Parasitology International 69 (2019) 13–16

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Puromycin selection for stable transfectants of the oyster-infecting parasite *Perkinsus marinus*

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ARTICLE INFO

Keywords:
Perkinsus marinus
Puromycin
Dual-transfection
Bleomycin

ABSTRACT

Perkinsus marinus is a marine protozoan parasite that infects natural and farmed oysters, attracting attention from researchers in both fisheries and evolutionary biology. The functions of almost all cellular components and organelles are, however, poorly understood even though a draft genome sequence of *P. marinus* is publicly available. One of the major obstacles for a functional study of the parasite is limited experimental means for genetic manipulation: a transfection method was established in 2008, and the first drug selection system with bleomycin was reported in 2016. We here introduce the second drug-selectable marker for selection of *P. marinus* transfectants. The parasite growth is efficiently inhibited by puromycin (IC_{50} = 4.96 μg/mL), and transfection of its resistance gene, puromycin-*N*-acetyl-transferase (*pac*), confers resistance to the drug on the parasite. Stable transfectants can be obtained within 2 months by treating with puromycin at 100 μg/mL. Furthermore, combining puromycin and bleomycin treatment can select transfectants co-expressing two marker genes. This dual-transfection method raises the possibility of using co-localization to identify the cellular localization of novel proteins in *P. marinus*, thereby contributing to the understanding of cellular functions and pathogenesis.

1. Background

Perkinsus is a marine unicellular eukaryote and a member of Perkinsozoa, which is a recently recognized group of parasites that is a sister to dinoflagellates, including free-living photosynthetic species [1,2]. They are notorious for infecting commercially important bivalve species such as clams and oysters worldwide [3]. For example, *P. marinus* causes Dermo disease in the wild and cultivated eastern oyster, *Crassostrea viginica*, in the Atlantic seaboard of the United States and in the Gulf of Mexico. *Perkinsus olseni* infects a large variety of clam species, including the Manila clam, *Ruditapes philippinarum*, and is widespread throughout the tropical and temperate Pacific Ocean as well as the Mediterranean Sea. Moreover, recent studies show that distribution of *P. marinus* is spreading to other oyster hosts in the Southern Hemisphere [4] and Pacific Ocean [5,6]. Thus, there is an urgent need to develop means to control diseases caused by these parasites. Besides the fisheries' viewpoint, *Perkinsus* spp. have unique importance on evolutionary biology, because independent loss of photosynthesis has occurred in Perkinsozoa and apicomplexan parasites [7]. As a result, comparative analyses between these parasites, especially their chloroplast remnants, will greatly help us to understand the transition from free-living phototrophs to parasites. Although a draft genome sequence (DDBJ/ENA/GenBank accession AAXJ00000000) and transcriptome data of *P. marinus* [8,9] are valuable resources, experimental molecular tools have been quite limited; only a small set of genetic manipulation techniques have been available since 2008 [10]. Recent introduction of a drug selection system using bleomycin [11] and plating cultures on ager-based media [12] both greatly improved the situation, but additional tools are still needed to promote molecular-based studies in *Perkinsus*.

2. Objective

In *Perkinsus* spp., the functions of almost all cellular components and organelles are poorly understood. One of the first steps for functional analysis of uncharacterized proteins is determining their subcellular

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https://doi.org/10.1016/j.parint.2018.10.011
Received 18 April 2018; Received in revised form 25 October 2018; Accepted 29 October 2018
Available online 30 October 2018
1383-5769/

localization. Methods now exist to establish parasite lines stably expressing genes for fluorescent markers with bleomycin selection [11]. Thus, if one could establish validated protein markers for known subcellular compartments, then with a multiple-drug selection system it would be possible to use co-localization to link poorly studied proteins to those validated markers. Such system will also open a doorway into in vivo protein-protein interaction analysis, e.g. Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) and split GFP system [13]. We therefore searched for a novel selective marker for *P. marinus*, and here describe a second drug selection system for *P. marinus* using puromycin, a marker used for malarial parasites [14,15], and a dual-selection system using both bleomycin and puromycin.

3. Methods

3.1. Parasites

Perkinsus spp. can be maintained as an axenic in vitro culture [16–18]. Many strains and respective culture methods are available from American Type Culture Collection (ATCC; Manassas, VA). We usually use *P. marinus* strain CRTW-3HE (ATCC 50439) and maintain it at 26 °C in ATCC medium 1886. Lipid mixture (1000 ×) (Sigma-Aldrich, Saint Louis, MO) can be used as a substitute for a 100× lipid concentrate indicated in the ATCC's instructions. Instant Ocean Sea Salt (Aquarium Systems, Sarrebourg, France) and Daigo's Artificial Seawater SP (Wako Pure Chemical Industries, Osaka, Japan) can be used as synthetic sea salt.

3.2. Drug preparation

Dissolve puromycin in water at 10 mg/mL, filter the solution with a 0.22-μm filter unit, and store the small aliquots at less than −20 °C until use. Bleomycin can be prepared similarly, but the aliquots should be stored at −80 °C.

3.3. Plasmid for transfection

The plasmids pMGB (stands for MOE-GFP-*ble*; RDB15789) and pMGP (MOE-GFP-*pac*; RDB15790) and their details including annotated sequences are available from RIKEN BioResource Center (Tsukuba, Japan). The plasmids were constructed as previously described for pMOE-mCherry-ble (RDB14303) [11], but the mCherry gene was replaced with the eGFP gene (*gfp*), which was derived from the pEGFP-N1 plasmid (Clontech, Mountain View, CA). In the case of pMGP, the gene coding for puromycin-*N*-acetyl-transferase (*pac*) was amplified from the pPUR plasmid (Clontech) and fused instead of the bleomycin resistance gene (*Sh-ble*). Consequently, the plasmid pMGP carries a puromycin-resistance gene *pac* 5′-terminally linked with *gfp* under control of homogenous *MOE* promoter [10], and the plasmid pMGB only differs in the drug selective marker.

These plasmids may be modified according to specific research interests by restriction/ligation, Gibson assembly [19], or any of other relevant technics. For transfection, prepare plasmids at a concentration > 1 μg/μL.

3.4. Drug selection of transfected cells

Perform transfection as described in previous studies with 5 μg of supercoiled plasmid DNA [10,11]. Transfer transfected cells into 2 mL of culture medium and divide it into two wells in a 12-well plate. After 2–3 days, confirm transiently expressed fluorescence signal, and then dilute 50 μL of the culture into 1 mL of the medium with 100 μg/mL of puromycin and/or bleomycin. Tightly seal the culture plate with a sterilized plate-sealing film, and incubate at 26 °C for up to 2 months without any medium changes. The cells sparsely distributed at the beginning will gradually occupy throughout the bottom of the well. After confirming cell growth, mix the culture gently and transfer 100 μL into a new well containing 1 mL of medium without drugs to evaluate the efficacy of the selection. Although fluorescence signal often attenuated probably due to long lasting stationary or dormancy state, the subculture to fresh media enables us to observe fluorescence positive cells after 3–7 days. If efficacy was insufficient for your purpose, repeat the treatment with 100 μg/ml of drug for 1–2 weeks. Transfectants would be obtained preferentially during the secondary selection. You can maintain the selected cells as usual methods if once transfectants were obtained. If clonal cell strains were required, limiting dilution or plating on agar-based media [12] would be performed.

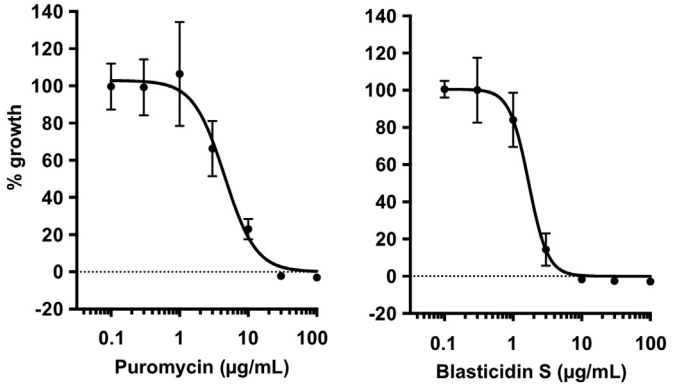

Fig. 1. Effect of protein synthesis inhibitors on *P. marinus* growth.
The 3-day growth of *P. marinus* strain CRTW-3HE (ATCC 50439) measured with a tetrazolium-based growth assay [11] was blocked by puromycin (IC_{50} = 4.69 μg/mL, 95% CI = 3.48 to 6.30 μg/mL) and blasticidin S (IC_{50} = 1.76 μg/mL, 95% CI = 1.30 to 2.39 μg/mL). The growth was indicated as relative values to the no-drug control. The mean of three independent assays, which were performed in triplicate, are plotted with error bars showing the standard deviation. IC_{50} and 95% CI were calculated as described in our previous report [25].

4. Special remarks/comments

4.1. Drug responses of P. marinus

Puromycin, a protein synthesis inhibitor, is effective in inhibiting growth of *P. marinus* strain CRTW-3HE (ATCC 50439) with an IC_{50} value of 4.69 μg/mL (ca. 10 μM) (Fig. 1). We also examined another protein synthesis inhibitor, blasticidin S, that showed stronger inhibition with an IC_{50} value of 1.76 μg/mL (ca. 4 μM) (Fig. 1). In the short-term assay [11], both drugs at concentrations > 30 μg/mL completely blocked *P. marinus* growth after 3-day exposure to the drug (Fig. 1). A few inhibitors of this class were previously examined against *P. marinus* growth; however, these were not strong inhibitors that have IC_{50} values > 100 μM [20] with the exception of cycloheximide (IC_{50} = 1.5 μM) [21]. Since the compounds with IC_{50} values < 10 μM are very limited [22], puromycin and blasticidin S are in the most potent growth inhibitors against *Perkinsus* spp.

4.2. Puromycin is a new selectable drug for P. marinus

Cells transfected with pMGP can be selected efficiently by puromycin at 100 μg/mL. After 2-month culture without drug, the GFP expression could be maintained > 70% of cells although the signal intensity varies depending on cells (Fig. 2). The selected cells showed strong resistance against puromycin, and the relative growth in the long-term sensitivity assay [11] was almost 100% at concentrations < 100 μg/mL and was 95.2 ± 2.8% even at 300 μg/mL puromycin.

In contrast, blasticidin-S deaminase, subcloned from the pSSPF3

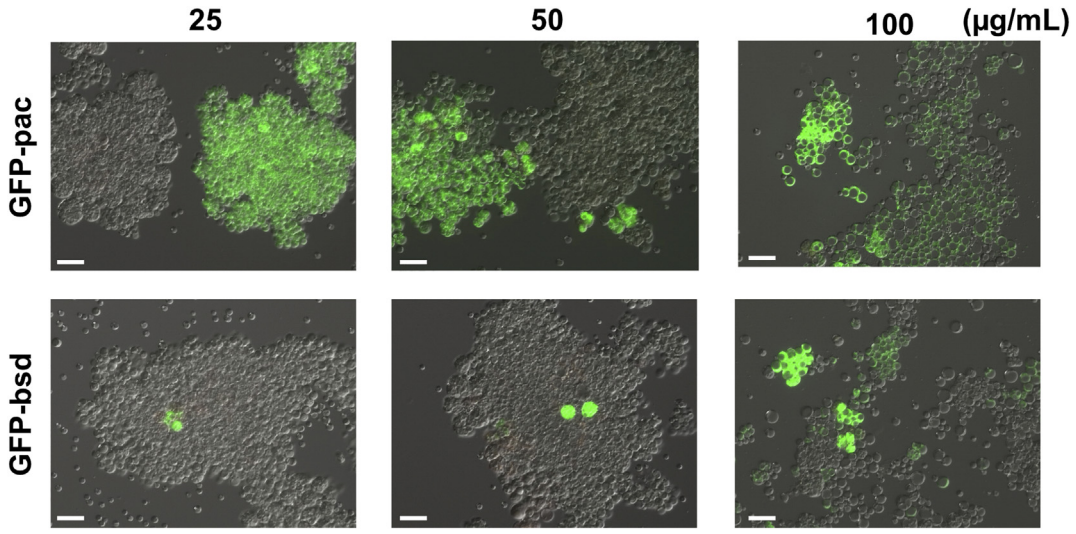

Fig. 2. Drug selection of transfectants.
Wild-type *P. marinus* parasites (ATCC 50439) transfected with plasmids encoding GFP-fused drug resistance genes followed by a 2-month treatment with different concentrations of the corresponding drugs. Upper panels, puromycin treatment on GFP-pac transfectant; lower panels, blasticidin S treatment on GFP-bsd transfectant. Merged images (DIC and GFP) are shown. The scale bars indicate 20 μm.

plasmid [23] and fused with eGFP, was not functional as a selectable marker, although blasticidin S is available for the selection of apicomplexan parasites [15,23,24]. Even under the highest concentration, only small fraction of the cells were GFP-positive (Fig. 2; < 30%) and this was reproducible. In the lower-concentration treatments, GFP-positive, thus drug-resistant cells were located at the center of large clumps of GFP-negative, drug-sensitive cells (Fig. 2). This observation suggests that a few drug-resistant cells can support growth of surrounding drug-sensitive cells. The cells treated with blasticidin S shrank and adhered on the bottom of the well, and this adhesive character will promote the clump formation. Probably, the combination of the adhesive character enhanced by blasticidin treatment and high degradation activity of blasticidin-S deaminase results in selection failure in *P. marinus*.

4.3. Independence of puromycin and bleomycin selection

The puromycin-selected cells (GFP-pac) were fully sensitive to bleomycin in the long-term sensitivity assay with an IC_{50} value comparable to that of wild-type cells (Table 1). The same was true for bleomycin-selected cells (GFP-ble) after transfection with pMGB; GFP-ble, which showed strong resistance to bleomycin, was fully sensitive to puromycin (Table 1). This indicates that resistance to one drug does not affect to the sensitivity to the other drug.

Indeed, we were able to obtain stable GFP/mCherry double-positive cells (Fig. 3) when we transfected the pMGP plasmid into the bleomycin-selected mCherry-ble cells, which was established in our previous report [11], and selected with 100 μg/mL of puromycin.

Table 1
Long-term drug sensitivity of wildtype and drug-selected lines.

Drug	Cell line	IC_{50} (μg/mL)	95%CI (μg/mL)
Bleomycin	wildtype	1.70	1.06–2.74
	GFP-ble	> 300	–
	GFP-pac	1.75	1.29–2.38
Puromycin	wildtype	24.1	19.4–29.8
	GFP-ble	22.9	21.7–24.1
	GFP-pac	> 300	–

P. marinus cell lines were incubated with indicated drug for 3 w, and cell growth was measured by tetrazolium-based growth assay [11]. IC_{50} and 95% CI were calculated as described in our previous report [25].

Simultaneous selection with the two drugs is also possible since the transfection efficacy is high (37.8% in ref. [10]). When we transfected the wild-type strain CRTW-3HE with 2.5 μg each of pMGP and pMOE-mCherry-ble plasmids [11], GFP/mCherry double-positive cells can be obtained after treatment with 100 μg/mL each of puromycin and bleomycin.

4.4. Further optimization

Although we have not rigolously tested, the drug selection procedure can be further optimized. If additional labor and cost are acceptable, stable transformants will be obtained earlier by routine medium change. Cell growth can be monitored by cell counting chamber, and completion of selection can be judged with restoring or saturating log-phase growth.

If transient transformant is used for experiments, we recommend 2–3 d after transfection, since proportion of fluorescence-positive cells saturates in this timespan. You may exploit fluorescence-activated cell sorting to enrich transfected cells; however, optimization of sorting condition for high-salinity sheath fluid will be required to avoid osmotic stress on sorted cells and cares should be taken to prevent salt clogging.

4.5. Conclusion

The method described here is the second drug selection system available in *P. marinus*, and, in combination with the bleomycin system previously reported [11], provides opportunities for co-localization analyses and protein interaction assays such as FRET, BRET, or split GFP system [13]. These tools allow for a new range of questions to be asked in *Perkinsus,* which is a potential model system for investigating not only its biology and pathology, but also fundamental principles of the evolution to parasite from phototroph, of degeneration/acquisition of cellular functions associated with the origin of parasitism, and of the early evolution of the dinoflagellate lineage and their many unusual characteristics.

Acknowledgments

We thank Dr. Shigeharu Sato for supplying the pSSPF3 plasmid. This work was supported by MEXT: Ministry of Education, Culture, Sports,

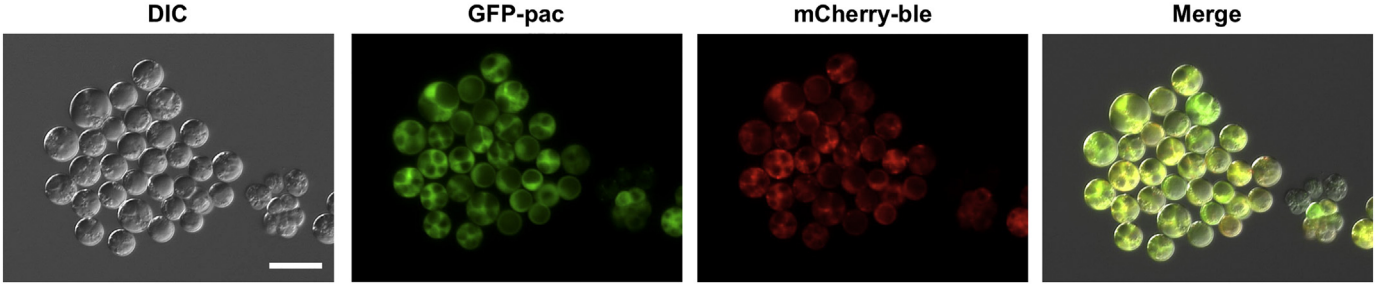

Fig. 3. Dual selection using puromycin and bleomycin.
The mCherry-ble cell line selected by bleomycin [11] was transfected with pMGP, then treated with 100 μg/mL of puromycin for 2 months. The scale bar indicates 20 μm.

Science and Technology, Japan KAKENHI Grant Number 23117004 and by the Marine Microbiology Initiative program of Gordon and Betty Moore Foundation (GBMF 4964).

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