A). Dividing cells showed labeling in both the preexisting and newly formed oral apparatus, indicating that as the new structure is formed, parasite antigens are rapidly inserted into the cell membrane (Fig. 3B). Control cells (transformed with *neo1*) showed no obvious labeling (Fig 3C). Thus, the i-antigen protein is targeted to the cell surface of transformed *Tetrahymena* cells.

**Table 1. Transformation of *T. thermophila* using biolistic bombardment.**

| Targeting fragment | Selection method | Frequency (transformants/μg DNA) |
|---|---|---|
| Experiment 1 | | |
| btu1-2::neo1 | tx | 115 |
| btu1-2::neo1 | pm | 204 |
| no DNA | tx | 0 |
| no DNA | pm | 0 |
| Experiment 2 | | |
| btu1-2::neo1 | tx | 37 |
| btu1-4::IAG[G1] | tx | 31 |
| no DNA | tx | 0 |

tx = 30 μM paclitaxel. pm = 120 μg/ml paromomycin.

## Discussion

Based on quantitative western blot analysis (Fig. 1), the level of expression of the NPT-II gene in *Tetrahymena* was equal to or greater than that for the same transgene expressed in cultured plant and animal cells[21,22]. The level of expression of the *I. multifiliis* surface antigen was roughly five times higher, placing it in the order of milligrams per liter of *T. thermophila* as all densities above $10^7$ cells/ml can be routinely achieved in bioreactors[3]. This is sufficient for the large-scale preparation of parasite antigens, but efforts to improve the overall levels of expression using alternative gene regulatory elements are currently under way.

The ability to express *Ichthyophthirius* i-antigens in *Tetrahymena* has immediate implications for vaccine development. As the etiologic agent of white spot disease in freshwater fish, *I. multifiliis* has a major impact on commercial aquaculture worldwide[23]. There is considerable evidence for the involvement of i-

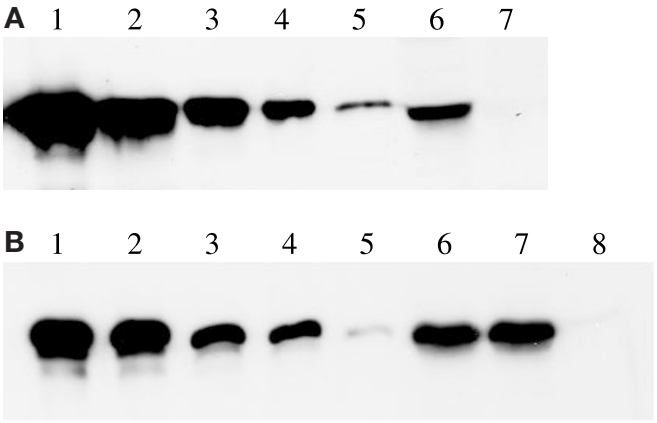


**Figure 1. Quantitative analysis of transgenic protein expression. (A) A western blot probed with anti–NPT-II antibodies. Lanes 1–5: purified NPT-II protein standard (25, 10, 5, 2.5, and 1 ng). Lane 6: total protein extract from a *btu1-2::neo1* transformant. Lane 7: extract from a *btu1-4::IAG48[G1]* transformant as a negative control. (B) A western blot probed with antibodies generated against purified i-antigens of *Ichthyophthirius multifiliis*. Lanes 1–5: purified i-antigen (40, 20, 10, 5, and 1 ng). Lanes 7 and 8: total protein extracts of two independent *btu1-4::IAG48[G1]* transformants. Lane 9: control extract from cells transformed by the *btu1-2::neo1* fragment. 5□$10^4$ cells were loaded per lane, equivalent to 50 μg of total cell protein[34,35].**

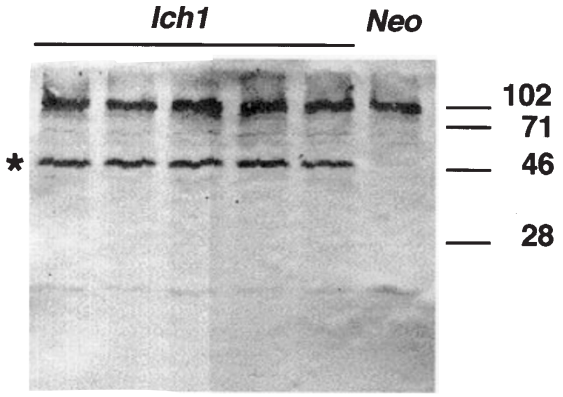


**Figure 2. Western blot analysis of total protein extracts of five strains transformed by the *btu1-4::IAG48[G1]* fragment and a single control strain transformed with the *btu1-2::neo1* fragment. Proteins were probed with rabbit anti–Ich i-antigen antibodies. All *btu1-4::IAG48[G1]* transformants show a protein band of the expected size of about 50 kDa (marked by a star), which is absent in the negative control transformant.**

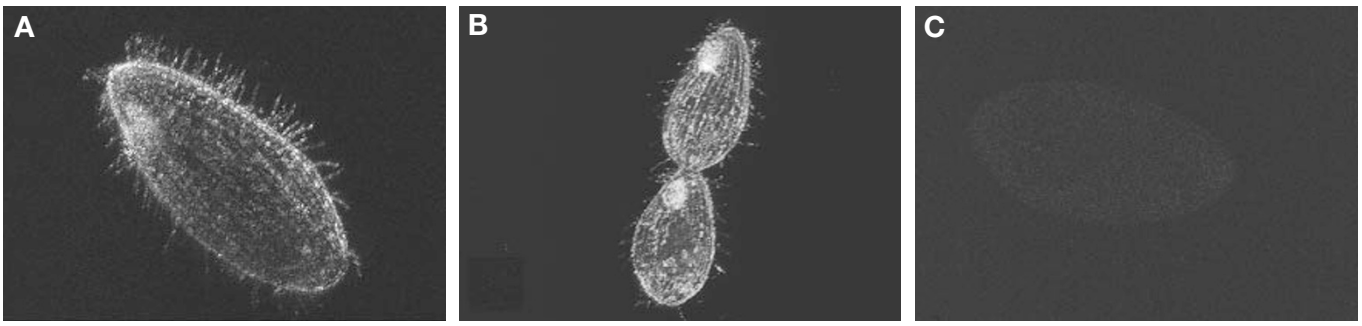


**Figure 3. Confocal immunofluorescent analysis of transformants. (A and B) Cells were transformed with the *btu1-4::IAG48[G1]*fragment. (C) is a negative control transformed with the *btu1-2::neo1* fragment. Prior to labeling, cells were fixed and further processed without permeabilization. (B) Cell in the final stage of cytokinesis.**

antigens in protective immunity[24,25]. However, *Ichthyophthirius* is an obligate parasite and can be grown only in association with its host. This precludes isolation of sufficient amounts of antigen for development of a subunit vaccine. Because *T. thermophila* shares the same codon usage as *Ichthyophthirius*[26,27], expression of parasite antigens in *Tetrahymena* may now make possible the large-scale production of a subunit vaccine. Indeed, because *T. thermophila* is normally present in freshwater ecosystems, macronuclear transformants expressing parasite antigens could potentially be used as live vaccines against *I. multifiliis*.

*Tetrahymena* may provide additional advantages as a host for the cloning and expression of genes from organisms with AT-rich genomes. A number of human pathogens (for example malarial plasmodia, mycoplasmas, etc) have extremely AT-rich genomes. Because of the inherent instability of AT tracts in conventional systems such as *Escherichia coli*, cloning genes from these organisms can sometimes prove difficult. *Tetrahymena* DNA consists of approximately 75% AT[28], and it would seem reasonable that *T. thermophila* would have little problem maintaining foreign DNA sequences having an equal bias in base composition.

One potential difficulty in expressing foreign genes in *Tetrahymena* may be codon usage. *T. thermophila* utilizes UAA and UAG triplets for glutamine, while in most other organisms these codons terminate translation[29]. Clearly, foreign genes whose coding regions terminate with UGA would be correctly expressed. In the case of UAA or UAG, site-directed mutagenesis could be used to replace these with UGA. Furthermore, while *Tetrahymena* codon usage is highly skewed, foreign genes with multiple codons rarely used in *Tetrahymena* have been successfully expressed[14].

Finally, the surface localization of *Ichthyophthirius* proteins in *btu1-4::IAG48[G1]* transformants indicates that the signal peptides associated with the parasite antigen function appropriately in *Tetrahymena*. Indeed, the overall distribution of *I. multifiliis* antigens in transformed cells bears striking resemblance to the pattern seen in the parasite itself where it is found in ciliary and plasma membranes, and in the cell cortex in association with secretory mucocysts[30]. Obviously, an important goal for the future will be to define the nature of the signal sequences required for membrane localization so that proteins from more distantly related species can be directed to the surface with high efficiency.

## Experimental protocol

**Construction of transformation plasmids.** The pBAB1 plasmid (kindly provided by M. Gorovsky, University of Rochester, NY) contains the *neo1* gene coding sequence[14], inserted in place of the coding sequence of the *BTU1* gene of *T. thermophila*[17]. pBAB1 was used to construct another derivative, pBICH3 in which the *neo* coding region was replaced with the entire coding sequence of the *Ichthyophthirius* i-antigen (isolate G1) preprotein[26]. To this end, the pBAB1 plasmid was amplified with the *NEO*257 primer (kindly provided by J. Bowen and M. Gorovsky), the sequence of which encodes the antisense strand of the *neo1* gene, and primer BTN3, the sequence of which corresponds to the 3′ noncoding region of *BTU1*. The BTN3 primer contains a *Bam*HI restriction site sequence at its 5′ end. A single *Hin*dIII site is located almost immediately downstream from the translation initiation codon, ATG, of the *neo1* gene[14]. The product of PCR amplification of pBAB1 was digested with *Bam*HI and *Hin*dIII to obtain a smaller fragment containing the entire flanking sequences of *BTU1* and the plasmid vector. The plasmid pH4T2-Ich, carrying a 2 kb *Eco*RI genomic fragment of the *Ichthyophthirius* i-antigen gene, was amplified. The entire coding sequence of the *Ichthyophthirius* i-antigen gene was amplified with primers IC5 and IC3, which also introduced *Hin*dIII, and *Bam*HI restriction sites at positions encoding the N-terminal and C-terminal end of the protein, respectively. After digestion with *Hin*dIII and *Bam*HI the i-antigen coding sequence was inserted into the *Bam*HI-*Hin*dIII sites of the amplified pBAB1 fragment to give pBICH3 plasmid.

**Culture growth and DNA-mediated transformation of *T. thermophila*.** A *Tetrahymena thermophila* strain, CU522 (kindly provided by Peter Bruns, Cornell University, Ithaca, NY) was used as a transformation host. This strain carries a single substitution (Lys350Met) in the β-tubulin *BTU1* gene, which confers increased resistance to several microtubule depolymerizing drugs, such as oryzalin and colchicine, and increased sensitivity to a microtubule-stabilizing agent, paclitaxel[18]. Cells were grown in 50 ml of SPP medium[31], supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (SPPA [medium), in 250 ml Erlenmeyer flasks with shaking at 150 rpm at 30°C. Prior to transformation, the CU522 cells were grown to a density of about $6\times10^5$ cells/ml in SPPA with shaking at 30°C. Twenty-four hours before transformation, 50 ml of growing cells were washed and suspended in 10 mM Tris-HCl (pH 7.5) buffer in the original volume. After 4–5 h starving cells were counted again, cell concentration was adjusted to $3\times10^5$ cells/ml, and cells were left at 30°C without shaking for another 18–20 h. To target the *BTU1* gene derivatives to the endogenous *BTU1* locus of *T. thermophila*, either pBAB1 or pBICH3 plasmids were digested with *Sac*I and *Sal*I restriction endonucleases to separate the insert (either *btu1-2::neo1* or *btu1-4::IAG48[G1]*) from the plasmid. Fifty micrograms of digested DNA were purified by a single phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by chloroform/isoamyl alcohol (24:1) extraction, precipitated with an equal volume of isopropyl alcohol in the presence of 0.15 M sodium acetate, dried and resuspended at 1 mg/ml. Ten micrograms of linearized plasmid DNA was used to coat 60 mg of 0.6 μm gold particles (Bio-Rad, Hercules, CA). $2.4\times10^7$ of starved cells were spun down at 600 G for 3 min, washed with 45 ml of 10 mM Tris (pH 7.5), and resuspended in 3 ml of Tris buffer. One milliliter of cells was bombarded using 10 μg of DNA-coated gold particles at 900 psi using DuPont Biolistic PDS-1000/He particle delivery system (Bio-Rad). Bombarded cells were resuspended in 50 ml of SPPA, left for 2–3 h at 30°C. Paclitaxel was added to a final concentration of 20 μM, cells were plated on microtiter plates using 100 μl cells/well, and plates were incubated in moist boxes at 30°C (in darkness to prevent photobreakdown of paclitaxel). Wells containing paclitaxel-resistant transformants were apparent after 2–3 days of selection. Complete replacement of all endogenous *BTU1* gene copies by transformed *BTU1* derivative was achieved using paclitaxel selection for phenotypic assortment as described[18].

**Immunocytochemistry.** For immunofluorescent detection of the *I. multifiliis* i-antigen in transgenic *Tetrahymena* cells, 10 ml of exponentially growing cultures ($2\times10^5$ cells/ml) were harvested, washed with 10 ml of 10 mM

Tris (pH. 7.5), and resuspended in 0.5 ml. Cells were fixed with 3.5 ml of 2% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH. 6.9) for 30 min at room temperature. Fixed cells were washed once with 3 ml PHEM buffer and twice with 3 ml modified phosphate buffered saline (PBS; 130 mM NaCl, 2 mM KCl, 8 mM $Na_2HPO_4$, 2 mM $KH_2PO4$, 10 mM EGTA, 2 mM $MgCl_2$, pH. 7.2) and resuspended in 0.5 ml. For antibody labeling, 100 □l of fixed cells were washed three times for 10 min with 3 ml PBS-BT (3% bovine serum albumin, 0.1% Tween 20 in PBS), and concentrated in 100 □l. One microliter of affinity-purified rabbit antiserum directed against *Ichthyophthirius* G1 i-antigen[32] was added, followed by incubation overnight at 4°C, three washes in PBS-BT, and incubation with the detection antibodies (at 1:100 dilution) for 45 min at room temperature. Secondary antibodies were goat antirabbit IgG coupled to FITC (Zymed, San Francisco, CA). Labeled cells were washed three times with PBS, concentrated in 0.1 ml of PBS plus 10 µl of DABCO mounting medium (100 mg/ml 1,4-diazobicyclo-[2,2,2]-octane [Sigma Chemical, St. Louis, MO], dissolved in 90% glycerol in PBS). To mount, 5 µl of cells were combined with 5 □l of DABCO medium, covered with a coverslip, and sealed with nail polish. Slides were examined with a Bio-Rad MRC 600 Laser Scanning Confocal Microscope at the University of Georgia Center for Advanced Ultrastructural Research (Athens, GA). Sets of optical sections of individual cells were processed to obtain complete three-dimensional reconstructions.

**Immunoblotting.** Protein extracts were prepared from 1–2 □10□ cells taken from exponentially growing cultures. Cells were spun down at 2000 rpm for 5 min, washed with 10 ml ice-cold 10 mM Tris, pH 7.5, and resuspended in 125 µl of ice-cold Tris buffer supplemented with a mixture of protease inhibitors, which included 0.5 µg/ml leupeptin, 10 µg/ml E-64, 10 □g/ml chymostatin, and 12.5 µg/ml antipain (all inhibitors from Sigma), combined with 125 □l of boiling 2 x SDS-PAGE sample buffer and boiled for 5–10 min. Ten microliters of extracts were loaded on a 10% SDS-PAGE minigel, and proteins were transferred on nitrocellulose using semidry transfer system (Bio-Rad). The filter was blocked for 2 h in PBS-T (PBS with 0.1% Tween-20) buffer containing 5% dried milk, followed by incubation with the anti-*I. multifiliis* i-antigen antibodies (1:10,000) overnight at 4°C. The filter was washed extensively with PBS-T and incubated in the same buffer containing the goat antirabbit IgG antibodies conjugated to alkaline phosphatase (Bio-Rad) for 1 h at room temperature. The membrane was washed in PBS-T and developed using NBT/BCIP (Bio-Rad) as described[33]. A quantitative analysis of the levels of expression of transgenic proteins in extracts of transformed *Tetrahymena* was performed using the ECL Western Blotting Analysis System (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. To quantify the amounts of NPT-II protein in the *neo1* transformants, we used the anti–NPT-II antibodies and a purified NPT-II protein as a standard (5 Prime-3 Prime, Boulder, CO).To quantify the amounts of the expressed i-antigens of *I. multifiliis,* we used anti–i-antigen antibodies and a purified i-antigen protein of *Ichthyophthirius* as a standard (kindly provided by Xuting Wang, University of Georgia). For each blot, a series of dilutions of the appropriate protein standard and transformant total protein extracts were electrophoresed, blotted, developed for chemiluminescence and exposed to film. The shortest exposure on which the lowest amount of the standard protein could just be detected was scanned using a densitometer (Molecular Dynamics, Sunnyvale, CA) and a standard curve was prepared. To determine the amount of transgene protein in tested extracts, dilutions that produced a signal that falls within the linear portion of the standard curve were chosen.

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