

Review

Identifying ‘prime suspects’: symbioses and the evolution of multicellularity[☆]

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Abstract

The possible involvement of symbioses in the evolution of multicellularity is explored. Evidence is drawn principally from the biology of present day associations of plants and animals with prokaryotes. A particular emphasis is placed on future research opportunities in this area of biology that have been provided by the advent of specific molecular techniques and new model systems. With the application of new approaches that result from these advances, a more holistic understanding of the biology of the coevolved communities, composed of animals or plants and their associated prokaryotes, is within the reach of biologists over the next few decades. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

The importance of microbial symbioses in macroevolutionary events has been a matter of interest and controversy for many years (for review, see Margulis and Fester, 1991). The evolution of multicellular organisms is usually considered to be independent of the microbes with which they associate, although most, if not all, animals and plants have specific relationships with prokaryotes and/or fungi, most commonly in

highly complex consortia (Savage, 1977, 1986; Ly-senko, 1985; McFall-Ngai, 1998). It is this complexity that has rendered the rigorous study of these associations largely unfeasible and, thus, has forced much of the community of experimental biologists to ignore the impact of microbes on the biology of their host animals and plants. However, technological breakthroughs in the last decade, principally in molecular biology, are now opening this frontier for investigation. Two specific approaches are being taken to the study of these interactions: the characterization of whole consortia and the experimental analysis of monospecific models of symbiosis. During the 21st century, the development of these two complementary approaches promises to provide a means

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by which we can make great strides in our understanding of the interactions between microbial cells and the cells of their animal and plant hosts.

Not only is the study of such associations now more feasible and timely, but also an urgent need to improve our understanding of these relationships has presented itself. New and re-emerging microbial diseases are developing and spreading at an alarming rate, compromising the health not only of humans, but also populations of other animal and plant species (Garrett, 1994; McCormick, 1998). Microbial resistances to both biologically and chemically derived antibiotics have resulted in unprecedented escalation of the evolutionary 'arms race' between host communities and their encroaching pathogens. In addition, bioweapons are on their way to superceding nuclear weapons as the principal looming global threat (Alibek and Handelman, 1999; Henderson, 1999; Kortepeter and Parker, 2000). On the scale of an individual unit of evolutionary selection, microbial pathogenesis, resulting from either natural or man-made disease, presents a potential danger to the health of the whole functioning community, i.e. the individual comprised of one principal set of multicellular eukaryotic plant or animal cells and its associated microbial consortium. Before we can understand what has gone awry with the intrusion of interlopers into the healthy relationship between an animal or plant and its beneficial microbial partners, we must better understand the nature of these normal relationships themselves. As Thomas (1974) wrote in his celebrated collection of essays, *The Lives of a Cell*: 'Disease usually results from inconclusive negotiations for symbiosis, an overstepping of the line by one side or the other, a biologic misunderstanding of borders.' To grasp the nature of disease caused by microbes, we must develop approaches to investigate the nature of these 'negotiations' that animals and plants have with their symbiotic partners, and we must characterize these 'borders'.

Although the characterization of symbiotic interactions may be important and timely, the study of their evolution and their impact on the origin and radiation of multicellular eukaryotes presents a particularly daunting task; i.e. we have been challenged in the analysis of present-day associations, let alone those of the past. However, what telltale signs are there in the past and present biology of prokaryotes and eukaryotes that would

suggest that these associations have been important in evolution? This contribution reviews some of the strongest available evidence that symbiosis has played, and continues to play, a significant role in the evolution of multicellular organisms. In addition, considered here will be an array of recently-developed experimental approaches to the study of this area of biology that hold great promise for future rigorous analysis of this problem. The treatment will be limited to associations between prokaryotes and multicellular animals or plants. The evidence will be presented as elements of a mystery or crime. What evidence is available suggesting that alliances between prokaryotic and eukaryotic cells are culpable — having motive, opportunity, means and a prior record that would lead a sleuth to conclude, beyond a reasonable doubt, that such alliances are guilty of imposing selection pressure on the evolution of multicellularity, and are thus 'prime suspects'?

2. Motive and opportunity — with premeditation and at the scene

If the hypothesis is true that the process of symbiosis participated in the evolution of multicellularity and the radiation of plant and animal taxa, evidence of 'motive' and 'opportunity' should be available to implicate it in these processes. Specifically, symbiotic partners should offer some special 'talents' to one another that would provide selection pressure for their association, i.e. the fitness of the partners should be increased by the formation of alliances. Also, the partners should have been, and should continue to be, a significant presence in one another's environment.

'Motives' for both partners have been well documented, and can be observed in present day associations. In many cases, the host provides the prokaryotic symbiont with a nutrient-rich environment in which to grow, and the control of that growth will be, at some level, responsible for whether the association is beneficial or pathogenic to the host. Conversely, by associating with microbes, a host would be afforded a variety of benefits. For example, an important selection pressure in favor of colonization of tissues by specific non-pathogenic microbes is likely to be the 'colonization resistance' that animals thus col-

onized would have against pathogens. In present day associations, not only do enteric symbionts compete for food with pathogens, they are known to produce anti-bacterial compounds that are active against encroaching pathogenic strains (Rolfe, 1996). In addition, the host may avail itself of a vast array of complex metabolic pathways characteristic of these microbes. Historically, it is possible that 'early' multicellular organisms had a broader array of metabolic capabilities, some of which were lost upon subsequent association with prokaryotes. Alternatively, in the evolution of a specific group, selection pressure for versatile metabolic potential may have been driven by the initial association of consortia of cells from different genetic backgrounds (i.e. unicellular eukaryotes and prokaryotes). Regardless of the evolutionary sequence of events, the involvement of microorganisms in the provision of vitamins and other nutrients for present-day multicellular organisms (particularly animals, as heterotrophs) has been well established, and our awareness of the extent of the dependence of animals and plants on bacteria for the synthesis of biomolecules continues to expand. For example, in recent years, bacteria have been implicated in the provision of key secondary metabolites, such as tetrodotoxin (Sumida et al., 1987; Ritchie et al., 2000) and guaiaicol (Dillon et al., 2000), which had been initially assumed to be synthesized solely by animal or plant metabolic pathways. The extent of 'motive' that animals and plants have for forming associations with bacteria continually deepens with our increasing knowledge of these interactions.

What about 'opportunity'? Studies of the evolution of life on earth have convincingly demonstrated the dominance of microorganisms in the history of the biosphere, and an examination of the interface between the biosphere and the physical environment has underscored the central role of microorganisms in the control of past and present-day ecological processes. Specifically, whereas geological evidence suggests that the first one-fifth of the earth's history was lifeless, the next three-fifths was an era in which the biosphere was inhabited almost exclusively by prokaryotes and unicellular eukaryotes. Only in the last one-fifth of the earth's history, or approximately 0.9 billion years, have multicellular organisms been a conspicuous addition. However, animals, plants, and fungi did not displace microor-

ganisms as the dominant component of the biosphere, but rather their evolution was supplemental to microbial evolution. Recent estimates of prokaryotic biomass have suggested that they continue today to be the predominant life forms on earth (Whitman et al., 1998). The co-occurrence of large numbers of microorganisms with the cells of present-day and ancestral multicellular organisms would provide ample opportunity for the formation of alliances.

3. Means — armed and predisposed for an encounter

Is evidence available to suggest that bacterial cells and the cells of multicellular organisms have the molecular, biochemical, and cellular means by which to communicate not only among themselves, but also with their symbiotic partner(s)?

3.1. Multicellular prokaryotes — the tendency toward gang-related behavior among microbial cells

3.1.1. Quorum sensing

Although it has long been accepted that microbes are abundant and key players in the biosphere, until recently, the cells of most microbial species were thought to behave as individuals and to respond solely to abiotic environmental cues, and not to other cells in their surroundings. A widespread ability among microbial cells to interact with one another would indicate that microbes exhibit behaviors generally attributed only to multicellular organisms. This proclivity among microbial cells to exhibit communal behavior would suggest that they may be predisposed to cooperation with animal and plant cells, i.e. have the molecular and biochemical mechanisms to decipher the signaling interactions that occur between the coordinated cells of animal and plant tissues. Recently, a type of intra- and inter-specific communication among populations of bacterial cells called 'quorum sensing' has been discovered to be a broadly occurring phenomenon (Fig. 1; Dunny and Winans, 1999). In this behavior, the bacteria constitutively produce low levels of one or more specific pheromone-like molecules, or autoinducers. When bacterial population densities are high, the autoinducer reaches a critical ambient concentration and diffuses back into the bacterial cell, positively regulating not only its

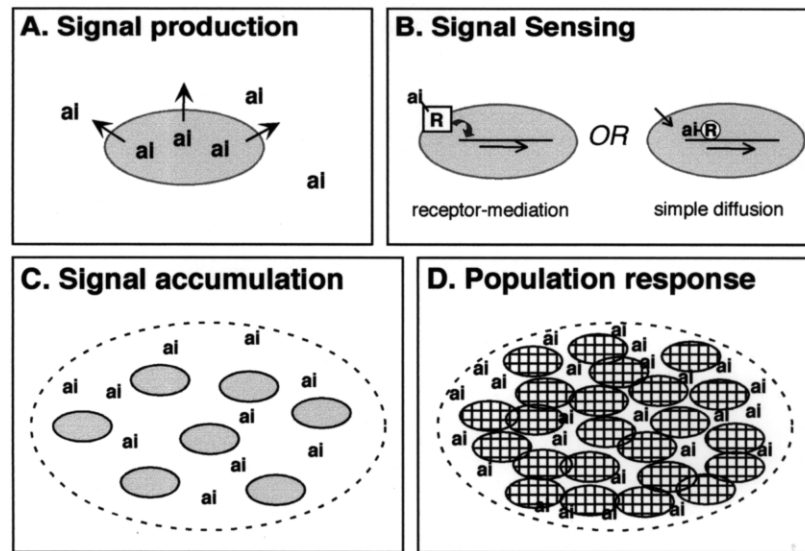


Fig. 1. Bacterial signaling through quorum sensing. (a) Autoinducer molecules that are produced by bacteria (light gray ovals) diffuse or are secreted into the surrounding environment. (b) The autoinductive signals are sensed through interaction with regulatory proteins (R) that are either cell-surface associated or cytoplasmic. In the former case, the autoinducers do not enter the cell, while in the latter they reach diffusion equilibrium across the cytoplasmic membrane. In either case, binding of autoinducer to its cognizant regulatory protein leads to a change in bacterial gene expression. (c) As bacterial population densities increase in confined environments (dashed oval), the signal accumulates. (d) At some critical bacterial density, sufficient autoinducer accumulates, producing a response in the population (hatched ovals). Such responses are useful to the bacteria only in environments of high cell density, such as in symbioses. (ai: autoinducer.)

own production, but also the transcription of a series of other genes organized as a regulon. Typically, the expression upregulated by autoinducers results in the synthesis of products that are used only at high population densities.

Does this behavior occur in microbes that are associating with animals and plants? One of the principal contexts in which bacteria reach high population densities is when they occur in symbioses with animals and plants and, in fact, quorum sensing was first described in *Vibrio fischeri*, a Gram-negative, marine luminous bacterium that forms luminescent symbioses with certain species of squids and fishes. Over 30 years ago, *V. fischeri* was found to produce luminescence in culture only after achieving a high population density (Nealson and Markovitz, 1970; Nealson et al., 1970; Nealson, 1999), i.e. at cell numbers characteristic of the conditions occurring within animal luminescent organs. Studies of quorum sensing in *V. fischeri* revealed that the bacteria sense the accumulation of an acylated homoserine lactone, the type of autoinducer now known to be most common among the Gram-negative bacterial species exhibiting quorum sensing. The *luxI* gene of the luminescence (or *lux*)

regulon of *V. fischeri* encodes the protein that directs synthesis of the major autoinducer, a 3-oxo-hexanoyl homoserine lactone (Eberhard et al., 1981). The product of the *luxR* gene, another component of the *lux* regulon, is a transcriptional activator that, when bound to autoinducer, directs the induction of the *luxCDABE* genes, which encode all the structural proteins required to drive the emission of bioluminescence (Engebrecht et al., 1983). In the last 5 years, at least one additional autoinducer has been described (Gilson et al., 1995) in *V. fischeri*, but the relative importance of these other molecules to the biology of this microorganism remains to be determined.

The behavior of quorum sensing, once thought to be a curiosity and specific to luminous bacteria, is now known to occur in many bacterial species that form associations with eukaryotic tissues, whether the relationship is pathogenic or beneficial (Dunny and Winans, 1999; Kievit and Iglewski, 2000). For example, over the past few years, homologues of *luxI* and *luxR* have been found in at least 15 other bacterial genera, including *Rhizobium*, *Pseudomonas* and *Agrobacterium*, that form associations with plants and animals. In addition, a variety of other types of quorum-sens-

ing systems have been identified that use a biochemically distinct group of compounds as signaling molecules. For example, a number of Gram-positive bacterial species have independently evolved a quorum-sensing mechanism that uses small peptides, analogous to animal hormones, as signal molecules. In some instances, the activity of these peptide autoinducers is specific for the symbiotic niche of the Gram-positive bacterial species. Thus, it is becoming increasingly clear that the involvement of quorum-sensing behaviors appears to be the rule rather than the exception in the associations of bacteria with multicellular organisms.

Does the behavior of quorum sensing affect host tissues, i.e. is quorum sensing not only a means of communication with the bacteria, but also with the host cells? Only a few examples of a direct effect of autoinducers on plant or animal host cells have been documented (DiMango et al., 1995; Rosemeyer et al., 1998; Telford et al., 1998). However, because autoinduction is linked to production of gene products essential to the activity of the bacterial population in their high-density niche, it is not surprising that this activity affects the host and, in some cases, may be essential for the onset and maintenance of the host–symbiont interaction. Examples of such effects have been noted in both pathogenic and cooperative associations. When the pathogen *Pseudomonas aeruginosa* occurs in high density, such as in the lung of an individual with cystic fibrosis, the auto-induction system directs the production of elastase, an enzyme that compromises the integrity of the extracellular matrix of the host's alveolar epithelium (Pesci and Iglewski, 1999). In the squid–vibrio association, bacterial luminescence is not only used by the host in its behavior, but the activity of the autoinduced *lux* operon is also essential for maintenance of the symbiosis. Mutants defective in genes of the *lux* operon do not induce normal host tissue development and fail to persist in the host's light organ (Visick et al., 2000).

3.1.2. Cell determination and differentiation in bacterial development

Similar to cooperation among cells of a population, developmental determination and differentiation has generally been considered the purview solely of multicellular organisms. However, the 'division of labor' among the cells of a population,

resulting from the processes of determination and differentiation, also occurs in a number of prokaryotic species (Saier, 2000), such as the cyanobacterium *Anabaena* sp., the fruiting bacterium *Myxococcus xanthus*, and the stalked bacterium *Caulobacter crescentus*. Analyses of present-day developmental programs in *Anabaena* spp. for example, reveal several developmental programs that yield different cell fates. These include cells in vegetative growth, resting cells, cells fixing nitrogen and cells undergoing apoptotic-like processes. Furthermore, the fossil record indicates that this type of developmental facility may have occurred as early as 3.5 billion years ago. Although such a precedent is itself not necessarily direct evidence for the role of bacteria in the evolution of multicellular organisms, the presence of such traits in bacteria suggests that they have the ability to process the types of signals responsible for directing complex development.

3.2. The cell biology of prokaryotic–eukaryotic interactions

The above-described characteristics of prokaryotes indicate that they have the ability to behave as multicellular organisms and, as such, may possess traits that predispose them to form cooperative associations with multicellular eukaryotes. Is there other, more direct evidence that bacteria can and do have the 'means' to interact with host cells?

3.2.1. The influence of bacteria on present-day, and perhaps ancient, animal and plant developmental programs

The impact of host–symbiont interactions on the development of symbioses between leguminous plants and their nitrogen-fixing bacterial partners has been well established by decades of research on this type of association (for review see Stougaard, 2000). These efforts have indicated that a complex dialogue between the partners is required for the development of functional root nodules and the transformation of the rhizobia, the bacterial partner, to the terminally differentiated bacteroid state. However, only in the last decade has strong experimental evidence been accumulating that bacterial symbionts can directly affect the development of the animal tissues with which they interface (McFall-Ngai, 1998). Most notably, biochemical and molecular

genetic manipulations of mammalian hosts and their enteric microbial partners have provided convincing evidence that the bacteria participate, through the induction of specific gene expression, in the remodeling of neonatal gastrointestinal tissue during the establishment of the natural microbiota (Hooper et al., 2000). Specific microbiota also appear to be essential components and active participants in the normal maturation of the vertebrate immune system (Rook and Stanford, 1998; Lanning et al., 2000). In addition, in the squid–vibrio association, the bacterial symbionts induce an array of developmental changes in host tissues, including cell death and cell swelling, that result in morphogenesis of the symbiotic organ (for review see McFall-Ngai, 1999). Reciprocally, interaction with host tissues induces changes in the morphology and gene expression of the bacterial symbiont (Ruby and Asato, 1993; Visick and Ruby, 1998).

The importance of symbiotic associations in the evolution of developmental processes can only be a matter of speculation. Biologists have postulated that the diversification of animal body plans may have been the result of some set of environmental influences, such as temperature change or increases in atmospheric oxygen. Wolpert (1994) speculates that the mechanism (responsible for the origin of animal developmental processes) ‘involves an environmentally produced effect becoming part of the developmental program. An environmental signal is replaced by a developmental one’. Significant environmental changes, both past and present, are often hypothesized to result from large-scale microbial metabolic activity (Atlas and Bartha, 1992) and, thus, may have indirectly affected the evolution of animal development. However, the involvement of microbes in the evolution of these processes, as a direct (or more proximate) biotic environmental influence, while rarely suggested, should not be entirely discounted. As discussed above, microbes were certainly a significant element in the environment of evolving metazoans. Within this context, it is interesting to consider the issue that microbes, as an environmental influence, would be capable of responding to and co-evolving with the metazoan host and its evolving developmental pathways, a feature obviously not characteristic of abiotic environmental influences.

In what tissues might the influence of microbes have affected the evolution of animal develop-

mental programs? Because we have good evidence that microbes participate in the development of the gastrointestinal and immune systems in the embryonic programs of extant animals, these systems provide good candidates. For example, in some cases, gastrulation results in the formation of a protected pocket, or archenteron, within an embryo. In addition, it is common for invertebrate embryos to develop without capsules or extraembryonic membranes to separate them from the biotic environment (Hadfield, personal communication). It is difficult to imagine that some subset of the environmental bacteria would not have colonized such nutrient-rich, sequestered niches as such habitats became available during the evolution of animal body plans. These microenvironments may have provided the first opportunity for ‘enteric’ microbial consortia to begin to co-evolve with their hosts. If bacteria were associated with these early metazoans and participating in the early evolution of development, the evolution of the immune system, which presumably arose to distinguish benign self and non-self from dangerous non-self, is also likely to have been influenced by any co-evolution of microbiota with their host animals. The extent to which such interactions participated in the selection of the traits that are primitive to the character of either endoderm or immune systems may never be resolved. However, the presence of ancient, shared pathways for the interaction of animals with prokaryotes, such as those mediated through the highly conserved Toll receptor family of molecules (see below), would provide evidence for such a co-evolution.

Although less dramatic than the significant developmental changes occurring in animals and plants, it is not uncommon for bacteria to undergo significant developmental changes when they associate with multicellular hosts. Very often, flagella are lost and the global regulation of metabolism is modified (Ottemann and Miller, 1997). With a wide variety of bacterial genomes being sequenced and characterized, one can predict an increased appreciation for the genetic potential that bacterial cells can use to respond to their hosts.

3.2.2. Cellular microbiology

In 1996, a piece appeared in the ‘Perspectives’ section of *Science* magazine announcing the emergence of a ‘new discipline’ called ‘cellular

microbiology' (Cossart et al., 1996). Although for many years, derivatives of microbial cells have been used as pharmacological agents for the study of eukaryotic cell biology, the academic treatment of prokaryotic and eukaryotic cell biology had principally been conducted independently of one another. Many textbooks have been written that cover either microbiology or eukaryotic cell biology. Where microbes are considered in the eukaryotic cell biology texts, they are included because they have some 'ancient' character that has been best analyzed by studying the more 'simple' prokaryotic or fungal cell. In microbiology, consideration of animal and plant cells is often restricted to their role in providing a niche (most often a pathogenic one) for a particular microbial species. Cellular microbiology presents a more holistic treatment that seeks to categorize and integrate the current knowledge of how both normal and pathogenic interactions occur between prokaryotic and eukaryotic cells, and how their biology is altered as a result of those dynamic and reciprocal interactions.

Two very recent textbooks, *Cellular Microbiology* by Henderson et al. (1999) and *Cellular Microbiology* by Cossart et al. (2000) have synthesized current knowledge in this field. Because of the bias in our present database, these treatments focus on the biology of pathogenic relationships between prokaryotes and eukaryotes. These texts categorize the array of shared pathways that characterize the responses of microbes to their hosts and hosts to their microbes. Thus, many data are available from the study of bacterial pathogenesis that have demonstrated sophisticated mechanisms of bacterial cell action and host response, some of which have also been reported in cooperative associations (for review, see Hentschel et al., 2000). From an evolutionary viewpoint, the presence of common pathways of response suggests molecular and biochemical mechanisms underlying prokaryotic–eukaryotic associations that are subject to selection pressure, regardless of whether the outcome of the relationship enhances or compromises the fitness of the partners.

An interesting example of shared mechanisms of host response is found in the involvement of the Toll/Rel family of receptor proteins, and the elements of the NFkappaB pathway, which is activated by the binding of the Toll receptor to its ligand (for review see Medzhitov and Janeway,

2000). Toll receptors were first described in *Drosophila melanogaster* as key players in body axis determination during embryogenesis and, more recently, were found to play a role in 'anti-microbial' responses in the late ontogeny of *D. melanogaster*. Several homologous receptors have been identified in the fruitfly and mutations in the genes that encode these proteins lead to susceptibility to specific types of pathogens (e.g. fungal or bacterial). The analyses of human genome sequences have revealed homologues of the Toll receptor in humans, called Toll-like receptors (TLR), which are also involved in responses to bacteria. In the last couple of years, over a dozen TLRs have been identified in the human genome. In one well-studied case, TLR-4 is responsive either directly or through an adaptor protein to the bacterial surface molecule lipopolysaccharide (LPS). Interaction with LPS leads, through a cascade of phosphorylation events, to the nuclear localization of the transcription factor NFkappaB. The binding of this transcription factor to the recognition sequence of specific target genes ultimately results in the synthesis of cytokines, anti-microbial peptides, and other bioactive molecules that mediate the cellular response to the presence of bacteria. Furthermore, homologues of the Toll-receptor family and elements of the NFkappaB pathway have been found in plants, where they are also implicated in the modulation of responses to microorganisms (Medzhitov and Janeway, 1998).

A common assumption in the field of cellular microbiology is that the identified host responses are anti-microbial, and the microbial responses are virulence determinants. However, in the context of the evolution of animal–microbial symbioses, these pathways may have evolved for interactions between the partners, and the nature of the relationship, whether pathogenic or cooperative, is the result of differential control of these responses. For example, all Gram-negative bacteria have the reactive surface molecule LPS, and the component of this molecule that confers 'virulence' onto the LPS, i.e. the lipid A portion of the molecule, is highly conserved. In light of the fact that these bacteria share a common virulence determinant, by what mechanisms are interactions with the consortium of beneficial Gram-negative intestinal symbionts fostered, while Gram-negative pathogens are dissuaded? In the monospecific beneficial associations that have

been studied, i.e. the legume–rhizobia and the squid–vibrio systems, LPS is a critical bacterial signaling molecule. Experiments with mutants in the LPS of rhizobia have indicated that LPS is important at all stages in the development of the mature nitrogen-fixing root nodule (Dazzo et al., 1991; Perotto et al., 1994; Lopez-Lara et al., 1995). In the squid–vibrio system, the LPS molecule of *V. fischeri* induces a program of cell death in the superficial epithelial field of the host's light organ, one of the principal events in light organ morphogenesis (Foster et al., 2000).

The frontier field of cellular microbiology should provide a context for the study of all types of interactions between microbes and their multicellular hosts. Establishing the legitimacy of this area has been a very significant contribution, as it provides a focus for research efforts. Studies of these shared pathways should allow biologists not only to distinguish between pathogens and beneficial associations, but also to define how these pathways have arisen and diverged.

4. Priors

A large body of data exists, suggesting that symbioses have been important in the radiation of certain taxa. An extensive review of this evidence will not be provided here. Instead, the focus will be on how new perspectives and findings might be integrated into this existing database.

The endosymbiotic origin of the organelles of eukaryotic cell is the most widely accepted circumstance of the involvement of symbiosis in macroevolutionary events. However, Margulis points out that the idea had been around for a long time, but providing solid evidence awaited her benchmark work in the 1970s that applied the new technology of electron microscopy to the ultrastructural analysis of the eukaryotic cell (Margulis, 1970). Thus, although much speculation had surrounded this issue, it was not until the technology was available to test the idea that this theory was incorporated into the mainstream of biological thought.

The evolution of the eukaryotic cell resulted in the formation of an integrated reproductive unit that included prokaryotic cells. Did the inclusion of prokaryotic cells in the further evolution of eukaryotes into multicellular organisms cease with the evolution of that first cell type? Or, instead, is

it possible that reproductive units of coevolved consortia of prokaryotic and eukaryotic cells also characterized the evolution of multicellular organisms, i.e. did multicellular organisms evolve not as individual genomes, but as complex communities? The study of this phenomenon has been largely restricted to animals with consortia that provide some unique metabolic potential that renders the animal economically important, most notably the rumen microbiota of ungulates (Hungate, 1966) and the paunch microbiota of termites (Breznak, 1982). Several monospecific symbioses have also provided evidence for coevolution of host and symbiont, and the possible involvement of the symbiosis in the radiation of a given clade (e.g. Distel et al., 1994; Doyle, 1994; Baumann et al., 1997; Nishiguchi et al., 1998). While these studies are limited, they provide data demonstrating that the bacterial symbionts can influence the pattern of radiation of multicellular organisms. With new advances in genome analysis, we now have the technology to determine whether a wide variety of organisms have coevolved with specific consortia. These data promise to provide the definitive evidence as to the extent of involvement of bacteria in the evolutionary patterns of all animals and plants.

5. The verdict: beyond a reasonable doubt

'It is an old maxim of mine that when you have excluded the impossible, whatever remains, however improbable, must be the truth.' Sir Arthur Conan Doyle (1859–1930).

The available data indicate that, since multicellularity arose, and throughout its evolutionary history, bacteria have been a constant and dominant force in the environment of these increasingly complex organisms. Bacteria have a propensity to occupy the niches that are created by the formation of multicellular tissues, and multicellular organisms take advantage of the vast array of complex pathways that characterize bacterial metabolism. Thus, the sum of the evidence suggests that symbiosis did indeed have 'motive, means, and opportunity', as well as a prior record, that would lead one to the conclusion that symbiotic associations are guilty of influencing the evolutionary patterns of multicellular organisms.

6. Horizons — new ‘forensic’ tools, methods, and models

The knowledge provided by the application of specific, newly developed molecular techniques should allow biologists over the next several decades to assess the impact that the microbial communities exert on all aspects of the biology of the multicellular organisms with which they associate. In the more distant future, as the techniques of genomic analysis continue to develop, the potential may exist for describing not only the genomes of all constituents of an inter-generationally stable consortium (both host and its microbes), but also the molecular basis that underlies their dynamic interactions. At present, we are only at the dawn of this era, and the studies of the more proximal future are likely to focus on two sets of questions:

1. Who are the members of specific consortial communities; what types and what proportion of the multicellular organisms are in association with a microbial community that is predictable between generations, i.e. with which they have coevolved; what are the activities of the constituents of the consortium as components of the community?
2. What models are available that would allow experimental manipulation of the partners; are the predictions of the general nature of associations between bacteria and multicellular organisms that are made through studies with these models supported by analysis of these features in the more common consortia interactions, i.e. which characters of the models are derived, and which are primitive?

A successful approach to the first set of questions relies on the ability to define and characterize the community. For years, ecologists have been identifying unculturable bacteria in various environments by sequencing the 16S rRNA genes obtained by PCR-amplification (Giovannoni et al., 1988). These techniques have more recently been applied to defining the bacterial community associated with a number of multicellular organisms (e.g. Paster et al., 1996; Thimm et al., 1998; Van der Maarel et al., 1998; Suau et al., 1999). The predictability of communities, both ontogenetically and between generations, is being studied by a variety of methods, such as denaturing or

temperature gradient gel electrophoresis (DGGE or TGGE; Muyzer and Smalla, 1998; Simpson et al., 1999) and terminal restriction fragment length polymorphism (TRFLP; Liu et al., 1997; Kerkhof et al., 2000; Marsh et al., 2000). Both of these methods, and several others, use the 16S rRNA genes of the consortium and have the potential to define the phylogenetic complexity of a community. TRFLP analyses can also provide insight into the relative abundance of the constituents of the community, whereas the PCR-based DGGE and TGGE are generally not used for this purpose. These approaches continue to be refined and remain controversial at this time. For example, it has recently been argued the gene encoding 16S rRNA is not an optimal gene for microbial community studies, because it displays significant intraspecies heterogeneity. Alternative genes, such as those for the RNA polymerase beta subunit (*rpoB*), are being suggested as more suitable candidates for these types of analyses (Dahllof et al., 2000).

In addition to methods designed to identify the constituents of the community, and to assess the changes in its composition, several approaches are being developed that promise to provide information about the physiological activity within the consortium. Methods for profiling the metabolism of the whole microbial community are undergoing rapid development. For example, the commercially available Biolog microtiter plates (Biolog, Hayward, CA, USA) are being used to determine community metabolic activity, as well as various environmentally-induced changes in this activity, within microbial consortia (Choi and Dobbs, 1999; Kerkhof et al., 2000; O’Connell et al., 2000). Other methods are being developed to determine the phenotypes of specific non-culturable bacteria within consortia, i.e. not only who they are, but also how they function. In one notable method, all the DNA of a given community of microorganisms, the ‘metagenome’, is isolated and large fragments of the DNA are cloned into *E. coli* using a BAC (bacterial artificial chromosome) vector (Osburne et al., 2000; Rondon et al., 2000). Genes, such as rDNA or *rpoB* can be used to identify the organisms, and by sequencing the DNA that flanks these genotypic markers, aspects of the metabolic potential of a given organism can be revealed. For example, in a recent study of the soil metagenome, sequences for a variety of metabolites, such as

antibiotic compounds, were found to be associated with specific non-culturable strains within the consortium (Rondon et al., 2000). This approach of 'metagenomic library' analysis will be particularly powerful in the characterization of symbiotic communities dominated by microorganisms that are refractory to laboratory culture.

Approaches to the second set of questions seek to determine what general principals can be learned by the study of experimental model systems that can be more broadly applied to the basic biology of symbiosis between bacteria and multicellular organisms. Similar to the study of other types of model systems in biology (e.g. *Drosophila melanogaster* and *Caenorhabditis elegans*), these systems seek to define the dynamics of the most 'simplified' systems available with the belief that some of the findings can be extrapolated to, and tested with, more complex associations. To be tractable and generally applicable to an understanding of the most common types of symbioses, the ideal model system should be a host with a monoculture of bacteria living extracellularly along polarized epithelia. Historically, our knowledge base has drawn from two very different arenas: the past enormous successes in the study of pathogenic microbes, which typically occur in monoculture, and research on the cooperative associations between leguminous plants and their nitrogen-fixing symbionts. The need to understand pathogenesis has been driven by the enormous impact that bacterial pathogens have historically had on human populations, and it is likely that some portion of our understanding of these harmful animal–microbial interactions will apply to beneficial associations. The legume–rhizobia symbioses, which are agriculturally important associations that lend themselves well to laboratory study, have offered until very recently the only well-developed model for the analyses of beneficial eukaryotic–prokaryotic interactions. However, many animal species and other plant taxa that entertain cooperative associations with monospecific cultures of microbes are presently being exploited as a means to understand how animals form stable, beneficial relationships with microbes (Ruby, 1999; Graf, 2000; Hooper et al., 2000). It is anticipated that the knowledge gained from studies of these very simple models can then be applied to a study of the more complex consortia interactions, so that the

primitive or derived nature of a particular feature can be assessed.

7. Conclusions

At present, the evidence that symbiosis has had an influence on the evolution of multicellular organisms is strong, yet circumstantial; the 'jury is still out'. The consideration of our current knowledge in this area can be viewed as a type of horizon analysis. From both conceptual and technological points of view, much information is available to suggest that we are at the dawn of a new synthesis in which all aspects of the biology of multicellular organisms will be studied from the viewpoint of the whole community, rather than only that of the host.

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