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REVIEW

Growing Ungrowable Bacteria: Overview and Perspectives on Insect Symbiont Culturability

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SUMMARY Insects are often involved in endosymbiosis, that is, the housing of symbiotic microbes within their tissues or within their cells. Endosymbionts are a major driving force in insects' evolution, because they dramatically affect their host physiology and allow them to adapt to new niches, for example, by complementing their diet or by protecting them against pathogens. Endosymbiotic bacteria are, however, fastidious and therefore difficult to manipulate outside of their hosts, especially intracellular species. The coevolution between hosts and endosymbionts leads to alterations in the genomes of endosymbionts, limiting their ability to cope with changing environments. Consequently, few insect endosymbionts are culturable *in vitro* and genetically tractable, making functional genetics studies impracticable on most endosymbiotic bacteria. However, recently, major progress has been made in manipulating several intracellular endosymbiont species *in vitro*, leading to astonishing discoveries on their physiology and the way they interact with their host. This review establishes a comprehensive picture of the *in vitro* tractability of insect endosymbiotic bacteria and addresses the reason why most species are not culturable. By compiling and discussing the latest developments in the design of custom media and genetic manipulation protocols, it aims at providing new leads to expand the range of tractable endosymbionts and foster genetic research on these models.

KEYWORDS insect, symbiosis, endosymbiont, *Wolbachia*, *in vitro* culture, endosymbiosis, *in vitro*

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INTRODUCTION

The term "symbiosis" was coined by Anton de Bary in 1879 to describe the intimate interaction between two species (1). In this historical context, microbes were synonyms for illness, and the focus of biological sciences was the individual seen as a whole (i.e., as a fully autonomous system), relegating symbiosis to the rank of a biological curiosity. Biologists have come a long way since the 19th century, identifying a growing number of nonpathogenic symbiotic interactions across all kingdoms of life and acknowledging them as major factors driving the evolution of life (2–4). The most famous example of such drive is, without a doubt, the evolution of eukaryotic cells from the symbiotic integration of prokaryotes, proposed by Mereschkowski in 1910 (5) and formulated by Margulis in 1967 as the endosymbiotic theory (6–8). This is an evidently extreme level of symbiont integration, and most associations have much milder consequences, yet microbiomes affect multiple aspects of their host physiology, including their development (9, 10), metabolism (11–13), immunity (11, 14), and behavior (15, 16). As a whole, they are recognized as a major source of ecological innovation and a compelling point in the study of biology (4, 17–19).

Insects, in particular, are often involved in endosymbiotic relationships. With an estimate of 4 to 10 million extant species (20), they can be considered the most successful taxon of multicellular organisms and are found across all terrestrial habitats, including the most extreme ones. Naturalists and entomologists reported the presence of bacteria living within insect tissues (i.e., "endosymbiotic" bacteria) since the 1930s (e.g., see references 21, 24, and 173), and modern investigation techniques progressively unraveled that endosymbiosis is actually the rule rather than the exception in insects: about half of them house endosymbiotic bacteria in their tissues (22, 23), and about 10% directly rely on endosymbiotic bacteria to ensure their development and reproduction (10, 17, 24).

The field of insect endosymbiosis received growing attention in the 2000s, leading to astonishing discoveries on insect physiology, ecology, and evolution. Research on insect endosymbionts is, however, hampered by their intractability. The vast majority of non-model insect hosts cannot be husbanded in a laboratory for long periods because of their complex nutritional or abiotic requirements, or simply because they are extremely difficult to collect in nature. Thus, host rearing is the first technical hurdle preventing the study of their endosymbionts. Some insect hosts became model systems in the field of symbiosis research, because they can be easily husbanded under laboratory conditions. Even if the insect host is tractable, however, most of the insect bacterial endosymbionts are unable to survive on artificial media (25), keeping functional studies on endosymbiont genetics and metabolism out of reach. Endosymbiont genetics can be approached only by the study of natural genetic variants (e.g., see references 174 and 175) or by genetically manipulating the insect host, when possible, to express bacterial genes (28–30). However, these approaches are slow, relying partly on chance, and can hardly lead to the full characterization of a significant number of endosymbiotic genes. Achieving *in vitro* culture is a groundbreaking step in the study of any bacterium, as it opens the way to faster and controlled genetic modifications for functional genetic studies and for practical applications of basic research. This work briefly reviews the diversity of insects' bacterial endosymbionts and their culturability status. It also presents an overview of the already existing *in vitro* culture and modification systems and discusses the way new systems could be developed to expand the range of tractable endosymbionts.

INSECT SYMBIONT DIVERSITY AND CLASSIFICATION

Establishing a classification of insect symbioses can be confusing given their diversity and the complexity of their interactions with their host. The most widely accepted classification is based on the degree of integration of the symbiont into the host physiology, regardless of the symbiont taxonomy. It outlines two major groups, the obligate and the facultative endosymbionts, depending on whether the host is able to survive under wild conditions without the endosymbiont ("aposymbiotic" host). How-

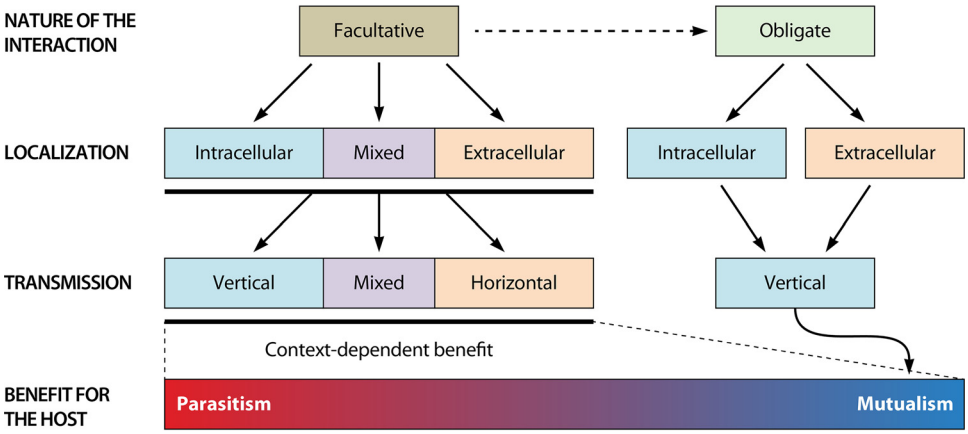

FIG 1 Classification of insect-bacterium symbioses. Insect symbionts can be obligate or facultative for their host. Transitions from facultative to obligate are possible over evolutionary time. Obligate symbionts can be either intracellular or extracellular. They are all vertically transmitted (usually by transovarial transfer for intracellular species and by brood contamination for extracellular species) and mutualistic. Facultative symbionts can have an intracellular, extracellular, or mixed lifestyle and can be horizontally or vertically transmitted. Both modes of transmissions have been suggested for a few species. Facultative symbionts have a maintenance cost for their host, but they also bring ecological advantages, hence their net impact can be either deleterious or beneficial depending on the environmental context.

ever, this simple classification is limited, as it does not account for the endosymbiont localization (intracellular or extracellular) or for the transmission mechanism (vertical or horizontal). Furthermore, a facultative endosymbiont can become locally obligate for an insect population if it confers a dramatic selective advantage on its carriers. Over evolutionary time, obligate symbionts can also be replaced by new bacterial partners, which in turn become obligates, while the ancestral obligate becomes dispensable and is progressively eliminated (31–34). Thus, naming and precisely classifying such diverse and dynamic arrays of interactions is always a challenge for the research community (Fig. 1).

In this review, a special focus will be given to bacterial endosymbionts having a fully or partly intracellular lifestyle. We will address these species depending on their impact on the host (i.e., obligate intracellular and facultative intracellular endosymbionts) and with a specific discussion on facultative intracellular endosymbionts that manipulate their host's reproduction. Extracellular symbionts, which include obligate and facultative symbionts with very diverse lifestyles and impacts on their host, will be briefly addressed in a separate section. We excluded from this review fungi (35) and yeast-like symbionts (36). The gut microbiota and other ectosymbionts (symbionts not being housed within host tissues) will also be excluded from this review, with the exception of a few species of interest because of their particularly intimate relationship with their host.

Intracellular Obligate Endosymbionts

Intracellular obligate endosymbionts are necessary for their host development under wild conditions and reach 100% prevalence in the host insect populations (10, 17), although some species can be maintained aposymbiotic under laboratory conditions (37, 38). Such endosymbionts are associated with insects thriving on nutritionally unbalanced diets. They complement the insect's diet by providing essential amino acids or vitamins required for the host's proper development (39) or by recycling nitrogen (40, 41). They are secluded inside bacteriocytes, which are symbiosis-dedicated cells composing the bacteriome (formerly called the mycetome), a highly polymorphic organ across endosymbiotic insect species. Most obligate endosymbionts live either free in the cytosol of bacteriocytes or inside vacuoles. They have a strict vertical transmission, most often by transovarial transfer of the bacteria from ovarian bacteriomes to the maternal germ line (39, 42). Their intimate relationship with their

host entails a coevolution between the two partners that sometimes leads to cospeciation events (26, 43, 44). Obligate endosymbiont genomes display very characteristic features: a small size (<1 Mb) and a bias toward A-T bases (45). The small size is explained by their intracellular lifestyle, i.e., in a highly stable environment with steady selective pressures toward genes that are beneficial for the symbiotic interaction. Genes that are either deleterious or neutral for the interaction and those that are dispensable for the bacteria are either gradually pseudogenized or excised by large deletions and chromosomal rearrangements (46). The most frequently lost genes include metabolic genes that are redundant with host-encoded metabolic pathways and genes that encode virulence factors or are involved in the production of immunogenic structures (e.g., peptidoglycan synthesis enzymes) (45). DNA repair and recombination genes are also frequently pseudogenized (47), which increases the genome mutation rate and, hence, the evolutionary adaptation speed of the endosymbiont to its host. Of note, these features are also observed in some extracellular obligate symbiont genomes (see below), which suggests that the intimacy of the host-symbiont relationship, rather than the intra- or extracellular localization of the symbiont, entails the symbiont genome reduction.

Most associations consist of a single obligate endosymbiont within one insect species, but some multiple-obligate endosymbioses have been reported. The Auchenorrhyncha, for instance, are infected with an ancestral *Bacteriodetes*, *Sulcia muelleri*, along with different alpha-, beta-, or gammaproteobacterial combinations proper to each insect lineage (48). Mealybugs also harbor a remarkable nested obligate endosymbiosis where a betaproteobacterial endosymbiont, *Tremblaya princeps*, contains a gammaproteobacterial endosymbiont, *Moranella endobia* (49), drawing an intricate network of metabolic pathways between the three symbiotic partners (27, 50).

Intracellular Facultative Endosymbionts

Facultative endosymbionts, on the other hand, are not necessary for host survival and can be found at variable prevalence in different populations of the same insect species. Multiple infections by several facultative endosymbionts are frequent (39). Unlike obligate endosymbionts, facultative endosymbionts have a wide tissue tropism and can be intracellular, extracellular, or both (39). Their effect on host physiology is not always well understood. Some species bring ecological advantages to their host, such as tolerance to heat (51), environmental specialization (52), reduction of predation risk (53), or protection against viruses (54) or parasites (55–59). Harboring the symbiont can be beneficial under some environmental conditions but costly under others, making them conditional mutualists (60). Their transmission is both vertical and horizontal (39, 61), which limits host-symbiont coevolution. Their genomes do not undergo such dramatic gene losses and, thus, are larger than those of obligate endosymbionts (Table 1 and Fig. 2), conferring on them a better adaptability to changing environments. Facultative endosymbionts can be a transitional step between free-living bacteria and obligate endosymbionts. As an example, the facultative endosymbiont of aphids, *Serratia symbiotica*, is divided into two clades, A and B (62). Clade A regroups bacteria that are both intra- and extracellular (63) and are not required for host development (64), while clade B, originally discovered in the Cedar bark aphid *Cinara cedri* and other members of the Lachninae family, is restricted to bacteriocytes and is coobligate for the host, along with the ancestral obligate of aphids, *Buchnera aphidicola* (62, 65). *B. aphidicola* organisms infecting *C. cedri* lost the ability to synthesize tryptophan, an essential amino acid provisioned by *B. aphidicola* in other aphid families. This metabolic function has been taken over by *S. symbiotica B*, illustrating a transition from a facultative to an obligate role (65). This transition occurred independently at least four times in aphid evolutionary history (66).

Reproductive Manipulators

A group of facultative, maternally inherited endosymbionts, including *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Cardinium*, and *Arsenophonus* genera, has evolved the ability to

TABLE 1 Characteristics and culturability status of the selected bacterial symbionts of insects[a]

Symbiont	Phylum or class	Host	Association	Transmission	Available genomes (complete, total)	Genome size (Mb)	%GC	Culturability	Genetic engineering	Host recolonization
Intracellular obligate endosymbionts										
Buchnera aphidicola	*Gammaproteobacteria*	Aphids	Obligate	Transovarial transfer	38, 69	0.58	24.7	–	–	–
Carsonella ruddii	*Gammaproteobacteria*	Psyllids	Obligate	Transovarial transfer	8, 9	0.17	15.4	–	–	–
Portiera aleyrodidarum	*Gammaproteobacteria*	Whiteflies	Obligate	Transovarial transfer	5, 6	0.33	25.1	–	–	–
Evansia muelleri	*Gammaproteobacteria*	Moss bugs	Obligate	Transovarial transfer	1	0.36	25.3	–	–	–
Tremblaya princeps	*Betaproteobacteria*	Scale insects	Obligate	Transovarial transfer	7, 8	0.14	58.9	–	–	–
Uzinura diaspidicola	*Bacteroidetes*	Scale insects	Obligate	Transovarial transfer	1	0.26	30.2	–	–	–
Sulcia muelleri	*Bacteroidetes*	Auchenorrhyncha	Obligate	Transovarial transfer	34, 39	0.25	22.8	–	–	–
Zinderia insecticola	*Betaproteobacteria*	Froghoppers	Obligate	Transovarial transfer	1	0.21	13.5	–	–	–
Nasuia deltocephalinicola	*Betaproteobacteria*	Leafhoppers	Obligate	Transovarial transfer	3	0.12	16.3	–	–	–
Baumannia cicadellinicola	*Gammaproteobacteria*	Leafhoppers	Obligate	Transovarial transfer	3, 4	0.69	34.6	–	–	–
Vidania fulgoroideae	*Betaproteobacteria*	Planthoppers	Obligate	Transovarial transfer	1	0.14	18.2	–	–	–
Purcelliella pentastirinorum	*Gammaproteobacteria*	Planthoppers	Obligate	Transovarial transfer	1	0.48	21.1	–	–	–
Hodgkinia cicadicola	*Alphaproteobacteria*	Cicadas	Obligate	Transovarial transfer	18, 34	0.13	46.5	–	–	–
Nardonella spp.	*Gammaproteobacteria*	Weevils	Obligate	Transovarial transfer	4	0.22	16.7	–	–	–
Sodalis pierantonius	*Gammaproteobacteria*	Weevils	Obligate	Transovarial transfer	1	4.5	56.1	–	–	–
Blochmania floridanus	*Gammaproteobacteria*	Ants	Obligate	Transovarial transfer	1	0.71	27.4	–	–	–
Blattabacterium spp.	*Bacteroidetes*	Cockroaches, termites	Obligate	Transovarial transfer	21, 22	0.62	25.2	–	–	–
Skilesia alterna	*Bacteroidetes*	*Geopemphigus* aphids	Obligate	Transovarial transfer	1	1.32	37	–	–	–
Wigglesworthia glossinidia	*Gammaproteobacteria*	Tsetse flies	Obligate	Milk gland	2	0.71	23.8	–	–	–
Arsenophonus melophagi	*Gammaproteobacteria*	Louse flies	Obligate	Milk gland	0, 1	1.16	32.2	–	–	–
Sodalis baculum	*Gammaproteobacteria*	Lygaeoid stinkbugs	Obligate	Plausible transovarial transfer	1	1.62	36.8	–	–	–
Serratia symbiotica clade B	*Gammaproteobacteria*	Lachinae aphids	Transitional obligate	Transovarial transfer, possible horizontal transfer	2, 3	0.65–1.76	20.9–32.2	–	–	–
Intracellular facultative endosymbionts										
Asaia spp.	*Gammaproteobacteria*	Mosquitos, leafhoppers	Facultative	Vertical and horizontal transmission	0, 6	3.55	60.2	✓	✓	✓
Fritschea spp.	*Chlamydiae*	Scale insects	Facultative	Vertical and possible horizontal transmission				–	–	–
Fukatsuia symbiotica	*Gammaproteobacteria*	Aphids	Facultative	Vertical and horizontal transmission	1, 2	2.96	43.6	✓	–	–
Hamiltonella defensa	*Gammaproteobacteria*	Aphids, whiteflies	Facultative	Vertical and horizontal transmission	8, 13	1.42–2.28	37.2–41.1	✓	–	–
Regiella insecticola	*Gammaproteobacteria*	Aphids	Facultative	Vertical and horizontal transmission	0, 2	2.04	42.5	(✓)	–	–
Rickettsiella viridis	*Gammaproteobacteria*	Aphids	Facultative	Vertical and probable horizontal transmission	1	1.58	39.3	–	–	–
Serratia symbiotica clade A	*Gammaproteobacteria*	Aphids	Facultative	Vertical and horizontal transmission	2, 12	1.53–3.58	40.3–52.5	✓	–	–
Sodalis glossinidius	*Gammaproteobacteria*	Tsetse flies	Facultative	Milk-gland	2	4.30	54.4	✓	✓	✓
Sodalis melophagi	*Gammaproteobacteria*	Louse flies	Facultative	Milk-gland	0, 1	4.15	50.8	✓	–	–

(Continued on next page)

TABLE 1 (Continued)

Symbiont	Phylum or class	Host	Association	Transmission	Available genomes (complete, total)	Genome size (Mb)	%GC	Culturability	Genetic engineering	Host recolonization
Reproductive manipulators										
Arsenophonus spp.	*Gammaproteobacteria*	Various insects	Reproductive manipulator	Vertical and horizontal transmission	8, 9	2.33–4.99	38.3	✓	✓	✓
Cardinium spp.	*Bacteroidetes*	Various arthropods and nematodes	Reproductive manipulator	Vertical and horizontal transmission	2, 8	0.89–1.48	33.7–39.2	(✓)	–	–
Rickettsia spp.	*Alphaproteobacteria*	Various arthropods	Reproductive manipulator	Vertical and horizontal transmission	0, 5	1.25–2.10	32.7	(✓)	✓	✓
Spiroplasma spp.	*Mollicutes*	Various arthropods and plants	Reproductive manipulator	Vertical and horizontal transmission	4, 9	0.71–2.30	23.7–29.3	✓	✓	(✓)
Wolbachia spp.	*Alphaproteobacteria*	Various arthropods and nematodes	Reproductive manipulator	Vertical and horizontal transmission	26, 1,249	0.47–2.55	26.4–38.3	(✓)	–	–
Extracellular symbionts										
Burkholderia gladioli	*Betaproteobacteria*	Lagria beetles	Context dependent	Egg smearing	0, 2	2.08–8.56	59–67.9	✓*	–	–
Burkholderia spp.	*Betaproteobacteria*	Alydid stinkbugs	Facultative	Environmental	1	6.96	63.15	✓*	✓	✓
Ishikawaella capsulata	*Gammaproteobacteria*	Plataspid stinkbugs	Obligate	Egg capsules	1	0.75	30.2	–	–	–
Pantoea spp.	*Gammaproteobacteria*	Pentatomid stinkbugs	Obligate	Egg smearing	2	1.18	30.53	✓*	–	–
Rhodococcus rhodnii	*Actinobacteria*	Assassin bugs	Facultative	Coprophagy	0, 3	4.44	69.7	✓	✓	✓
Snodgrassella alvi	*Betaproteobacteria*	Bees, bumble bees	Facultative	Coprophagy	1, 65	2.43	41.81	✓	✓	✓
Streptomyces philanthi	*Actinobacteria*	Beewolves	Context dependent	Brood smearing	0, 1	7.66	72.2	✓*	–	–
Tachikawaea gelatinosa	*Gammaproteobacteria*	Urostylidid stinkbugs	Obligate	Egg jelly	1	0.71	25.1	–	–	–

[a]The list is not exhaustive. Genome availability indicates the number of genomes published in the NCBI GenBank database in March 2020 and serves as an indication of the research attention for the model. A genome is considered complete if the assembly yielded a fully circularized chromosome with sufficient coverage. Indicated genome sizes and %GC are the average values of all complete published genomes of the species. Culturability status reads are the following: ✓, culturable in axenic medium; (✓), culturable on insect cell lines; –, not culturable *in vitro*. An asterisk indicates that the culturability status is valid for at least one strain of one species but might not be valid for the whole genus. Genetic engineering status reads are the following: ✓, at least a transformation with an exogenous plasmid has been achieved; –, no transformation has been reported in the literature. Host recolonization status reads are the following: ✓, *in vitro*-cultured endosymbionts can recolonize their natural host; (✓), *in vitro*-cultured wild-type endosymbionts can recolonize their natural host, but genetically modified endosymbionts have a colonization defect; –, recolonization has not been reported in the literature.

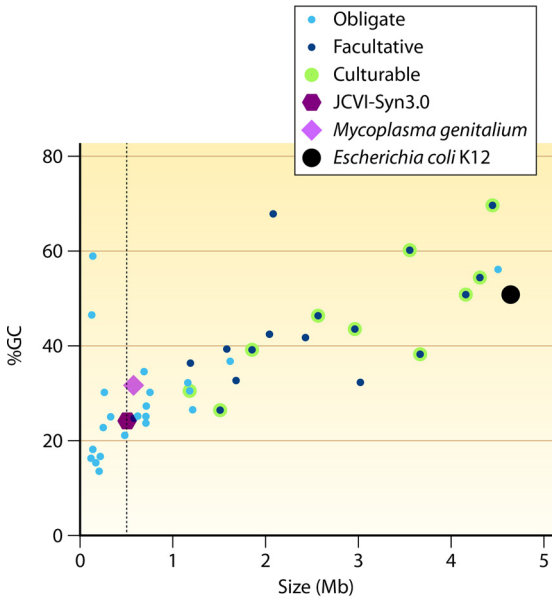

FIG 2 Genome size and AT bias of selected insect endosymbiont genomes and free-living bacteria with minimal genomes. The *Escherichia coli* genome is indicated as a free-living representative. *Mycoplasma genitalium* is indicated as a host-associated bacterium with a minimal genome and JCVI-Syn3.0 as an artificial, free-living bacterium with a minimal genome. The empirical genome size limit of 0.5 Mb, below which bacteria are not culturable yet, is marked with a dotted line.

manipulate their host's reproduction (67). They can be intracellular, extracellular, or both in the adult host, but their vertical transmission by transovarial transfer makes them experience an intracellular lifestyle in the oocyte of their host. Four manipulation mechanisms have been reported so far, including male feminization, male killing, cytoplasmic incompatibility, and parthenogenesis induction (67, 68), that all create an evolutionary drive favoring infected individuals over uninfected ones, thereby increasing the spread of the endosymbiont within insect populations. Reproductive manipulators are the most frequently encountered insect endosymbionts (22, 23) and, unlike the aforementioned obligate and facultative endosymbionts, are not host specialized. Most notably, *Wolbachia* form a wide range of phylogenetic groups that infect insects and crustaceans across diverse orders (22, 68, 69) and filarial nematodes, where they act as obligate endosymbionts (68, 70).

Extracellular Symbionts

The gut of most insects harbors a stable microbiota that can contain species with an intimate metabolic interaction with the host, reminiscent of those of intracellular endosymbionts. The gut microbiota of the insect models *Apis mellifera* and *Drosophila melanogaster*, for example, have been the focus of extended investigation (71), and some of their representative species have been successfully cultured and genetically engineered (e.g., see references 72 and 73). However, given the vast diversity of microbiota among insects (and sometimes among individuals of the same species), we chose not to discuss further the insect gut microbiota as a whole in order to limit the scope of this review. We will, however, present some select species of interest, either because of their particularly intimate interaction with their host or because they allowed a technical breakthrough in the development of culture media or genetic engineering methods for other species (Table 1). Select species include the honeybee gut symbiont *Snodgrassella alvi* (74), the stinkbug symbionts *Ishikawaella capsulata* (75), *Tachikawaea gelatinosa* (76), *Pantoea* spp. (77), and *Burkholderia* spp. (78), and the assassin bug symbiont *Rhodococcus rhodnii* (79). These species show variable levels of coadaptation with their host and variable levels of genome erosion (Table 1) (44, 75, 80, 81). Finally, some insects harbor extracellular symbionts in remarkable specialized

structures, such as *Lagria* beetles, which harbor extracellular *Burkholderia gladioli* in dorsal compartments in larvae and in accessory glands in adult females (82), or beewolves, which house brood-protective *Streptomyces* symbionts in the reservoir of their antennal glands (83). Extracellular symbionts can be obligate or facultative. Some of them are facultative under laboratory conditions but nearly obligate in the wild, as they protect their host against extremely widespread pathogens. Their transmission is environment mediated (i.e., the bacteria experience a passage through the external environment between each generation) (84).

WHY ARE MOST ENDOSYMBIONTS UNCULTURABLE?

The vast majority of the bacterial diversity is not culturable, meaning that the cells do not multiply on artificial media (85). Among several dozen insect endosymbiont species described so far, only a few of them can be grown *in vitro* on axenic media (i.e., culture media in which the bacteria of interest is the only live being), and a few more can be grown on insect cell lines only (Table 1). No intracellular obligate insect endosymbiont has been grown *in vitro* to date.

The Concept of Minimal Gene Set

Remarkably, the possibility to grow endosymbionts *in vitro* correlates with the sizes of their genomes and, hence, with the extent of genome erosion they underwent (Fig. 2); the larger the genome, the more likely the endosymbiont is to be culturable. This empirical observation reminds us of the concept of minimal gene set (or minimal genome), developed in the 1990s, which is the set of essential (strictly required) genes for an organism to live (86, 87). Defining a minimal genome proved to be challenging, because the essentiality of a gene is context dependent (87) and because noncoding regions can also be essential, although they are often overlooked in systematic gene inactivation screenings (88). The self-replicating bacteria with the smallest genome that can be grown *in vitro* are species of the *Mycoplasma* genus, with a genome size between 0.5 and 1 Mb. For instance, *Mycoplasma genitalium*, one of the first models used to experimentally determine a minimal gene set, has a genome of 0.58 Mb coding for 482 genes, of which 382 are essential (89), and JCVI-syn3.0 ("*Mycoplasma laboratorium*"), a fully synthetic bacteria derived from *Mycoplasma mycoides*, contains a 0.51-Mb genome coding for 473 genes (90). Remarkably, the minimal gene set of JCVI-syn3.0 includes genes involved in core functions (transcription, translation, and division), cell structure, and metabolic pathways, as well as 149 genes of unknown function (90). This suggests that predicting the culturability of a bacterium goes beyond metabolic and structural prediction and necessarily implies empirical assays.

Genome Limitations for the Culture of Insect Endosymbionts

A size of 0.5 Mb makes an empirical threshold below which we are currently not able to cultivate bacteria (Fig. 2). This limit is not fixed and can evolve along with technical advances, and it also does not imply that a bacterium with a larger genome will be easily culturable, if at all. Because of their gradual erosion, the genomes of ancient obligate insect endosymbionts (whether they are intra- or extracellular) often had sizes below 0.5 Mb (Table 1), sometimes below 0.2 Mb, and retained fewer genes than some eukaryotic organelles (91). Such endosymbionts retained a context-dependent minimal gene set that allows them to live inside bacteriocytes or in their specific gut niche, where they benefit from a stable environment and nutritional support from the host but are very unlikely to adapt to any environmental change. Thus, having them growing *in vitro* will be a major challenge, as they would require an *in vitro* environment that mimics exactly what they experience within their host in terms of nutrient availability but also in terms of physicochemical microenvironment. A notable exception among obligates in their genome size is *Sodalis pierantonius*, the obligate endosymbiont of the cereal weevil *Sitophilus* sp. (92, 93). *S. pierantonius* established a symbiotic relationship with its host through the replacement of a weevil ancestral symbiont, *Nardonella* sp. (33, 94). While *Nardonella* cospeciated with Dryophthorinae

and Molytinae weevil 125 million years ago (33, 94, 95), its replacement by *S. pierantonius* happened only circa 28,000 years ago (96), making it the "youngest" known obligate insect endosymbiont. The *S. pierantonius* genome has not undergone significant erosion and has a size comparable to that of free-living bacteria (97), which suggests a good ability to cope with environmental changes and makes it a good candidate for developing *in vitro* culture.

A few other obligates with genomes over 0.5 Mb also have been successfully maintained live *ex vivo* for a few hours to a few days. *Nardonella* can be maintained in bacteriome cultures for a couple of hours (98), and *Buchnera* can be extracted from the host bacteriocytes and survive for a few hours in isotonic buffer (99, 100). *Wigglesworthia*, the obligate endosymbiont of Tsetse flies, can be extracted and maintained live for several days in a medium based on pleuropneumonia-like organism medium (PPLO), a broth for the cultivation of *Mycoplasma* spp. (101). *Wigglesworthia* could divide once in PPLO-based medium when supplemented with nucleotides but did not grow further after this unique division, although it survived for several months. Interestingly, *Wigglesworthia* does not survive in rich insect cell medium even when supplemented with nucleotides, vitamins, and hormones, illustrating that the presence of various and abundant nutrients is not sufficient to sustain its growth *in vitro* (101).

Facultative endosymbionts, on the other hand, have genomes that are above 0.5 Mb in size, mostly above 1 Mb (Table 1), indicating that they retained a richer gene set that entails a better adaptability to change. In theory, facultative insect endosymbionts are more prone to becoming culturable, the challenge being to recreate *in vitro* an environment that is close enough to what they experience in-host.

ENDOSYMBIONT CULTURE ON INSECT CELL LINES

Facing the observation that insect endosymbionts were not easily adaptable to axenic culture media, several research groups turned to insect cell cultures as a cradle to seed these bacteria (Fig. 3). The rationale behind this approach is that the living host cells would condition the medium by releasing any uncharacterized growth factor required for the endosymbiont to grow. This conditioned medium would sustain the bacterial growth for a few generations, during which the population would undergo a selection toward *in vitro*-adapted individuals and eventually be able to survive without the insect cells. The earliest reports of an arthropod endosymbiont growing on a cultured cell line concerned *Wolbachia*, but the difficulty of classifying bacteria at the time, based on biochemical and morphological observations, led to identification mistakes. A famous example is the cultivation in axenic medium in 1961 of *Wolbachia persica*, a rickettsia-like bacterium isolated from ticks (102). Phylogenetic analyses performed decades later indicated that *W. persica* was related to gammaproteobacteria rather than *Rickettsiales* (103) and led to its recent reclassification in the *Francisella* genus (104). Such examples are grounds for caution when considering culture attempts performed before the rise of the genomics era.

A wide range of properly identified facultative insect endosymbionts have been cultured on insect cell lines, such as *Sodalis glossinidus* (facultative endosymbiont of Tsetse flies [105]), *Regiella insecticola* and *Hamiltonella defensa* (facultative endosymbionts of aphids [106]), and several reproductive manipulators belonging to the *Wolbachia*, *Spiroplasma*, *Arsenophonus*, and *Cardinium* genera (107–111). Most of these endosymbionts are intracellular during at least a part of their life cycle, which could explain their ability to thrive in cells *in vitro*. An intracellular lifestyle is, however, not sufficient to entail *in vitro* growth on cultured cells, as no obligate endosymbiont has been cultured this way so far.

Interestingly, these endosymbionts were sometimes cultivated on cells isolated from their native host but also on cell lines derived from other insects, which the endosymbiont cannot infect *in vivo* (Table 2). This indicates (i) that nonhost cells do not show any adverse effect against the symbiont and (ii) that facultative endosymbionts require a medium conditioning that is not specific to their host cells, hence, the conditioning is unlikely to consist of the release of highly species-specific molecules. Most notably, the

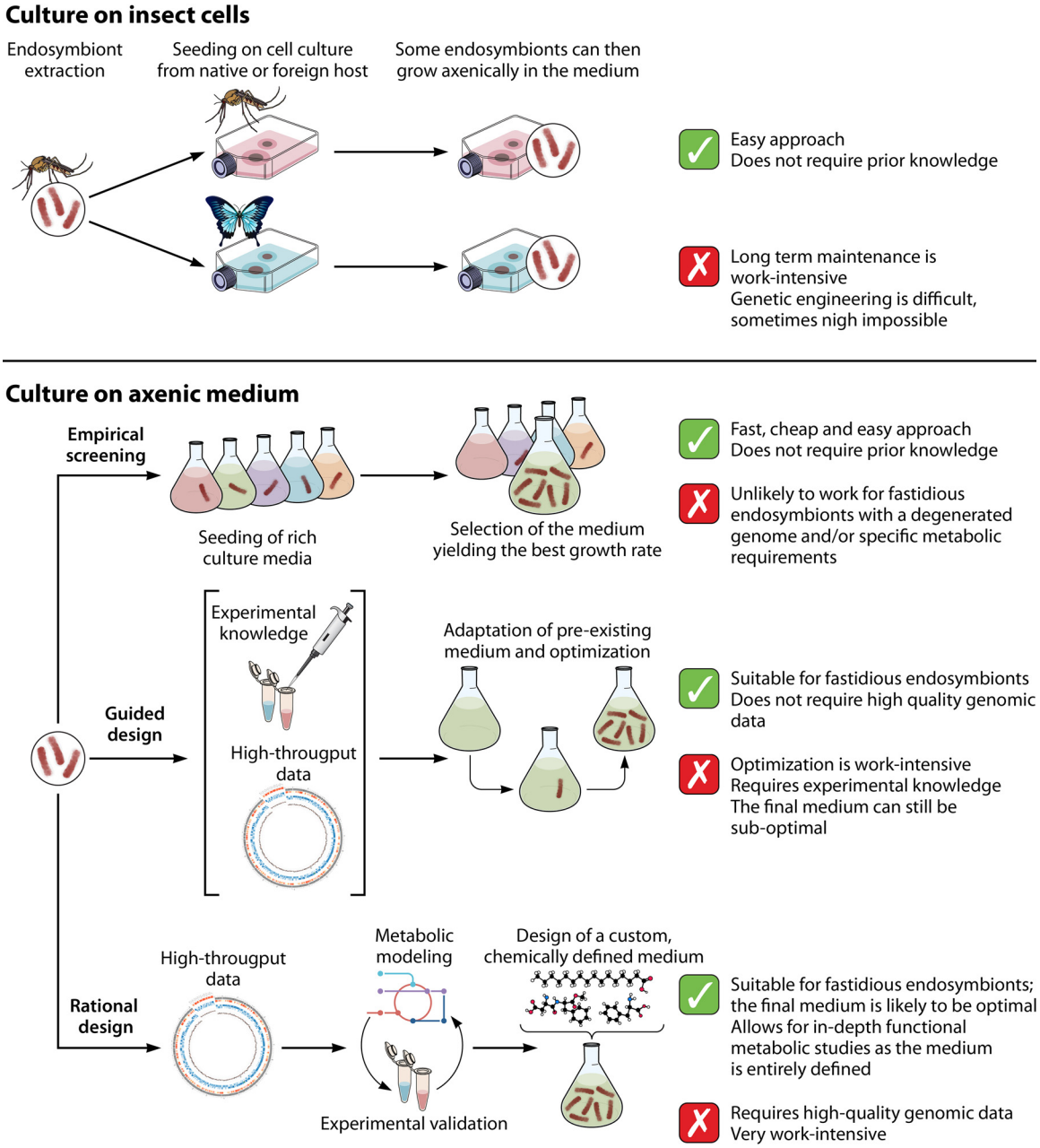

FIG 3 Approaches for the development of endosymbiont *in vitro* culture.

cell line C6/36, derived from the mosquito *Aedes albopictus*, can support the growth of facultative endosymbionts of distantly related insects, such as *Hamiltonella defensa* and *Regiella insecticola*, from aphids as well as *Arsenophonus trioatominarum* extracted from kissing bugs (106, 109). However, this might not be the case for any *Aedes albopictus* cell lines, as evidenced by *in vitro* infection experiments performed with *Wolbachia* that revealed a great disparity in the way different cell lines cope with the presence of the endosymbiont (112). Remarkably, *Wolbachia* growth can also be sustained by a mammalian cell line (110), although this observation has not been supported by other studies so far. The ability to grow on noninsect cells is likely due to its relatedness to mammalian pathogenic *Rickettsiales*. *Wolbachia* is, however, likely to be an exception, as no other insect endosymbiont has been cultured in nonarthropod cell lines so far.

The culture of endosymbionts on insect cell lines has allowed significant discoveries

TABLE 2 Culture conditions of insect endosymbionts on insect and mammalian cell lines[a]

Endosymbiont	Host	Cell line	Cell line origin	Infection	Reference
Arsenophonus triatominarum	*Triatoma infestans* (kissing bug)	C6/36	*Aedes albopictus*	Artificial	109
Cardinium spp.	*Ixodes scapularis*, *Rhipicephalus appendiculatus* (ticks)	RAE25, ISE6, ISE25	*Ixodes scapularis*, *Rhipicephalus appendiculatus* (ticks)	Natural	111
Regiella insecticola, *Hamiltonella defensa*	Aphids	C6/36 S2 Sf9	*Aedes albopictus* *Drosophila melanogaster* *Spodoptera frugiperda* (*Lepidoptera*)	Artificial	102
Rickettsia monacensis	*Ixodes scapularis* (tick)	ISE6	*Ixodes scapularis* (tick)	Artificial	118
Sodalis glossinidus	Tsetse fly, *Glossina* sp.	Unknown	*Aedes albopictus*	Artificial	105
Spiroplasma poulsonii	*Drosophila willistoni*	IPLBTN-R2	*Trichoplusia ni* (*Lepidoptera*)	Artificial	108
Wolbachia pipientis	*Aedes albopictus*	Aa23	*Aedes albopictus*	Natural	107
Wolbachia spp.	*Laodelphax striatellus* (planthopper)	NIAS-AeAl-2 BCIRL-HZ-AM1 L929	*Aedes albopictus* *Heliothis zea* (*Lepidoptera*) *Mus musculus* (mouse)	Artificial	110

[a]A natural infection indicates that the endosymbiont was grown on cells originating from its natural host, while an artificial infection indicates that the endosymbiont was grown on cells from an organism other than its natural host.

on the way they interact with their host cells (113–115) and their mechanisms of infection (116), and it allowed researchers to perform drug screenings *in vitro* (117). It is, however, a palliative method, as it is a technically fastidious process compared to culture in an axenic medium. Furthermore, very few genetic manipulations on bacteria cultured on cell lines have been reported so far (118), illustrating how technically challenging it can be.

EMPIRICAL DEVELOPMENT OF AXENIC MEDIA

Some insect symbiont genera have been successfully cultured in axenic media, mostly extracellular or intra-/extracellular mixed lifestyles (Table 3). Some extracellular symbionts, such as *Bukholderia* sp., *Pantoea* sp., and *Rhodococcus rhodnii*, are easily cultivated (77–79, 119, 120). Their unstable environment, relative to that of intracellular endosymbionts, did not entail a significant erosion of their genomes (Table 1), and they adapt well to standard rich microbiology media, such as Luria-Bertani (LB) broth or brain heart infusion (BHI). Their optimal growth temperature is within the 20 to 30°C range, consistent with the temperatures at which their respective host thrive (77–79). *Asaia* sp., a facultative endosymbiont of mosquitos (121) and leafhoppers (122), has a mixed lifestyle. It infects massively the gut lumen but also the salivary glands and reproductive tract of its host (121, 123). Consequently, it is not a demanding bacterium and grows well on media that have been designed for free-living relatives of the same genus (121, 124).

Other symbionts can be grown on media designed for insect cell culture, which replicate the environment they experience in-host (Table 3). The Mitsuhashi and Maramorosch (MM) medium (125), originally designed for leafhopper cell culture but also suitable for dipteran, homopteran, and lepidopteran cell lines, was used to grow *Arsenophonus arthropodicus* from louse flies (126), *Sodalis glossinidius* from Tsetse flies (79, 127), and *Sodalis melophagi* from sheep keds (128). The TC-100 medium (129), a modified Grace's medium optimized for Sf9 (*Spodoptera frugiperda* 9) cell growth and baculovirus expression, is also suitable to grow the aphid facultative endosymbionts *Hamiltonella defensa* (130) and *Fukatsuia symbiotica* (131) and the beewolf symbiont *Streptomyces philanthi* (132). Lastly, the medium 863 has been used for *Serratia symbiotica* culture (133, 134), although it was demonstrated later that it can be replaced by Trypticase soy broth, a standard microbiology medium (135). Remarkably, a high variability in the temperature requirements of various strains of the same endosymbiont species has sometimes been reported (136), inviting caution regarding this parameter when trying to cultivate new species.

Some species require being cultured on insect cell lines in an adaptation step before being shifted to an axenic medium (e.g., see reference 130), while others can be directly cultured in axenic medium after isolation from their host. For instance, *Spiroplasma poulsonii* and *Sodalis glossinidius* were historically cultured axenically after an insect cell

TABLE 3 Culture conditions of selected insect symbionts in axenic media

Endosymbiont	Host	Medium design	Base medium	Supplementation	Atmosphere	Optimal temperature (°C)	Shaking (rpm)	Reference
Arsenophonus arthropodicus	*Pseudolynchia canariensis* (louse fly)	Insect cell line medium	Mitsuhashi and Maramorosch insect (MMI) medium		Microaerophilic	25	No	118
Arsenophonus nasoaniae	*Nasionia vitripennis* (parasitoid wasp)	Standard microbiology media	Brain heart infusion		Aerobic	30	250	164
Asaia spp.	*Anopheles* sp. (mosquito)	Standard microbiology media	Mannitol broth		Aerobic	30	Not reported	121, 124
Burkholderia spp.	*Riptortus clavatus* (stinkbug)	Insect cell medium and standard microbiology media	Mitsuhashi and Maramorosch insect (MMI) medium		Aerobic	25–30	100–150	78
	Lagria beetles		Luria-Bertani broth					82
			YG broth					119
			MGY liquid medium					
Fukatsuia symbiotica	*Acyrthosiphon pisum* (aphid)	Insect cell line medium	TC-100 insect medium	Fetal bovine serum	Aerobic	20–27	No	131
Hamiltonella defensa	*Acyrthosiphon pisum* (aphid)	Insect cell line medium	TC-100 insect medium	Fetal bovine serum	Aerobic	20–27	No	130
Pantoea spp.	*Plautia stali* (stinkbug)	Standard microbiology media	Luria-Bertani broth		Aerobic	25	Not reported	77
Rhodococcus rhodnii	*Rhodnius prolixus* (Triatominae)	Standard microbiology media	Brain heart infusion		Aerobic	28	150	79
Serratia symbiotica	*Aphis fabae* (aphid)	Empirical	863 medium		Aerobic	20–28	150–200	133
		Preexisting medium designed for a free-living relative	Trypticase soy broth		Microaerophilic			135
		Insect cell line medium	Mitsuhashi and Maramorosch insect (MMI) medium	Fetal bovine serum				137
Sodalis glossinidius	*Glossina* sp. (Tsetse fly)	Standard microbiology media, rational design	Luria-Bertani broth, brain heart infusion		Microaerophilic	25	No	127, 144, 145
Sodalis melophagi	*Melophagus ovinus* (sheep ked)	Insect cell line medium	Mitsuhashi and Maramorosch insect (MMI) medium	Fetal bovine serum	Microaerophilic	27	No	127
Streptomyces philanthi	Solitary digger wasps	Insect cell line medium	Grace's and others	Fetal bovine serum	Aerobic	27–30	No	132
Spiroplasma poulsonii	*Drosophila* sp. (fruit fly)	Insect cell line medium	H-2 medium		Aerobic	25	No	108
		Prior-based screening and optimization	BSK-H-spiro	Rabbit serum, fly extract and lipids	Microaerophilic			138

culture adaptation step (108, 137) and before methods for direct culture were established (127, 138), indicating that the insect cell line step can facilitate the adaptation to the *in vitro* condition but is probably not necessary for most bacteria if a suitable medium is designed. A direct axenic culture also means that the bacterium is readily available in a host-like *in vitro* environment after extraction from its host, reducing the extent of any selection or adaptation process that could bias *in vitro* versus *in vivo* comparative studies.

RATIONAL DESIGN OF AXENIC MEDIA

Empirical approaches using insect cell lines or standard microbiology media allowed significant progress in rendering facultative endosymbionts culturable; however, sometimes they are inefficient. Even though their genomes are not dramatically eroded, some endosymbionts do not grow in defined medium containing all amino acids and vitamins that would theoretically support the growth of any bacteria. This is reported for *Cardinium* sp. and *Spiroplasma poulsonii* (111, 138, 139), and numerous research laboratories tried to culture other endosymbionts using empirical trials and went unsuccessful without publishing (personal communications). This can be explained by a requirement for specific structural lipids or sugars, metabolites produced by host anaplerotic reactions, or unexpected carbons sources. An alternative approach that accounts for this possibility consists of designing a custom culture medium by leveraging knowledge on the bacterium from experimental results from databases (Fig. 3). The first successful cultures using rational design were historically established for the human pathogen *Tropheryma whipplei* (140). The design was based solely on a genome analysis that revealed incomplete metabolic pathways. A selective complementation of these pathways allowed the bacterium to grow. Such an approach was further developed for intracellular bacteria with small genomes, leveraging genomics but also experimental knowledge. An illustrative example is the medium design of *Coxiella burnetii*, another human pathogen (141). A base medium sustains the bacterium for 24 h, which allowed the experimental testing of genomics-based predictions regarding the nutritional requirements of the bacteria and the atmosphere composition (i.e., the O_2 and CO_2 balance) in which it grows best. Transcriptomics data are also easy to obtain from unculturable bacteria and can be leveraged to develop culture media. For example, a metatranscriptomics analysis revealed the unexpected requirement for host mucins as a carbon source for a *Rikenella*-like bacterium living in the leech gut (142). Replacing the glucose of EG medium (a standard microbiology medium) with bovine mucins was sufficient to allow the *Rikenella*-like bacterium to grow *in vitro*.

Rational designs have only recently been performed for endosymbiotic bacteria, with the culture of *Spiroplasma poulsonii* and *Sodalis glossinidius* in a partially and completely rationally designed medium, respectively. *Spiroplasma poulsonii* was cultured axenically with an insect cell line adaptation step in 1986 (108), but this achievement was never reproduced outside of the original laboratory. A possible explanation relies on the variability between *S. poulsonii* strains, some of which are less fastidious than others and are more able to grow in simpler media. More than 30 years later, a medium was designed using a partially rational approach (138). The design relied on an extremely rich base medium, the Barbour-Stoenner-Kelly H (BSK-H) medium, that was adapted based on the prior experimental knowledge that *S. poulsonii* massively takes up host lipids (143) and on the hypothesis that *S. poulsonii* needs other, unknown, host molecules and a microaerophilic environment. Optimization was then made using a statistics-based method derived from the industrial process optimization field. Briefly, the ability of the bacterium to grow was measured in a set of test media that were prepared with various levels of insect extract and lipid supplementation, as well as different oxygen partial pressures. The effect on growth was then computed for each of these parameters using a statistical model, which allowed predicting which level should be optimal for each parameter, even if the optimal combination of all parameters had not been tested experimentally. A final experimental assay of the predicted optimal medium, the BSK-H-spiro medium, validated it as supporting *S. poulsonii*

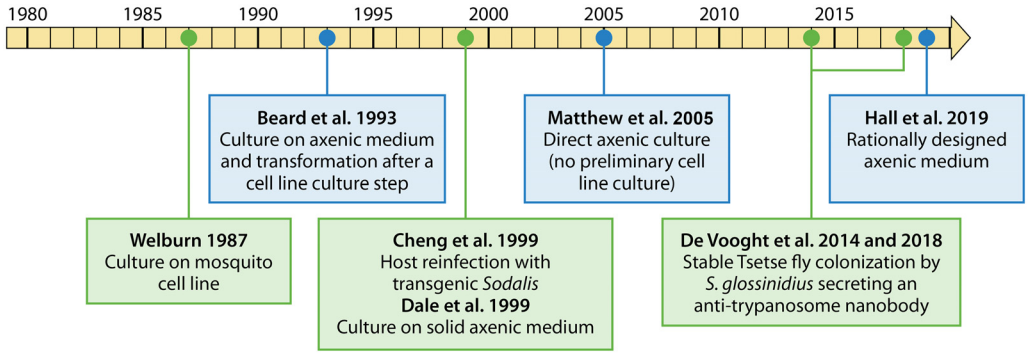

FIG 4 Milestones in the technical development of *Sodalis glossinidius in vitro* manipulation. See references 105, 127, 137, 145, 154, 156, 176, and 177.

growth better than any other tested combination of parameters (138). This method is straightforward and applicable to any endosymbiont than can at least survive in an already existing medium. Its main limitation is that only tested parameters can be optimized, meaning that researchers must have knowledge available (or prejudices) about the endosymbiont requirements in order to choose the most appropriate parameters to optimize (Fig. 3). If the endosymbiont requires nutrients that are not suspected from experimental data, this approach cannot predict it and will not yield any result.

Unlike *S. poulsonii*, *S. glossinidius* is relatively easy to grow *in vitro* and settles for insect cell line media or standard microbiology media, such as LB and BHI (127, 137, 144). All these media are, however, chemically undefined, which hampers *in vitro* metabolic studies based on the selective depletion of nutrients. A further step was taken in the rational design of culture medium with the development of SGM11, an entirely defined medium for the axenic culture of *S. glossinidius* (145) (Fig. 3 and 4). A whole-genome metabolic model has been established (146) and refined (145) to predict which metabolites are essential for *S. glossinidius* growth (*N*-acetylglucosamine, trehalose, L-serine, L-arginine, L-proline, L-glutamate, L-aspartate, nicotinamide, α-ketoglutarate, fumarate, and thiamine monophosphate). An M9 minimal medium supplemented with these metabolites supported *S. glossinidius* growth better than a rich undefined medium (145). This entirely defined medium is a valuable tool for metabolic studies of *S. glossidinius* and already offered new perspectives on the way it interacts with its hosts by taking up *N*-acetylglucosamine derived from the breakdown of the tsetse peritrophic membrane, favoring host infection by trypanosome parasites (145, 147).

This approach has the massive advantage of not requiring any prior knowledge of the endosymbiont metabolism and theoretically can be applied to any sequenced endosymbiont (Fig. 3). One of its main limitations is the requirement for very-high-quality genomic data to ensure the validity of the metabolic model. However, the steady improvement of sequencing technologies and the consequent growing number of good-quality insect endosymbiont genomes made available (Table 1) make this approach highly promising, especially for endosymbionts with highly degenerated genomes that require multiple supplementations.

GENETIC TRACTABILITY OF CULTURED SYMBIONTS

Growing an endosymbiont *in vitro* already brings interesting opportunities to study its metabolism, make *in vitro* versus *in vivo* comparative analyses, or even perform experimental evolution studies that would be complicated to run in the host. This is also the first step toward achieving genetic tractability of the bacteria, which in turn would unlock functional genetic studies. Among axenically culturable insect endosymbiont species, only a few have also been genetically modified and resettled in their host. The pipeline is relatively straightforward and includes the *in vitro* isolation and culture of the endosymbiont, its transformation and selection of clones, and the

recolonization of the host with the transformant or recombinant population for *in vivo* studies.

Genetic Tractability of Extracellular Symbionts

Some symbionts with an extracellular or mixed lifestyle can be readily modified, such as *Burkholderia insecticola* (148, 149), *Rhodococcus rhodnii* (79, 150, 151), and *Asaia* sp. (121, 152, 153), using protocols and plasmids or chromosomally knocked in constructs designed for closely related free-living bacteria. With these models, no major issues have been raised regarding the transformation efficiency, the selection of modified clonal populations, or host recolonization by feeding.

Sodalis glossinidius as a Case Study for Facultative Endosymbiont Genetic Engineering

Undoubtedly the most advanced model for genetic modification among facultative endosymbionts that do not experience an extraorganismal phase in their lifestyle is *S. glossinidius*. This endosymbiont has been transformed multiple times using a wide range of constructs, including free or integrative plasmids that carry a green fluorescent protein (GFP) marker (137, 154, 155) and, more recently, a cassette for the expression and secretion of a functional nanobody *in host* (156, 157). Transposon-based mutagenesis screenings have been successfully carried out, leading to the identification of the type III secretion system as a crucial requirement for host cell invasion (158). Targeted knockouts and reporter fusions were also used to point out the involvement of the two-component system PhoP-PhoQ in regulating the type III secretion system and in resistance against host antimicrobial peptides (159) and the role of the outer membrane protein A (OmpA) in biofilm formation and host gut colonization (160) and to extensively characterize the iron acquisition mechanism of *S. glossinidius* and its implications in endosymbiotic homeostasis (161–163).

Spiroplasma poulsonii and *Arsenophonus nasoniae* as Emergently Tractable Models

More recently, *Spiroplasma poulsonii* and *Arsenophonus nasoniae*, two reproductive manipulators, have also been transformed by plasmids carrying a fluorescent marker (164, 165). *A. nasoniae* has been isolated *in vitro* from its host, the parasitoid wasp *Nasonia vitripennis*, and transformed with pOM1-*gfp*, a shuttle vector originating from the pathogenic gammaproteobacteria *Francisella tularensis* (166). This plasmid was transformed previously in other gammaproteobacteria (167), and its ability to replicate in *A. nasoniae* suggests that it is a tool of major interest for the transformation of other culturable endosymbiotic gammaproteobacteria, such as the aphid facultatives *Hamiltonella*, *Fukatsuia*, and *Serratia*, as well as a majority of obligate endosymbionts, should they become culturable. The transformation of *A. nasoniae* with a GFP-coding plasmid and its successful resettlement in the *N. vitripennis* host allowed for the *in vivo* tracking of the transmission route of the endosymbiont with an unprecedented level of detail (164).

Unlike most endosymbiont transformation reports that use plasmids with a broad host range, such as pOM1, the approach for transforming *Spiroplasma poulsonii* relies on the use of a shuttle vector derived from a natural plasmid found in the closely related species *Spiroplasma citri* (165). Although the plasmid could be transformed in *S. poulsonii*, the selection of positive clones and recolonization of the host proved to be challenging. Because of its extreme thinness (100 to 200 nm thick) and strong motility, *Spiroplasma poulsonii* swims across gelled media and, thus, does not grow colonies, preventing easy clone selection. Furthermore, adult host recolonization with a mixed population of transformant and wild-type bacteria leads to infection-induced phenotypes (most notably, male killing in the offspring of injected females), but the transformant bacteria remain undetectable in the host (165), indicating a poor recolonization ability.

Host Recolonization with Genetically Modified Endosymbionts

Interestingly, colonization difficulties have also been reported with recombinant *S. glossinidius*, which must be injected into antibiotic-treated Tsetse fly adults or pupae devoid of wild-type *S. glossinidius* (e.g., see references 155 and 156). These concomitant data, with that obtained with *S. poulsonii*, suggest that manipulating the endosymbionts *in vitro* can lead to decreased fitness *in vivo* compared to that of their wild-type counterparts, and, in some cases, irreversible changes happen *in vitro* that cause the loss of their ability to recolonize the host at all. Thus, the *in vitro* isolation, transformation, selection, and recolonization steps should be performed in the shortest possible time frame to limit the risk of such changes happening.

In any case, host recolonization is a delicate step in the process, and the optimal protocol can vary a lot across models. For example, injecting adult *Drosophila* with an *in vitro* culture of *Spiroplasma poulsonii* leads to an efficient vertical transmission (165), while recombinant *S. glossinidius* must be injected into larvae to observe an efficient vertical transmission (156). *A. nasoniae*, the parasitoid wasp symbiont, is not strictly vertically transmitted, as transmission occurs through the infection of host fly pupae by the wasp. When the parasitoid wasp female lays an egg in a fly pupa, it also contaminates the fly pupal tissues with the endosymbiont. The wasp larva subsequently eats infected fly pupa tissues and gets infected in turn (168). Thus, the best way to recolonize parasitoid wasps with transformant *A. nasoniae* is to inject the bacteria into fly pupae and let wasps parasitize them, leading to an efficiently infected next generation of wasps (164). These examples illustrate how the host recolonization method needs to be tailored individually for each model, accounting for its ecological features (e.g., the extracellular, mixed, or intracellular lifestyle of the endosymbiont and its transmission mechanism).

Looking Forward: Engineering of Recombination-Deficient Endosymbionts

Achieving the transformation of a plasmid is already a great step forward in endosymbiont genetic engineering, as it opens the possibility, for instance, of marking them for *in vivo* tracking or of adding foreign gene copies. However, complete genetic tractability requires the editing of the endosymbiont chromosome through recombination. Chromosomal recombination of a plasmid lifts the requirement for antibiotics for plasmid selection, which avoids antibiotics-related biases during *in vivo* studies. Recombination is also the key for targeted gene knockouts, as opposed to random mutagenesis approaches that are more labor-intensive and have an uncontrolled outcome. Recombination will also be required for the modification of symbionts for field applications in insect population control, such as *Wolbachia*, as modifications for this purpose need to be finely controlled and stable over time.

During their genomes' evolution, most obligate endosymbionts lose recombination and DNA repair genes (47), which could be a future hurdle for genetic engineering (should *in vitro* culture become available for them). This issue also concerns some endosymbionts with a low level of genome erosion, for instance, *S. poulsonii*, which has a pseudogenized copy of *recA* and no *recF*, two crucial recombination genes (138, 169). A solution to overcome this could be to use plasmid-encoded recombination facilitators. A first possibility is the use of phage-derived sequences, such as the integrative elements of the L1 mycobacteriophage, the attachment site *attP*, and the *integrase* coding sequence (170). Having these elements on a plasmid significantly favored its site-specific (although uncontrolled) and stable integration in the chromosome of *Rhodococcus rhodnii* (150) and can be a helpful approach for the development of knock-in plasmids for other symbionts. The addition of an exogenous *recA* coding sequence has also been successfully used to increase the recombination frequency in *Mycoplasma mycoides* (171) and could be adapted for symbiotic species as well. Lastly, CRISPR interference (CRISPRi) can be used to knock down bacterial genes using constructs borne on a nonintegrative plasmid (172). This could be a solution to studying symbiont gene function if recombination proves to be unachievable, although

it has the downside of requiring antibiotic selection to maintain the construct and cannot be used for field applications.

CONCLUSIONS

Culturing a symbiont is a major step forward in the understanding of its biology as a bacterium but also in the understanding of the way it interacts and affects its host's biology. Besides the obvious interest of having genetically modified symbionts as tools for basic research, it would represent a promising source of innovation for the development of paratransgenetic techniques (that is, affecting the host by genetically modifying its symbionts) to control insect pest or disease vector populations in the field. The past decade has seen a large number of technical breakthroughs in the field regarding the axenic culture of so-far unculturable species and regarding the development of genetic manipulation tools and their application for *in vivo* studies. These new developments allowed for a more comprehensive picture of the key factors that allow endosymbionts to grow and get modified *in vitro* and will hopefully snowball toward expanding the range of culturable species in the coming years.

APPENDIX

GLOSSARY

anaplerotic reaction Metabolic reaction that forms intermediate products of a metabolic pathway.

aposymbiotic Devoid of symbionts.

axenic medium Culture medium in which the cultured species is the only living organism.

bacteriocyte Specialized cells dedicated to the intracellular housing of endosymbionts.

bacteriome In most insect species, bacteriocytes group together to form a distinct organ called the bacteriome. In some species, bacteriocytes remain scattered throughout tissues and do not form a proper bacteriome structure.

endosymbiosis Symbiotic interaction in which the symbiont lives within host tissues (hemolymph) or cells (bacteriocytes or nonspecialized cells). It is opposed to ectosymbiosis, in which the symbiont lives outside the host organism (e.g., on the insect cuticle or in the close environment).

facultative A symbiont is facultative if it is not required for the host development or survival.

M9 minimal medium Minimal growth medium commonly used for bacterial cultures, composed of inorganics salts that can be complemented with select carbon sources.

microbiota All microorganism found in and on a host organism. The microbiota includes endosymbionts but also other species that can be mutualistic, commensal, or pathogenic.

obligate Obligate symbionts are required by their host to develop and survive under wild conditions. Some hosts can survive under laboratory conditions without their obligate symbionts, but their fitness is severely impaired.

shuttle vector Vectors (plasmids) that can propagate in at least two different species. Most shuttle vectors are designed to propagate in the species of interest and in *Escherichia coli* for easy cloning and amplification.

transmission (horizontal/vertical) A vertical transmission occurs when symbionts are transmitted from parents to offspring (most often by females, sometimes by males), as opposed to horizontal transmission, where the symbiont can be transmitted between individuals of the same generation.

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