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Molecular diagnostics for monitoring contaminants in algal cultivation

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ABSTRACT

There is currently great interest in mass cultivation of microalgae for production of fuels and other high value products. Since algae have not previously been grown at the scales and with the precision required for these endeavors, sensitive methods are needed for enumeration of elite algal varieties relative to "weedy" invader strains that are ubiquitous in the environment and a common issue with culture management. The ideal monitoring strategy would be inexpensive and identify weedy algae long before they become prominent in cultures of elite varieties. Herein, multiple polymerase chain reaction (PCR)-based tools for monitoring contaminants are presented. These include resources to identify unknown strains, to routinely monitor dominant constituents in cultures, and to detect contaminants constituting as little as one in 10^8 cells in a culture. Quantitative PCR was shown to be 10^4 times more sensitive for detecting weeds than flow cytometry. During characterization of these tools, it was demonstrated that contamination is a common phenomenon and that early detection is necessary for informed decision making during culture selection for subculturing or scale-up. Thus, implementation of strategies for monitoring contaminants in algal cultivation is a critical component of culture management for optimal productivity.

1. Introduction

Microalgae (herein, "algae") comprise a highly diverse set of photosynthetic eukaryotes that arose via independent endosymbiotic events [1,2]. Because strains from divergent taxa produce oils appropriate for use in production of renewable biofuel, general interest in algae has increased significantly [3]. Oil productivity in some algal varieties is significantly greater than even the most robust oil-producing traditional crops [4], and genetic modification is now common in multiple relevant algal strains and thus may be used to further enhance high-oil-productivity strains [5–8]. Following agricultural convention, these desired algal varieties with high oil productivity and other inherent or engineered qualities that make them suitable crops for commercial production may be generically referred to as "elite" lines. Algae have not historically been cultivated at the scales nor with the technical precision required for affordable, reliable mass cultivation and quality-controlled fuel production. Major barriers that currently limit the potential of algal biofuels include proven, stable, large-scale (>1000 ha) cultivation methods for appropriate high-oil-content algal strains and an understanding of culture maintenance and pest management strategies [9].

Because algae are ubiquitous in the environment, there are constant opportunities for low oil content algae to contaminate cultures and compete with elite strains for sunlight and nutrients. Such contaminants are appropriately referred to as "weeds" and must be managed as such to minimize their impact on crop productivity and resulting fuel quality. Because lipids are more reduced than carbohydrates and proteins, high-oil elite algae require more photosynthetically derived reductant per unit biomass than weedy strains containing less oil. Thus, weedy algae may grow faster than elite strains and have the potential to become abundant or dominant in a culture [10]. Both open pond and closed photobioreactor systems are known to be invaded by weedy species, grazers and pathogens [11–13], so such invasions must be expected regardless of the cultivation system. Clearly, algal culture monitoring methods will be needed along with pest management programs for algae-based biofuel production, and culture monitoring is equally important for production facilities, research laboratories and culture collections [14]. To be included as part of a routine culture monitoring regime, these tools and related protocols should be of low or moderate cost, versatile for adaptation to various algal communities, able to be implemented immediately, require only limited technical expertise, and be informative.

Current culture monitoring methods vary in throughput, instrumentation, degree of experience required and cost. Growers may use microscopy to manually observe cultures and identify algae based on morphology and pigmentation. This methodology is low throughput

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and requires considerable expertise distinguishing strains. Microalgae are small (1–100 μm in diameter), and distinct genera may have nearly identical overall appearances [15,16]. Furthermore, algal strains of the same species may be morphologically indistinguishable, yet harbor cryptic genetic diversity that affects crop value [17]. In addition to standard microscopy, it is common to use flow cytometry and imaging flow cytometry to group cells based on phenotypes such as size and chlorophyll content [18]. Despite its increased throughput, flow cytometry has limited ability to identify algae with certainty or to distinguish strains with similar phenotypes.

Nucleic-acid-based methods may be used to unambiguously identify algae, for example by sequencing or otherwise characterizing a portion of algal genomes. Genes encoding RNA subunits of prokaryotic or eukaryotic ribosomes are commonly characterized for taxonomic and phylogeny purposes. Relevant to the work presented herein, there are evolutionarily constrained regions of rRNA genes ideal for design of PCR primers of broad specificity or for comparison of distantly related organisms, as well as interspersed variable regions that may be used to distinguish more closely related organisms [19–21]. Additionally, there are millions of rRNA sequences deposited in general nucleotide databases (i.e., Genbank, http://www.ncbi.nlm.nih.gov/genbank/) and specialized rRNA databases (i.e., SILVA, http://www.arb-silva.de).

In this work, molecular tools were developed for routine monitoring of elite and weedy algae in laboratory and production cultures. The various tools and procedures involved characterization of 18S rRNA genes. In the analyses presented, the polymorphism among algal 18S rRNA genes was sufficient to distinguish different genera, species of the same genus, and geographic isolates seemingly of a single species. Specifically, PCR primers were designed to amplify an approximately 1500 nt region of 18S rRNA genes from three classes of algae: *Bacillariophyceae*, *Eustigmatophyceae*, and *Chlorophyceae* (herein referred to as "BEC"). These amplicons can be sequenced for definitive identification of strains, or they can be digested with a restriction enzyme to generate allele-specific fragmentation patterns for rapid, inexpensive characterization of strains and cultures (Fig. 1, left panel). Two strategies for culture monitoring based on quantitative PCR (QPCR) were also compared for their ability to detect weedy algae at low abundance in elite cultures (i.e., allele-specific QPCR probes and allele-specific QPCR primers; Fig. 1, middle and right panels, respectively). We chose the more promising allele-specific QPCR primer method and compared its sensitivity and specificity to that of flow cytometry for detecting weedy algae at low abundance in cultures. In addition to clarifying the utility and limitations of these tools, we demonstrate the importance of sensitive and accurate weed detection during selection of potential innocula for scale-up or subculturing.

2. Materials and methods

2.1. Sampling

Samples were collected (Solix Biosystems; [11]). Approximately 1.5 mL of culture was sampled from cultures ranging in biomass density between 0.5 and 5 g(dry weight)/L, equivalent to 9×10^7 and 1×10^9 cells/mL, respectively. Other samples came from agar plates where single colonies or numerous colonies were picked using a pipette tip and placed into F/2 media. The samples were centrifuged at 6000 ×*g* for 10 min at room temperature and the supernatant was decanted. Cell pellets were less than 100 mg and were stored at −20 °C until DNA extraction.

2.2. Flow cytometry

Samples were analyzed using a guava easyCyte HT + flow cytometer (EMD Millipore) equipped with an argon laser (488 nm) and 680/30 nm bandpass filter. For each sample, 20,000 events (i.e., cells) were scored for red fluorescence to identify chlorophyll-positive cells and for low-angle forward scatter to determine approximate diameter. Algal cells were identified as chlorophyll-positive events, and populations of algal genera were distinguished by size.

2.3. DNA extraction

Total DNA was isolated from frozen cell pellets. Cells were disrupted by grinding in liquid nitrogen with a mortar and pestle for 5 min or by mechanical disruption using a bead beater (BioSpec Products) or paint shaker (Fluid Management). Frozen cell pellets in microcentrifuge tubes were shaken 3 × 1 min in the presence of 0.5 mm zirconia/silica beads (BioSpec Products Inc.). Prior to and between each round of shaking, the biomass was flash frozen in liquid nitrogen. Following

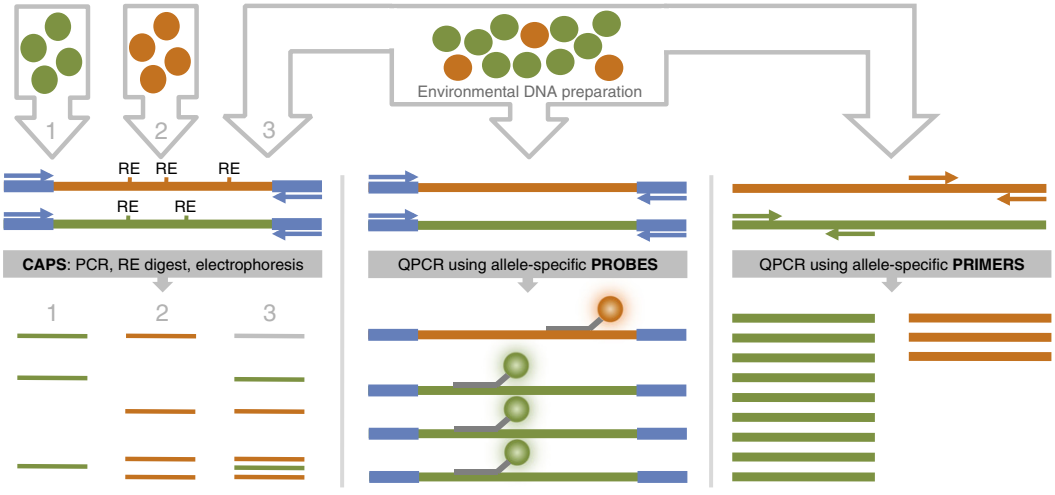

Fig. 1. Nucleic acid-based diagnostics for monitoring algal cultures. Schematic overview contrasting three strategies for monitoring algal cultures. Left panel: Using cleaved amplified polymorphic sequences (CAPS), a portion of the 18S rRNA gene is amplified from different algae (represented as green or orange cells) using a single set of primers with broad specificity (blue arrows). Amplicons are digested with an appropriate restriction enzyme (RE, restriction enzyme cut sites) and restriction fragments are resolved by electrophoresis. Allele-specific fragmentation patterns may be used to identify algae in unialgal cultures (e.g., inputs 1 & 2) or mixed cultures (input 3). Some restriction fragments may be shared by multiple organisms and are not useful for diagnostic purposes (e.g., gray fragment in restriction pattern 3). Middle panel: fluorescent probes in QPCR reactions detect allele-specific polymorphisms within 18S rRNA amplicons produced using primers with broad specificity (blue arrows). Relative fluorescence intensity from multiplexed probes with distinct fluorophores may be used to estimate relative abundances of organisms in cultures. Right panel: allele-specific QPCR primers amplify 18S rRNA gene regions from specific organisms in a culture and estimate their relative abundances.