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Puromycin resistance gene as an effective selection marker for ciliate *Tetrahymena*

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ABSTRACT

A puromycin-*N*-acetyltransferase gene (*pac*) is widely used as a selection marker for eukaryotic gene manipulation. However, it has never been utilized for molecular studies in the ciliate *Tetrahymena thermophila*, in spite of the limited number of selection markers available for this organism. To utilize *pac* as a maker gene for *T. thermophila*, the nucleotide sequence of the *pac* gene was altered to accord with the most preferred codon-usage in *T. thermophila*. This codon-optimized *pac* gene expressed in *T. thermophila* conferred a resistance to transformed cells against 2000 μg/ml of puromycin dihydrochloride, whereas the growth of wild-type cells was completely inhibited by 200 μg/ml. Furthermore, an expression cassette constructed with the codon-optimized *pac* and an *MTT1* promoter was effectively utilized for experiments to tag endogenous proteins of interest by fusing the cassette into the target gene locus. These results indicate that *pac* can be used as a selection marker in molecular studies of *T. thermophila*.

1. Introduction

The ciliated protozoan *Tetrahymena thermophila* has been an experimental model organism for biochemistry, physiology, genetics, molecular biology, and cell biology (Collins, 2012; Elliot, 1973). After the macronuclear genome project was completed (Eisen et al., 2006), it has become a powerful model cell system. *T. thermophila* genes can be modified by exogenous sequences using targeting strategies based on homologous recombination (Chalker, 2012 and references cited therein). When disrupting or tagging *T. thermophila* genes, neomycin-resistant gene (*neo*) (Gaertig et al., 1994; Kahn et al., 1993; Shang et al., 2002) or blasticidin-resistant gene (*bsr*) (Brown et al., 1999; Elde et al., 2005) expression cassettes are generally used as exogenous selection markers. The most popular marker gene, *neo*, confers resistance to paromomycin, an anti-protozoa drug. However, paromomycin is also used as a selection drug for very popular autonomously replicating vectors composed of *T. thermophila*'s mutant rRNA gene (Chalker, 2012). Hence, the choice of drugs is limited when multiple manipulations are attempted.

Puromycin, an analog of aminoacyl-tRNA, inhibits the incorporation of aminoacyl-tRNA into the C-terminal of a synthesizing polypeptide on a ribosome, resulting in premature termination of the polypeptide chain (Nathans, 1964). Since puromycin is toxic to the growth of various eukaryote cells including mammalian cells, a puromycin-resistant gene (puromycin-*N*-acetyltransferase gene (*pac*)) is commonly used as a selection marker gene in mammalian cells (de la Luna et al., 1988; Vara et al., 1986). Because puromycin also exhibits strong toxicity for the growth of *Tetrahymena* cell (Cerroni and Zeuthen, 1962; Eckert, 1977; Frankel, 1967), it is expected that *pac* could be used as a selection marker gene in *Tetrahymena*. However, it has never been used in the study of *Tetrahymena*, due to lack of an efficient and easy to use DNA vector suitable for this organism. In this study, we have developed a DNA vector utilizing *pac* as a selection marker for gene manipulation in *Tetrahymena*. We altered the nucleotide sequence of the *pac* gene to accord with the most preferred codon-usage in *T. thermophila*. *Tetrahymena* transformed with the codon-optimized *pac* showed high resistance to puromycin, and codon-optimized *pac* also efficiently worked in gene targeting experiments. This study demonstrates the efficacy of *pac* as an exogenous selection marker gene for molecular studies of *Tetrahymena*.

Abbreviations: *bsr*, blasticidin S-resistant gene; *BTU2*, beta-tubulin gene; *chx1-1*, gene mutation expressing cycloheximide sensitive; cy-s, cycloheximide sensitive; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *HHF1*, histone H4 gene; *HHO1*, histone H1 gene; IC90, 90% inhibitory concentration; *mpr1-1*, gene mutation expressing 6-methylpurine sensitive; mp-s, 6-methylpurine sensitive; *MTT1*, metallothionein gene; *neo*, neomycin-resistant gene; *SEH1*, nucleoporin Seh1 gene; *pac*, puromycin-N-acetyltransferase gene; pVGF1, plasmid vector for the expression of GFP fusion protein in vegetative *Tetrahymena* cells; 3′ UTR, three prime untranslated region.

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2. Materials and methods

2.1. Cells, culture conditions, and induction of mating activity

T. thermophila inbred B strains, CU427.4 [*chx1-1*/*chx1-1* (CHX1; cy-s, VI)] and CU428.2 [*mpr1-1*/*mpr1-1* (mp-s, VII)], were obtained from the *Tetrahymena* Stock Center at Cornell University. Cells were maintained in shallow culture medium, containing 1.5% proteose peptone (BD Difco, Tokyo, Japan), 0.5% yeast extract (BD Difco) and 0.5% D-glucose (Wako, Osaka, Japan), in a plastic dish at 30 °C without shaking.

For induction of mating activity, logarithmically growing cells were harvested by centrifugation at 700 *g* for 1 min, washed twice with starvation medium (10 mM Tris–HCl, 40 nM calcium chloride, pH 7.5) and then incubated in the starvation medium at 2×10^5 cells/ml for 12–18 h at 30 °C. After incubation, two cell strains with different mating types were mixed and further incubated for 10 h. The mating efficiency of this procedure is approximately 90%. The cells were then transformed with exogenous DNA.

2.2. Counting cells for growth curves

For growth curves, cell numbers were counted by a corpuscle density analyzer CDA-1000 (MP JAPAN, Tokyo, Japan) with an aperture size of 100 μm in diameter. Because this system detects and counts particles in suspension, dead cells could not be eliminated from the counts. Therefore, the growth curves include some dead cells.

2.3. Codon optimization of pac

To construct a codon-optimized *pac* DNA sequence, four regions of *pac* corresponding to the fragments 1–4 (see Table 1 and Fig. 2D) were synthesized as follows: The single stranded oligo nucleotides listed in Table 1 were chemically synthesized (Life Technologies, Tokyo, Japan). To make a double stranded DNA of fragment 1, the same amount of puro1/F (*Sac*II) (a forward strand) and puro1/R (a reverse strand), which have a short stretch of complementary sequences in their 3′ ends (see Fig. 2D), were mixed and then incubated at 72 °C with PrimeSTAR reagent (Takara, Kyoto, Japan). The double stranded DNAs of the fragments 2–4 were synthesized similarly using the forward and reverse strands corresponding to each fragment listed in Table 1. To ligate each fragment into a pBlueScript KS vector, first the fragments 1 and 2 were ligated into the vector as follows: Fragment 1 was digested with *Sac*II and phosphorylated with T4 kinase (Takara); fragment 2 was digested with *Xba*I; pBlueScript KS was digested with *Sac*II and *Xba*I; and fragments 1 and 2 and the vector were ligated to generate a vector carrying fragments 1 and 2. Second, fragments 3 and 4 were ligated into the vector as follows: Fragment 3 was digested with *Xba*I and phosphorylated with T4 kinase (Takara); fragment 4 was digested with *Apa*I; the vector carrying fragments 1 and 2 was digested with *Xba*I and *Apa*I; and fragments 3 and 4 and the vector were ligated to generate a vector carrying full-length codon-optimized *pac*. The amino acid sequence of this codon-optimized pac protein is same as that of the original pac protein.

2.4. Construction of DNA plasmids

All DNA fragments used for plasmid construction were amplified by PCR with PrimeSTAR reagent. Temperatures for denaturation, annealing and extension were 98, 55 and 72 °C, respectively. Extension time was approximately 10 s/kb, and the reaction was repeated for 33 cycles.

To integrate the *pac* gene at the endogenous *MTT1* gene locus (as shown in Fig. 3A), *pac* flanked by 5′- and 3′-flanking sequences of the *MTT1* gene was constructed as follows: The codon-optimized *pac* gene (opt-*pac*) or the original *pac* gene (ori-*pac*) was amplified from plasmid DNA with a forward primer making a blunt end and a reverse primer having an *Spe*I site. A 1963-bp 5′ flanking sequence of the *MTT1* gene, including the *MTT1* promoter, was amplified from total genome DNA with a forward primer having a *Sac*I site and a reverse primer making a blunt end, and then digested with *Sac*I and phosphorylated with T4 kinase. This 5′ flanking sequence and the *pac* gene sequence digested with *Spe*I were ligated together into a pBlueScript KS vector at the *Sac*I and *Spe*I sites. Subsequently, a 2110-bp 3′ flanking sequence of the *MTT1* gene, including the *MTT1* 3′ UTR, was amplified with a forward primer having a *Spe*I site and a reverse primer having a *Kpn*I site. After digestion with restriction enzymes, the 3′ flanking sequence was inserted into the 3′ region of the *pac* sequence at *Spe*I and *Kpn*I. Transfection of *Tetrahymena* with the resulting plasmid replaces the endogenous *MTT1* gene with the *pac* gene (Fig. 3A).

A opt-*pac*-expression cassette was constructed with the optimized *pac* gene as follows: The codon-optimized *pac* gene was amplified from a plasmid with a forward primer making blunt-end and a reverse primer having an *Spe*I site, and then phosphorylated with T4 kinase and digested with *Spe*I. This DNA was ligated to a DNA fragment of an *MTT1* promoter sequence (938 bp) by blunt-end ligation, and then to a DNA fragment of a *BTU2* 3′ UTR sequence (306 bp) using *Spe*I. The expression cassette region was inserted into pBluescript KS; it can be cut out of the vector by *Eco*RI. This expression cassette with the optimized *pac* gene was designated *pur4*: this name accords with the nomenclature of *neo* cassettes for *T. thermophila* as the 4th generation of a codon-optimized neomycin-resistant gene (GI:188039077) with a cadmium-inducible *MTT1* promoter (Mochizuki, 2008).

To construct the DNA plasmid used to fuse the mCherry-coding sequence to the 3′ end of *SEH1* (designated pmCherry-*SEH1*), the marker cassette region (*neo4*) of pmCherry-neo4 (a gift from Dr. Mochizuki) was replaced with the *pur4* expression cassette using *Eco*RI sites. The resultant plasmid (pmCherry-pur4) was fused with a 2066-bp 5′ flanking sequence of *SEH1*, which includes a large part of the *SEH1* coding region with a disrupted stop codon at *Sac*I and *Nhe*I sites. Subsequently, a 1960-bp sequence of a 3′ flanking sequence of *SEH1* starting at 613 bp from the stop codon of *SEH1* was inserted into the plasmid at the *Sal*I and *Kpn*I sites (as shown in Fig. 4A).

To construct the targeting DNA plasmid used to fuse a GFP-coding sequence to the 3′ end of *HHO1* (designated pEGFP-*HHO1*), the marker

Table 1
Oligo nucleotides used for codon-optimization of *pac*.

DNA fragment	Name	Direction	Sequence (5′ → 3′)
Fragment 1	Puro1/F(SacII)	Forward	GGCCGCGGATGACTGAATATAAGCCTACTGTTAGATTAGCTACTAGAGATGATGTTCCTAGAGCTGTTAGAACTTTAGCTGCTGCTTTCGCT
	Puro1/R	Reverse	ATTCTTATAATTCAGTAACTCTTTCAATGTGTCTATCAGGATCAACAGTGTGTCTAGTAGCAGGATAATCAGCGAAAGCAGCAGCTAAAG
Fragment 2	Puro2/F	Forward	TATTCTTAACTAGAGTTGGTTTAGATATTGGTAAGGTTTGGGTTGCTGATGATGGTGCTGCTGTTGCTGTTTGGACTACTCCTGAATCTG
	Puro2/R(XbaI)	Reverse	GGTCTAGAACCAGATAATTCAGCCATTCTAGGACCAATTTCAGCGAAAACAGCACCAGCTTCAACAGATTCAGGAGTAGTCCAAAC
Fragment 3	Puro3/F(XbaI)	Forward	GGTCTAGATTAGCTGCTTAATAATAAATGGAAGGTTTATTAGCTCCTCACAGACCTAAGGAACCTGCTTGGTTCTTAGCTACTGTTGGTG
	Puro3/R	Reverse	CTCTTTCAGCAGCTTCAACACCAGGTAAAACAACAGCAGAACCTAAACCCTTACCTTAGTGATCAGGAGAAACACCAACAGTAGCTAAGAAC
Fragment 4	Puro4/F	Forward	CTGGTGTTCCTGCTTTCTTAGAAACTTCTGCTCCTAGAAATTTACCTTTCTATGAAAGATTAGGTTTCACTGTTACTGCTG
	Puro4/R(ApaI)	Reverse	CCGGGCCCTCAAGCACCAGGCTTTCTAGTCATACACCAAGTTCTAGGACCTTCAGGAACTTCAACATCAGCAGTAACAGTGAAACCTAATC

cassette region (*neo4*) of pEGFP-neo4 (a gift from Dr. Mochizuki) was replaced with a *bsr* cassette using *Sma*I and *Sal*I sites. The resultant plasmid (pEGFP-bsr) was fused with a 1693-bp 5′ flanking sequence of *HHO1*, including the *HHO1* coding region, using *Xba*I and *Bam*HI sites. Subsequently, a 1486-bp 3′ flanking sequence of *HHO1* was inserted into the plasmid using *Sal*I and *Kpn*I sites. The *bsr* cassette carrying a blasticidin-resistant gene with an *HHF1* promoter and the 3′ UTR of the *BTU2* gene was constructed according to Xia et al. (2000).

These DNA plasmids, which were to be integrated into the macronuclear genome, were linearized by digestion with *Sac*I and *Kpn*I prior to transfection by electroporation.

cDNA of a vacuole membrane protein TTHERM_00300090 was amplified from cDNA library prepared from vegetative *T. thermophila* according to Iwamoto et al. (2009) and inserted into the autonomously replicating rDNA vector pVGF1 (Wiley et al., 2000) using *Xho*I and *Apa*I sites.

All DNA plasmids were amplified in competent cells of DH5α, purified with a plasmid purification kit (Qiagen KK, Tokyo, Japan), and sequencing using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.5. Transfection of DNA plasmids

Mating pairs of the cells at 10 h after mixing complementary strains were washed once with 10 mM Hepes-NaOH (pH 7.5) and then resuspended in 10 mM Hepes-NaOH (pH 7.5) at approximately 3.5×10^6 pairs/ml. The cells (mating pairs) were subjected to transfection by electroporation: a mixture of 230 μl of cell suspension and 20 μl of DNA solution (containing 20 μg of pVGF1 carrying TTHERM_00300090 cDNA or 50 μg of all other integration plasmids) was transferred into a cuvette with a 0.2 cm gap and pulsed using a Gene Pulser II (Bio-Rad, Hercules, CA) with 220 V, 25 μF and 200 Ω at room temperature. The resulting cell suspension was diluted with the culture medium and an aliquot (100 μl) was added to each well of 96-well plates. After 18–24 h cultivation, cells (100 μl) were treated with selection drugs: puromycin dihydrochloride (Fermentek, Jerusalem, Israel) plus cadmium chloride at final concentrations of 200 μg/ml and 1.0 μg/ml, respectively; paromomycin sulfate (Sigma Aldrich Co., St. Louis, MO) at 120 μg/ml; or blasticidin S (InvivoGen, San Diego, CA) at 60 μg/ml. Resistant cells usually appeared 2–4 days after the drug was added.

After the selection with puromycin, diagnosing PCR was performed to test if wild-type *MTT1* gene loci were replaced with opt-*pac* integrated loci. Whole genome DNA purified from puromycin-resistant cells was subjected to PCR with three primers (F-TACCAAACTTCTATGCATCTTGTATGTG, R1-ATTAACAAGTCTAACACTCTTGC and R2-ACAGTAGGCTTATATTCAGTC) and SpeedSTAR reagent (Takara). Primers F and R1 amplify a 2.6-kb fragment from the wild-type *MTT1* gene, and primers F and R2 amplify a 2.2-kp fragment from the *pac*-integrated locus. Reactions were repeated for 33 cycles with 100 ng of genomic DNA as the template.

To select cells with resistance to all three drugs, we first selected cells with resistance to paromomycin and blasticidin: Cells were co-transfected with two DNA plasmids, a pVGF1 DNA plasmid (paromomycin resistance) carrying TTHERM_00300090 gene and a DNA fragment carrying *bsr* cassette sequence (blasticidin S resistance) (generated by linearization of pEGFP-*HHO1* with *Sac*I and *Kpn*I as described above), and cultured for 3 days in the presence of 120 μg/ml of paromomycin sulfate and 60 μg/ml of blasticidin S. The "double positive" cells growing under this condition were isolated and transformed with a DNA fragment carrying a *pur4* cassette (puromycin resistance) (generated by linearization of pmCherry-*SEH1* with *Sac*I and *Kpn*I as described above). The cells were then cultured in the presence of 400 μg/ml puromycin together with 0.5 μg/ml $CdCl_2$ for 3 days. The cells were then treated with higher concentrations of all three drugs (up to 300 μg/ml paromomycin, up to 500 μg/ml blasticidin S, up to 1200 μg/ml puromycin in this case) to select for triple-drug resistant cells.

2.6. Fluorescence observation

A small aliquot of cell suspension was placed between two coverslips to immobilize and to flatten the cells. Fluorescence images of the cells were obtained using fluorescence microscope IX-70 (Olympus, Tokyo, Japan) and an oil-immersion objective lens UApo40 (NA = 1.35) (Olympus) equipped on the DeltaVision microscope system (Applied Precision, Issaquah, WA). Images were processed with denoising software developed by Boulanger and his colleagues (Boulanger et al., 2009).

3. Results and discussion

3.1. Tetrahymena is sensitive to puromycin

To estimate a concentration of puromycin to effectively inhibit the cell growth of *T. thermophila*, we examined growth rates of cells in the presence of various concentrations of puromycin dihydrochloride (hereafter, puromycin) (Fig. 1A). Fifty micrograms per milliliter of puromycin reduced the growth rate by 80% in the first 24 h, but did not completely block cell growth; there was a gradual increase in cell numbers until 72 h. Initially, cell growth was completely blocked at 100 μg/ml, however, under this condition, while almost all of the cells died in the first 48 h, surviving cells grew rapidly after 48 h. At 150 μg/ml, cell growth was completely inhibited for 72 h. However, while the majority of cells died by 72 h, surviving cells grew after 72 h. (not shown). At 200 μg/ml, almost all the cells died by 48 h, and no survivors appeared at later time points. These results suggest that a concentration of 200 μg/ml or more is required for selecting for transformed cells of *Tetrahymena* with puromycin.

Cadmium chloride ($CdCl_2$) is often used to activate the cadmium-inducible *MTT1* promoter for expression of exogenously introduced genes. Thus, we examined whether $CaCl_2$ affects the efficacy of puromycin on cell growth. Growth rates were determined in the presence of 0.5 μg/ml (approximately 2.7 μM) $CdCl_2$, a concentration high enough to induce the promoter activity of the *MTT1* gene (Fig. 1B). Growth curves obtained in the presence of $CdCl_2$ were similar to those obtained in the absence of $CdCl_2$ (compare Fig. 1A and B), suggesting that $CdCl_2$, at least at a concentration of 0.5 μg/ml, did not affect the growth rates of *Tetrahymena* or their tolerance to puromycin.

Concentrations of puromycin sufficient to inhibit cell growth of mammalian cells are 0.5–10 μg/ml (Lieberman and Ove, 1959; Studzinski and Ellem, 1966; Thomas et al., 1973). These concentrations are much lower than those required to inhibit the growth of *Tetrahymena* (Fig. 1). Protozoa other than ciliates also exhibit low tolerance to puromycin: the pathogenic protozoa *Plasmodium falciparum* (puromycin-IC90 is 60 ng/ml; de Koning-Ward et al., 2001), *Trypanosoma cruzi* (1 μg/ml inhibits infection of host cell; Piras et al., 1982) and *Giardia lamblia* (puromycin-IC90 is 54 μg/ml; Su et al., 2007). The tolerance to puromycin displayed by ciliates may be caused by their surface structure; ciliates live freely in water and must prevent the invasion into the cell of hydrophilic molecules present in the environment.

3.2. Codon-optimization of puromycin resistance gene for Tetrahymena

A puromycin-*N*-acetyltransferase gene (*pac*, GI:763525), 600-bp in length, is derived from *Streptomyces alboniger* (Vara et al., 1985, 1986). The *S. alboniger pac* gene is GC-rich and uses GC-rich codons: the GC content of the *pac* sequence is 73.3% (Fig. 2A and B), and the third base of each codon is biased to C or G (Fig. 2C). In contrast, *Tetrahymena* prefers AT-rich codons (Eisen et al., 2006; Wuitschick and Karrer, 1999): the third base of each codon is biased to T in the *Tetrahymena* genome (Fig. 2C). As the codon-

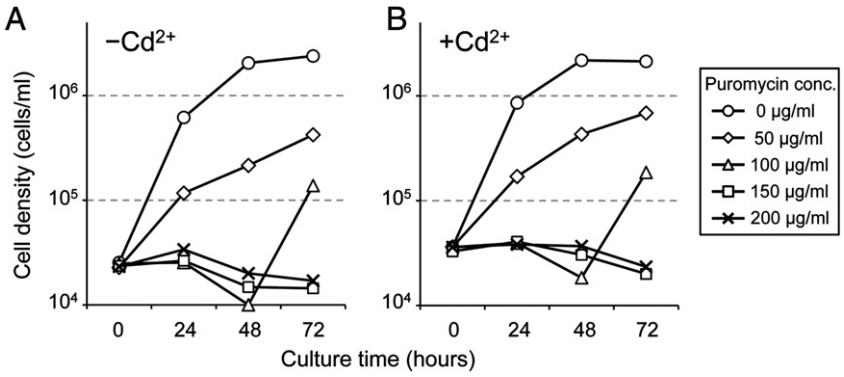


Fig. 1. Growth curves of *T. thermophila* cells cultured with puromycin. (A) The cell strain CU427 exhibiting a wild-type phenotype was cultured in medium with various concentrations of puromycin for the indicated periods. Puromycin was added to the culture at 0 h. (B) The same as panel A except that 0.5 μg/ml $CdCl_2$ was added to the culture medium 24 h prior to the addition of puromycin.

usage affects the translational efficiency in several organisms (Akashi, 2003; Novoa and Ribas de Pouplana, 2012; Redemann et al., 2011), the translational efficiency of the *S. alboniger pac* gene might be too low for selection of drug-resistant clones when expressed in *Tetrahymena*. To get higher translation efficiency of *pac* in *Tetrahymena*, we optimized the codon-usage of *pac* to the most preferred one suitable

A

```
Original ATGACCGAGTACAAGCCCACGGTGCGCCTCGCCACCCGCGACGACGTCCCCCGGGCCGTA  60
Optimized -----T--A--T-----T--T--TA-AT-A--T--TA-A--T--T--T--TA-A--T--T  60
Translated  M  T  E  Y  K  P  T  V  R  L  A  T  R  D  D  V  P  R  A  V

           CGCACCCTCGCCGCCGCGTTCGCCGACTACCCCGCCACGCGCCACACCGTCGACCCGGAC 120
           A-A--TT-A--T--T--T-----T--T--T--T--T--TA-A-----T--T--T--T--T 120
            R  T  L  A  A  A  F  A  D  Y  P  A  T  R  H  T  V  D  P  D

           CGCCACATCGAGCGGGTCACCGAGCTGCAAGAACTCTTCCTCACGCGCGTCGGGCTCGAC 180
           A-A-----T--AA-A--T--T--AT-AT-----T-A---T-A--TA-A--T--TT-A--T 180
            R  H  I  E  R  V  T  E  L  Q  E  L  F  L  T  R  V  G  L  D

           ATCGGCAAGGTGTGGGTCGCGGACGACGGCGCCGCGGTGGCGGTCTGGACCACGCCGGAG 240
           --T--T-----T-----T--T--T--T--T--T--T--T--T--T-----T--T--T--A 240
            I  G  K  V  W  V  A  D  D  G  A  A  V  A  V  W  T  T  P  E

           AGCGTCGAAGCGGGGGGCGGTGTTCGCCGAGATCGGCCCCGCGCATGGCCGAGTTGAGCGGT 300
           TCT--T-----T--T--T--T-----T--A--T--T--TA-A-----T--A--ATCT--- 300
            S  V  E  A  G  A  V  F  A  E  I  G  P  R  M  A  E  L  S  G

           TCCCGGCTGGCCGCGCAGCAACAGATGGAAGGCCTCCTGGCGCCGCACCGGCCCAAGGAG 360
           --TA-AT-A--T--TT-AT--T-A--------TT-AT-A--T--T---A-A--T-----A 360
            S  R  L  A  A  Q  Q  Q  M  E  G  L  L  A  P  H  R  P  K  E

           CCCGCGTGGTTCCTGGCCACCGTCGGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGGC 420
           --T--T------T-A--T--T--T--T--T--T--T---T-A--T------T-A--T 420
            P  A  W  F  L  A  T  V  G  V  S  P  D  H  Q  G  K  G  L  G

           AGCGCCGTCGTGCTCCCCGGAGTGGAGGCGGCCGAGCGCGCCGGGGTGCCCGCCTTCCTG 480
           TCT--T--T--TT-A--T--T--T--A--T--T--AA-A--T--T--T--T---T-A 480
            S  A  V  V  L  P  G  V  E  A  A  E  R  A  G  V  P  A  F  L

           GAGACCTCCGCGCCCCGCAACCTCCCCTTCTACGAGCGGCTCGGCTTCACCGTCACCGCC 540
           --A--T--T--T--TA-A--TT-A--T------T--AA-AT-A--T------T--T--T--T 540
            E  T  S  A  P  R  N  L  P  F  Y  E  R  L  G  F  T  V  T  A

           GACGTCGAGGTGCCCGAAGGACCGCGCACCTGGTGCATGACCCGCAAGCCCGGTGCCTGA 600
           --T--T--A--T--T-----T--TA-A--T-----T-----TA-A-----T-----T--- 600
            D  V  E  V  P  E  G  P  R  T  W  C  M  T  R  K  P  G  A  *
```

B

	Original *pac*	Optimized *pac*
A	86	143
C	236	99
G	204	142
T	74	216
%GC	*73.3*	*40.2*

C

		Usage in *Tetrahymena* genome	Original *pac*	Optimized *pac*
Ala	GCA	13%	0% (-)	0% (-)
	GCC	20%	61% (17)	0% (-)
	GCG	<1%	39% (11)	0% (-)
	GCT	66%	0% (-)	100% (28)
Val	GTA	17%	5% (1)	0% (-)
	GTC	29%	57% (12)	0% (-)
	GTG	3%	38% (8)	0% (-)
	GTT	51%	0% (-)	100% (21)
Pro	CCA	15%	0% (-)	0% (-)
	CCC	25%	71% (12)	0% (-)
	CCG	1%	29% (5)	0% (-)
	CCT	59%	0% (-)	100% (17)

D

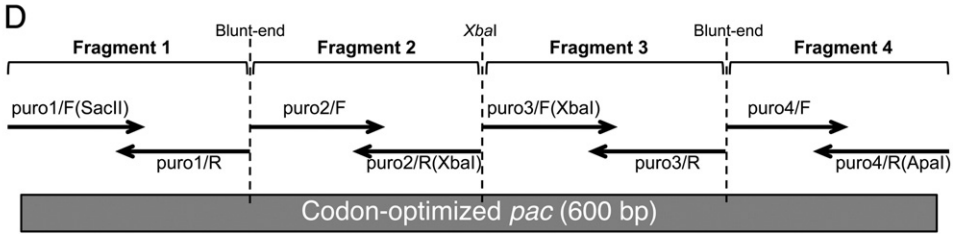
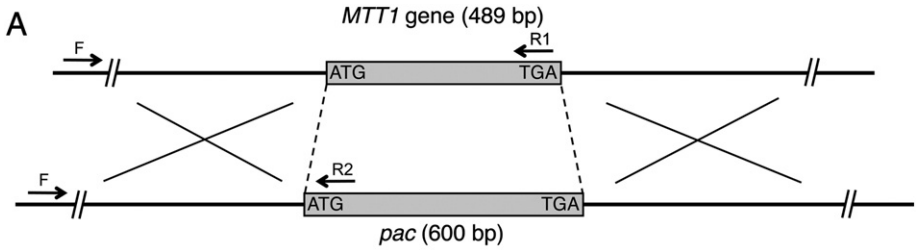


Fig. 2. Sequence comparison between the original and codon-optimized puromycin N-acetyltransferase (*pac*) genes. (A) Nucleotide and amino-acid sequences of *pac*. In the codon-optimized *pac* sequence, only changed nucleotides are shown. Amino-acid sequence is not altered after codon-optimization. (B) Comparison of the numbers of each nucleotide residue between before and after codon-optimization. The GC contents are shown in the bottom as a percentage (%GC). (C) Codon-usage for selected amino acid residues, Ala, Val, and Pro, in the *Tetrahymena* genome and the original-*pac* and optimized-*pac* proteins. The numbers in parentheses indicate the number of each codon used. (D) Single strand oligo nucleotides used for codon-optimization are mapped on the optimized-*pac* gene. The nucleotide sequence of each oligo nucleotide is shown in Table 1. See Materials and methods for detailed procedure.



B

	Codon-optimized *pac*	Original *pac*
Exp. 1	130	12
Exp. 2	148	17

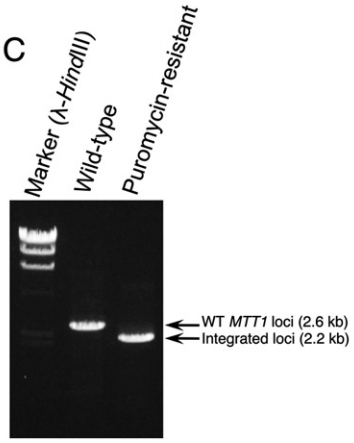


Fig. 3. Integration of the *pac* ORF into the *MTT1* locus in the macronuclear genome. (A) Schematic representation of the procedure. Arrows indicate the annealing positions of PCR primers used in (C). (B) Number of wells with puromycin-resistant cells (total wells = 288). After transformation, cells were aliquoted into 288 wells (3 × 96-well plates), and the number of positive clones was counted for codon-optimized *pac* and original *pac*. Results from two independent experiments are shown. (C) DNA fragments of the *MTT1* gene locus amplified by diagnostic PCR using primers F, R1, and R2, indicated by arrows in (A), from wild type cells (middle lane) or puromycin-resistant cells selected with puromycin up to 2000 μg/ml (right lane). Left lane shows DNA size markers (λ phage DNA digested with *Hind*III). All 3 primers (F, R1, and R2) were added to the diagnostic PCR.

for *T. thermophila* (Fig. 2A), according to a previous report for the use of *neo* as a selection marker in *Tetrahymena* (Mochizuki, 2008). Four oligo nucleotides covering the full length *pac* sequence were synthesized and joined together (see Materials and methods and Fig. 2D), resulting in modification of one hundred sixty-eight codons out of 200 and reduction of the total GC content to 40.2% (Fig. 2B).

To test if codon-optimization effectively increases gene expression of *pac*, the codon-optimized *pac* gene (opt-*pac*; GenBank Accession: AA845356) and the original *pac* gene (ori-*pac*) were integrated into the endogenous *MTT1* loci in the macronuclear genome of *T. thermophila* by homologous recombination (Fig. 3A): *MTT1* is a gene coding for metallothionein (TTHERM_00241640, GI:118383466). The frequency of appearance of puromycin-resistant clones was measured for cells cultured in the presence of 200 μg/ml of puromycin: $CdCl_2$ was also added to the culture medium to a final concentration of 0.5 μg/ml to activate the *MTT1* promoter. Puromycin-resistant clones emerged in 130–148 out of 288 wells for opt-*pac* whereas puromycin-resistant clones emerged in only 12–17 wells for ori-*pac* (Fig. 3B), indicating that codon-optimization is highly effective in raising puromycin tolerance in *Tetrahymena* cells.

Puromycin-resistant clones with opt-*pac* were continuously selected by increased concentrations of puromycin up to 2000 μg/ml in the culture medium, with 0.5–1.0 μg/ml $CdCl_2$. The macronuclear genome in *T. thermophila* is polyploid, consists of approximately 45-multiplied copies; therefore, we determined how much macronuclear genomic DNA was replaced with opt-*pac* in the puromycin-resistant clones. For this purpose, genomic DNA was isolated from the cells resistant to 2000 μg/ml puromycin, and *MTT1* loci were amplified by PCR. In the puromycin-resistant cells, a DNA fragment corresponding to opt-*pac* was detected, but the fragment corresponding to the wild-type *MTT1* was not (Fig. 3C), suggesting that most of macronuclear *MTT1* gene loci were replaced by opt-*pac* by phenotypic assortment (Sonneborn, 1974). Puromycin-resistant clones sometimes grew with up to 2200 μg/ml puromycin, although in the most cases they could not. These results suggest that the maximum concentration of puromycin appropriate for selection with opt-*pac* is around 2000 μg/ml when driven by the *MTT1* promoter activated with 0.5–1.0 μg/ml $CdCl_2$.

3.3. Application of the opt-pac-expression cassette (pur4)

To utilize the opt-*pac* gene as a selection marker for gene targeting experiments, we constructed a DNA plasmid carrying the opt-*pac*-expression cassette, *pur4*, as described in Materials and methods. *pur4* is 1.9 kb in length and composed of an *MTT1* promoter (Shang et al., 2002), the opt-*pac* gene (this study), and a *BTU2* terminator (Gaertig et al., 1994). The *pur4* cassette was used to construct a DNA plasmid for tagging a gene of interest with an mCherry gene (mCherry-pur4) (Fig. 4A). The mCherry-pur4 construct was integrated into the 3′ end of the macronuclear *SEH1* gene (TTHERM_00954180, GI:289576326), which encodes the 42-kDa nucleoporin Seh1, a component of the nuclear pore complex (Iwamoto et al., 2009). After cultivation under gradually increasing concentrations of puromycin up to 600 μg/ml, Seh1-mCherry fluorescence was located at the NPCs (data not shown; see Fig. 4B, right panel, for Seh1-mCherry expressed with two other components). This result indicates that *pur4* can be used as a selection marker for gene manipulation in *T. thermophila*.

Similar to the results described above with opt-*pac*, cells carrying *pur4* could grow in the presence of puromycin at concentrations of up to 2000 μg/ml puromycin, but not at puromycin concentrations greater than 2000 μg/ml. We also examined how many copies of the *SEH1* gene were replaced with the tagged gene. As for opt-*pac* and the *MTT1* locus, the whole genome was purified from cells exhibiting resistance to 2000 μg/ml puromycin and occupation of the *SEH1* gene loci by the tagged gene was analyzed by PCR. Wild-type *SEH1* gene loci were barely detectable (data not shown), suggesting that in the presence of 2000 μg/ml of puromycin most of the *SEH1* gene loci were replaced with the tagged gene. This result is consistent with the result from integration of opt-*pac* into the *MTT1* locus shown in Fig. 3.

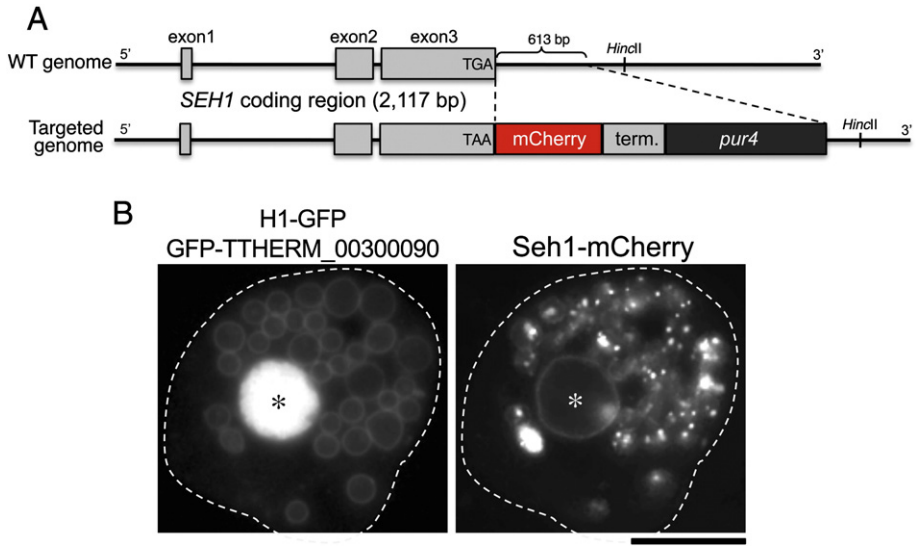


Fig. 4. Simultaneous expression of three target proteins independently-tagged with fluorescent proteins. (A) Schematic representation of the gene tagging of the nucleoporin Seh1 gene (*SEH1*) with an mCherry gene using the *pur4* cassette. (B) Fluorescence images of a single *Tetrahymena* cell simultaneously expressing three different target protein tagged with GFP or mCherry: histone H1-GFP (asterisk in left panel), GFP-TTHERM_00300090 (bubble-like structures located in the cytoplasm in the left panel), and Seh1-mCherry (see the nuclear rim of the macronucleus indicated by the asterisk in the right panel). The cells expressing a *SEH1* gene fused with an mCherry gene were selected with approximately 1200 μg/ml puromycin in the presence of 0.5 μg/ml $CdCl_2$. The cells expressing macronuclear histone H1 gene (*HHO1*) fused with a GFP gene were selected with approximately 500 μg/ml blasticidin S. The cells expressing GFP-TTHERM_00300090 cloned into a pVGF1 vector were selected with approximately 300 μg/ml paromomycin. The asterisk indicates the macronucleus, and the dotted line represents the outline of a cell. Scale bar represents 20 μm. In the left panel, H1-GFP and GFP-TTHERM_00300090 were localized in the macronucleus and on the membranes of numerous food vacuoles dispersed in the cytoplasm, respectively. In the right panel, Seh1-mCherry was observed in the macronuclear envelope. The micronucleus was invisible because it positioned behind the macronucleus in this cell. The numerous collateral fluorescent structures observed with the excitation channel for mCherry are the contents of food vacuoles. They are probably surplus Seh1-mCherry being digested by autophagocytosis.

Next, we examined the use of *pur4* simultaneously with *bsr* (blasticidin S resistance) and rDNA-based pVGF1 (paromomycin resistance). *SEH1* in cells in which *HHO1* (histone H1, GI:161794) had been tagged with GFP using the *bsr* cassette and in which GFP-TTHERM_00300090 (a vacuole membrane protein, GI:118382760) had been introduced using the autonomously replicating vector pVGF1 were tagged with mCherry using *pur4* as described above. These three fluorescence-labeled proteins were expressed simultaneously in a single cell (Fig. 4B). This result indicates that the puromycin-resistant gene, *pur4*, can act as an additional marker and can be used together with paromomycin and/or blasticidin S resistant genes.

In the presence of paromomycin and blasticidin S resistant genes, however, 150–200 μg/ml of puromycin was insufficient for the first screening for puromycin-resistant cells; all wells of the plate contained survivor cells at these concentrations. This unexpected high-resistance to puromycin in multiple drug resistant cells was probably due to cross-resistance of other drug resistant genes to puromycin. Consequently, higher puromycin concentrations, 400 μg/ml, were required for the first selection of the cells shown in Fig. 4B. It should be noted that addition of very high concentrations of puromycin (over 2500 μg/ml) to the culture medium containing yeast extracts made the medium cloudy (data not shown). This may be caused by unknown reactions of puromycin with some substances originated from the yeast extracts. To avoid this problem, when very high concentrations of puromycin are required, the use of proteose peptone medium, instead of medium containing yeast extract, is recommended: Cells can grow with a normal doubling time in proteose peptone medium supplemented with Fe ion (Orias et al., 1999).

For selection of puromycin-resistant cells transformed with *pur4*, the effective concentrations of puromycin range from 200 μg/ml (the concentration for the initial selection) to 2000 μg/ml (for replacement of most macronuclear gene copies). This range is narrower than that used for selection of paromomycin-resistant cells transformed with *neo4*, which ranges from 100 μg/ml to approximately 80,000 μg/ml of paromomycin. Puromycin seems to be more effective than paromomycin, and thus may shorten the selection period required for replacement of macronuclear gene loci. In addition to this potential advantage, the use of puromycin has other advantages: Blasticidin, unlike puromycin, is mutagenic, so cells selected with blasticidin will have an unknown number of non-lethal mutations. Puromycin is also much less deleterious than blasticidin S to accidently exposed humans. These facts make puromycin a valuable additional selection marker for *Tetrahymena*.

Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

Conceived and designed the experiments: MI. Performed the experiments: MI CM. Analyzed the data: MI YH TH. Wrote the paper: MI YH TH.

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