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REVIEW

Harnessing the Power of Model Organisms To Unravel Microbial Functions in the Coral Holobiont

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SUMMARY Stony corals build the framework of coral reefs, ecosystems of immense ecological and economic importance. The existence of these ecosystems is threatened by climate change and other anthropogenic stressors that manifest in microbial dysbiosis such as coral bleaching and disease, often leading to coral mortality. Despite a significant amount of research, the mechanisms ultimately underlying these destructive phenomena, and what could prevent or mitigate them, remain to be resolved. This is mostly due to practical challenges in experimentation on corals and the highly complex nature of the coral holobiont that also includes bacteria, archaea, protists, and viruses. While the overall importance of these partners is well recognized, their

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specific contributions to holobiont functioning and their interspecific dynamics remain largely unexplored. Here, we review the potential of adopting model organisms as more tractable systems to address these knowledge gaps. We draw on parallels from the broader biological and biomedical fields to guide the establishment, implementation, and integration of new and emerging model organisms with the aim of addressing the specific needs of coral research. We evaluate the cnidarian models Hydra, Aiptasia, Cassiopea, and *Astrangia poculata*; review the fast-evolving field of coral tissue and cell cultures; and propose a framework for the establishment of "true" tropical reef-building coral models. Based on this assessment, we also suggest future research to address key aspects limiting our ability to understand and hence improve the response of reef-building corals to future ocean conditions.

KEYWORDS model organisms, metaorganism, reef-building corals, microbial functions

INTRODUCTION

Scleractinian or stony corals build the framework of coral reefs, which are the most biodiverse and productive marine ecosystems (1). Coral reefs provide important ecosystem services and a livelihood to over 500 million people globally (2). Climate change, together with the local stressors of pollution and overexploitation have heavily impacted coral reefs around the world, causing major habitat loss and threatening the survival of these ecosystems (3–5) (Fig. 1). Preserving the biological and ecological functions of coral reefs requires drastic reductions of global and local stressors (6) together with active conservation and restoration interventions (7, 8). The effectiveness of such interventions depends upon a deep, accurate, and comprehensive understanding of coral biology (9, 10). However, while speed is imperative, research progress is challenged by the difficulties associated with working with corals.

Holobiont Diversity and Complexity

Corals are complex metaorganisms. The coral animal hosts a vast array of microorganisms encompassing unicellular algae of the family *Symbiodiniaceae*, bacteria, archaea, fungi, and other protists, as well as viruses, which collectively constitute the so-called coral holobiont (see Box 1) (11–13). Each coral colony represents a rich and diverse microecosystem often hosting several *Symbiodiniaceae* species (14, 15), hundreds to tens of thousands of bacterial taxa (16, 17), and probably at least as many archaea, viruses,

BOX 1: CORAL HOLOBIONT MEMBERS AND FUNCTIONS—AN OVERVIEW OF CURRENT KNOWLEDGE

***Symbiodiniaceae* and coral bleaching.** The endosymbiotic dinoflagellate algae (*Symbiodiniaceae*) are the most extensively studied and better characterized members of the coral holobiont (13). The coral-algal symbiosis is obligate and based around nutritional exchange, where the metabolic contribution of the photosynthetic algae supports high productivity under oligotrophic conditions—far beyond the capacity of the coral animal alone (24, 25). This symbiosis supports the building of the structural foundation of coral reefs and represents the engine of these ecosystems (26). However, this symbiosis is under threat, primarily due to global warming and other anthropogenic stressors on both a local and a global scale. The loss of the algae from coral tissue—coral bleaching—weakens the coral host and often leads to its death (27). Increasing frequency of and decreasing recovery time between bleaching events (28) make coral bleaching the largest challenge for the persistence of reef ecosystems, which has received much attention over the last 3 decades (29). Nonetheless, a complete and detailed understanding of the underlying cellular mechanisms is still lacking.

Bacteria and coral disease. Bacteria are the best studied of the microbial coral holobiont members and are known to play an important role in holobiont health (30). They are known to exist in highly diverse communities, which appear to vary in composition depending on coral and *Symbiodiniaceae* genotype (31, 32), environmental conditions (33), anatomical compartments (21, 34), and even colony age (35). They have been accredited as controlling or governing key functions for the coral host including, nutrient cycling (36, 37), and immunity (38, 39), and they have even been hypothesized to facilitate rapid environmental adaptation (40).

Imbalances in the microbiome, or dysbioses, compromise coral health and can lead to the emergence of disease (30). Due to climate change and anthropogenic activities, coral diseases are increasing in frequency and number, e.g., black band disease, now occurring in coral reefs around the world (41), and gray patch disease in the Indo-Pacific (42). Some researchers are now arguing that disease rivals coral bleaching as a major cause of coral reef decline on a global scale (43, 44).

Manipulating the coral microbiome has recently been shown to increase the tolerance of corals to a number of stressors, for example, by enriching the holobiont in members with beneficial functions and traits (45–48). Research on coral probiotics aims at understanding how to perform such manipulations to accelerate the rate of coral adaptation to global change (45, 47, 49). However, although our understanding of the likely functional role many bacteria play in coral health is rapidly advancing, we still lack a mechanistic understanding of the dynamics and functions of the majority of the coral associates (reviewed in reference 50). While there are limitations inherent to culture-based methods, a recent study has shown that diverse members across many phyla can and have already been cultured and highlighted how these could be further expanded in the coming years from the adoption of more diverse culturing approaches (51).

Understudied microbial partners. The remainder of the coral's microbiome—i.e., the "other" (endolithic) microalgae, protists, archaea, fungi, and viruses—is comparatively less well understood. However, these microbes constitute a nonnegligible proportion of the coral microbiome. Archaea were found to constitute up to half of the prokaryotic fraction in absolute abundance, fungi were the most abundant microorganism in metagenomes of *Porites astreoides*, for example (18, 19), and viruses have been shown to be present in abundances of upwards of $\sim 10^7$ viruses per mL of mucus (52). Fungi and endolithic algae specifically appear to at least spatially dominate in the coral aragonite skeleton, where they have been shown to be directly involved in carbon and nitrogen cycling and may metabolically interact with each other and the coral host (53, 54). Archaea also appear to be involved in nutrient metabolism, in particular ammonia oxidation, carbon metabolism, and the synthesis of essential vitamins (55). Viruses, however, remain the most elusive members of the coral holobiont, and both their pathogenic and their beneficial roles are currently being investigated (reviewed in reference 56).

and protists (18–20). This high diversity of microorganisms can be partially explained by coral colony morphology consisting of a dynamic surface mucus layer, the coral gastrodermal and epidermal tissues, the mesoglea, and the skeleton. Each of these represents a microhabitat or niche populated by distinct microbial communities (21–23).

Many of the associated microorganisms are likely to be involved in holobiont metabolism, immunity, and environmental adaptation and may therefore contribute to the health and performance of the metaorganism (reviewed in references 30 and 50). The holobiont phenotype thus results from the combination of the long-term stable host genotype and the more flexible genotypes of the associated microbes (57–60). In addition, the environment (e.g., temperature, light, and salinity) and host characteristics (e.g., trophic state and

FIG 1 Coral bleaching and coral diseases as major threats to coral reefs. (A) Aerial view of coral bleaching in the Great Barrier Reef (Australia) during the 2017 mass bleaching event. (B) *Acropora cytherea* affected by white syndrome (WS), a tissue loss disease of unknown etiology. (C) *Orbicella annularis* suffering from stony coral tissue loss disease (SCTLD), a new lethal disease alarmingly spreading through the Caribbean. (D) *Goniopora* sp. infected with black band disease (BBD) during a bleaching event (visible loss of pigmentation). BBD is caused by a microbial consortium dominated by filamentous cyanobacteria. Image credits: A, Ed Roberts/ARC Centre of Excellence for Coral Reef Studies; B, C, and D, Dr. Greta Aeby.

age) modulate the cross-kingdom interactions between holobiont members (i.e., host-microbe and microbe-microbe) in a complex and underexplored framework (35, 61, 62).

Our understanding of what makes a coral "tick" has recently expanded exponentially and now the majority of researchers acknowledge the importance of the holobiont as a whole rather than focus on any one aspect (30). However, we still struggle to disentangle holobiont complexity and fall short in our understanding of coral functioning from a holistic perspective (Fig. 2 and Table 1). Several fundamental questions, such as those listed below, therefore remain either fully or partially unanswered.

Who is there and where? The coral microbiome has certainly not been fully characterized yet. The current knowledge of the coral microbiome is highly skewed toward *Symbiodiniaceae* and bacterial members, and less is known about the other microbial partners that constitute a large proportion of the microbiome in both biomass and absolute abundance (18, 19, 53). Thousands of taxa are likely yet to even be described and characterized.

Who does what? While it is well recognized that the microbiome plays an important role in fundamental physiological functions such as nutrition, development, and immunity, the exact contribution and involvement of each microbial taxon remains to be resolved.

Who interacts with whom? Besides coral-*Symbiodiniaceae* dynamics, very little is known about interactions between other members of the holobiont (63, 64). For instance, do bacteria interact with each other, the coral host, the *Symbiodiniaceae*, archaea, fungi, and/or viruses? Furthermore, can microbial communities in different anatomical compartments interact with each other? If so, then our next question would be as follows.

How do they interact with each other? Individuals may affect or impact others within the community via positive (e.g., mutualistic symbiosis and facilitation), negative (e.g.,

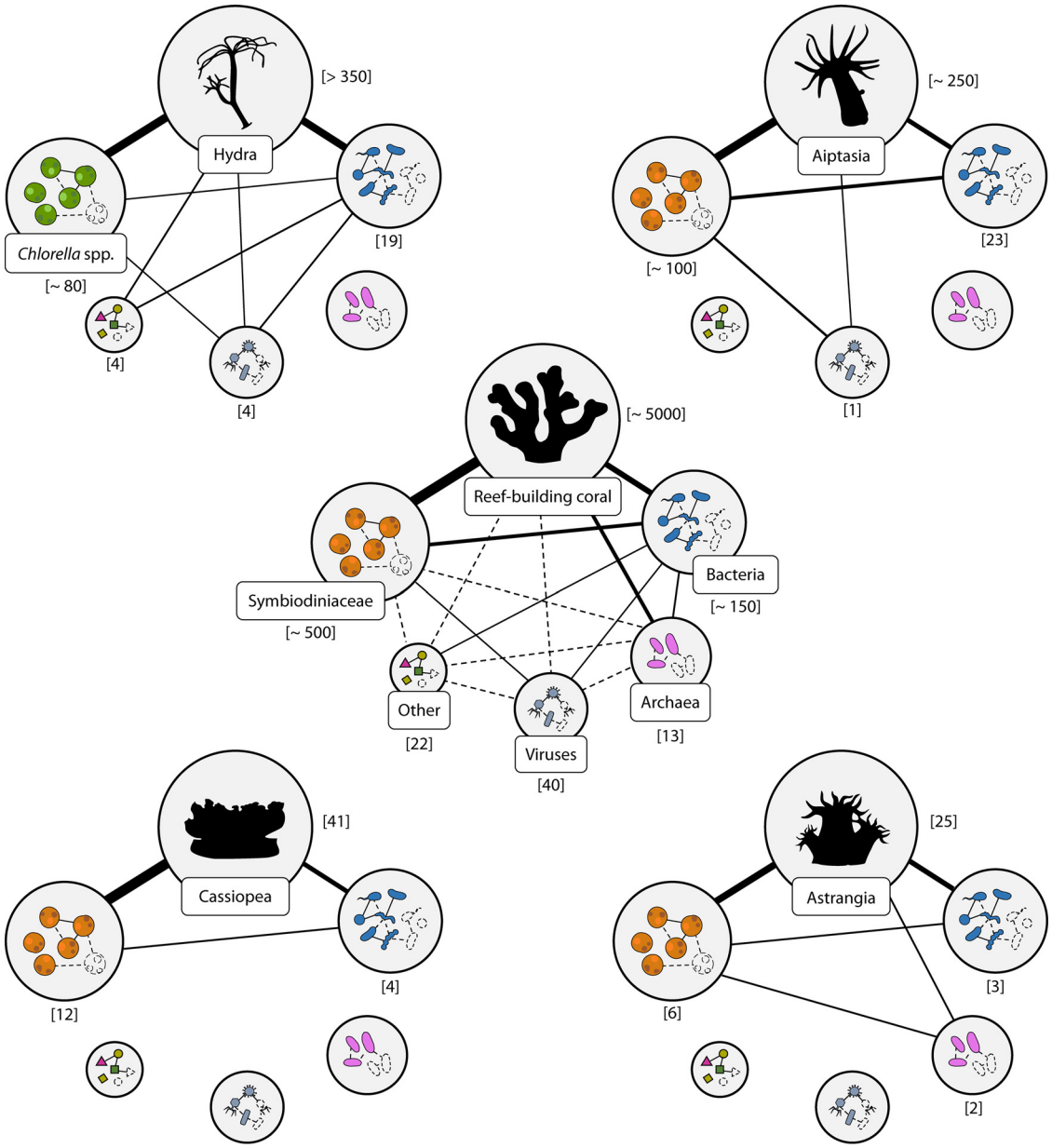

FIG 2 Overview of the state of knowledge on cnidarian holobionts regarding composition and functional interactions among their members. Cnidarian holobionts are disassembled into their major taxonomic compartments (gray circles). Within each taxonomic compartment, dashed-outlined microbes represent hypothesized, yet currently unidentified, taxa. Lines connecting compartments indicate known relationships, while absence of connecting lines indicate lack of information. Lines connecting individual microbes indicate known (solid), hypothesized (dashed), and presumed lack of functional relationships (no lines). The size of the taxonomic compartments and the thickness of the connecting lines approximate the assumed importance of each holobiont member or relationship. "Other" includes fungi and other protists (e.g., unicellular algae other than *Symbiodiniaceae* and *Chlorella* spp.). Numbers in square brackets report the numbers of peer-reviewed publications on the model organisms and their compartments (see the supplemental material for additional details).

competition, predation, and parasitism), or neutral (e.g., commensalism) interactions. Resolving the network of interactions between individual members of the microbiome and across Kingdoms is extremely challenging, yet it will yield very valuable information on ecological and coevolutionary processes (42, 65).

Model Organisms Unravel Complex Biological Principles

Model organisms by their very nature facilitate research because they are practically and/or ethically "more convenient to study" than the organisms of interest, and at the same time they are similar enough so that discoveries can be meaningfully transferred.

TABLE 1 Summary of the most relevant literature on functional interactions between members of the cnidarian model organisms

	Observation(s)[a]			
Interaction	**Hydra**	**Aiptasia**	**Cassiopea**	***Astrangia* spp.**
Host-symbiotic algae	↔ Facultative endosymbiosis with *Chlorella* spp. (only *H. viridissima*); metabolic complementarity. The host provides CO_2, N, P, and S. The algae provide photosynthates and amino acids (reviewed in references 84, 85, and 296). → Host controls *Chlorella* population size through algal cell cycle modulation, expulsion, or digestion (reviewed in references 84, 85, and 296).	↔ Bleaching is associated with cellular and molecular responses in both partners (127, 297–300). ↔Onset of symbiosis as a modulation of the host immune response (137, 301–304). ↔ Species specific; association with non-native algal types results in altered expression patterns for metabolic exchanges, oxidative stress response, and immunity processes (119, 122, 136). ↔ Metabolic complementarity; Transfer of org. and inorg. nutrients between partners (135, 140, 305–307). ← Species-specific; association with non-native algal types is less stable and results in lower host growth and reproduction rates (132, 133).	↔ Partner specificity: Association with non-native thermotolerant algal strain produces more heat-sensitive holobionts (164) ↔ Coupling of host and symbiont metabolism through translocation and recycling of C and N compounds (308, 309). → Host can restrict N (nitrate) availability to symbiont (170, 173). ← Symbiosis-driven development (161, 169, 171). ← Unable to heterotrophically compensate lack of alga-derived nutrients under low illumination (310) or in aposymbiotic state (162). ← Alga-fixed and translocated C satisfies >100% of host metabolic demand (311).	↔ Facultative association; the symbiont provides mild advantages (nutrition, host growth and healing) or nondetectable effects (calcification rate) (180, 190, 312–314). → Host regulates symbiont population density through expulsion (315). → Host trophic conditions drive mutualistic/parasitic shift (181).
Host-bacteria	→ Host influences bacterial microbiome through antimicrobial compounds and by altering bacterial quorum-sensing signaling (95, 97–99, 106). ← Bacteria protect the *Hydra* host against fungal infections (100). ← Bacterial involvement in host body developmental regulation (101). ← Bacterial role in spontaneous body contraction regulation (105).	↔ Species specificity in the host-bacterium association (111). → Host state linked to bacterial microbiome structure and composition (141, 145). ← Potential role of bacteria in host defensive tissues (316).	← Bacteria as larvae settlement cue (161, 317).	
Host-virus	↔ Viromes are species specific; heat stress linked to altered viromes and altered predicted cellular functions involved in (DNA and C) metabolism and defense (318).	↔ Diverse but stable viral assemblage (319).		
Host-fungi	← Lethal fungal pathogen (100).			

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TABLE 1 (Continued)

	Observation(s)[a]			
Interaction	**Hydra**	**Aiptasia**	**Cassiopea**	***Astrangia* spp.**
Symbiotic algae-bacteria	→ Symbiotic algae provide host with colonization resistance and community-immunity against an invasive bacterium (320).		↔ Symbiotic state linked to bacterial community composition; potential bacterial involvement in modulating N availability (170).	→ Recovery of prokaryotic microbiota following antibiotic treatment is more consistent among symbiotic (than aposymbiotic) individuals (195). × Prokaryotic microbiome unaffected by seasonality or symbiotic state (77, 321).
Symbiotic algae-archaea				→ Recovery of prokaryotic microbiota following antibiotic treatment is more consistent among symbiotic thann aposymbiotic individuals (195).
Symbiotic algae-virus	←? Consistent presence of virus in Chlorella suggests functional role (322).	→ Relative abundance of viral taxa linked to symbiotic state (319).		
Bacterium-virus	← Bacteriophages dominate *Hydra* virome (318). ← Bacteriophage role in bacterial population dynamics (323, 324).			

[a]References are indicated parenthetically where applicable.

Interestingly, there is no apparent fixed set of rules to define a model organism or its validity (66). Instead, the models are usually chosen based on the suitability of the organism to investigate a specific phenomenon or set of questions needing to be addressed, namely, its tractability and its informative power (67, 68). For example, tractability is typically associated with small size, fast growth rate, short reproductive cycle, broad availability, ease of maintenance under laboratory settings, and simplicity of some traits (e.g., small number of genes or simple body plan) (68). In addition to tractability, many models possess what could be perceived as "odd" or "unusual" features, which make them stand out among similar organisms. Typically, these oddities have a great informative power if harnessed for research purposes (67, 68). For example, the high regenerative capacity of Hydra or the ability to survive extreme conditions of tardigrades helped shed light on the mechanisms of aging, transdifferentiation, and *de novo* generation of biological patterns (69, 70) and on protection against damage of biological structures (71, 72).

Simplicity is also a very important feature for biological investigation that traditionally follows a reductionist approach. Compared to more complex or derived systems, simpler systems possess all of the fundamental features but lack much of the "extra noise", such as additional biochemical pathways or regulatory processes, and therefore facilitate understanding of fundamental biological mechanisms. Thus, researchers exploring the mechanisms of gene expression and regulation found it more convenient to study the yeast *Saccharomyces cerevisiae*, a single-celled eukaryote with a simple genomic structure and comparably little non-coding DNA (73), rather than something as complex as a human cell. The same fundamental principles apply to both, to the point that essential yeast genes can be replaced by their human orthologs (74).

As scientific knowledge grows and new questions arise, new model organisms are being added to a growing list (75). Technological progress is increasing the insight from each model organism rather than replacing them. Easy access to high-throughput sequencing technologies expands the number of sequenced genomes available and facilitates the development of new and customized molecular tools (67, 68). Model organisms are not only growing in number, but they are also used in new and more varied applications. While model organisms have been extensively used to investigate fundamental biological principles (e.g., organismal development, behavior, and evolution [69]), phenomena directly affecting human health (i.e., to understand and treat diseases) or to generate economic benefits (agricultural crops and livestock), more recently, nature and biodiversity conservation represents a new niche for model organism-based research (76).

Coral research is challenged by a set of important questions that could benefit from the use of model organisms (76–79) (Fig. 2). Disentangling holobiont complexity to shed light on the mechanisms underlying coral responses to global change (e.g., coral bleaching and diseases; see Box 1 and Fig. 1) and how these can be prevented or mitigated, is one of the most pressing challenges faced today (8). To reverse the decline of coral reefs (and maintain the services they provide), scientists have been embracing efforts to increase corals resilience through approaches that span many levels of intervention, from the microscopic level (cellular and molecular, i.e., assisted evolution) to the macroscopic level (ecosystem scale, i.e., assisted gene flow), and from laboratory-based to field deployment (7). Here, we provide a comprehensive overview of the state-of-play of model organisms and systems that can be utilized to move coral holobiont research into the next stage. Our aim for this review is to (i) highlight each model system's advantages and disadvantages and (ii) synthesize open research questions and how establishment of new model systems could address them. We review established and emerging cnidarian model systems, including the freshwater hydroid Hydra, the anemone Aiptasia, the jellyfish Cassiopea, and the temperate coral *Astrangia poculata* (Fig. 3). Moreover, we provide a comparative overview of their attributes, including distribution, ease of rearing in aquaria, life cycle, amenability to manipulate symbiotic states, existing knowledge base, and resources. We further introduce the different approaches that can facilitate direct experimentation on tropical stony corals, a

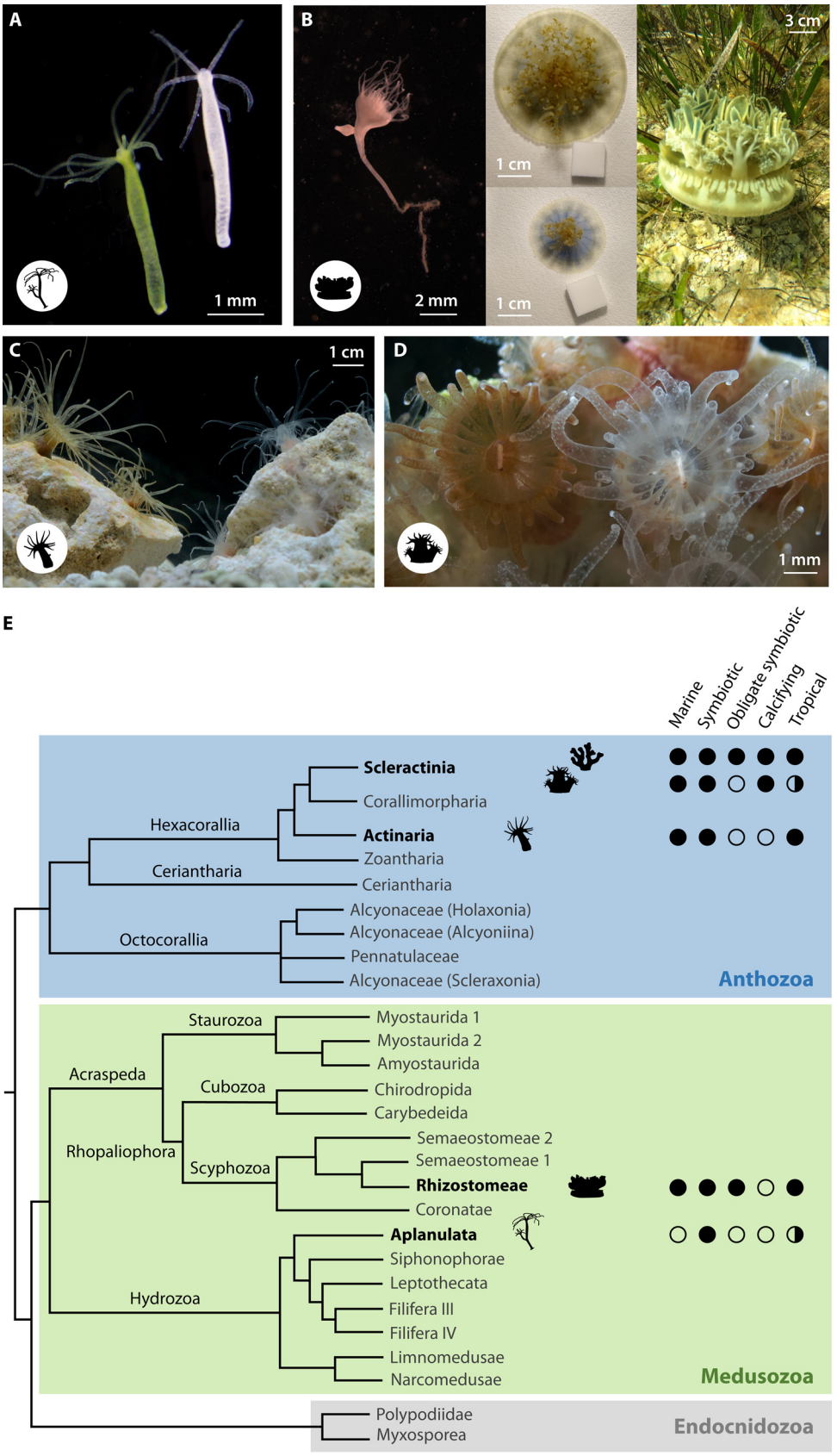

FIG 3 Cnidarian model organisms and systems for reef-building corals. (A) Freshwater hydroid Hydra, symbiotic with the chlorophyte *Chlorella* spp. (left) and aposymbiotic (right). (B) *Cassiopea xamachana* scyphistoma (polyp,

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necessary step to validate discoveries made on laboratory model systems (10, 80). These approaches include the establishment of tropical stony coral species as model organisms, as well as simplified systems such as cell and tissue cultures.

CNIDARIAN MODEL ORGANISMS AS CORAL HOLOBIONT MODELS

Freshwater Hydroid Hydra

Hydra is the oldest cnidarian model organism and arguably the best known. Hydra species belong to the class Hydrozoa, in the anthozoan sister subphylum Medusozoa (Fig. 3E). They inhabit freshwater ecosystems worldwide (81–83) and while most do not associate with microalgae, one species (*H. viridissima*) can establish facultative endosymbiosis with the chlorophyte *Chlorella* spp. (84, 85). Hydra has a simple life cycle that can be easily completed in the lab. Under normal laboratory conditions, solitary polyps reproduce asexually by budding, while environmental shifts in temperature or food induce sexual reproduction (86, 87). This ease of rearing combined with its exceptional regenerative capacity, was already appreciated in the 1700s (88) and contributed to the birth of experimental zoology (89). This is evidenced by early fundamental discoveries using the Hydra model, such as Ethel Browne's discovery of induced formation of a secondary axis by transplanting a head onto a polyp (90), 15 years before Mangold and Spemann published their observation of the organizer activity of the dorsal lip of the amphibian embryo (91). Hydra then grew into a model for developmental biology that helped answer questions of pattern formation at the theoretical (92) and molecular levels (93). Later, the adoption of *H. viridissima* as a model organism contributed to the understanding of fundamental processes in cnidarian-algal nutrient exchange and symbiosis regulation (see reference 85 and references therein).

Over the last 15 years, Hydra has become an important model for host-bacterium research. Initial analyses of the bacterial microbiome of different Hydra species revealed a high degree of species specificity reflecting the phylogenetic relationships of its Hydra host species (94, 95). Subsequent research identified that the epithelial cells produce specific antimicrobial peptides (96–99) which act as key innate immune factors responsible for shaping the species-specific bacterial associations (95).

The Hydra model has shed light on the involvement of the bacterial microbiome in shaping the holobiont phenotype. For example, the removal of the intact microbiome revealed that bacteria protect the Hydra host against fungal infections (100). Other recent results indicate that bacteria associated with Hydra are able to modify the Wnt-signaling pathway, a central signaling cascade in development (101). This pathway is involved in several developmental processes in Hydra, such as head (93) and bud (102, 103) formation, and the differentiation of stem cells (104). Functional analyses of bacterium-regulated genes revealed that the corresponding peptides have an antagonistic function to Wnt-signaling and influence stem cell differentiation (101). Studies have also shown that specific bacterial species are involved in regulating the frequency of spontaneous body contractions, which is reduced by ~50% in germ-free animals (105). Although the mechanisms underlying these responses are still unclear, studying the host-microbe signaling in detail revealed a direct interaction between Hydra and its associated bacteria based on quorum-sensing signaling molecules (106). This investigation identified a fundamental mechanism whereby the host-modified bacterial signal molecule promotes symbiosis establishment, while the non-modified signal molecule

FIG 3 Legend (Continued)
early life stage, left), young symbiotic medusa (center top), young aposymbiotic medusa (center-bottom), and adult symbiotic medusa (right). (C) Sea anemone Aiptasia, symbiotic (left) and aposymbiotic (right) polyps. (D) *Astrangia poculata* naturally occurring in symbiotic (left) and aposymbiotic (right) state. (E) Phylogeny showing the relative phylogenetic distance within cnidarians and between tropical scleractinians (including reef-building corals) and the cnidarian model organisms discussed in this review. Specifically, from top to bottom are shown: tropical stony corals (Scleractinia), *Astrangia* spp. (Scleractinia), *Exaiptasia* spp. (Actinaria), *Cassiopea xamachana* (Rhizostomeae), and *Hydra* spp. (Aplanulata). Phylogeny was modified from reference 159. Image credits: A, Jay Bathia; B, Victoria Sharp, Claudia Tatiana Galindo, and Andre Morandini; C, Samuel Begood; D, Alicia Schickle.

represses it (106). This demonstrates that Hydra is able to alter quorum-sensing controlled behavior of its bacterial symbionts to promote metaorganism assembly and resilience (106).

Within the bacterial communities of Hydra, one particular bacterium (a *Curvibacter* sp.) stands out as it is the most abundant member (95). *Curvibacter* sp. appears to populate and accumulate in a mucus-like layer of the ectodermis (100) and can easily be cultivated and reproduced in the lab (100). Genomic and transcriptomic data are available for both *Curvibacter* and its Hydra host (106–108), which facilitates functional studies in both symbiosis partners (109–111). Importantly, due to the transparent appearance of the animals, the host's cells and those of the *Curvibacter* can be transgenically labeled (109, 110). This means microscopic analysis can be achieved in an *in vivo* context on single cells, as well as whole tissue levels across space and time. It is now even possible to generate germ-free Hydra polyps via antibiotic treatment and repopulate them with single or multiple bacterial strains (95, 100, 112). Despite differences in the surface topography between Hydra and stony corals (113), their relatively distant phylogenetic relationships (Fig. 3E), and differences in life history (Table 2), the Hydra model can help to understand mucosal host-microbe interactions in corals by providing a roadmap to controlled symbiosis-reestablishment experiments in cnidarians.

Sea Anemone Aiptasia

The sea anemone Aiptasia is also among the original models utilized for the study of coral-dinoflagellate symbiosis, with some publications dating back to the 1960s and early 70s (e.g., reference [114]). The name Aiptasia is a common name for *Exaiptasia diaphana* (Rapp, 1829), which was previously named *Exaiptasia pallida* (115). Similar to Hydra, Aiptasia is fast growing, hardy, clonal, and it is extremely amenable to laboratory culture. Indeed, it is considered an aquarium pest by the hobby industry due to its rapid growth and propagation, often quickly overgrowing corals and other sessile reef fauna. Animals can be purchased from animal supply companies, collected from nature with relative ease, and acquired from the growing global network of Aiptasia laboratories that commonly share strains. Its strengths and limitations as a model have been extensively documented elsewhere (e.g., see references 76, 116, and 117), so we will only summarize them here.

With Aiptasia's popularity growing, the model has been adopted by an increasing number of laboratories around the world (117, 118). Historically, studies were primarily performed on animals of unknown or mixed genetic background, with different laboratories using different populations, strains, and possibly even different species (115). Since the early 2000s, however, the Aiptasia community has rallied and increasingly turned to clonal populations, allowing for the control (to some degree) of genetic background noise (119, 120). Two specific clonal lines, CC7 and H2 (originating from Florida and Hawaii, respectively), are now shared widely among research groups in the United States, Europe, and the Middle East (118, 121). However, different clonal populations are in laboratory culture in both Australia (117) and New Zealand (122) since these countries have strict limitations on importing non-native organisms. The genomes of CC7 (123), H2, and a clonal line from the Red Sea are available, as are numerous transcriptomes from other clonal lines (see Table 2).

The Aiptasia-*Symbiodiniaceae* model is a powerful system to unravel the cellular and molecular mechanism underlying coral bleaching. In contrast to tropical corals, Aiptasia can be easily bleached experimentally in a standardized and controlled manner, maintained in this aposymbiotic state indefinitely (typically under dark conditions to prevent repopulation from accidentally introduced algae) and subsequently repopulated with *Symbiodiniaceae* algae. Aposymbiotic animals will grow and continue to undergo pedal laceration to reproduce asexually if fed frequently (124), suggesting that the health of the animal is not impeded to any major degree. Bleaching can be achieved via a number of methods, including heat stress (125), cold shock (often in combination with the herbicide DCMU [126]), or by incubation in menthol (126). Examining aspects of the heat stress response and accompanying loss of symbionts

TABLE 2 Summary of relevant features of cnidarian model organisms[a]

Feature	Observation(s)					
	Hydra	Aiptasia	Cassiopea (polyp)	Cassiopea (medusa)	Astrangia	Tropical scleractinians
Species	*Hydra* spp.	*Exaiptasia diaphana* (Rapp, 1829)	*C. xamachana* (Bigelow, 1892)		*A. poculata* (Ellis & Solander, 1786)	~800 species (symbiotic)
Body features						
Form	Sessile	Sessile	Benthic	Benthic but not sessile	Benthic	Sessile
Motility	Limited	Limited	No	Yes	No	No
Calcifying	No	No	No	No	Yes	Yes
Colonial	No	No	No	No	Yes	Yes
Polyp size	5 to 15 mm	~1 mm to 5 cm	~0.1 to 2 mm	~2 mm to 20 cm	<10 mm	~1 mm to 20 cm
Environment and availability						
Water/medium	Freshwater	Seawater	Seawater		Seawater	Seawater
Latitudinal distribution	Temperate	Tropical and subtropical	Tropical and subtropical		Temperate to tropical	Tropical and subtropical
Trophic environment	Medium nutrients (mesotrophic)	Low nutrients (oligotrophic)	High nutrients (eutrophic)		Medium nutrients (mesotrophic)	Low nutrients (oligotrophic)
Habitat	Streams and lakes	Mangroves, coral reefs	Mangroves	Mangroves, coral reefs, seagrass beds	Shallow to deep hard substrates	Coral reefs
Geographic area	Circumglobal	Shallow seas in the intertropical belt	Caribbean and Gulf of Mexico		Atlantic	Shallow seas in the intertropical belt
Life cycle and propagation						
Full/closed life cycle in lab (*ex situ*)	Yes	No	Yes		Yes	Yes
Mode of clonal propagation	Budding	Pedal laceration	Budding	Regeneration	Fragmentation	Budding and fragmentation
Time to maturity or (sexual) generation time	Wks	NA	Mos		Mos	Yrs
Rearing and maintenance in artificial settings	Easy	Easy	Easy	Moderate	Moderate/demanding	Demanding
Algal symbiont						
Algal symbiont: identity	*Chlorella*	*Symbiodiniaceae*	*Symbiodiniaceae*		*Symbiodiniaceae*	*Symbiodiniaceae*
Algal symbiont: culturable	No	Yes	Yes		Yes	Yes
Needs algal symbiont to complete development	No	NA	Yes		No	Yes
Obligate symbiosis at adult stage	No	Yes	Yes		No	Yes
Survival in aposymbiotic state	Yrs	Indefinitely	Indefinitely	>3 wks	Indefinitely	Wks
Algal symbiont acquisition	Mixed	Horizontal (env)	Horizontal (env)		Horizontal (env)	Depends on species

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TABLE 2 (Continued)

Feature	Observation(s)					
	Hydra	Aiptasia	Cassiopea (polyp)	Cassiopea (medusa)	Astrangia	Tropical scleractinians
Practical aspects						
Consortium	OpenHydra	Aiptasia Symbiosis Resource	CassiopeaBase		Temperate Coral Research Working Group	No
Online open access resources	Hydra 2.0 Genome Project Portal	protocols.io, Reefgenomics	Medina Lab		Coral Microbiome Portal	Reefgenomics, SymPortal
Established strain(s)	Yes	CC7, H2, EM5, JK, JKA2, VW9, VWA12, PLF3, PLF5, and PLF8	12 strains: T1–A, B, C, D, E, and F and T2–A, B, C, D, E, and F		No	No
Genome(s) available	Assembled	3	Casxa1		Draft	>45 (05.10.2022)
Cites regulation	No	No	No		No	Appendix II

[a]This summary can help evaluate the suitability of a system of interest based on similarities and dissimilarities with tropical scleractinian corals. NA, not applicable; mos, months; wks, weeks; yrs, years.

over time in Aiptasia provides a powerful analog to coral bleaching responses (e.g., references 125, 127, and 128). However, for rendering animals aposymbiotic for use in experiments on symbiosis reestablishment, menthol bleaching is the most rapid and the most effective at eliminating essentially all symbionts from host tissues (126).

Depending on host origin, Aiptasia harbors different *Symbiodiniaceae* species. Most laboratory animals contain *Breviolum minutum* (including host strain H2), or *Symbiodinium linuchae* (including host strain CC7). Both of these symbiont species have been successfully brought into culture and the genomes sequenced (129, 130). In addition, Aiptasia is tolerant of a variety of non-native symbiont species, including *Symbiodinium microadriaticum* and *Durusdinium trenchii*, two species with differing susceptibility to environmental perturbation (131–134). These species can enter hosts but are less successful than native species at populating them, and the metabolic exchange and interpartner homeostasis is perturbed (122, 135, 136). Similar to polyps, Aiptasia larvae can take up a variety of algal species and subsequent persistence and proliferation appears to depend on the ability of the microalgae to suppress host innate immune response and escape expulsion (vomocytosis) (137). This diversity in specificity allows for the study of comparative *Symbiodiniaceae* repopulation dynamics, mechanisms of recognition, specificity and regulation, and differential susceptibility to heat stress or other perturbation (e.g., see references 133 and 138–140).

The Aiptasia microbiome has now been described in a variety of strains from around the world (141–144). Overall, there is congruence between the different studies, showing a similar microbiome makeup among animals in culture and similarity in the taxonomic diversity of microbiomes between Aiptasia and corals. These baseline descriptions of the Aiptasia microbiome set the stage for future studies on the effect of heat stress and other environmental perturbation on the Aiptasia holobiont, the interaction of the algal symbionts with the microbiome, and the possibility of manipulating the microbiome to aid in building coral resilience. Indeed, some of this work has already begun (113, 145).

The life history characteristics of Aiptasia contain both key strengths, and at present, significant limitations to its value as a model system. For example, asexual reproduction (pedal laceration) facilitates clonal propagation, but sexual reproduction has not yet been achieved in captivity. Aiptasia is gonochoristic, although there is some evidence that animals can switch sex (146). Animals spawn non-symbiotic gametes and therefore onset of symbiosis must occur anew with each host generation. Further, researchers have now developed culturing conditions that result in predictable and repeated spawning of gametes, successful fertilization and subsequent rearing of F1 larvae (147–150). These larvae can establish symbiosis with algae from culture, which again provides a powerful system to examine mechanisms of recognition and specificity. To date however, despite considerable effort by several research groups, there has been no success in achieving larval settlement and metamorphosis into juvenile polyps (unpublished data). This presents a major barrier to conventional genetics, gene editing, or other gene knockdown techniques in Aiptasia (151) that would revolutionize our ability to discern host gene function in the symbiosis.

There are other aspects of Aiptasia biology that warrant further development to cement this organism as a key model. The host processes of pedal laceration and subsequent patterning that results in development of clonal juveniles has been described (152–154). However, the role of symbiosis in these processes is just beginning to be described (155) and is a topic ripe for future work. Finally, although we believe animals harboring *B. minutum* occur pan-tropically, whereas those containing *S. linuchae* appear restricted to the Florida Keys (156, 157), there is a lack of global sampling studies describing natural symbiosis states. Such surveys could be further expanded to include other symbiotic anemone species within the Aiptasiidae. This would extend the relevance of the model system into a comparative genomic and ecological framework and explore conservation in the molecular evolution of marine endosymbioses.

Jellyfish Cassiopea

The Upside-down Jellyfish *Cassiopea xamachana* (Bigelow, 1892) has been a powerful model to study developmental symbiosis for more than four decades (reviewed in

reference 158). In recent years, the "Cassiopea" model has gained increasing attention as a system to study cnidarian symbiosis (78, 158), with a complete genome now available (159, 160). All nine species in the genus are found in tropical and subtropical waters around the world, although the distribution range of *C. xamachana* is limited to the Caribbean and Gulf of Mexico (summarized in reference 158), and therefore the adoption of this species as a model organism might need to overcome transport restrictions (as previously discussed for Aiptasia). Like corals, Cassiopea establishes an obligatory association with *Symbiodiniaceae* algae (161, 162) and is therefore employed to study the bleaching response of cnidarian-algal symbiosis to heat stress (163, 164). Cassiopea is suited to laboratory rearing and investigations since it can tolerate a broad range of environmental conditions (163, 165, 166), is noncalcifying, and has a short life cycle that can be completed in the laboratory in four to six months. Embryos can be collected daily from the brooding region of female medusae, a trait which facilitates genetic (e.g., microinjection) and developmental studies (e.g., embryogenesis) (reviewed in reference 158).

The Cassiopea life cycle allows easy access to different life-stages that also differ in their dependence on *Symbiodiniaceae*. Female medusae constantly release brooded swimming larvae that settle and metamorphose into polyps upon encountering microbial settlement cues (161). These polyps (scyphistomae) reproduce by budding, and large clonal aposymbiotic populations can easily be maintained under laboratory conditions with regular feeding as "immortal lines" (158). The establishment of symbiosis with *S. microadriaticum* triggers strobilation, a metamorphic transition into sexual ephyra (i.e., free-living juvenile medusae), termed symbiosis-driven development (167). Interestingly, polyps can establish symbiosis with a broad range of *Symbiodiniaceae* species (168), but only some elicit metamorphosis (169). This system enables developmental, genetic, and physiological comparisons of the onset of symbiosis in the same host genetic background. On the contrary, adult medusae depend on their *Symbiodiniaceae* partner (162, 170) toward which they show a high degree of selectivity and specificity (164, 169, 171), not unlike differences in symbiotic specificity between juvenile and adult corals (172).

These characteristics make Cassiopea particularly suitable for symbiosis manipulations. Both polyps and medusae can be bleached through temperature stress (162, 171) or menthol treatment (170) and, although lack of the algal symbionts eventually leads to death (162), aposymbiotic medusae can survive for more than 3 weeks (170). Comparison between symbiotic states can help unravel each partner's contribution to holobiont functioning, such as nutrient uptake and dynamics, and the effect of *Symbiodiniaceae* presence or absence on the bacterial microbiome (170). Further, aposymbiotic polyps can reestablish symbiosis with native or non-native *Symbiodiniaceae* strains (164, 169, 171). The ability to obtain different polyp-*Symbiodiniaceae* associations will in turn allow the production of clonal polyps that harbor different microbiomes. This, together with efforts to develop axenic and gnotobiotic animals, will also open doors to systematically explore host-microbiome interactions with the Cassiopea model system (158).

Another outstanding feature of Cassiopea is its ability to maintain a functional symbiosis across a wide range of environmental stressors (163, 165, 166). This trait can therefore help identify mechanisms that confer tolerance to changing environmental conditions in reef-building corals (170, 173). For example, Cassiopea can withstand high temperatures, showing onset of bleaching between 37 and 40°C (163). Also, high nutrient loads, that typically destabilize the coral-algal symbiosis and lower their bleaching threshold (174), are well tolerated by Cassiopea (175). Tracking of uptake and translocation of isotopically labeled nutrients suggest that Cassiopea is able to exert control over its algal symbionts' capacity to access N, specifically by restricting nitrate (170, 173), the N species linked to decreased heat tolerance in corals (176).

The Cassiopea system is marked by peculiarities that further distinguish it from stony corals and that contribute to its large environmental tolerance. While corals host *Symbiodiniaceae* in their gastrodermal cells, in Cassiopea these are predominantly located inside amoebocytes (177). Amoebocytes are motile cells found in the

mesoglea, which can be actively redistributed to meet energetic demands across different body parts (173). In addition, Cassiopea has a greater capacity to enrich its nutrient environment compared to corals (178). Rather than relying on currents to transport particle and solutes, Cassiopea uses bell pulsation to generate flows that draw particles (e.g., zooplankton) from the surrounding seawater to its feeding appendages and mobilize nutrients from the underneath sediments (178). Discoveries made on Cassiopea therefore need to be contextualized considering these aspects.

Temperate Coral *Astrangia poculata*

The temperate coral *Astrangia poculata* (Ellis & Solander, 1786) is one of the very few calcifying cnidarian model organisms currently available. Since this species is more amenable to rearing in aquaria (compared to the majority of tropical scleractinians), it represents an attractive, and increasingly popular, model system. Colonies are easy to collect since they are abundant in coastal, easily accessible locations across the western Atlantic. On average, colonies carry about 20 to 100 polyps, can grow to ~10 cm in diameter, and are gonochoric (carry separate sexes). Spawning is synchronous and inducible in the laboratory throughout the period of late July to early October, mirroring patterns of gametogenesis (179). In addition, *A. poculata* are gaining attention not only as an emerging model system but also as an emblem for coral and climate change research, demonstrated by its designation in 2021 as the Official State Coral of RI, USA.

Above all, two aspects (and their implications) are particularly remarkable about *A. poculata*: the nature of its photosymbiosis and its outstanding thermotolerance. *A. poculata* facultatively engages in symbiosis with the photosymbiont *Breviolum psygmophilum*. Sympatric colonies can be found in different symbiotic states (symbiotic, aposymbiotic, and patchy/mixed) across all seasons (77, 180), and photosymbiont density can be artificially manipulated (increased with high light intensity; decreased with low light intensity) (R. Rotjan, unpublished data). Because tropical corals often cannot be decoupled from *Symbiodiniaceae* without imposing stress, many critical, basal questions regarding this symbiosis are difficult to address in corals directly. Here, the *A. poculata* model can be particularly advantageous. Tracking nitrogen uptake and translocation in both symbiotic and aposymbiotic *A. poculata*, for example, helped elucidate how nutrient availability modulates the coral-algal relationship, specifically suggesting that nitrogen and carbon limitation shift the coral-photosymbiont mutualism toward parasitism (181). Similar to Cassiopea, *A. poculata* can tolerate extreme temperatures—withstanding what is among the largest temperature ranges that any hard coral has been documented to experience in its natural habitat. In the species' northernmost distribution (southern New England), seawater temperature seasonally fluctuates over a range exceeding 20°C, with average temperatures spanning from 4 to 29°C (77). This annual temperature range compares to that of the Persian Gulf, the region with the most extreme environmental conditions where tropical reef-building corals persist (with recorded extremes spanning from ~11 to 36°C [182, 183]). Although the thermal environment of *A. poculata* is much colder than that of coral reefs, this ability to cope with such a large temperature range makes *A. poculata* an excellent experimental system for identification of genes and critical mechanisms of thermal tolerance. For example, comparison of gene expression between symbiotic states of *A. poculata* under thermal stress demonstrated that many stress-response genes previously identified in tropical corals likely belong to the host, as these were also present in aposymbiotic specimens (184), while transcriptional profiles of *Symbiodiniaceae* remain relatively unaffected by heat stress in corals (185). These experiments mirror physiological and metabolic patterns of the coral holobiont under stress (186, 187) and underline the potential to directly transfer insights gained from studying *A. poculata* to tropical reef-building corals. Furthermore, explorations of *A. poculata* in its natural environment across seasons and along latitudinal gradients can be used to test the influence of symbiosis and seasonality on microbe-microbe interactions within the holobiont. Across the year, *A. poculata* experiences shifts in photosymbiont density similar to those described for stony corals (188, 189), and the onset of a state of cold-induced

quiescence (dormancy) during the winter months (190–192). Recent research efforts have utilized individuals from these naturally occurring gradients to identify microbes and multipartner (*Symbiodiniaceae*-bacterial) interactions important in the cnidarian response to environmental changes. Recent work on wild *A. poculata* colonies showed that the influence of photosymbiont density on the taxonomic structure and activity of the bacterial and archaeal community was smaller than that of seasonality (77). These findings largely agree with the stable bacterial communities found in cold shock bleached Aiptasia (141) and heat-stress bleached *Porites lobata* and *Pocillopora acuta* corals (193, 194) and support the generalization that external (environmental) factors have a stronger effect on microbiome structuring than photosymbiont density alone. Interestingly however, the presence of *B. psygmophilum* appears to facilitate consistent recovery of the bacterial and archaeal communities in *A. poculata* after antibiotics treatment (195). In these studies, the *A. poculata* bacterial community shares similarities in taxonomic structure with those of tropical corals but is remarkably less species-rich and more predictable (51, 77). As next steps, development of protocols is under way for spawning, embryonic development, larval rearing, larval settlement, and post-settlement growth to enable experimental examination of processes governing multipartner symbioses, including symbiont recruitment, establishment, and succession.

Increasing the Power of Cnidarian Model Systems

The traits that make the discussed cnidarian model organisms convenient study systems (and ecologically successful species) also set them apart from tropical reef-building corals. For example, features that greatly facilitate experimental investigation such as the lack of a carbonate skeleton, facultative photosymbiosis, and broad environmental tolerance (or "hardiness" of a species) have relevant physiological implications, and ignoring them might leave important biological mechanisms unaddressed. Therefore, to be informative for coral reef conservation, discoveries made from model systems should not be viewed as standalones but contextualized within a broader framework. To increase the power of these experimental model organisms, it is therefore necessary to adopt combined research approaches that use multiple models chosen for the complementarity of their features and that rely on multi-institutional collaborations (see "A Trait-Based Approach To Identify Suitable Coral Species" below and Table 2 for a summary of similarities and dissimilarities between the discussed organisms).

DIRECT TESTING AND EXPERIMENTATION ON CORALS THROUGH HOLOBIONT SIMPLIFICATION

Tissue Cultures as Structural Simplification

Structural simplifications offered by tissue cultures and cell lines allow for direct miniaturization of tropical stony coral systems. They eliminate skeletal components, which increases optical transparency aiding visualization, and liberates sample processing from the interferences of the aragonite particles and Ca^{2+} ions. As in the examples of the cnidarian model systems, a small sample size here aids high replication, translates into faster and less expensive workflows, and uses available live material efficiently.

The first step of structural simplification of the coral host involves the isolation or explantation of tissues or cells and maintenance of these as "primary cultures" or "tissue explants." This can be undertaken in a number of ways (Table 3). So-called "destructive approaches" affect tissue organization by breaking down cell-cell or cell-ECM (extracellular matrix) adhesion, through the removal of divalent cations (Ca^{2+}, Mg^{2+}), enzymatic digestion of ECM components, and single-cell isolation through gravimetric fractionation or sieving of digested tissues (reviewed in reference 196). Isolated coral cells then reaggregate into multicellular structures. Nondestructive approaches preserve the original tissue organization and can be achieved mechanically by cutting (197, 198) or peeling off coral tissues (199, 200), or physiologically by inducing a stress response mechanism called polyp bail-out (201–203). Destructive approaches remain prone to microbial contamination that hinders long-term survival (196) (Table 3).

TABLE 3 Chronological overview of studies on coral cell and tissue cultures with synthesis of culture origin and type, viability, and use of antimicrobials

Tropical scleractinian coral species group(s)	Other cnidarian species	Life stage	Suspended and/or adherent[a]	Viability[b]	Proliferation[c]	Isolation approach[d]	Antibiotic and/or antimycotic[e]	Year of publication	Reference(s)
Fungia scutaria, Pocillopora damicornis	*Boloceroides sp., Cassiopea xamachana, Exaiptasia diaphana (formerly Aiptasia pulchella), Zoanthus sociatus*	Adult	S	5 h	NO	Des		1992	252
Acropora microphthalma, Montipora digitata, Pocillopora damicornis, Porites sp., Seriatopora hystrix, Stylophora pistillata		Adult	S	12 days	NO	Des	AB, AM	1999	204
Pocillopora damicornis		Adult	A, S	7 to 12 days	NO	Des	AB, AM	2001	207
Pocillopora damicornis, Stylophora pistillata		Adult	A	7 days	NO	Des	AB, AM	2004	202, 211
Montipora digitata	*Xenia elongata*	Adult	A	21 days	NO	Des	AB, AM	2007	208
Acropora millepora		Planula	S	10 wks	CP	Des	AB, AM	2009	214
Fungia sp.,Pavona divaricata		Adult	S	4 days	NO	Des		2009	205
Pocillopora damicornis		Adult	S	9 days to 2 mos	NO	Des		2010	325
Favia favus, Fungia granulosa	*Oculina patagonica*	Adult	A, S	3 mos to >3 yrs	After	NDes		2011	199
Stylophora pistillata		Adult	A	6 to 8 wks	NA	Des	AB, AM	2012	209
Pocillopora damicornis		Adult	S	>2 days	NS	Des	AB, AM	2013	206
Pocillopora damicornis		Adult	S	5 to 11 days	NA	NDes		2014	197
Fungia granulosa		Adult	S	>2 mos	NA	NDes	AB	2015	200
Pocillopora damicornis, Seriatopora hystrix, Stylophora pistillata		Adult	A, S	Wks to mos	After	NDes		2016	203
Stylophora pistillata		Adult	A	>12 days	NA	Des	AB, AM	2017	210
Goniopora lobata		Adult	S	NA	NA	NDes		2017	198
Pocillopora damicornis	*Nematostella vectensis*	Adult	S	12 to 13 days	Reported	Des	AB	2021	216
Pocillopora acuta		Adult	S	7 to 10 days	NO	Des	AB, AM	2021	213
Acropora tenuis		Planula	S	>8 mos	CP	Des	AB, AM	2021	212

[a]S, suspended; A, adherent.
[b]wks, weeks; mos, months; yrs, years.
[c]NA, not applicable; CP, clear proliferation; NS, not significant; NO, not observed; After, after settlement and differentiation; Reported, reported but time limited.
[d]Des, destructive; NDes, nondestructive.
[e]AB, antibiotic; AM, antimycotic.

Nondestructive approaches only require minimal treatment to control contamination and have, on average, longer viability (Table 3).

Coral tissue explants can be maintained in either suspended (204–206) or adherent cultures (202, 207–209). Suspended aggregates are ball-shaped and present a tissue organization similar to coral colonies (204–206). These also maintain a photophysiology comparable to that of the parental colony (200). They have been used to investigate the involvement of light and oxidative stress in coral bleaching (205) and to study ecotoxicology of cryoprotectants (197). Interestingly, the method developed by Vizel et al. (199) produced explants that can be kept for several months in an undeveloped state or induced to develop into a polyp that calcifies and ultimately regrows into a colony. In addition, *Symbiodiniaceae*-free tissue balls potentially provide a tool to complement investigation on the coral-algal symbiosis (198).

Adherent aggregates are generally flat, but spatial relationships between the diverse cell types are not well resolved between isolation protocols (202, 207–209). Only these adherent aggregates (as opposed to tissue balls) calcify *in vitro*, likely owing to the presence of the ECM and skeletal organic matrix which are secreted *de novo* (207, 208). The easy access to the site of calcification in adherent cultures has allowed researchers to reveal important details of the calcification process, such as its conditional independence from photosynthetic activity of the *Symbiodiniaceae* partner (209), the intracellular commencement of the biomineralization process (209), and its dependence on multicellularity (e.g., see references 207, 208, and 210). The use of adherent cultures also produced the first evidence of interactions between coral cells and an associated endolithic fungus (211). Although it appears to be limited to pocilloporid corals, polyp bail-out induced through controlled salinity stress is arguably the most successful approach for obtaining adherent coral micropropagates (201, 203). The resulting micropropagated polyps fit inside a microfluidic platform, the "coral-on-a-chip", representing one of the most advanced tools for live study of coral physiology (203). This made it possible to observe microscopic phenomena in real time, such as calcification, coral-pathogen interactions, and coral bleaching (203).

Untapped Potential of Coral Cell Cultures

The next stage in structural simplification of the coral host can be achieved with secondary cultures and cell lines (cultures that can be propagated indefinitely). These are less-representative versions of the original biological system because they filter out some cell types, but they overcome the time limitation of primary cultures and the need to continuously source from living organisms. To date, most coral-derived cell cultures have only short viability and coral cell lines have been established only very recently (212, 213) (Table 3). The most remarkable achievements in terms of culture longevity rely on cells originating from larvae (212, 214, 215). This approach resulted in mixed cell cultures visibly proliferating and remaining viable for between 10 weeks (214) and upwards of 8 months, with the possibility of restarting the culture after cryopreservation and rewarming (212, 215). To this point proliferating cultures either contained mixed, often unidentified, cell types (214, 216), or homogeneous, but not *a priori* specifically selected for, cell populations (212). Nevertheless, cell lines with specific properties could be selected *a posteriori* from the pool of available established cell lines, each of which reportedly expressed specific and consistent sets of genes (over time) reminiscent of different cell types (e.g., gastrodermis, epidermis, and secretory, undifferentiated, and neuronal cells) (212). This was demonstrated (in principle) after cells from a line showing endoderm-like properties successfully established endosymbiosis (*in vitro*) when exposed to cultured *Symbiodiniaceae* (215).

Among the various cell types, stem-cell like cells are particularly sought after because of their potential to initiate persistent cell lines. Currently all immortal animal cell lines originate from tumors or from experimentally reprogrammed cells (217). These behave substantially differently than physiological cell populations (218). For that matter, tropical corals (and other cnidarians) are an attractive subject for cell cultures, owing to their longevity (219) and regenerative capacity, which suggests that stem-cell like cell populations remain abundant throughout the life of these organisms (220). While adult somatic cells of hydrozoans have been described to dedifferentiate (i.e., return to a pluri- or totipotent state) and

transdifferentiate (i.e., differentiate into different cell types) (220–222), a mechanistic understanding of coral self-renewal is still lacking (223). Stem-cell like cell lineages have been identified and characterized in cnidarians (class Hydrozoa) as interstitial cell lines (or I cells) (220, 224). In corals (class Anthozoa), protease-treated larval-derived cells assumed amorphous shapes with extended pseudopodia capable of proliferating *in vitro* indefinitely. These appeared morphologically similar to endoderm precursor cells (212) and resemble amoebocytes, which are hypothesized to play a similar role to I cells in non-hydrozoan cnidarians (223). Gene expression patterns, enzymatic activity assays such as applied to human hematopoietic stem cells based on aldehyde dehydrogenase, and fluorescence-activated cell sorting (FACS) could help locate stem-cell like cells in corals (225, 226), and some coral cell lines have indeed shown properties of progenitor cells (212). However, even single-cell RNA sequencing of FACS-sorted cells from swimming larvae, primary polyps, and adult colonies of *Stylophora pistillata* could not identify any stem-cell like cell populations (227). The cellular basis underlying coral regenerative capacity thus remains elusive.

Another limitation in establishing coral cell cultures is the lack of coral-specific culturing formulations (213). The culture media used presently seem to favor growth of contaminants rather than of coral cells, and dilution leads to better results (196). In addition, for many coral cell types, proliferation likely requires initial adhesion to a substrate (196, 213). Therefore, lack of knowledge about the structure and function of regenerative systems and of appropriate culturing conditions hinder the culturability of coral tissues and cells (196, 213). This field might benefit from a change of perspective that focuses on the peculiarity of corals rather than on their similarity with terrestrial metazoans. Culturing techniques could be improved through reverse-engineered approaches that use (meta)genomic, transcriptomic, and proteomic information to guide the design of culturing media and protocols, as pioneered in bacterial culturing (228, 229).

Simplifying the Coral Holobiont by Disassembling Its Members

In the intact coral holobiont, the interdependence and complementarity of processes underlying fundamental functions hamper the understanding of contributions of individual members (80). Hence, as a complementary strategy to structural simplification, the complexity of the coral holobiont can also be simplified by disassembling and isolating its members (host and microorganisms, respectively). Such approaches can guide studies aimed at clarifying partner dynamics, and generate predictions that will then ultimately need validation through the controlled reassembly of metaorganism components (80, 100).

Possibility of gnotobiotic coral hosts. Axenic (germ-free) or gnotobiotic hosts (where all associated microbes are known), are powerful tools to study the role of the microbiome in health and fitness (as reviewed in reference 80). Comparison between symbiotic and axenic individuals allows us to explore the contribution of the microbiome to host physiology, and targeted inoculation with selected microorganisms can help identify causative links to their function (100, 101, 105). By combining established *Symbiodiniaceae* and bacterial depletion protocols, the generation of gnotobiotic coral hosts can be broken down into sequential steps.

The removal of *Symbiodiniaceae* from the gastrodermal tissue by bleaching produces aposymbiotic hosts and can be seen as a first step in holobiont simplification. Ideal methods maximize bleaching efficacy while they minimize the impact on the host and the remaining microbiota. Temperature stress was among the first methods utilized to bleach corals in artificial systems (27); however, it may result in high host mortality, not be fully effective, and potentially influence thermotolerance in subsequent studies (126, 230). The herbicide Diuron or DCMU [*N*′-(3,4-dichlorophenyl,-*N*,*N*-dimethylurea)] overcomes most of these limitations, but it also does not lead to complete bleaching and it is a hazardous substance (231, 232). Menthol is considered a more "gentle," yet effective, bleaching agent that has now been applied to many cnidarians (170, 232, 233). However, the exact mechanism triggering the bleaching response remains to be fully elucidated (232, 234). Menthol-bleached hosts remain aposymbiotic (for at least 15 weeks, in Aiptasia) after the cessation

of the treatment (126) and can subsequently be employed in experiments and reestablish symbiosis with *Symbiodiniaceae* (126, 232). Of note, while a fully aposymbiotic state can be difficult to achieve (and prove), removing ~98% of the symbiont population appears sufficient to allow inoculated non-native symbionts to establish and repopulate the host (235). How these chemical bleaching agents affect other microorganisms in the coral holobiont, however, remains unknown.

Completing a fully sterile life cycle is the gold standard of developing true axenic animals (236). Similarly, protocols for creating axenic Hydra polyps make use of sterile rearing techniques and its closed life cycle in the laboratory (112), a prospect that may become attainable in research on corals given advancements in artificially producing larvae from brooding species (237, 238) and *ex situ* techniques for spawning coral species (239, 240). Among cnidarians, Hydra, Nematostella, and Aiptasia adult polyps can be rendered gnotobiotic through antimicrobial treatment (100, 113, 241). This represents a complementary or alternative approach, which has been successful in eliminating more than 99% of the microbial load of corals (242), when a closed life cycle is not (yet) available (100, 236, 241). However, microbial load can recover in as little as 96 h once dosing stops (242). Although no protocol for long-term maintenance of gnotobiotic corals after antimicrobial treatment is available yet, it could be argued that organisms that are allowed to develop naturally (i.e., with their native microbiome), and only later undergo microbial depletion, represent more realistic models of wild-type organisms (100, 236).

The efficacy of the holobiont disassembly process will vary between coral life histories. First, coral species have different strategies of acquiring their microbiome and vary in microbiome flexibility at early life stages (243) and as adults (33). The main division occurs between broadcast spawners and brooders (244), with the former acquiring *Symbiodiniaceae* and likely bacteria horizontally from the environment (244, 245), while the latter mostly inherit them vertically from the parental colony (172). Second, in contrast to axenic mice and Hydra, raising axenic corals may be challenging as it remains unclear whether corals require the presence of specific microorganisms to complete development (246). Finally, antimicrobial treatment might be detrimental as it was shown to cause disaggregation of tissue in coral larvae (214) and adults in some cases (216). Since this might be a feature of the specific antimicrobials employed, testing substances with different mechanisms of action (e.g., azoles) is warranted. Nevertheless, antimicrobial treatment may not be effective in the long-term due to difficulties of antibiotics reaching the inner skeleton (21, 242). Early-life stages after settlement may therefore be particularly suitable for manipulation, as they have more dynamic microbial communities than do adult corals (45, 243).

***Symbiodiniaceae* cultures.** *Symbiodiniaceae* cultures represent the first established and most advanced cell culturing technique in coral research. *Symbiodiniaceae* are routinely studied for their properties in culture (247, 248) and are used to study early symbiont acquisition by larvae, symbiosis establishment and reestablishment (e.g., following bleaching) dynamics, and comparative physiology in the host (133, 149, 169). While historically only a small proportion of coral-associated *Symbiodiniaceae* have been considered culturable (249), new approaches such as isolation and culturing from single cells promise innovation in the field (250).

Symbiodiniaceae in culture experience substantially different conditions compared to those *in hospite* (within the host) and therefore do not perfectly replicate endosymbiotic dynamics (215, 251, 252). Many cultures utilize antibiotics to keep bacterial contamination to a minimum, but such practices could affect *Symbiodiniaceae*, either through side effects of the antibiotics or through the induced loss of their bacterial associates (253, 254).

Bacterial cultures. The majority of our knowledge on coral-associated bacteria is based on 16S rRNA gene amplicon sequencing, while metabolic pathways and interactions in the holobiont are less well-explored (55). The use of culture-based approaches may be one option which will likely provide additional insight into microbial functions (e.g., reference 255), but which has largely been forgotten about in favor of next generation culture-independent methods. Sweet et al. (51) recently curated data of the

diversity and function of cultured bacteria (both published and unpublished) from tropical, temperate, and cold-water corals. This resulted in a catalog (isolates.reefgenomics.org) of 3,055 unique isolates that spanned 138 species and 12 putatively novel bacterial genera across the *Pseudomonadota* (*Proteobacteria*), *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla. Available genomes from these bacteria were considerably sparse, but those available (74 at the time of writing) allowed the researchers to analyze biosynthetic gene clusters underlying the production of secondary metabolites important in host health and symbiosis (51, 256). Despite this promising start, most bacteria have yet to be cultured, and some have even been deemed unculturable (257). Indeed, a metadata analysis of SSU rRNA gene sequences from bacteria and archaea associated with corals found that only 6.5% of these were generated from cultured isolates (258). Unlocking at least part of this additional diversity is likely to be achieved with alternative isolation and cultivation procedures, inspired by advancements in the broader microbiological field (259, 260). For example, the gradients of physicochemical growth conditions could be widened within the 'culturomics' framework (238), and implementing microfluidics systems (261–263). The growth of obligate symbiotic or syntrophic bacteria could be achieved through coculturing (264), and growth media and sorting methods could be developed through omics-guided approaches (228, 229).

Microbial contaminants in cultures: friends or foes? Invasion of cell cultures by microbial contaminants is a universal issue. Antimicrobial agents routinely employed in terrestrial animal cell cultures are largely ineffective against coral microbial contaminants, which are known to overgrow and cause the termination of coral cell cultures (200, 214, 216, 265). While there is clearly a need for coral-specific antimicrobial treatments, corals are exceptional in hosting microbes not only on external surfaces and mucosa but also in all other tissue compartments and in the skeleton (21, 53). In addition, although cultured *Symbiodiniaceae* strains are often treated with antimicrobials (249) and can in some cases be maintained axenically (118), *Symbiodiniaceae* cultures also harbor abundant and characteristic bacterial microbiomes (266). Recently, bacteria have also been reported to associate with *Symbiodiniaceae* intracellularly as well as extracellularly (267). This suggests a tight involvement of the coral microbiome in holobiont functioning and regulation. Indeed, the longest viability of coral explants was achieved from protocols that did not use antimicrobials (199, 203) (Table 3). In contrast, the first coral cell lines were grown in media containing antimicrobials (212, 215). This indicates a potential connection between the presence of associated bacteria and the formation of complex structures (tissues), and represents an incentive to investigate whether so-called microbial contaminants might comprise key coral associates.

Reassembling the Metaorganism for Hypothesis Testing

The study of isolated holobiont members is necessary to generate hypotheses on their functions and the dynamics of their interactions (100, 241); however, these hypotheses then need to be tested in a metaorganism context (80). For this purpose, reassembling metaorganisms represents the ultimate testing ground. Practically speaking, the inoculation of axenic or gnotobiotic hosts with cultured or "transplanted" (46, 268) microbial isolates will elucidate the intra- and interkingdom interactions underpinning holobiont functioning. This approach borrows from the field of human gut microbiome, where studies on animal models could demonstrate causative links between the presence of specific bacteria and the host phenotype. For example, the introduction of a single gut-residing bacteria in axenic mice led to the development of autoimmune arthritis (269) and the presence or absence of a bacterial consortium modulated food allergy in the host (270). More recently, several authors have proposed to adopt this type of approach based on success in other cnidarian models (100, 105, 110, 113, 241, 271, 272) and on promising first applications in some reef-building coral species (46).

TROPICAL STONY CORALS AS CANDIDATE MODEL SPECIES

The validation of laboratory results from model systems on true corals remains irreplaceable in the transition from controlled experiments to practical implementation of

conservation activities. Non-coral model organisms lack important features such as the aragonite skeleton, obligate nature of the symbiosis with *Symbiodiniaceae*, and adaptation to oligotrophic conditions. Although the term "coral model" is found in the literature and attributed to a number of species (see reference 213 and references therein), to date there is no formally established or universally agreed-upon true coral model organism. Establishment of such models should start by identifying a group of promising species which possess characteristics that maximize amenability to experimentation (tractability) and informative power (transferability of knowledge to ecologically relevant contexts). Because tractability and transferability often can be antithetic, we discuss the most relevant factors that affect these two properties in selected candidate coral model species (see, e.g., Table 4).

Trait-Based Approach To Identify Suitable Coral Species

Tractability. Tractability is the sum of a set of characteristics that make a coral species a practically more convenient study subject (Table 4). These characteristics include: (i) broad availability in geographic distribution and abundance in the reef (however, this aspect may only be relevant in the initial exploratory stage: once the species becomes established as a model organism, research will focus on a few selected strains/clonal lines shared between research groups, overcoming the need to source colonies from the wild); (ii) compatibility with growth under aquarium conditions to maintain long-term cultures or living collections (this includes for example colonies of *Stylophora pistillata* that have been kept in aquarium culture for more than 3 decades) (273); (iii) amenability to bleaching, maintenance in a bleached state, and symbiosis reestablishment to disentangle host and *Symbiodiniaceae* contribution to holobiont functioning (85); and (iv) larger polyps (e.g., in *Galaxea fascicularis*) offer the option to isolate a single polyp, reducing complexity from the colony to the individual level (274, 275). Corals with larger polyps are also easier to visually examine (276, 277), not to mention separating the tissue from the skeletal matrix (197–200). (v) Finally, further case-specific advantageous traits could also include the continuous release of larvae (237, 238) with the long-term prospect of achieving a closed axenic life cycle or a consistent budding and polyp bailout response to reliably produce tissue cultures (203, 278, 279). Some of these traits are correlated and tend to co-occur. For example, survival in the bleached state is linked to polyp size because it generally correlates with heterotrophic feeding capacity (280).

Transferability. Coral model species with high tractability will be representative of only a subsample of the scleractinian taxon. Therefore, their representativeness and transferability should be considered targeting a diverse suite of model systems. We suggest identifying coral model species that aim at collectively covering the broadest range possible of the following aspects (Table 4): (i) geographic distribution, where at both larger and smaller scales, different regions are dominated by different species of both coral host and associated *Symbiodiniaceae* (15, 281), and (ii) phylogeny, where metabolism and microbiome composition differ between the two major coral clades (282, 283) and thermal tolerance varies between coral families (284). Of the hundreds of extant tropical reef-building coral species, only a few have been extensively investigated, with the majority of studies focusing on members of just a few families (29). Although this might seem like an underrepresentation, it is noteworthy that the three most abundant families (Acroporidae, Pocilloporidae, and Poritidae) make up ~70% of corals on the coral reefs worldwide (see reference 285 and references therein). In addition, *Symbiodiniaceae* identity should also be considered, since it is known to affect metabolism and thermotolerance (286, 287). The following aspects should also be taken into account: (iii) colony morphology, which plays an important role in thermotolerance (284, 288) and is influenced by external conditions (289); (iv) trophic strategy, which can range from more autotrophic to more heterotrophic and also affects holobiont thermal tolerance (280); (v) habitat preference, which determines the exposure and acclimatization potential to stressors (290, 291) and can vary with depth, distance from shore, and reef type and topography, among others; (vi) reproductive mode,

TABLE 4 Relevant features to identify and evaluate the suitability of candidate coral species to the establishment of true coral model organisms[a]

	Observation(s)[b]				
Characteristics	***Pocillopora damicornis***	***Stylophora pistillata***	***Acropora millepora***	***Galaxea fascicularis***	***Orbicella faveolata***
Tractability					
Distribution (% of global ecoregions according to CotW)	Very broad (85.3)	Broad (68.7)	Broad (56.7)	Broad (67.3)	Localized (5.3)
Suitability to aquarium rearing	High, aquarium culture >10 yrs (326)	High, aquarium culture >30 yrs (273)	High, completed life cycle *ex situ* (240)	High (327, 328)	High (329)
Amenability to experimental bleaching	Unknown	High, effective with menthol (232)	Unknown	High, effective with menthol (328)	Unknown
Polyp size range in mm (corallite diam)	0.8–1 (WoRMS, CTD)	0.9–1.4 (CTD)	0.4–1.6 (CTD)	5–10 (CotW)	2–3 (CotW)
Others	Polyp-bailout stress response (203, 330, 331)	Polyp-bailout stress response (203)	First broadcast spawning coral to produce F2 fully *ex situ* (240)	Protruding corallites allow easy isolation of individual polyps; polyps remain extended during daytime; large egg size (332)	Major reef builder in the Caribbean (333)
Transferability					
Natural occurrence: oceanic basin	IndoPacific (CotW)	IndoPacific (CotW)	IndoPacific (CotW)	IndoPacific (CotW)	Atlantic (CotW)
Taxonomy: host clade	Robusta (334)	Robusta (335)	Complexa (334)	Complexa (334)	Robusta (336)
Taxonomy: host family	Pocilloporidae (WoRMS)	Pocilloporidae (WoRMS)	Acroporidae (WoRMS)	Euphylliidae (WoRMS)	Merulinidae (WoRMS)
Taxonomy: *Symbiodiniaceae* genus	*Cladocopium* spp. most common, *Symbidinium* and *Durusdinium* spp. also found (CTD)	*Cladocopium* spp. (CTD)	*Cladocopium* spp. (CTD)	*Cladocopium* and/or *Durusdinium* spp. (337–341)	*Brevioulum* spp. predominant; *Symbidinium*, *Cladocopium*, and *Durusdinium* spp. also found (CTD)
Colony morphology	Branched, usually <30 cm tall (WoRMS)	Branching to submassive (CofW)	Corymbose cushions or clumps (CotW)	Massive (often dome-shaped) or columnar (CotW)	Massive, sizes up to 10 m (CotW)
Trophic strategy			Relatively autotrophic (280)	Mixotrophic but with great variability (342)	
Habitat preference depth	0 to >40 m (particularly abundant at 5 to 20 m) (WoRMS)	1 to 65 m (343)	2 to 30 m (CTD) (344)	2 to 20 m (CotW, WoRMS)	0.5 to 40 m (CTD)
Reproductive mode	Brooder (predominantly) and broadcast spawner (244)	Brooder (peculiarity: protandrous simultaneous hermaphrodites) (345)	Broadcast spawner (244, 346)	Broadcast spawner (peculiarity: pseudogynodioecious) (347)	Broadcast spawner (348)

[a]Tractability refers to traits that facilitate experimental work, while transferability indicates the most relevant aspects to consider to address the broad variation encompassed by the scleractinian taxon. This list can be considered a template or guide to be applied beyond the species listed here.

[b]Abbreviations: CotW, Corals of the World (www.coralsoftheworld.org); CTD, Coral Trait Database (https://www.coraltraits.org/); WoRMS, World Register of Marine Species (https://www.marinespecies.org). Reference sources are indicated parenthetically where applicable.

which determines the mode of microbiome transmission between generations and affects the stability of the microbiome and the potential for transgenerational adaptation (23, 292, 293); and (vii) host-symbiont flexibility, since the ability of the coral host to associate with different *Symbiodiniaceae* strains and species has been linked to adaptive capacity (281, 294). However, to allow for comparisons between host species, a framework for the quantification of this trait is needed. Namely, it is necessary to define a methodology for symbiont characterization together with a metric to quantify flexibility (or specificity), as well as to explore within-species variation through balanced sampling efforts that account for temporal, spatial, and environmental variability (295). Further, this approach could be expanded to the other components of the microbiome (e.g., bacteria [33]).

JOINING FORCES: EXPANDING COMMUNITY-BASED APPROACHES TO CNIDARIAN MODEL ORGANISMS

While it is necessary to establish multiple and diverse model systems (67, 68), strategic focus on a limited number of species will increase efficiency of resource use. A clearly stated or universally agreed-upon selection of "best candidate" species for the establishment of true coral model organisms seems to be lacking; however, the coral field clearly has its favorites. For example, over the last 30 years the three species *Pocillopora damicornis*, *Stylophora pistillata*, and *Acropora millepora* were preferentially used in coral heat stress experiments (29). Although these may be excellent candidates for a true coral model, preexisting knowledge is not *per se* a requirement nor an indicator of the validity of species as model organisms (68), but a widespread appreciation might be a good indicator of their practical advantages that should be taken into account.

Cnidarian model systems can accelerate the rate of discovery necessary to develop solutions for coral reef preservation, and these tools can become particularly powerful when part of a structured framework with defined goals and strategies (10, 76, 80). The idea of a community-based coordinated effort to develop and establish cnidarian model systems was put forward with a "call to arms" (10, 76). The main recommendations included (i) a reframing of researchers' attitude to put collective achievements above individual accomplishments, together with (ii) cooperation between working groups to improve efficiency, through active communication and resource sharing, and (iii) the adoption of a targeted approach that prioritizes the most pressing issues pertinent to corals and coral reef adaptation to future ocean conditions.

Examples that show encouraging progress in this direction can be found among the existing cnidarian model organism working groups and open resource networks. Researchers and educators interested in working with Hydra can find a comprehensive collection of best practices and resources through the OpenHydra (http://openhydra.org/) platform, and the Hydra 2.0 Genome Project Portal (https://research.nhgri.nih.gov/hydra/) provides easy access to the data generated from the Hydra genome sequencing projects. The Aiptasia community has developed a web site (https://aiptasia-resource.org/) and a Protocols.io group (https://www.protocols.io/workspaces/aiptasiasymbiodiniaceae-model-system) for researchers and educators who are interested in using Aiptasia in the laboratory and classroom (Table 2). Current resources for the Cassiopea model include a publicly available draft genome (https://mycocosm.jgi.doe.gov/Casxa1/Casxa1.home.html) and 10 clonal lines available upon request (http://medinalab.org/new/). The genome line T1-A is kept by multiple labs around the world (https://cassiopeabase.org/). The bacterial species, *Pseudoalteromonas* sp., that is an active inducer of settlement is available upon request as well as other *Cassiopea* spp. bacterial isolates from different developmental stages (medinalab.org [A. H. Kerwin et al., unpublished data]). Research on *A. poculata* has accelerated rapidly in recent years, thanks to the development of a large research collaborative focused on the temperate coral genera *Astrangia* and *Oculina*. The Temperate Coral Research Working Group (https://sites.bu.edu/astrangia/), which now exceeds 100 researchers, has met annually since 2016 (with the exception of 2020) and consists of researchers, educators, and journalists who work with *Astrangia* and *Oculina*, all of whom are addressing long-standing questions of coral-microbe symbiosis, as well as climate change education

and outreach. A draft genome of the host has been assembled and is currently under annotation. *Breviolum psygmophilum*, the intracellular photosymbiont thought to be present in all *A. poculata* populations, is in culture, and draft transcriptomes of *B. psygmophilum* and other congeners exist. 16S rRNA sequences from *A. poculata* specimens from two populations in the United States (Jamestown, RI, and Woods Hole, MA) have been deposited and are publicly available on the Coral Microbiome Portal (https://www2.whoi.edu/site/amy-apprill/coral-microbiome-portal/). Protocols for spawning, embryonic development, larval rearing, larval settlement, and CRISPR protocols have been developed and will be shared so that they are publicly available for researchers. Regarding the establishment of true coral models, however, there is still a general lack of coordination, and we can draw inspiration from the work done on the other cnidarian model systems.

Community-based and coordinated efforts may further include the integrated use of the different model systems, for example, in a validation sequence where first explorations are conducted on more tractable systems and are subsequently validated on systems with higher transferability, improving both speed and efficiency. In this context, the protocols for drug development and approval provide a valuable example of integrated approaches that combine the use of several model systems selected for the complementarity of their features. Similar to preclinical trials in human medical applications, test series are performed on model systems of increasing complexity starting from *in vitro* assays, and proceeding on to animals that possess anatomical and physiological features comparable to humans regarding the effects of a particular drug. Only drugs that pass all of the sequential validations are considered for testing on humans in clinical trials. In analogy, coral-related biological features can first be assayed in tissue or cell cultures and/or non-coral models, to be later corroborated by direct testing on corals (80). This process has already been successfully implemented to explore the involvement of cellular and immunity responses in coral bleaching, by first using Aiptasia and subsequently verifying on stony corals (reviewed in reference 10). Therefore, joining forces and coordinating efforts among groups working on different cnidarian model systems represents a promising approach to accelerate the development of solutions for coral reef conservation.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

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