Reasons to build dinoflagellate *Symbiodinium* BAC libraries

Thomas G. Doak, Princeton, USA. <tdoak@princeton.edu>
Robert B. Moore, University of Sydney, AU. <r.moore@mmb.usyd.edu.au>
Ove Hoegh-Guldberg, University of Queensland, AU. <OveH@uq.edu.au>
Mary Alice Coffroth, State University of New York at Buffalo, USA. <coffroth@buffalo.edu>

Abstract

Dinoflagellates are ubiquitous marine and freshwater protists. As free-living photosynthetic plankton, they account for ~50% of the primary productivity of oceans and lakes. As photosynthetic symbionts, they provide essential nutrients to most corals and numerous other marine invertebrates, supporting coral reefs, one of the most diverse ecosystems on earth, a rich food source, and a potential source of future pharmaceuticals. When they are expelled from their coral hosts during mass coral bleaching events, coral reefs and the surrounding ecosystems rapidly decline and die. Population explosions of dinoflagellates are the cause of red tides, extensive fish kills, and paralytic shellfish poisoning. As parasites and predators, they threaten numerous fisheries. As bioluminescent organisms, they are a spectacular feature of nighttime oceans and an established model for luminescence chemistry.

Dinoflagellates are alveolates and a sister group to the apicomplexans–important human and animal pathogens. The apicomplexans are obligate intracellular parasites, *e.g. Plasmodium* spp. the agents of malaria. Describing a dinoflagellate alveolate that is an intracellular symbiont will aid us in understanding the capacities, weaknesses and evolution of the apicomplexans, and will inform the apicomplexan genomes in a way directly relevant to the development of antimalarial and antiapicomplexan drugs.

Dinoflagellates, because of these roles, impact human health and life on many levels. We have proposed in a separate white paper that the *Symbiodinium* genome be sequenced, to increase our understanding of these important organisms. *Symbiodinium* is the primary symbiont of marine invertebrates, is a well studied dinoflagellate, has a very small genome for a dinoflagellate (the vegetative cells are haploid and contain ~2.5 x 10^9 bp of DNA), and is representative of the metabolic capabilities of dinoflagellates as a group. Here we request that a BAC library be made for a common Caribbean *Symbiodinium* species, to serve as a basis for genome sequences.

Rational

1. Introduction

The ~2000 living species of dinoflagellates, classified in ~125 genera, form one of the three main phyla of alveolates, the other two being the entirely parasitic apicomplexans, and the largely free-living ciliates. Currently there are genome projects for the apicomplexans *Cryptosporidium parvum*, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium yoelii*, and *Toxoplasma gondii*; the ciliates *Tetrahymena thermophila*, *Oxytricha trifallax*, and *Paramecium tetraurelia*; and one genus basal to dinoflagellates, *Perkinsus marinus;* but none for dinoflagellates.

Dinoflagellates are typically unicellular, photosynthetic, free-swimming, biflagellate organisms that constitute an important component of freshwater, brackish, and marine phytoplanktonic communities. While most are autotrophic (photosynthetic), some are heterotrophic, saprophytic, or symbiotic. Some are highly modified parasites. The autotrophic dinoflagellates are either free-living, or associated with a broad range of hosts as endosymbionts. The dominant modern coral group, the Scleractinia, contain dinoflagellates–almost exclusively of the genus *Symbiodinium*–as endosymbiotic "zooxanthellae." Reef-building scleractinian corals have an absolute need for intracellular zooxanthellae, as providers of photosynthate, which serves to fuel the energetically expensive deposition of calcium carbonate (Trench 1987).

2. Dinoflagellate biology

2.1 Dinoflagellate groups. Dinoflagellates have been grouped by the types of pigments they produce. The two major groups are the peridinin and the fucoxanthin containing dinoflagellates. The peridinin-containing group is the most prevalent and includes *Symbiodinium* and the various species that cause red tides.

2.2 Dinoflagellate life cycles. Most dinoflagellate life cycles include sexual and asexual, motile and non-motile stages. Asexual reproduction takes place by fission. In the sexual reproduction of a typical dinoflagellate, each haploid vegetative cell differentiates without division to form a gamete. Pairs of gametes fuse to form a non-dividing diploid zygote, and after a short resting period or a long dormancy, the zygote undergoes meiosis to produce the next generation of haploid vegetative cells.

2.3 The dinoflagellate nucleus. Dinoflagellates possess a unique nuclear structure, the dinokaryotic nucleus. Chromosomes are continuously condensed throughout mitosis and interphase, although they do unwind for DNA replication. During prophase, metaphase, and anaphase chromosomes are membrane attached: the nuclear membrane remains intact during mitosis and the mitotic spindle develops in channels that permeate the nucleus, and coordinate the segregation of chromosomes *via* membrane attachments (Bhaud *et al*. 2000; Soyer-Gobillard *et al*. 1999).

Dinoflagellates lack histones H-2, -3, -4, and so do not have nucleosomes, but rather the DNA is in 2.5 nm fibers, as in bacteria, not in 25 nm fibers as in most eukaryotes (Loeblich 1984). They do have a possible homologue of histone H1 (Kasinsky *et al*. 2001), which is thought to perform a DNA compaction function.

Dinoflagellate genome sizes are frequently greater than those of other eukaryotes (up to 200 pg/cell). Renaturation experiments (Allen *et al*. 1975; Tripplet *et al*. 1993; Davies *et al.* 1988; Hinnebusch *et al.* 1980; Kite *et al.* 1988) have shown that even in dinoflagellates with large genomes there are high percentages of unique sequence: *e.g. Heterocapsa pygmaea* (9.1 pg) 75%; *Crypthecodinium cohnii* (7.3 pg) 40-45%; *Glenodinium foliaceum* (75 pg) 56%.

Dinoflagellate DNA composition is also distinctive. While dinoflagellates have methyl C (mCpG), as do most eukaryotes, in several species more than 60% of thymines are replaced by hydroxymethyluracil (hmdU, as hmUpA and hmUpC; Steele & Rae, 1980). In *Symbiodinium* spp. hmdU ranges from 5% to 12% (Blank *et al.* 1988). This modification destabilizes the double helix, but the adaptive value is not known.

2.4 Dinoflagellate plastids. Chloroplasts (plastids) are accepted to have originated as a cyanobacterium that was endocytosed, but not digested, by a protist, becoming a symbiotic organelle (endosymbiosis). The double membrane around green algal and higher plant plastids derives from the two membranes of the cyanobacteria (Palmer 2003). However, in peridinin-possessing dinoflagellates the plastid has a triple membrane (van den Hoek *et al.* 1995) and is probably derived from an endocytosed red alga (Yoon *et al*. 2002a).

The plastid genome of peridinin-possessing dinoflagellates is divided into 13 mini-circles (B. Green, pers. comm.), each of which carries a single gene, including the 16S and 26S genes (Zhang *et al*. 2002). This is an entirely unique situation among plastid genomes, both to have the genome consist of unigene minicircles and to be so highly reduced in gene number. Dinoflagellates are also unique in that Rubisco, an essential enzyme for carbon fixation, is nuclear-encoded, and of the prokaryotic type, rather than the eukaryotic type (Morse *et al.* 1995, Rowan *et al.* 1996).

3. Dinoflagellate evolutionary placement.

The alveolates have been proposed to form a super-group, the chromalveolates, along with the chromists (Cavalier-Smith, 1999). The chromists (roughly equivalent to the previously defined Stramenopile, Heterokonta, and Chromobionta groups) include not only algae, but also diatoms, kelps, water molds and downy mildews. It appears that the chromalveolates were ancestrally photosynthetic, by virtue of a secondary plastid that was acquired once in a common ancestor, derived from a red alga (Archibald & Keeling, 2002). If this is true, many lineages, such as the water molds and ciliates, have secondarily lost their plastids, to become heterotrophic.

4. Important areas of research in dinoflagellates.

4.1 Evolutionary transitions from symbiosis to parasitism. Symbiotic dinoflagellates illustrate this hypothesis at three levels. Firstly, dinoflagellates have a sister relationship to the exclusively parasitic apicomplexans. A great deal can be learned by comparing the intracellular adaptations of apicomplexans with those of symbiotic dinoflagellates*:* specifically, what genes have been lost, gained, and maintained in the apicomplexan lineage.

Second is the relationships within the dinoflagellates. Symbiotic dinoflagellates seem to represent at least a conceptual transition from the free-living autotrophic dinoflagellate, to the heterotrophic, sometimes

parasitic, dinoflagellates. For example, the parasitic dinoflagellates often have reduced plastids just as apicomplexans have (van den Hoek *et al.* 1995).

Third, the infection processes of symbiotic dinoflagellates and apicomplexans may be postulated to share common mechanisms. The mechanism of host specificity in cnidarian-zooxanthellae associations requires the host's cell-adhesion molecule Sym-32 (Weis *et al.* 1999). Sym-32 is in the fasciclin-1 super family of cell adhesion molecules (Reynolds *et al*. 2000), which also have recurring roles in other symbioses: lichen-cyanobacterium and plant-rhizobium (Oke & Long 1999; Paulsrud & Lindblad 2002). Interestingly, *Plasmodium* proteins involved in host specificity include an integrin homologue (Sultan 1999): integrins are postulated as the partners of the fas-1/Sym-32 family (Kim *et al.* 2002).

4.2 Dinoflagellates as models for apicomplexan infection Dinoflagellate alveoli, comprised of a membrane complex, are homologues of the inner membrane complex of apicomplexans (Leander & Keeling 2003). The inner membrane complex is a vital part of the machinery that allows apicomplexan parasites to invade their host (Bannister *et al.* 1990). Additionally, the apical apparatus of some dinoflagellates contains structures homologous to the apical complex of apicomplexan parasites (Leander & Keeling 2003; Kuvardina *et al.* 2002). Moreover, dinoflagellate ejectisomes are likely homologous to the rhoptries and micronemes of apicomplexans (Leander *et al.* 2003). These latter structures are also part of the parasite invasion apparatus and harbor many antigens that are currently being trialed as malaria vaccines (Chauhan & Bhardwaj 2003).

4.3 Integration of organellar and nuclear genomes. The dinoflagellate plastid is an example of the interaction of two organisms, however ancient. Peridinin-possessing dinoflagellates have a chloroplast genome consisting of multiple mini-circles encoding only a few genes (~13). We expect that the genome sequence of *Symbiodinium* will reveal the rest of the algal derived genes, just as apicomplexan genomes have revealed the algal origin of the apicoplast (Wilson 2002).

The apicomplexan apicoplast is one of the few organellar genomes to have revealed that a component of its DNA was derived from another organelle, in this case from the mitochondrion (Obornik *et al*. 2002). Separately, there is a wide body of evidence that multiple endosymbionts have donated genes to apicomplexan nuclear genomes (Striepen *et al.* 2002; Dzierszinski *et al.* 2001; J. Kissinger, manuscript in preparation). These findings support the hypothesis that inter-compartmental DNA traffic has occurred in the alveolate lineage.

4.4 Genome structure, regulation, and evolutionary significance. Dinoflagellate genomes are unique in a number of ways: dinoflagellate chromosomes are condensed throughout the cell cycle, except in S-phase, without the benefit of nucleosomes and dinoflagellate genomes possess both a high level of mC and the unusual methylated base hydroxymethyluracil (Rizzo 1987). The nature of the gene sequences that are methylated is unknown, and it is not known what function the unique hydroxymethyluracil serves.

Applications of a model dinoflagellate to human biology and health

Dinoflagellates have direct impacts on human biology and health, and the photosynthetic members are the only autotrophic representatives of the autotrophic ancestor of all alveolates. They will serve as workhorses to improve human health, in three ways.

First, as a sister group to the exclusively parasitic apicomplexans, dinoflagellates can serve to inform the search for antimalarial compounds, because their plastid functions may overlap with those of the apicoplast, and the latter organelle is essential to survival in *Plasmodium* (Ralph *et al.* 2001, Waller *et al.* 2003). The genomic sequence comparison of dinoflagellates and apicomplexans is expected to yield insights into apicoplast evolution and alveolate vulnerabilities.

Second, the economic and medical impacts of dinoflagellates on human food sources can be reduced by this research. Toxic and parasitic dinoflagellates decimate seafood supplies, killing the catch, or rendering it toxic when eaten (Durborow 1999). A deeper understanding of dinoflagellate biology, especially their sexual cycles (Anderson 2003), will help in the prediction of outbreaks. A host of different lifestyles are found within the harmful dinoflagellates, and we argue that *Symbiodinium* represents aspects of all these lifestyles.

Third, the prospects for exploiting coral reef ecosystems as a source of pharmaceuticals (*e.g.* Fenical 1997; Luiten *et al.* 2003) will be enhanced, by understanding the factors contributing to reef degradation.

Breakdown of the *Symbiodinium*-coral symbiosis impacts directly on reef biodiversity and on reef integrity beyond the host and symbiont themselves, and is an early indicator of a strong decline in marine and terrestrial biodiversity that is expected in the coming decades due to global warming (Walther *et al.* 2002).

Symbiodinium is the dinoflagellate of choice

1. Introduction. *Symbiodinium* is an emerging model for many aspects of dinoflagellate biology and by inference alveolate biology. It is not yet a well-developed genetic system–nor is any dinoflagellate–and so genomic information offers the best and most immediate entry into the physiology of this group. Any one dinoflagellate genome will energize all dinoflagellate research, and we believe that *Symbiodinium* is the most usefully placed within the phylum. In this context, the small genome size and broad set of metabolic capabilities of *Symbiodinium* suggest that it will be a relevant model for all dinoflagellates. The fact that the vast majority of zooxanthellate marine invertebrates choose *Symbiodinium* indicates that *Symbiodinium* possesses pathways to take advantage of host resources, and is hypothesized to share these pathways with the parasitic dinoflagellates. *Symbiodinium* are also periodically free-living (Trench 1987; Rowan 1998; La Jeunesse 2001; Baker 2003), making this genus a suitable model for free-living HAB dinoflagellates.

2. *Symbiodinium's* ecological roles. *Symbiodinium* is the dominant algal symbiont in all reef-building corals and in many other invertebrates in the world's equatorial, tropical, and temperate oceans. As such, *Symbiodinium* is not an ecologically incidental model. When corals are stressed by environmental perturbations such as declining water quality or climate change, the symbiotic relationship between host and dinoflagellate symbiont breaks down (Brown 1997; Hoegh-Guldberg 1999; Downs *et al*. 2002; Douglas 2003; Warner *et al*. 1999; Walther *et al*. 2002; Baker 2003; Hughes *et al.* 2003). The reasons for this symbiotic dysfunction are only poorly understood. By underpinning the most diverse marine ecosystem on earth, *Symbiodinium* carries all of that diversity linked to its fate, along with the ecosystem's undiscovered pharmaceuticals and unexplored scientific potential.

5. Suitability of *Symbiodinium* for experimentation.

5.1 Introduction. Despite being periodically sequestered in hosts in the wild, *Symbiodinium* live well in culture. We will exploit this to obtain DNA from axenic cell lines for the BAC project. Cultured *Symbiodinium* strains can be grown from single cells to colonies on agar (*e.g.* via simple dilution streaking), which can't be done for most of the larger, more fragile, HAB species. Population genetic studies have verified that *Symbiodinium* populations are sexual (Baillie *et al*. 1998, 2000a, 2000b, La Jeunesse 2001; Goulet & Coffroth 2003; Santos *et al.* 2003), although gametogenesis has not been reproducibly observed (Blank 1987).

5.3 Transformation and selectable markers. In 1998, genetic transformation of dinoflagellates was achieved for both *Symbiodinium* and *Amphidinium* (ten Lohuis & Miller 1998b), showing the general applicability of the method. The selectable markers used were neomycin and hygromycin B phosphotransferases, driven by the *Agrobacterium* p1'2' and nos promoters. A recorder construct was also successfully used, coupling the b-glucoronidase gene with the p1'2' promoter, or the cauliflower mosaic virus 35S promoter.

siRNA has not been tried in dinoflagellates, but given that dinoflagellates can be transformed and that both ciliates and apicomplexans respond to siRNA, it is expected that lost of function treatments can be developed in dinoflagellates and be a major way forward from the EST microarray and genome projects.

6. The *Symbiodinium* research community

The *Symbiodinium* community has been growing exponentially since the naming of the genus in 1959, and is currently composed mainly of researchers who have been using *Symbiodinium* for fifteen years or less. Most of the interest is in molecular population genetics (biodiversity of the genus) and cell biology. A feature that unites these two camps is a desire to understand the extent of host-symbiont specificity, and to establish broad rules for why this occurs, and whether there are obligate partnerships that are not adaptable (Baker 2001, 2003; Hoegh-Guldberg *et al.* 2002; Buddemeier *et al.* in press).

The Coffroth, Medina and Rizzo labs will be the US bases for the genome project and will carry out the DNA purifications required for library creation and all algal culturing required for the genome project.

Additional zooxanthellae will be provided to the US labs by the Hoegh-Guldberg lab. In a separate project, the McFadden lab (alveolate evolution, Melbourne Australia) intends to characterize the *Symbiodinium* genome using pulsed field gel electrophoresis.

7. The *Symbiodinium* genome and the status of its "pre-genomics"

7.1 The genome. While many dinoflagellates have huge genomes, as much as 20-200 pg per cell, *Symbiodinium* has a much smaller genome: average ~2.4 pg across a number of isolates from multiple sources (measured by DAPI staining and flow cytometry; G. Lambert & D. Galbraith pers. comm.). [Measurements by R. Gregor (using Feulgen image analyses densitometry) of a small number of samples have given much lower values (~0.6 pg/cell), and we are working to resolve this discrepancy.] The GC content has been measured at 43-55% for *Symbiodinium* (Blank *et al.* 1988; Blank & Huss 1989). Preliminary renaturation analyses suggested that at least 20% of the genome was repetitive (Blank & Huss 1989). We are not aware of an estimate of dinoflagellate gene number, however, their nearest characterized free-living relatives, the ciliates, are estimated to have ~25,000 genes.

Symbiodinium DNA is easily isolated, and there are established protocols to produce intact nuclei (*e.g.* Rowan *et al.* 1996), and thus intact chromosomes, to produce the mega-base DNA needed for BAC libraries.

7.2 ESTs/Microarrays. The Hoegh-Guldberg group is funded to develop a microarray for *Symbiodinium* and its Indo-Pacific coral host (*Acropora aspera*), which will be used to identify key stress responses within the two organisms. Monica Medina (Joint Genome Institute, University of California), who will also work with the *Symbiodinium* Genome project, is directing a similar project at the Department of Energy's JGI center, in collaboration with Mary Alice Coffroth. This project will use Caribbean strains of *Symbiodinium* and the Caribbean coral *Montastrea faveolata*. As well, an EST project–without microarrays–is being conducted by NOAA, and led by Cheryl Woodley.

Proposal

1. Choice of *Symbiodinium* strains. We have proposed in a separate white paper that a finished genome sequence be generated for a *Symbiodinium* isolate, plus lower coverage sequence of three other isolates, to allow a complete catalog of *Symbiodinium's* genes to be generated. Here we request that BAC libraries be made for the initial isolate.

We proposed that the primary strain for the genome sequence project (~10x plus closing) be a clade B isolate from *Montastrea faveolata* because 1) its genome has been measured at 2.1 pg (David Gailbraith pers. comm.); 2) the lifecycle of clade B has been well characterized, as haploid and sexual (Goulet & Coffroth 2003; Santos & Coffroth 2003, Santos *et al* 2003); 3) cultures are on hand; 4) this is the strain that M. Medina will use for her EST project; 5) clade B is already the workhorse of intracellular zooxanthellae symbiosis research, as they are found in anemones (*e.g. Aiptasia* spp.) which are easily maintained in aquaria.

We have also proposed that the clade C isolate from *Acropora aspera,* used in the Australian EST project, be sequenced to 5x. Clade B and C are separated by ~65 million years. The clade C sequence will begin to characterize the most widespread clade, it will compliment the Australian EST effort, and it will aid in ORF identification for all *Symbiodinium* spp.

We propose that a 15-fold coverage BAC library, average insert size 200 kbp, be constructed, for the primary B isolate , and hope eventually to obtain funding for the quite diverged C isolate.

2. Uses of the genome sequence. The primary purpose we foresee for the BAC clones is to aid the sequencing projects. End sequences of a BAC library will allow long-range assembly of the projects, and can also be used for the closure of major gaps. Perhaps the most immediate and notable effect of this project will be to allow all dinoflagellate researchers to go from EST sequences to full gene sequences, including the entire open reading frame and promoters. In addition to the three *Symbiodinium* EST, we know of eight dinoflagellate EST projects. These EST projects address the issues of plastid and nuclear evolution, alveolate evolution, toxin chemistry, lifecycles and cell division, symbiotic integration, gene transfer, coral bleaching, stress markers and circadian rhythms.

The phylogenetic shadowing strategy will uncover structural and regulatory regions in non-coding DNA, including genome wide regulators of diel cycle, sexual differentiation, and nutrient toggling, which would not be possible to identify fully in a smaller project.

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