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New Selectable Markers for *Volvox carteri* Transformation

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***Volvox carteri* is an excellent model for investigating the evolution of multicellularity and cell differentiation, and the rate of future progress with this system will depend on improved molecular genetic tools. Several selectable markers for nuclear transformation of *V. carteri* have been developed, including the nitrate reductase (*nitA*) gene, but it would be useful to have additional markers to multiplex transgenes in this species. To further facilitate molecular genetic analyses of *V. carteri*, we developed two new selectable markers that provide rapid, easily selected, and stable resistance to the antibiotics hygromycin and blasticidin. We generated constructs with Volvox-specific regulatory sequences and codon-optimized hygromycin (VcHyg) and blasticidin (VcBlast) resistance genes from *Coccidioides posadasii* and *Bacillus cereus*, respectively. With these constructs, transformants were obtained via biolistic bombardment at rates of 0.5–13 per million target cells bombarded. Antibiotic-resistant survivors were readily isolated 7 days post bombardment. VcHyg and VcBlast transgenes and transcripts were detected in transformants. Co-transformation rates using the VcHyg or VcBlast markers with unselected genes were comparable to those obtained with *nitA*. These results indicate that the pVcHyg and pVcBlast plasmids are highly efficient and convenient for transforming and co-transforming a broad range of *V. carteri* strains.**

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Key words: Blasticidin S; green algae; hygromycin B; selectable marker; transformation; *Volvox carteri*.

Introduction

The volvocine green algae are a group of haploid, facultatively sexual eukaryotes that span the gamut of developmental complexity from unicellular to colonial to truly multicellular lifestyles. Collectively, these algae comprise an excellent system to study the molecular genetic factors important for the origin of cell differentiation and multicellularity (Prochnik et al. 2010) because they include species with various grades of developmental complexity, and because they evolved from a common unicellular ancestor much more recently than did animals or land plants (Herron et al. 2009). One well-studied member of this family, *Volvox carteri*, is an excellent model system in its own right; it has a short life cycle, advanced molecular genetic tools exist to facilitate its analysis, and its genome has been sequenced (Herron 2016; Matt and Umen 2016; Nishii and Miller 2010; Prochnik et al. 2010; Umen and Olson 2012). To date, most analyses of developmental complexity in the volvocine algae have involved investigations into *V. carteri* genes

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essential for multicellularity traits, such as asymmetric cell division, embryo inversion, cell adhesion (via extracellular matrix), cell differentiation, and oogamy (Kirk 2001; Kirk et al. 1999; Miller and Kirk 1999; Nishii et al. 2003; Schiedlmeier et al. 1994; Hallmann 2006; Geng et al. 2018).

One very important tool for functional analyses of gene function is stable nuclear transformation. Transformation of *V. carteri* was first achieved over two decades ago by complementing a mutant defective for the nitrate reductase gene (*nitA*), with a plasmid bearing that gene via microparticle bombardment (Schiedlmeier et al. 1994). *Volvox* transformation using the endogenous *nitA* gene has been very useful for genetic analyses (Geng et al. 2014; Kirk et al. 1999; Miller and Kirk 1999; Nishii et al. 2003; Ueki and Nishii 2009) but requires transformation into a *nitA*$^{-}$ host and selecting for growth with nitrate as sole nitrogen source, constraints that are sometimes problematic. Additionally, selected transformants could not be subjected to a second transformation. Experiments involving transformation of heterologous genes into *V. carteri* have been limited somewhat by the availability of efficient dominant selectable marker genes. Antibiotic resistance genes, like *ble* (provides resistance to zeocin and other members of the bleomycin family) and *aphH* (encodes an aminoglycoside phosphotransferase that provides resistance to paromomycin), are an alternative to *nitA* for *V. carteri* co-transformation experiments (Hallmann and Rappel 1999; Jakobiak et al. 2004). A major advantage of using these genes is that they are dominant markers that can be used in all strain backgrounds. There are, however, some drawbacks to using these selectable marker genes. For instance, the most effective *ble*-gene vector contains a 16-mer repeat of the *ble* gene, making amplification of that plasmid in bacteria problematic, and Ble selection requires multiple applications of the antibiotic (Hallmann and Rappel 1999). On the other hand, selection for paromomycin resistance in *V. carteri* using the *aphH* gene from *Streptomyces rimosus* (Jakobiak et al. 2004) can require extensive screening for transformants expressing a co-transformed gene (our unpublished results).

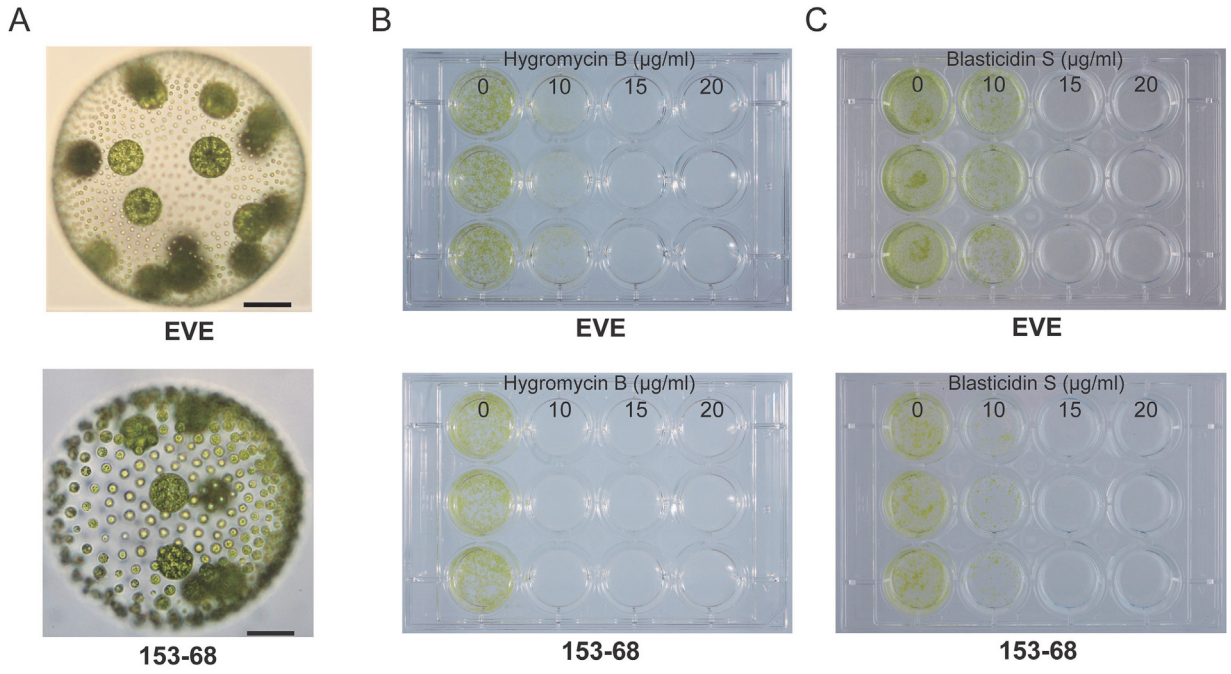
Two possible alternatives to Ble and paromomycin dominant selectable markers for *V. carteri* transformation are genes for hygromycin and blasticidin resistance. Like many other herbicides/antibiotics, hygromycin B and blasticidin S target the ribosome and interfere with different steps of protein synthesis and are active against both prokaryotic and eukaryotic cells (Brodersen et al. 2000; Svidritskiy et al. 2013). Hygromycin B primarily inhibits the translocation step of elongation and, to a lesser extent, causes mRNA misreading by binding the A (acceptor) site of the ribosome (Brodersen et al. 2000). By contrast, blasticidin S binds to the P (peptidyl) site and inhibits the peptidyl-transferase reaction (Svidritskiy et al. 2013). Resistance to hygromycin B can be provided by one of several bacterial aminoglycoside phosphotransferase (aph) activities, while resistance to blasticidin S can be provided by blasticidin deaminase activity. Because these antibiotics are inactivated by distinct mechanisms, they can be used independently of each other, such that the resistance genes can be stacked (Goyard and Beverley 2000).

Here, we report two new selectable marker vectors for use in *V. carteri* that are based on the *Coccidioides posadasii aph7* gene and the *bsr* gene from *Bacillus cereus*. The two engineered plasmids contain *V. carteri*-specific regulatory sequences (hybrid *hsp70A*/*rbcS3* promoter/5′-UTR and *rbcS2* 3′ UTR) and codon-optimized hygromycin (HygR) and blasticidin (BlastR) resistance genes containing the first intron of the *C. reinhardtii RBCS2* gene inserted into the coding regions of these markers. These markers yield transformants at relatively high rates and permit easy and rapid selection of transformants that maintain stable expression of resistance for at least many months. Both markers also yield co-transformants that express non-selected genes of interest in a large number of resistant transformants.

Results

Selection Conditions and Construction of Selectable Marker Plasmids

To determine whether hygromycin B and blasticidin S might be useful selection antibiotics for *V. carteri* transformation, we first tested the sensitivity of two commonly used *V. carteri* strains to these agents. Minimal inhibitory concentrations (MIC; defined as the lowest antibiotic concentration at which no growth was observed) of hygromycin B and blasticidin S were determined for wild type strain EVE and for Somatic Regenerator (Reg) mutant 153-68 in liquid media (Fig. 1A); Reg mutants contain many more reproductive cells than the wild type because their somatic cells dedifferentiate then become gonidia. Wells of microtiter plates containing ~4-mL Standard Volvox Medium (SVM) supplemented with antibiotic at concentra-



D

Strain	Hygromycin B concentration (µg/ml)					Blasticidin S concentration (µg/ml)				
	0	5	10	15	20	0	5	10	15	20
EVE	2	2	1	0	0	2	2	1	0	0
153-68	2	2	0	0	0	2	2	1	0	0

Figure 1. Analysis of antibiotic resistance in strains EVE and 153-68. **A**, Adult spheroids of transformation recipient strains EVE (wild type, top) and 153-68 (Reg mutant, bottom) (scale bar, 100 µm). EVE adults contain ~2000 small, non-reproductive somatic cells at surface of spheroid, and ~16 reproductive cells (gonidia) that ultimately cleave as embryos. In Reg mutants, somatic cells dedifferentiate and enlarge to become gonidia that cleave as embryos, so that every cell has the potential to reproduce. Approximately 500 and 1000 spheroids per well from vegetative cultures of EVE and 153-68, respectively, were inoculated into SVM containing hygromycin B (**B**) or blasticidin S (**C**) at concentrations ranging from 0–20 µg/mL and photographed 10 days later. **D**, Survival/growth were scored (2 = normal growth, 1 = impaired growth, 0 = no growth) following incubation in a Percival lighted growth chamber for 10 days. For both strains and both antibiotics, the minimal inhibitory concentrations ranged from 10–15 µg/mL.

tions ranging from 0–20 µg/mL were inoculated with ~500–1000 spheroids and inspected daily after day 3 (Fig. 1B, C). Both strains were sensitive to both antibiotics, at relatively low concentrations, even with a smaller number of spheroids tested in the same volume (data not shown); for both strains the MIC was 15 µg/mL (Fig. 1B–D) and killing was complete by 7 days post inoculation. 15 µg/mL was initially used for selection of transformants, but since 10 µg/mL worked equally well in terms of selection time and effectiveness, most transformation experiments were done with 10 µg/mL hygromycin.

To generate selectable marker plasmids for *V. carteri* that provide resistance against hygromycin B and blasticidin S, we designed chimeric genes with *V. carteri* regulatory sequences (*hsp70A/rbcS3* upstream regulatory region and 5′ UTR, and *rbcS2* 3′ UTR) flanking hygromycin and blasticidin resistance gene coding sequences that were codon optimized for *V. carteri* (Fig. 2). In addition, the 145-bp intron 1 of the *RBCS2* gene from the related alga *Chlamydomonas reinhardtii* was inte-

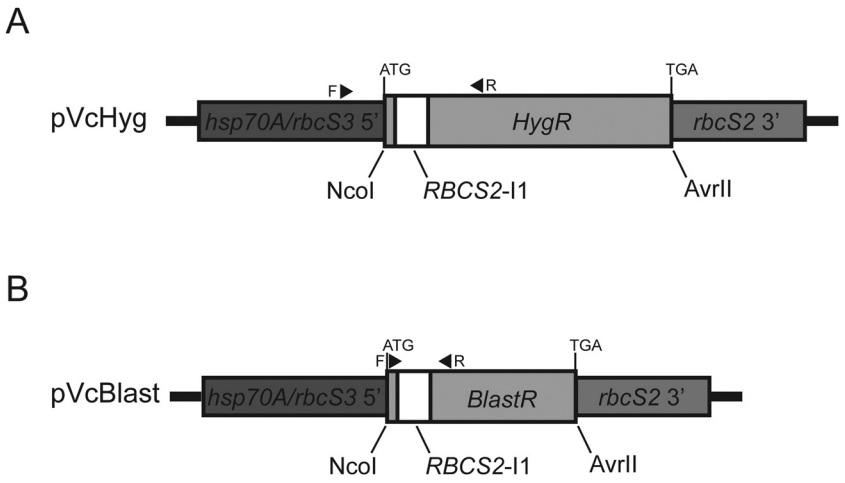


Figure 2. Selectable marker plasmids. Chimeric genes with *V. carteri* regulatory sequences (*hsp70A/rbcS3* upstream regulatory region and *5′* UTR, and *rbcS2 3′* UTR) flanking hygromycin (**A**) and blasticidin (**B**) resistance gene coding sequences were designed as shown. Coding sequences for the *Coccidioides posadasii aphH* gene (hygromycin resistance) and *Bacillus cereus bsr* gene (blasticidin resistance) were codon optimized for *V. carteri* and contain the 145-bp intron 1 of the *RBCS2* gene from *Chlamydomonas reinhardtii*. NcoI and AvrII restriction sites were inserted after the start and stop codons, respectively, to facilitate exchange of coding region sequences.

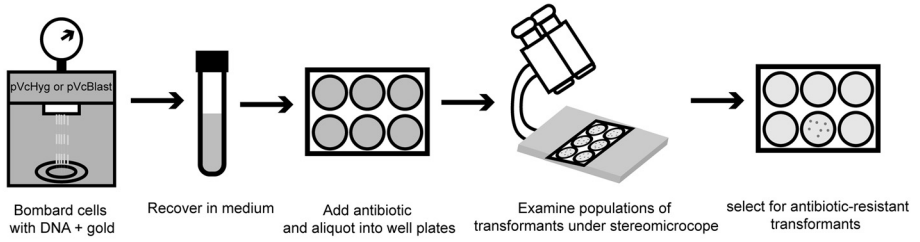
grated into the resistance gene sequences (Fig. 2), because some *V. carteri* and *C. reinhardtii* genes require at least one intron for efficient expression (Berthold et al. 2002; Gruber et al. 1996). The position of the intron was chosen to match consensus flanking sequences for green algal gene introns (Wolfgang Mages, personal communication). The plasmids generated from synthesized DNA fragments based on these designs, that contain the hygromycin resistance (HygR) or blasticidin resistance (BlastR) genes, were named pVcHyg and pVcBlast, respectively (Fig. 2).

Transformation and Co-transformation Efficiencies

To test the ability of pVcHyg and pVcBlast to provide resistance to hygromycin B and blasticidin S, we first attempted biolistic transformation of 153-68 strain (Fig. 1A), because Reg mutants are generally easier to transform than the wild type, since they produce substantially more reproductive cells and cultures do not need to be synchronized. We bombarded spheroids of 153-68 with microcarriers (gold particles) coated with the plasmids, then after a recovery period cultured the algae in the presence of antibiotic (transformation and selection scheme depicted in Figure. 3A). The results of these experiments are summarized in Figure 3B. After spheroids were bombarded with pVcHyg, hygromycin-resistant individuals were obvious within about 5–7 days because the antibiotic killed nearly all cells by that time, and ultimately survivors were recovered at an average rate of 6.8 resistant transformants per shot ($\sim 10^7$ gonidia/embryos; Fig. 3B, C), whereas we never obtained survivors from control experiments (no pVcHyg plasmid). In parallel experiments involving 153-68 and pVcBlast, we observed blasticidin-resistant survivors also within about 7 days of selection, and at an average rate of 5.1 per shot (Fig. 3B, C), but no blasticidin survivors were recovered from controls that did not receive pVcBlast. The rates of recovery of hygromycin and blasticidin survivors were higher than for Nit^+ survivors obtained from parallel experiments in which 153-68 spheroids were bombarded with the nitrate reductase gene (*nitA*) plasmid pVcNR15 (1.4 per shot) (Fig. 3B, C). Notably, selection for hygromycin B and blasticidin S resistance was much more rapid than for nitrate prototrophy; compared to 5–7 days for antibiotic resistance, pVcNR15 transformants could not be identified until at least 14 days after bombardment.

Because many important *V. carteri* experiments are performed with morphologically wild type strains, we next attempted to transform morphologically wild type strain EVE (Fig. 1A) with plasmid pVcHyg. As was the case for transformation of Reg mutant 153-68, antibiotic-resistant survivors were obtained following bombardment with pVcHyg, but not for unbombarded controls. Hygromycin-

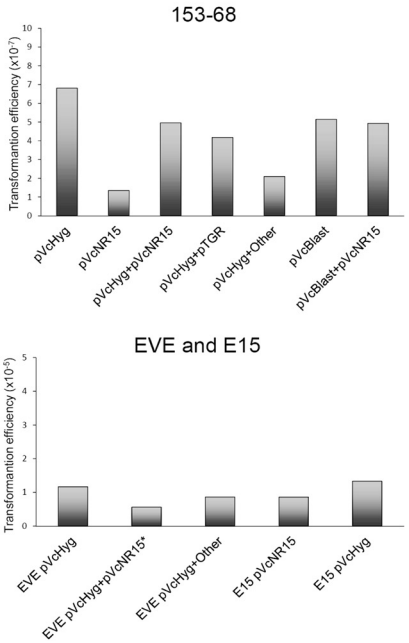
A



B

	Plasmid	Selection	Total number of transformants	Total number of recipient cells	Transformation efficiency	% co-transformation frequency
153-68	pVcHyg	Hyg^R	361	53×10^7	6.8×10^{-7}	-
	pVcNR15	Nit^+	196	145×10^7	1.4×10^{-7}	-
	pVcHyg+pVcNR15	Hyg^R	124	25×10^7	5.0×10^{-7}	-
	pVcHyg+pTGR	Hyg^R	472	113×10^7	4.2×10^{-7}	9 (13/147)
	pVcHyg+Other	Hyg^R	155	74×10^7	2.1×10^{-7}	33 (3/9)
	pVcBlast	$Blast^R$	72	14×10^7	5.1×10^{-7}	-
	pVcBlast+pVcNR15	$Blast^R$	69	14×10^7	4.9×10^{-7}	33 (5/15)
EVE	pVcHyg	Hyg^R	7	6×10^5	1.2×10^{-5}	-
	pVcHyg+pVcNR15*	Hyg^R	31	55×10^5	0.6×10^{-5}	23 (3/13)
	pVcHyg+Other	Hyg^R	39	45×10^5	0.9×10^{-5}	58 (11/19)
E15	pVcNR15	Nit^+	55	64×10^5	0.9×10^{-5}	-
	pVcHyg	Hyg^R	4	3×10^5	1.3×10^{-5}	-

C



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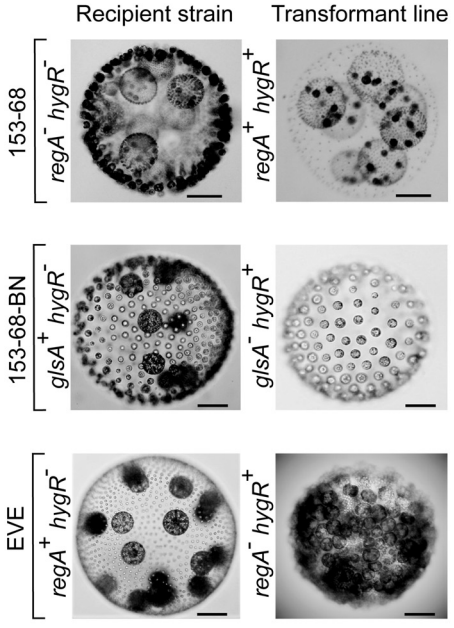

Figure 3. Transformation of pVcHyg and pVcBlast markers into *V. carteri*. **A**, Workflow for biolistic transformation in *V. carteri*. **B**, Transformation efficiencies for selectable marker plasmids pVcNR15, pVcHyg, pVcBlast, and combinations of pVcHyg or pVcBlast with other plasmids containing unselected genes. Transformation efficiencies are calculated as total number of transformants obtained per total number of recipient cells/embryos. For transformations, $\sim 10^7$ cells/embryos per shot (153-68) and $\sim 10^5$ gonidia/embryos per shot (EVE and E15)

resistant survivors were obtained at an average rate of 1.2 per shot (10^5 gonidia/embryos; Fig. 3B, C). EVE transformants cannot be selected following bombardment with the *nitA* gene (pVcNR15 plasmid) because EVE has a wild type *nitA* gene and is Nit$^+$, so strain E15, a Nit$^-$ derivative of EVE, was used to compare pVcNR15 and pVcHyg transformation efficiencies in the morphologically wild type background. Nit$^+$ transformants in E15 were obtained at a frequency of 0.9 per shot, while pVcHyg transformants were obtained at a rate of 1.3 per shot. We did not attempt to transform pVcBlast into EVE.

Most *V. carteri* transformation experiments involve not only a selectable marker plasmid, but also co-transformation of a non-selectable gene such as a gene encoding a screenable marker or a gene whose function is being tested. In *C. reinhardtii* and *V. carteri*, co-transformation frequencies of 10–80% have been reported (Berthold et al. 2002; Hallmann and Rappel 1999; Jakobiak et al. 2004; Schiedlmeier et al. 1994). Therefore, it was of interest to test co-transformation of pVcHyg and pVcBlast or derivatives with a second, unselected marker that can be screened following selection, such as the *nitA* gene or a gene that rescues/produces a morphological mutant phenotype. In such experiments with 153-68 (or its Nit$^+$ derivative 153-68-BN) as the recipient strain, transformations were performed with an equal mass mixture of pVcHyg and pTGR (encodes a GFP-tagged version of RegA, which complements the *regA*$^-$ mutation and Reg phenotype in 153-68), or of pVsg-glsA4 (pVcHyg with a CRISPR sgRNA cassette targeting the *glsA* gene) and pVCas9-1 (Cas9 gene with *V. carteri* regulatory sequences) and selected for hygromycin resistance. Expression of GFP-RegA or Cas9 plus sgRNA glsA4 in 153-68 are easy to recognize based on morphology of transformants (or their progeny) (Fig. 3D). For the HygR marker, we observed co-transformation efficiencies of ~9-33%, depending on the unselected plasmid (Fig. 3B). In experiments involving transformations of 153-68 with pVcBlast and pVcNR15, and initial selection for blasticidin resistance and then for ability to grow without nitrate (Nit$^+$), the co-transformation efficiency was ~33% (Fig. 3B). Notably, one such transformant (153-68-BN) was itself used as recipient in a co-transformation experiment with the HygR marker (Fig. 3D), demonstrating that the HygR and BlastR selection markers can be used to sequentially co-transform unselected genes into *V. carteri*.

In the case of EVE, co-transformation experiments were done with pVcHyg or an sgRNA-gene-containing pVcHyg derivative that targets either *regA* or *invA*, plus either pVCas9, pVcNR15-hp-regA (a hairpin RNA plasmid that targets *regA*), or pDOE-g (a plasmid that overexpresses the *V. carteri rlsD* gene). Co-expression of pVCas9 with *regA* or *invA* sgRNA genes generates Reg (*regA*$^-$) or Inversionless (Inv; *invA*$^-$) mutant transformant progeny, respectively, while pVcNR15-hp-regA knocks down *regA* expression and generates a Reg phenotype (Fig. 3D), and pDOE-g cause a distinct growth defect (results to be described elsewhere). We detected co-transformation efficiencies of ~23–58%, depending on the unselected plasmid (Fig. 3B), demonstrating that pVcHyg works well in co-transformation experiments.

Under our laboratory conditions, the pVcHyg and pVcBlast transformation efficiencies were higher than those for pVcNR15 when we used 153-68 as recipient, and the pVcHyg transformation efficiency was greater than the pVcNR15 transformation efficiency when we used E15 as host strain. Overall, the rates at which antibiotic-resistant transformants were obtained for these two plasmids were similar, whether transformations were performed with just

were used. Numbers in parentheses in final column indicate total number of co-transformants obtained with indicated marker. pVcNR15* indicates versions of pVcNR15 with inverted repeats inserted into the *nitA* 3′ UTR to generate RNA hairpins targeting developmental genes. “Other” indicates one or more unselected plasmids encoding products that generated morphological phenotypes, as described in text. **C**, Bar graph comparison of transformation efficiencies reported in (B). **D**, Representative co-transformant individuals. In all cases the recipient strain was hygromycin sensitive (*hygR*$^-$). Reg mutant 153-68 (*regA*$^-$ left column, top) was rescued by co-transformation of pVcHyg with *regA* gene-containing plasmid pTGR (right column, top); the *glsA* gene in 153-68-BN (*blastR*$^+$/*nitA*$^+$) (left column, middle) was mutated using CRISPR/Cas9 mutagenesis by co-transforming a pVcHyg-based *glsA* sgRNA plasmid with Cas9 plasmid pVcas9-1, producing individuals with the Gonidialess phenotype (*glsA*$^-$ right column, middle); the *regA* gene in wild type strain EVE (left column, bottom) was mutated using CRISPR/Cas9 mutagenesis by co-transforming a pVcHyg-based *regA* sgRNA plasmid with Cas9 plasmid pVcas9-1, producing individuals with the Reg phenotype (*regA*$^-$ right column, bottom). Scale bar, 100 μm.

pVcHyg or pVcBlast, or with either marker plus an unselected marker (Fig. 3B, C).

Stability and Limits of Antibiotic Resistance

The stability of the hygromycin and blasticidin resistance phenotypes was assessed in representative transformants after 6 or more months of cultivation without selection. When tested in medium containing 15 μg/mL of hygromycin B or blasticidin S, all transformants that had been maintained without selection exhibited normal growth, suggesting that expression of the transgenes is stable in *V. carteri* transformants for many generations.

We also tested selected transformant lines at higher concentrations of hygromycin or blasticidin (25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL), since it might be useful to know the upper limits of resistance when generating new lines. Surprisingly, the four independent hygromycin-resistant lines we assayed all survived and were able to reproduce normally at even 200 μg/mL antibiotic. Similarly, one of three blasticidin-resistant lines we tested was able to survive and reproduce at 200 μg/mL blasticidin, while the other two were slightly less resistant; one was able to survive at 75 μg/mL but not at 100 μg/mL, while the other survived 100 μg/mL but not 150 μg/mL. So both antibiotic resistance genes provide high levels of resistance, though some transformants likely express the genes at higher levels than other transformants, and/or contain more copies of them.

Molecular Analysis of Transformants

Since all transformants were selected based on resistance to either hygromycin B or blasticidin S after transformation, it was expected that transformants should contain and express the respective resistance gene. To determine if this were the case, first we analyzed several randomly selected hygromycin and blasticidin-resistant transformants to test for the presence of the antibiotic resistance genes. To test for presence of the HygR gene, genomic DNA was isolated from HygR transformants and used as a template for PCR with primers that spanned a 724-bp transgene fragment containing 216-bp of the *hsp70A/rbcS3* promoter region and 508-bp of the HygR gene coding sequence. PCR fragments of the expected size were obtained from all 153-68 and EVE transformants tested (Fig. 4A), indicating that these transformants all carry at least a part of the HygR gene. Similarly, PCR of pVcBlast-derived transformant DNAs using primers that amplify both 5′ regulatory sequence and BlastR gene coding sequence yielded an ~300-bp fragment for all lines analyzed (Fig. 4B). We sequenced the HygR and BlastR transformant PCR products and found them to be exactly as expected for amplification of the HygR and BlastR genes fragments, indicating that the transformants contain the transgenes.

To test for expression of the HygR and BlastR genes, we performed RT PCR on selected transformants that had been analyzed by genomic PCR (Fig. 4A, B). We isolated RNA from the transformants, reverse transcribed it into cDNA, and amplified using primer sets that span the artificial intron in each gene. We expected amplicons of 579-bp and 158-bp for properly spliced HygR and BlastR gene transcripts, respectively, and fragments corresponding to these sizes were obtained (Fig. 4C, D). Sequencing of the PCR products confirmed that, as expected, the antibiotic resistance genes were expressed in the transformants.

All co-transformation experiments reported above involved rescue of a developmental or nutritional phenotype (for instance, rescue of the *regA* and *nitA* mutations in 153-68 by the pTGR and pVcNR15 vectors, respectively) or generation of developmental phenotypes by expression of CRISPR components, or overexpression of developmental genes (Fig. 3). To test for the presence of unselected plasmids in these transformant lines, we analyzed selected candidate HygR and BlastR co-transformants by genomic PCR, using primers specific for those co-transformed plasmids, namely pTGR, pVcCas9-1, pVcNR15, and pDOE-g. In every case, we detected PCR products of the expected size in the co-transformed lines but not in the recipient controls (Supplementary Material Fig. S3), consistent with the conclusion that these co-transformed plasmids were present in these lines.

Discussion

Here we report the development of two new antibiotic-based selectable marker genes for *V. carteri* nuclear transformation. In our hands, the pVcHyg and pVcBlast plasmids permitted recovery of transformants in ½ to ⅓ the time required for Nit^+ selection with the *nitA* gene, and at 1.4–5-fold higher rates, in multiple strain backgrounds. In addition, both markers allowed co-transformation of non-selected plasmids at rates comparable to those reported previously for *nitA* vectors (Jakobiak

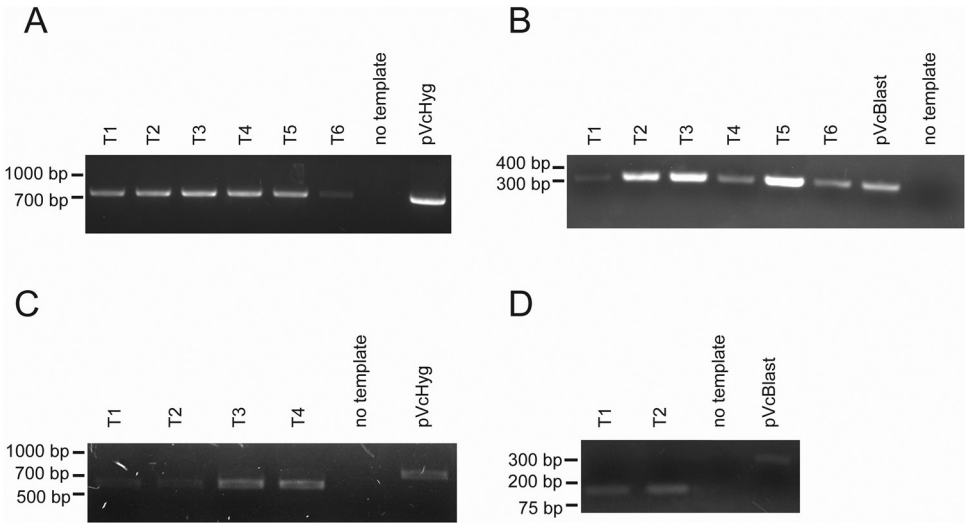

Figure 4. Detection of antibiotic resistance genes in transformants and demonstration of their transcription. **A**, Hygromycin resistant transformants were analyzed for the presence of the HygR transgene by genomic PCR. A fragment slightly larger than 700 bp (724-bp expected size) was amplified from all transformants tested. **B**, Blasticidin resistant transformants were analyzed for the presence of the BlastR transgene by PCR. A product of the expected size (~300 bp) was amplified from all transformants tested. **C**, RT-PCR analysis of HygR gene expression. RNA from hygromycin resistant transformants was reverse transcribed and products were amplified by PCR using primers that span the intron in the HygR gene. A product near the expected size (~580 bp) for the spliced transcript was detected for all transformants tested. **D**, RT-PCR analysis of blasticidin S resistance gene expression. RNA samples were reverse transcribed and amplified using primers that span the intron in the BlastR gene. A product near the expected size (~150-bp) for the spliced transcript was amplified for the 2 transformants analyzed. The pVcHyg and pVcBlast plasmids were used as positive controls and water as a non-template negative control for the genomic PCR and RT PCR. All transformant lines used for the analysis shown here were derived from 153-68 as recipient strain. In all cases, sequencing confirmed that intended products had been amplified.

et al. 2004; Schiedlmeier et al. 1994). Importantly, pVcHyg and pVcBlast can be used to transform strains with an intact nitrate reductase gene, making them more versatile than *nitA* for *V. carteri* transformations. In addition, pVcHyg and pVcBlast can be used to introduce multiple unselected transgenes into *V. carteri* in sequential transformation experiments.

Design and expression of HygR and BlastR genes. The design of the HygR and BlastR marker genes reported here was guided largely by the outcome of green algal cis- and transgene experiments reported by others. First, in the related alga *C. reinhardtii*, native promoters have been shown to perform better than foreign gene promoters, and a chimeric *HSP70A/RBCS2* promoter fusion (AR; 5′ regulatory sequences from the *C. reinhardtii* heat shock 70 protein and RuBISCO genes) is widely used in this alga because transformants can be obtained at higher rates than with single, native promoters (Jinkerson and Jonikas 2015; Schroda et al. 2002). Since a chimeric *hsp70A/rbcS3* promoter fusion was previously developed for *V. carteri* and worked better than a tubulin gene promoter when incorporated into a paromomycin resistance (PmR) gene marker (Jakobiak et al. 2004), we chose this promoter for our experiments. Second, since transgene expression and/or rates of transformation can be substantially improved in both *C. reinhardtii* and *V. carteri* by incorporating at least one intron into the transgene (Berthold et al. 2002; Gruber et al. 1996; Hallmann and Rappel 1999), we designed the HygR and BlastR coding regions to each be interrupted by the short intron 1 from the *C. reinhardtii RBCS2* gene. We positioned the intron based on consensus exon/intron junction sequences for *C. reinhardtii* (ddnrmgGT at 5′ junction and AGdhvkdb at 3′ junction; lower case and upper-case letters indicate exon and intron nucleotides, respectively) derived by others and used to create a *C. reinhardtii* HygR marker (Berthold et al. 2002) (W. Mages, personal communication). Third, proper codon bias is known to be important for efficient expression of foreign algal genes (Berthold et al. 2002; Jakobiak et al. 2004), so we synthesized the HygR and

BlastR coding regions to contain codons most commonly used in *V. carteri* genes.

Clearly the design of the HygR and BlastR markers was successful, as both genes were expressed and worked to provide stable antibiotic resistance to transgenic lines (Figs 3B, 4C, D). While transformation rates with these markers were higher than those obtained with the long-established *nitA* vector pVcNR15 (Fig. 3B), it is possible these rates could be improved by incorporating regulatory sequences that work even better than the *hsp70A/rbcS3* chimeric promoter and *rbcS* 3′ UTR. For instance, after this study was initiated, others used a data-mining strategy to identify *C. reinhardtii* regulatory sequences that outperform the AR promoter plus *RBCS3* 3′UTR, and that provide higher transformation rates driving the bleR resistance marker (Lopez-Paz et al. 2017). A similar strategy might be used to improve the HygR, BlastR, and other selectable markers in *V. carteri*.

Comparison of transformation efficiencies. We obtained HygR transformants of the morphologically wild type strain EVE at a frequency of 6.8 per shot (Fig. 3B), which was about 4-fold lower than reported for transformation of the *nitA* vector pVcNR15 into a GlsReg (Gonidialess and Somatic Regenerator) double mutant (Schiedlmeier et al. 1994). GlsReg mutants, like EVE, can be synchronized like EVE can be synchronized, making it easy to isolate large numbers of gonidia and early-cleavage embryos for transformation. As noted above, our experiments yielded 1.4–5× higher rates for pVcHyg transformation of EVE than for pVcNR15; presumably EVE is not as good a recipient as the GlsReg strain used in the previous study, and/or our transformation conditions are not as robust as those used in that previous investigation. Transformation rates of EVE have not been reported for other selectable marker vectors, but rates for transforming 153-68 with three different PmR vectors (and with pVcNR15) have been reported (Gruber et al. 1996; Jakobiak et al. 2004). Vectors pPmr3 (the most efficient PmR vector) and pVcNR15 yielded PmR and Nit$^+$ transformants, respectively, at very similar rates (7.3×10^{-6} vs 6.3×10^{-6}). The rates at which we obtained HygR and BlastR transformants in 153-68 were more than 10-fold lower than these, and our Nit$^+$ transformants were obtained at ∼40-fold lower rates. We believe these discrepancies are best explained by the fact that our recipient cell counts for 153-68 transformation included many cells that were not mature gonidia or early-cleavage stage embryos, and so a large majority of cells in our 153-68 experiments were likely not competent for transformation.

It is worth noting here that while we obtained similar transformation rates for the pVcHyg and pVcBlast vectors, blasticidin is significantly more expensive than hygromycin, so if hygromycin selection is possible, pVcHyg might be the preferred vector based on cost of the antibiotics.

Conclusions

The HygR and BlastR antibiotic resistant genes permit rapid, simple, and efficient transformation and co-transformation of *V. carteri*. They should facilitate and improve existing and developing molecular genetic methods such as those involving genetic complementation, RNAi, and CRISPR/Cas9 mutagenesis and genome editing. These advances should in turn expedite progress toward better understanding Volvox biology.

Methods

Volvox strains and cultivation conditions: *V. carteri* strains 153-68 (contains a short deletion that causes a frameshift and premature stop codon in *regA*), EVE (wild type), and E15 (Nit$^-$ version of EVE with uncharacterized mutation in *nitA*) were described previously (Geng et al. 2014; Kirk et al. 1999). 153-68-BN was generated by co-transforming 153-68 with the BlastR vector (described here) and nitrate-reductase-gene (*nitA*) vector pVcNR15 (Gruber et al. 1996). Transformant and control lines propagated for DNA and RNA preparation were cultured in 300-mL standard Volvox medium (SVM) or urea-free medium (UF-SVM) aerated in 500-mL flasks at 32 °C with a 16 h light/8 h dark regimen (Kirk et al. 1999). For tests of hygromycin and blasticidin toxicity and resistance stability, algae were cultured in SVM supplemented with antibiotic in wells of microtiter plates incubated at 32 °C with 16 h light/8 h dark regimen, in a Percival growth chamber (Percival Scientific AR-60L, Boone. IA).

Antibiotic toxicity and resistance tests: Antibiotic resistance levels of recipient strains were determined by inoculating spheroids in well-plates (∼500 and ∼1000 spheroids per well for EVE and 153-68, respectively) with SVM medium containing hygromycin B and blasticidin S (both purchased from Thermofisher Scientific, Waltham, MA) at concentrations ranging from 0–20 μg/mL and scoring for growth/survival (2 = normal growth, 1 = impaired growth, 0 = no growth) following incubation in a Percival lighted growth chamber for 10 days. Additionally, incidence of spontaneous resistance of Volvox to hygromycin B or blasticidin S was tested at densities of $\sim 10^3$–10^5 gonidia/embryos/mL (for EVE and 153-68, respectively). No spontaneous survivors were ever observed in any of ∼25 experiments with hygromycin and 8 experiments with blasticidin, involving $>10^8$ gonidia/embryos tested for hygromycin resistance and $>10^7$ gonidia/embryos tested for blasticidin resistance. Long-term resistance of individual transformants against either hygromycin B or blasticidin S following periods without selection were determined as follows. Transformant spheroids were grown in flasks containing

300-mL SVM without selection for 1 week, then harvested and resuspended in fresh SVM; this process was repeated for several weeks as necessary to test antibiotic resistance. For long term storage, transformants were kept at low density in media without selection in tubes, transferred every month and transferred to flasks containing 300-mL SVM right before testing the resistance to antibiotics. Two hundred microliters of cell suspensions were inoculated into wells containing 15 μg/mL of hygromycin B or blasticidin S. After 14 days of incubation with antibiotic, survival was assessed qualitatively by appearance of growth. The limits of antibiotic resistance of transformant lines was tested in triplicate experiments by inoculating 3-5 spheroids per well (12-well microtiter plate) containing 25, 50, 75, 100, 150, or 200 μg/mL hygromycin or blasticidin, and scoring for growth as described above for analysis of EVE and 153-68 resistance levels. For each transformant line, all three replicates displayed the same level of resistance.

Construction of chimeric selectable marker genes: Plasmids pVcHyg and pVcBlast, conferring resistance to hygromycin B and blasticidin S, respectively, were generated as follows. First, a 2.53-kb DNA fragment containing the *Coccidioides posadasii aph7* (hygromycin resistance gene) coding region flanked by *V. carteri* regulatory sequences was gene synthesized by Genscript (Piscataway, NJ) to contain the following features: 1) unique NcoI and AvrII restriction sites immediately after the start and stop codons, respectively; 2) the 145-bp *C. reinhardtii RBCS2* intron inserted 39 bp after the start codon; 3) *V. carteri* codon bias within the coding region; and 4) *V. carteri hsp70A/rbcS3* chimeric regulatory sequence immediately upstream of the start codon and *rbcS2* 3′ regulatory sequence immediately downstream of the stop codon. This fragment was subcloned into the EcoRV site of vector pUC57 by Genscript to produce plasmid pVcHyg. pVcBlast was generated by digesting pVcHyg with NcoI and AvrII to remove the *aphH* sequence and inserting a *V. carteri* codon-optimized *Bacillus cereus* blasticidin resistance (*bsr*) synthesized fragment (Genscript) containing NcoI and AvrII restriction sites just downstream of the start codon and upstream of the stop codon. Like the *aphH* gene fragment, this fragment was also synthesized to contain the 145-bp *C. reinhardtii RBCS2* intron and *V. carteri* codon bias within its coding region.

Nuclear transformation of *V. carteri*: *V. carteri* strains 153-68, 153-68-BN, EVE, and E15 were used as recipients. Plasmids were mixed with gold and used to bombard cells according to a protocol described previously (Schiedlmeier et al. 1994) for custom gene gun transformations (153-68 transformations) and according to the Seashell Technology™ (San Diego) protocol described for transformation using the Helios gene gun (EVE and E15 strains transformations; Model PDS-1000/He, Bio-Rad). For 153-68, whole spheroids were harvested by filtration as described above then resuspended in fresh medium to a density of ~10–20 × 10^6 cells/embryos per shot. If necessary, gonidia/embryos were centrifuged and resuspended in fresh medium as previously described. In both cases, harvested material was pipetted onto sterile 55 mm Whatman filter circles (catalog number 1450-055; GE Healthcare, UK), then bombarded with 10–20 μL of a suspension containing ~1 μg DNA precipitated onto 500 μg 1.5–3.0 μm spherical gold particles (Sigma-Aldrich, St. Louis, MO; catalog # 326585) resuspended in 100% ethanol. Unbombarded cells served as negative control. For EVE and E15, cultures were synchronized, and spheroids were collected in a magnetic filter funnel fitted with a 30-μm nitex mesh filter (Sefar, America Inc.). Gonidia and embryos were harvested by disrupting spheroids with a 50-mL dounce homogenizer using a loose-fitting pestle (B. Braun, Melsugen, Germany) and centrifuged at 500 RPM (100 × *g*) for 4 min (Eppendorf centrifuge 5702, brake off) after adding Percoll (Sigma; St. Louis, MO) to a final concentration of 7%. Pelleted gonidia/embryos were resuspended in fresh medium and centrifuged again at 300 RPM (60 × *g*). Pellets were resuspended to a final cell density of ~1 × 10^5 cells/embryos per mL (shot). Cells/embryos were bombarded with DNA/gold precipitates (1 μg DNA + 250 μg gold; Sigma Aldrich) spread on macrocarriers (Inbio Gold, Hurstbridge, Australia), using an 1100 psi rupture disk (Inbio Gold), and a chamber vacuum of 25 inches Hg with stopping screen positioned ~1 cm from macrocarrier and target embryos positioned 5 cm below macrocarrier (2nd and 4th slot positions in chamber). For co-transformation experiments, pVcNR15 (containing *V. carteri* nitrate reductase-gene) (Gruber et al. 1996) or another unselected plasmid was mixed in equal amounts (1 μg per shot) with either pVcHyg or pVcBlast. After transformation, bombarded cells were quickly transferred to tubes with 25 mL of medium and those tubes incubated for 2 days at 32 °C with 16 h:8 h light dark regimen. Contents of the tubes were transferred to tissue culture well-plates and additional medium containing 10 μg/mL of antibiotic was added. Plates were incubated at 32 °C until resistant colonies could be isolated and transferred to medium containing the same concentration of antibiotic for a second round of selection. Transformation efficiencies were calculated for both EVE and 153-68 recipient strains as the number of total transformant lines obtained after antibiotic selection per estimated total number of recipient cells treated/bombarded.

DNA methods: *V. carteri* DNA for genomic PCR was extracted using a method previously reported for isolation of genomic DNA from *Chlamydomonas reinhardtii* cells (Dejtisakdi and Miller 2016; Newman et al. 1990), with the following modifications. *V. carteri* spheroids were grown in 300 mL of SVM and harvested by Pasteur pipette after settling to the bottom of the flask. Cells were transferred to 1.5-mL microfuge tubes, pelleted at 13,000 × *g* for 3 min, then resuspended in 500 μL of TEN buffer (10 mM Tris–HCl, 10 mM EDTA, 150 mM NaCl) by vortexing, and centrifuged at 13,000 × *g* for 10 s. The supernatant was discarded, and the pellet was resuspended in 150 μL of water on ice. 300 μL of SDS-SB buffer (2% SDS, 400 mM NaCl, 40 mM EDTA, 100 mM Tris–HCl pH 8.0) was added and the suspension vortexed to mix. The sample was first extracted with 350 μL of saturated phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) by vortexing for 5 min at room temperature then centrifuging at 13,000 × *g* for 5 min before transferring the top aqueous phase into a new 1.5-mL microcentrifuge tube. A second extraction was performed twice by adding 300 μL chloroform:isoamyl alcohol (24:1, v:v) then following the same steps as for the first extraction. Genomic DNA was precipitated by adding 2 volumes of 95% cold ethanol at −20 °C for 30 min and pelleted by centrifugation at 13,000 × *g* for 10 min, washed with 70% cold ethanol, dried and resuspended in 40 μL of water, treated with 2.5 μL of 10 mg/mL RNase and stored at −20 °C. PCR to determine presence of the antibiotic resistance genes in transformants was by standard protocols. For genomic PCR to detect pVcHyg and pVcBlast, each 50-μL reaction contained 1 μL of the extracted genomic DNA and the primers Hyg fwd set1 (5′-AAAGTCAACACGTCCATGAACC-3′) and Hyg rev set1 (5′-CATAGCCTCAGCGACTGGCT-3′) for pVcHyg transformants and OKBLASTF (5′-CATTTCGCAGCAGGATCTGGAG-3′) and OKBLASTR (5′-CAGTCACACGGCCAATGTAGG-3′) for pVcBlast transformants. For genomic PCR to detect

pVcCas9-1, pVcNR15, pTGR, and pDOE-g, each 20 μL reaction contained 1 μL of a 1/10 dilution of extracted genomic DNA and plasmid-specific primers: for pVcCas9-1, Cas9-intron F (5′-ACGCGACCAGTAACTTTTG-3′) and Cas9-intron R (5′-CTTCTTGATCGAGTGGCGG-3′); for pVcNR15, nitA-F (5′- CATCGAGTTGAACCGCAATA-3′) and nitA-R (5′-CACCAGGCTAATCTGCGTCT-3′); for pTGR, TGR-F (5′-GCTGCTGCCCGACAACCACT-3′) and TGR-F (5′-CGCTAGTGCCACCCCCTCCT-3′), then using 1 μL of a 1/10 dilution of that product as template in a semi-nested PCR using primers TGR-FN (5′-CAGTCCGCCCTGTCCAAGGA-3′) and TGR-R; for pDOE-g, nitAU-F (5′- GGATCACTGGCTCCCGCTGC-3′) and rlsD-R (5′-TAGCGCCGAGAGTGGGAGCA-3′), then using 1 μL of a 1/10 dilution of that PCR product as template in a semi-nested PCR with nitAU-FN (5′-GGCGCAATTACTATTCAGCACA-3′) and rlsD-R, then then using 1 μL of a 1/10 dilution of that PCR product as template in a nested PCR with nitAU-FN and rlsD-RN (5′- GAGGTCCTTGAGACTTGGCT-3′). For all PCR, the following thermal cycler parameters were used in a T100™ Thermal Cycler (Bio-Rad laboratories Inc.): 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 20 s, and 72 °C for 20 s, with a final extension at 72 °C for 10 min.

RNA methods: RNA was extracted by centrifuging spheroids from a 300-mL culture at 200 × *g* for 5 min, transferring the loose pellet (~200 μL) to 1.5-mL microfuge tubes, and resuspending in 1 mL of TRIzol (Molecular Research Center, Inc.). Cells were disrupted with a bead beater system twice for 25 seconds using level 6 (FastPrep FP120 Cell Disrupter). The mixture was incubated for 5 min at room temperature, then 200 μL chloroform was added and the samples were shaken then put on ice for 3 min. Samples were then centrifuged 15 min at 12,000 × *g* at 4 °C and the supernatant was transferred to new tubes and precipitated overnight by adding 500 μL of isopropanol and incubating at −20 °C. The precipitated RNA was centrifuged at 12,000 × *g* for 10 min at 4 °C and supernatant removed. 1 mL of cold 70% ethanol was added for wash and the samples were centrifuged at 7,500 × *g* for 5 min at 4 °C. The supernatant was removed, and the pellets were air dried for ~10 min then resuspended in 200 μL of RNAse-free water (Fisher Scientific, Inc.). A Nanodrop 1000 (ND-1000, NanoDrop) was used to measure RNA concentration and aliquots were stored at −80 °C. Two to 5 μg of each RNA sample was treated with 2 units of RNAse-free DNAse following the Ambion Turbo DNA-Free Kit protocol (Life Technologies) and cDNA was generated by standard methods using random hexamer primers from the iScript Select cDNA Synthesis kit (Bio-Rad). RT reaction mixes were incubated at 25 °C for 5 min, then 42 °C for 30 min, then inactivated at 85 °C for 5 min. For each RT PCR, 1 μL of the resultant cDNA reaction mix was used in a 50-μL PCR under the conditions described above for genomic PCR. The primers used for genomic PCR were used for RT-PCR. Products of amplification were ligated into pGEM®-T Easy vector (Promega Co, USA) and sequenced by Sanger sequencing (GENEWIZ LLC, Southplainfield, NJ).

Data Statement

Raw data for transformation efficiency and genomic PCR/RT PCR experiments generated during this study will be made available upon request.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.protis.2018.11.002.

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