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New Insights into the Nature of Symbiotic Associations in Aphids: Infection Process, Biological Effects, and Transmission Mode of Cultivable *Serratia symbiotica* Bacteria

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ABSTRACT Symbiotic microorganisms are widespread in nature and can play a major role in the ecology and evolution of animals. The aphid-*Serratia symbiotica* bacterium interaction provides a valuable model to study the mechanisms behind these symbiotic associations. The recent discovery of cultivable *S. symbiotica* strains with a free-living lifestyle allowed us to simulate their environmental acquisition by aphids to examine the mechanisms involved in this infection pathway. Here, after oral ingestion, we analyzed the infection dynamics of cultivable *S. symbiotica* during the host's lifetime using quantitative PCR and fluorescence techniques and determined the immediate fitness consequences of these bacteria on their new host. We further examined the transmission behavior and phylogenetic position of cultivable strains. Our study revealed that cultivable *S. symbiotica* bacteria are predisposed to establish a symbiotic association with a new aphid host, settling in its gut. We show that cultivable *S. symbiotica* bacteria colonize the entire aphid digestive tract following infection, after which the bacteria multiply exponentially during aphid development. Our results further reveal that gut colonization by the bacteria induces a fitness cost to their hosts. Nevertheless, it appeared that the bacteria also offer an immediate protection against parasitoids. Interestingly, cultivable *S. symbiotica* strains seem to be extracellularly transmitted, possibly through the honeydew, while *S. symbiotica* is generally considered a maternally transmitted bacterium living within the aphid body cavity and bringing some benefits to its hosts, despite its costs. These findings provide new insights into the nature of symbiosis in aphids and the mechanisms underpinning these interactions.

IMPORTANCE *S. symbiotica* is one of the most common symbionts among aphid populations and includes a wide variety of strains whose degree of interdependence on the host may vary considerably. *S. symbiotica* strains with a free-living capacity have recently been isolated from aphids. By using these strains, we established artificial associations by simulating new bacterial acquisitions involved in aphid gut infections to decipher their infection processes and biological effects on their new hosts. Our results showed the early stages involved in this route of infection. So far, *S. symbiotica* has been considered a maternally transmitted aphid endosymbiont. Nevertheless, we show that our cultivable *S. symbiotica* strains occupy and replicate in the aphid gut and seem to be transmitted over generations through an environmental transmission mechanism. Moreover, cultivable *S. symbiotica* bacteria are both parasites and mutualists given the context, as are many aphid endosymbionts. Our findings give new perception of the associations involved in bacterial mutualism in aphids.

KEYWORDS benefits, fitness consequences, gut bacteria, infection dynamics, insects, life history evolution, symbiosis, transfer route

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Symbiotic associations between animals and microorganisms are ubiquitous in nature (1). These symbiotic microorganisms can exert profound influences on host ecology and evolution (2–4). As a source of evolutionary innovation, some symbionts have allowed their hosts to colonize otherwise uninhabitable environments (5). This is the case for many insects that feed exclusively on nutritionally restricted diets, such as plant sap and blood, but that harbor obligate symbionts that supply essential nutrients lacking in the insect diets (6–8).

Obligate insect-microbe associations exhibit host-symbiont phylogenetic congruence, indicating that they are ancient and stable associations maintained by a strict vertical transmission of symbionts from mother to progeny (9–11). Because of the long-term association with their host, the genomes of these symbionts have been drastically reduced, which severely hampers their ability to survive outside their host (12). In addition to obligate microbial partners, insects can host facultative symbionts that have been acquired more recently and that are either beneficial, commensal, or parasitic, depending on the ecological and environmental context (2, 3, 13). Unlike obligate symbionts which most of the time are hosted in specialized cells called bacteriocytes, facultative partners can inhabit the gut or exist inside various cells and tissues within the body cavity, such as sheath cells, the hemolymph, and secondary bacteriocytes (6, 14). Such facultative symbionts can also experience occasional horizontal transfers (15, 16) and can be acquired from environmental sources (2, 17–19). Mutualistic bacteria generally form long-term associations with their hosts and have diverged from bacteria that were not dependent on a host, losing their capacity to live independently (12). This process is, however, poorly understood, partly because symbiosis is mainly studied using coevolved associations rather than associations in their early stages and partly because it is difficult to cultivate bacteria that have extreme host dependence (20).

Aphids (Hemiptera: Aphididae) offer a valuable model to investigate the evolutionary processes that shape microbial symbiosis in insects. Almost all aphid species harbor an ancient obligate symbiont, *Buchnera aphidicola*, that provides essential amino acids lacking in the aphid imbalanced diets (21). These phloem sap-feeding insects can also harbor various facultative endosymbionts that are involved in more recent associations (22). More erratically distributed in aphid populations than obligate partners, facultative partners are not essential for aphid host growth and reproduction but may lead to the acquisition of ecologically important traits by aphids (4, 23–26). However, while facultative symbionts can have beneficial fitness consequences for aphid hosts, they can also impose a fitness cost on their host that makes them parasitic (22, 27–30).

Serratia symbiotica is one of the most common facultative symbionts found in natural aphid populations (20). In the literature, *S. symbiotica* is known for being an aphid endosymbiont containing a variety of strains carrying contrasting genomes with different tissue tropisms and lifestyles (31–33). While *S. symbiotica* strains found in the subfamily *Lachninae* exhibit highly eroded genomes and are involved in co-obligate nutritional associations (32, 34), strains found in the pea aphid (*Acyrthosiphon pisum*) are of a facultative nature, have a less eroded genome, and have been depicted as being associated with heat stress tolerance and parasitoid resistance (24, 35). The state of genome erosion of the strains reflects their level of integration into the symbiotic system: the smaller that the genome is, the more that it hampers the capability of the bacteria to survive outside the host (33). The existence of strains with different degrees of interdependence on the host makes *S. symbiotica* a valuable model to study the evolution of bacterial mutualism in insects (32, 33).

Recently, several strains of *S. symbiotica* have been successfully isolated from aphids in the *Aphis* genus and cultivated freely in an axenic pure medium without insect cell lines and fetal bovine serum (FBS) (36, 37). A new field study just revealed that these cultivable strains belong to the same clade as the *S. symbiotica* strains residing in the gut of field-collected aphids, suggesting that they are most likely gut-associated symbionts (38). This tissue tropism and lack of complete interdependence with the aphid host raise questions about the actual endosymbiotic status generally attributed

to *S. symbiotica* and open up new perspectives to study the nature of symbiotic associations existing in aphids. Compared to other sequenced *S. symbiotica* strains, the cultivable strain CWBI-2.3^T (39) has a larger genome, contains more coding DNA sequences (CDSs), and has conserved a broader repertoire of genes related to metabolism as well as an array of molecular tools facilitating the invasion of tissues in new hosts (i.e., virulence factors) (33, 39, 40). The cultivable *S. symbiotica* strains are thus of great interest because their potential independence with respect to their hosts as well as the genomic features of the cultivable strain CWBI-2.3^T suggest that (i) they are at the beginning of their symbiotic life with aphids (41) and may represent a kind of missing link in the evolution from a free-living bacterium toward a host-dependent mutualistic symbiont and (ii) they may constitute a reservoir from which new mutualistic associations arise. However, despite evidence that the cultivable strain CWBI-2.3^T has no immediate antagonistic effect on a newly infected host (42), the associated biological effects of *S. symbiotica* cultivable strains and, hence, their symbiotic status, as well as the way in which they infect aphids, have never been examined.

Here, we investigated experimentally the initial steps involved in aphid gut infection by cultivable *S. symbiotica* strains to (i) the apprehend mechanisms underpinning this new association and (ii) determine the symbiotic status of these strains. To address this, we examined the infection dynamics of the bacteria during the host's lifetime, using fluorescence and quantitative PCR (qPCR) techniques. We then analyzed the immediate host fitness costs and benefits resulting from that formed interaction. We also studied the transmission mode of the bacteria and assessed the relatedness of these cultivable strains. Finally, on the basis of the results obtained in this and previous studies, we suggest that gut colonization by bacteria is likely a new type of interaction in aphids and/or represents a possible evolutionary step toward an endosymbiotic association with aphids.

RESULTS

Infection dynamic of cultivable *S. symbiotica* during aphid development. Fluorescence analyses revealed that cultivable *S. symbiotica* strain S1+ (CWBI-2.3^T) (Fig. 1, in red) multiplies and persists through the whole digestive tract of infected aphids. In the first infection step (0 days postingestion), *S. symbiotica* formed an aggregate in the stomach (Fig. 1B). At 2 days after infection, bacterial cells migrated into the intestine (Fig. 1C), after which the population of bacteria increased and dispersed into the whole gut at 5 and 10 days postinfection (Fig. 1D and E). After 15 days of infection, *S. symbiotica* continued to multiply strongly and further diffused along the digestive tract to reach the hindgut (Fig. 1F to H). A large mass that seemed to correspond to digestive tract swelling could be observed. At each time, all the localization patterns were consistent across individuals. However, they could be variable, for example, because of the different positions of aphids during fluorescence *in situ* hybridization (FISH) observations. The negative control showed only the obligatory symbiont *B. aphidicola* in bacteriocytes (Fig. 2).

Quantitative PCR confirmed the infection dynamic of cultivable *S. symbiotica* strain S1+ quantitatively. The density of cultivable *S. symbiotica* increased exponentially with aphid age (Fig. 3). The copy numbers of the *Aphis fabae EF1*α gene increased until 10 days postinfection (15 days of age) and then declined very slowly with aphid age (see Fig. S1A in the supplemental material). During oral ingestion, aphids acquired about 10^4 copies of the *S. symbiotica dnaK* gene, and the copy numbers of this gene increased rapidly up to 5 days postinfection (10 days of age). Then, the gene copy numbers continued to increase more slowly with aphid age to reach about 10^7 copies of the *S. symbiotica dnaK* gene (30 days of age) (Fig. S1B).

Costs associated with cultivable *S. symbiotica*. All cultivable *S. symbiotica* strains had a significant negative effect on the total number of offspring (generalized linear model [GLM], $F = 20.35$, degrees of freedom [df] $= 3$, $P < 0.001$), the adult mass (general linear model, $F = 5.39$, df $= 3$, $P = 0.003$), and the duration of reproduction (GLM, $F = 19.98$, df $= 3$, $P < 0.001$) of *A. fabae*. Fecundity was lowered by about half for

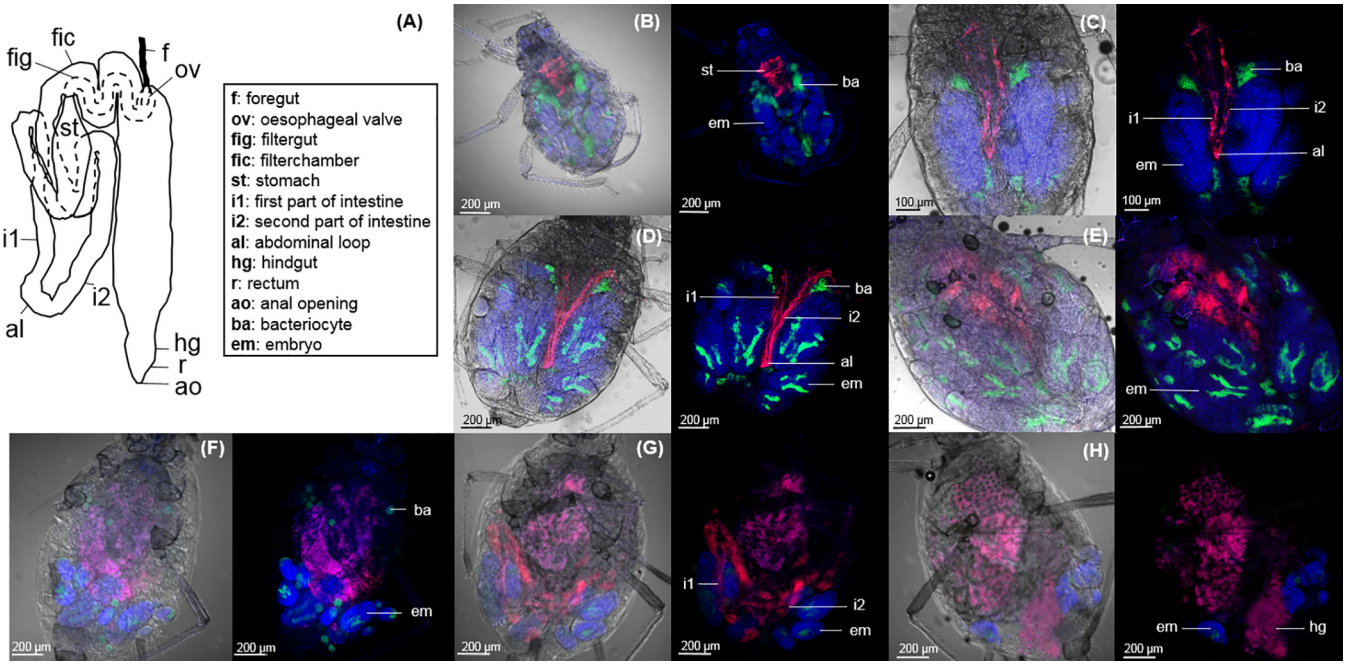

FIG 1 Infection processes of cultivable *S. symbiotica* strain CWBI-2.3^T (S1+) in *A. fabae* visualized by fluorescence *in situ* hybridization. (A) Semischematic representation of the digestive system of aphids inspired by a previous publication (47). (B to H) Ingestion of *S. symbiotica* after feeding of a contaminated diet (5 days old) (B) and at 2 days postinfection (7 days old) (C), 5 days postinfection (10 days old) (D), 10 days postinfection (15 days old) (E), 15 days postinfection (20 days old) (F), 20 days postinfection (25 days old) (G), and 30 days postinfection (35 days old) (H). In panels B to H, the photo on the right is without bright field and that on the left is with bright field. Red Cy3 signals correspond to *S. symbiotica*, green Cys5 signals correspond to *B. aphidicola*, and blue SYTOX green signals correspond to host tissues.

aphids infected with any one of the three strains of *S. symbiotica* compared to that for uninfected aphids (Fig. 4E). Fecundity was, however, less affected in aphids harboring the S1+ strain than in aphids harboring the other strains (S2+ and S3+). Adult mass and the duration of reproduction strongly declined when aphids were infected (Fig. 4C and F). Infections also significantly affected the survival of host aphids (Cox's model, $\chi^2 = 68.7$, df = 3, $P < 0.001$). A reduction of about 20 days in the longevity of infected aphids compared to that of uninfected aphids was observed (Fig. 4D; Table 1). Never-

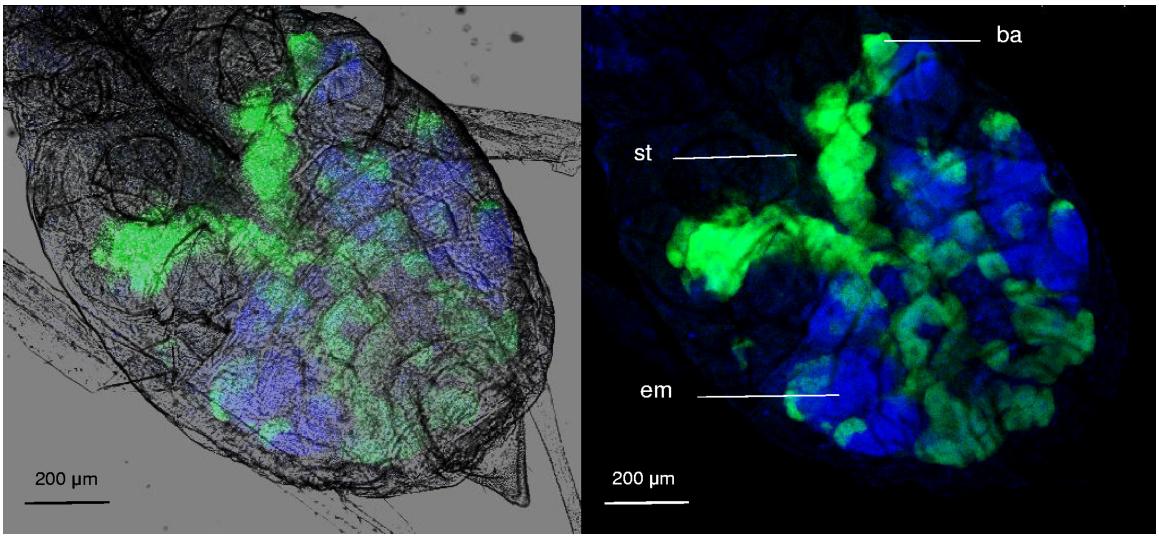

FIG 2 An uninfected *A. fabae* aphid visualized by fluorescence *in situ* hybridization. The photo on the right is without bright field, and the photo on the left is with bright field. Red Cy3 signals correspond to *S. symbiotica*, green Cys5 signals correspond to *B. aphidicola*, and blue SYTOX green signals correspond to host tissues. ba, bacteriocyte; st, stomach; em, embryo.

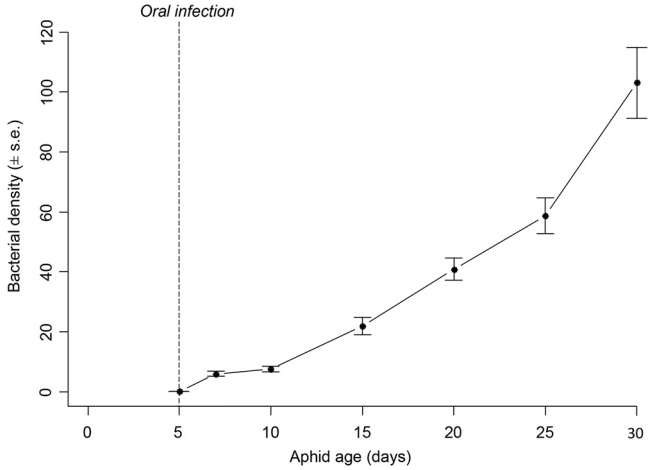

FIG 3 Population growth of cultivable *S. symbiotica* strain CWBI-2.3^T (S1+) during aphid development, estimated by TaqMan real-time quantitative PCR. Bacterial density is expressed as the *S. symbiotica dnaK* gene copy number divided by the *A. fabae EF1*α gene copy number (to correct for aphid body size). Six replicates were collected at different time points. Error bars depict the standard error.

theless, infection with cultivable *S. symbiotica* strains did not significantly affect the nymph survival rate (GLM, $\chi^2 = 1.59$, df = 3, $P = 0.058$; Fig. 4B) or development time (GLM, $F = 0.25$, df = 3, $P = 0.86$; Fig. 4A) of aphids.

Protective phenotype associated with cultivable *S. symbiotica*. Cultivable *S. symbiotica* strain S1+ had a significant negative effect on the parasitism rate (i.e., the number of parasitized aphids that became mummies) of *A. fabae* following *Aphidius colemani* parasitoid attack (generalized linear mixed model [GLMM], $\chi^2 = 28.48$, df = 1, $P < 0.001$). The formation of mummies was lowered by about 40% for infected aphids with cultivable *S. symbiotica* (30%) compared to that for uninfected aphids (71%) (Fig. 5A). The unmummified aphids were found either alive or dead (dead aphids were not generally found on the plant). The cultivable *S. symbiotica* bacteria had a significant effect on the death of attacked aphids (GLMM, $\chi^2 = 21.21$, df = 1, $P < 0.001$), with 47% of infected aphids but 21% of uninfected aphids found dead. Nevertheless, the rate of alive aphids was significantly greater for infected aphids (24%) than for uninfected aphids (8%) (GLMM, $\chi^2 = 8.07$, df = 1, $P = 0.0045$). The bacterial strain also had a significant negative effect on the emergence rate (i.e., the number of adult parasitoids that emerged from mummies/total number of mummies) of parasitoids from *A. fabae* (GLMM, $\chi^2 = 18.78$, df = 1, $P < 0.001$) and on the weight of the emerged parasitoids (general linear mixed model, $\chi^2 = 5.76$, df = 1, $P = 0.016$). The emergence rate of parasitoids from infected aphids (60%) was observed to be reduced by about 35% compared to that of parasitoids from uninfected aphids (95%) (Fig. 5B). After dissection of the nonemerged mummies, the presence of a dead parasitoid larva was systematically observed. The weight of the parasitoids that emerged from infected aphids was lower than that of the parasitoids that emerged from uninfected aphids (Fig. 5C).

Cultivable *S. symbiotica* transmission mode. A high transmission rate of cultivable *S. symbiotica* strains was observed in the first aphid generation (Table 2), but the rate varied significantly between strains (GLM, $\chi^2 = 12.83$, df = 2, $P = 0.0016$; Table 2). The transmission rate of strains S1+ and S2+ was higher than that of strain S3+ (Table 2). A high transmission rate of cultivable *S. symbiotica* strain S1+ was also observed in the second and the third generations, and the transmission rates over three generations were significantly similar (GLM, $\chi^2 = 0.57$, df = 2, $P = 0.75$; Table 2). For the other experiment, when aphids were taken off the plants immediately after birth (from adults infected by the S1+ strain), none were infected with the bacteria, while 7 out of 10 offspring were infected with the bacteria when they remained on the plant with infected adults. We further detected cultivable *S. symbiotica* strain S1+ in honeydew

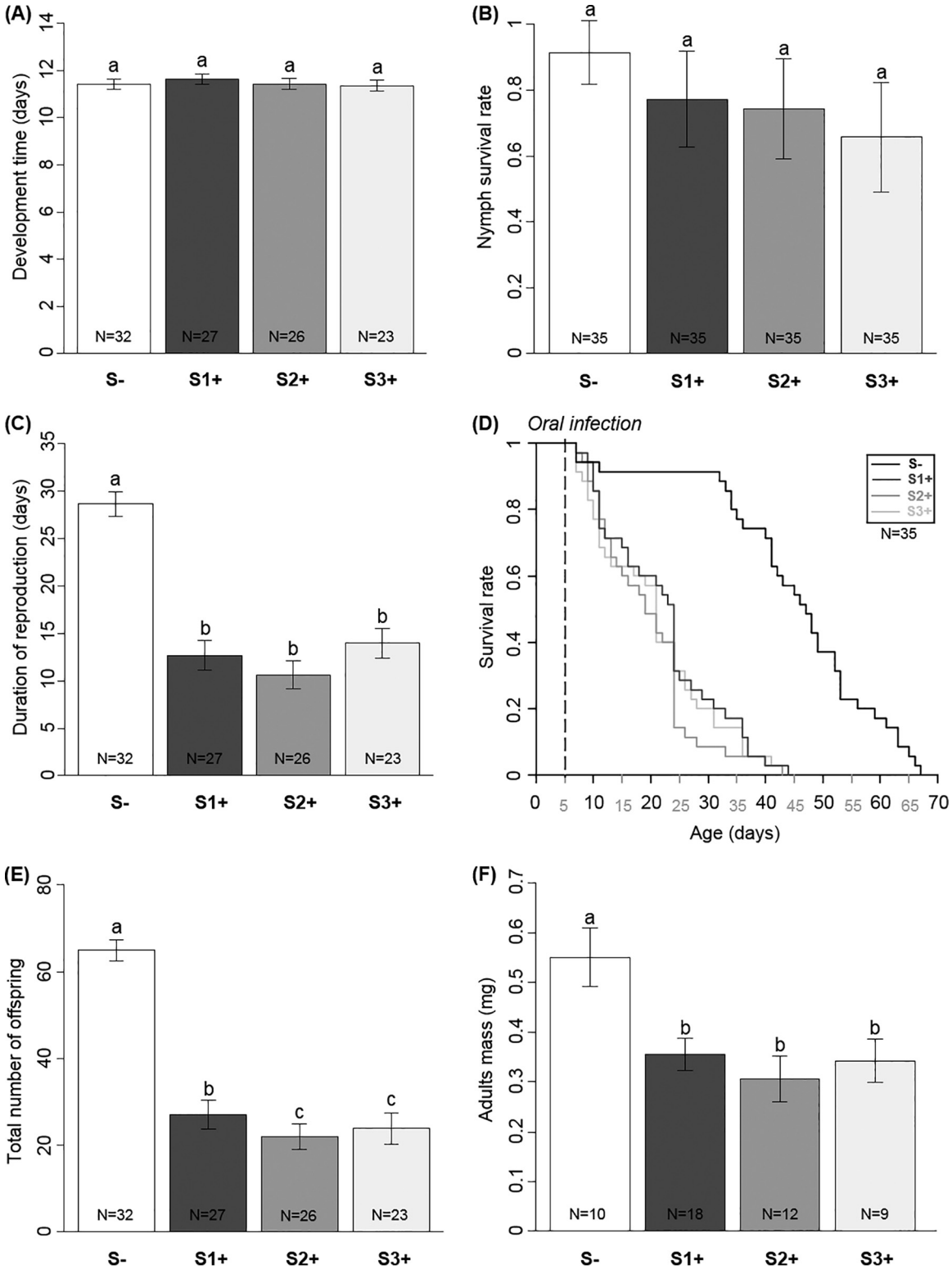

FIG 4 Effect of cultivable *S. symbiotica* strains on development time (A), nymph survival rate (B), duration of reproduction (C), percentage of survivors (D), total number of offspring (E), and mass of adults (F) of the aphid *A. fabae*. Four host treatments were used: aphids uninfected with cultivable *S. symbiotica* (S−) and aphids infected with cultivable *S. symbiotica* (S1+, strain CWBI-2.3^T; S2+, strain 24.1; S3+, strain Apa8 A1). *N*, number of aphids tested. Error bars depict the confidence interval for the nymph survival rate variable and the standard error for the other variables. The different lowercase letters show significant differences.

TABLE 1 Effect of cultivable *S. symbiotica* on the survival rate of the aphid *A. fabae*[a]

Treatment	β	Exp (β)	SE (β)	z	P
No infection with cultivable *S. symbiotica* (S−)	0.000	1.000	0.000		
Infection with cultivable *S. symbiotica* CWBI-2.3^T (S1+)	2.064	7.878	0.328	6.30	<0.001
Infection with cultivable *S. symbiotica* 24.1 (S2+)	2.367	10.664	0.333	7.10	<0.001
Infection with cultivable *S. symbiotica* Apa8 A1 (S3+)	2.197	8.998	0.329	6.68	<0.001

[a]β, estimated regression coefficient of Cox's proportional hazard model; Exp (β); hazard ratio; SE (β), coefficient standard error; *z*, *z*-test value; *P*, significance of the coefficient.

samples collected from all 13 aphids that were artificially infected in the digestive tract. These bacteria developed on the medium, indicating that living bacteria were potentially transferred from mother to offspring.

Cultivable *S. symbiotica* phylogeny. Phylogenetic analysis of concatenated sequences of the *accD*, *gyrB*, *murE*, and *recJ* genes revealed three distinct clades. Clade A was associated with the co-obligate endosymbiont *S. symbiotica* strains from *Cinara cedri* and *Tuberolachnus salignus* aphids (subfamily *Lachninae*; Fig. 6) which exhibit a long-term coevolutionary history with their host. Clade B consisted of the co-obligate endosymbiont *S. symbiotica* strain from *Cinara tujafilina* and the facultative endosymbiont *S. symbiotica* strain from *Acyrthosiphon pisum* (Fig. 6). Clade C included our three cultivable *S. symbiotica* strains (Fig. 6). From an evolutionary point of view, the cultivable strains (clade C) were closer to the clade of facultative endosymbionts (clade B) than the other clades. We also noticed that the evolutionary time separating our cultivable strains and *Serratia* species living independently of hosts is less important than the evolutionary time separating the co-obligate endosymbionts (clade A) and the free-living species.

DISCUSSION

Insects exhibit diverse relationships with a wide range of symbiotic bacteria that can deeply affect their ecology and evolution. Understanding how these symbiotic associations arise and spread across insect populations remains one of the most elusive challenges within the field of symbiosis research. The aphid-associated bacterium *S. symbiotica* has typically been described to be a strict endosymbiont, but the discovery of cultivable strains that are potentially capable of growing outside aphid hosts shows a different aspect of this symbiont species and raises questions about its associated biological effects, as well as the nature and the durability of the associations in which it is involved. These strains have been isolated from *Aphis* species but have not been clearly localized in their original hosts (36, 37). Nevertheless, a new field study revealed that these cultivable strains belong to the same clade as other *S. symbiotica* strains that reside in the aphid gut harvested in the field (38), suggesting that they would derive

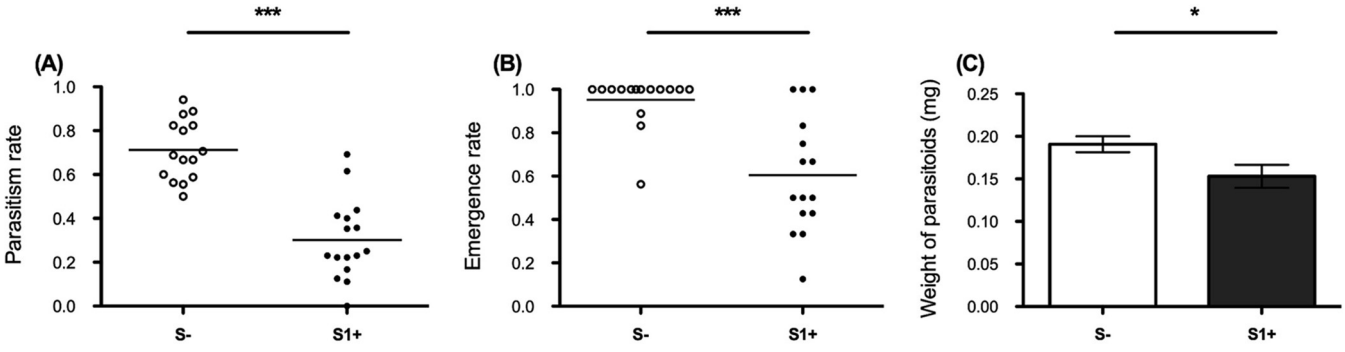

FIG 5 Effect of cultivable *S. symbiotica* strain CWBI-2.3^T (S1+) on parasitism rate of *A. fabae* following *A. colemani* attack (A), emergence rate of *A. colemani* from *A. fabae* (B), and weight of emerged parasitoids (C). The parasitism rate is the number of parasitized aphids that became mummies, and the emergence rate is the number of adult parasitoids that emerged from mummies. Two host treatments were used: no infection with cultivable *S. symbiotica* (S−) and infection with cultivable *S. symbiotica* (S1+). Data are for 16 parasitoids for infected aphid treatment and 15 parasitoids for uninfected aphid treatment. Significant differences are shown (*, $P < 0.05$; ***, $P < 0.001$).

TABLE 2 Cultivable *S. symbiotica* transmission rate in first, second, and third generations of *A. fabae*

Serratia symbiotica strain	Generation	Total no. of aphids tested	No. of aphids infected by *Serratia symbiotica*	*Serratia symbiotica* infection rate (%)[a]
S1+	1	57	51	89.47 (78.48–96.04)
S1+	2	40	37	92.5 (79.61–98.43)
S1+	3	40	35	87.5 (73.20–95.81)
S2+	1	59	49	83.05 (71.03–91.56)
S3+	1	46	28	60.87 (45.37–74.91)

[a]The infection rate is expressed as the percentage of *S. symbiotica*-positive individuals in relation to the total number of specimens tested. Values in parentheses are the confidence interval.

from the aphid gut. Here, we explored the infection process of cultivable *S. symbiotica* bacteria in the aphid gut and examined their biological effects on the new symbiotic system.

Our results indicate that the oral infection was reliable and that the aphid gut was suitable for harboring cultivable *S. symbiotica* strain S1+ (CWBI-2.3^T). This bacterium multiplied and spread throughout the whole digestive tract, with bacterial densities increasing exponentially with aphid age (similar to the findings for aphid endosymbionts) (24, 43, 44). This infection pattern is similar to that observed by Renoz and collaborators (38) in field-collected aphids, where *S. symbiotica* was naturally found in the aphid gut. Bacteria do not appear to be found in specialized structures, as is the case for the gut-associated *Burkholderia* symbiont in the bean bug *Riptortus pedestris* (45) or for *Sodalis glossinidius* in *Glossina morsitans* (46). Indeed, because of their plant sap diet, aphids have very distinct guts that are unlikely to have crypts (47). To establish an association with a host insect, bacteria must be able to pass through the host's defense mechanisms and have a battery of molecular tools to facilitate infection (13). Our results suggest that the cultivable *S. symbiotica* strain exhibits suitable mechanisms to invade the aphid gut, which is consistent with the findings of a previous study that provided evidence that cultivable *S. symbiotica* strain CWBI-2.3^T is well armed to grow

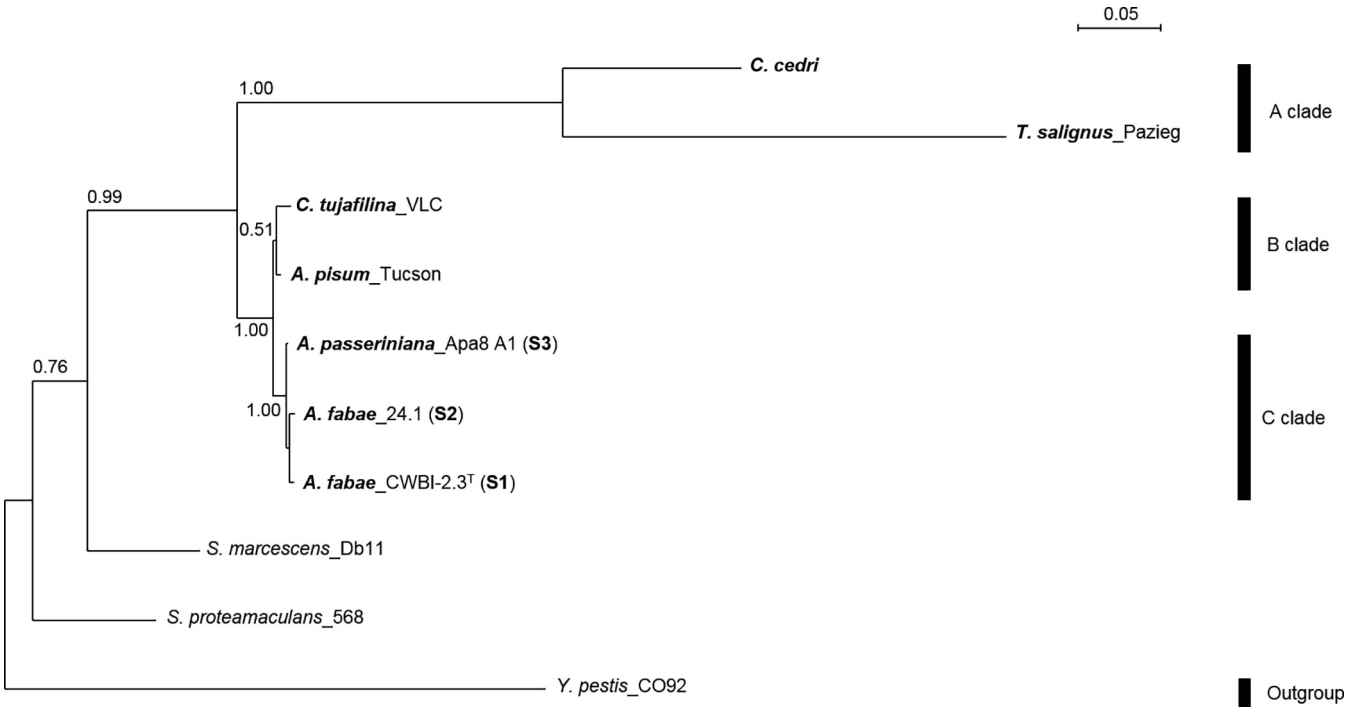

FIG 6 Phylogeny of *S. symbiotica* strains from different aphid species based on concatenated sequences of the genes *accD*, *gyrB*, *murE*, and *recJ* and constructed using Bayesian inferences. Name labels represent the aphid host of *S. symbiotica* followed by the *S. symbiotica* strain. *Serratia marcescens* and *Serratia proteamaculans* correspond to *Serratia* species living independently of hosts. *Yersinia pestis* was used as the outgroup. Branch labels indicate marginal posterior probabilities.

in the aphid gut (40). Another study showed that, unlike the entomopathogenic *Serratia marcescens*, cultivable *S. symbiotica* strain CWBI-2.3^T is not perceived to be an antagonist by the host immune system (42), allowing the bacterium to multiply and persist in the new host.

Once the infection has occurred, the aphid host must not succumb to the bacteria to allow the initiation of a long-standing interaction. Our results demonstrated that, once in the gut, cultivable *S. symbiotica* infections have negative effects on some *A. fabae* host life history traits in the absence of environmental stressors. These effects are similar to those found in previous studies that emphasize the costs associated with the presence of facultative endosymbionts for aphids, including the defensive endosymbiont *Hamiltonella defensa* (23, 27–30, 48). There may be several potential causes of virulence of cultivable *S. symbiotica* strains. The important proliferation of the bacteria may explain the observed fitness costs. Indeed, a correlation between virulence and bacterial density has been documented when *Wolbachia* infects *Drosophila melanogaster*, but the density and fitness costs associated with the infection rapidly decline over generations (49, 50). The detrimental effects associated with the establishment of cultivable *S. symbiotica* could also decline in subsequent generations. Indeed, according to genomic and experimental data, one of the scenarios that would explain the formation of mutualistic symbiosis between insects and bacteria in nature is the acquisition by these invertebrates of free-living bacteria that have progressively lost their antagonistic properties during the processes of evolutionary degeneration (12, 18). It is also possible that some virulence factors can hurt the new host (51). Indeed, cultivable *S. symbiotica* strain CWBI-2.3^T has conserved an array of molecular tools facilitating the invasion of new hosts (39, 40). In addition, the magnitude of the costs may depend on the combination of aphid genotype and symbiont strain (48), and harboring a nonadapted bacterium can have negative fitness consequences for the host (52). These findings thus demonstrate that cultivable strains of *S. symbiotica* exhibit parasitic effects during the early stages of infection by reducing the fitness of their aphid host.

Depending on the ecological context, facultative symbionts can be either beneficial, commensal, or parasitic (4, 22, 48, 53). There are many cases of endosymbionts whose presence is associated with costs for their host but that are mutualistic partners when the symbiotic system is subjected to another ecological context with specific selection pressures (23, 24, 54). To investigate this paradigm, we tested for the existence of protective effects against parasitoids associated with these cultivable strains using strain S1+ (CWBI-2.3^T). Indeed, defensive symbiosis in aphids has been reported for several species of endosymbionts, including *S. symbiotica*. We showed that the aphids infected by cultivable *S. symbiotica* at the gut level exhibited higher resistance to parasitism by *A. colemani* than uninfected aphids, in terms of mummy formation, the emergence rate, and the weight of adult parasitoids. Previous studies have already highlighted the protective effect of endosymbiotic *S. symbiotica* against parasitoids in *A. pisum* (35, 54), but comparatively, the effects seem to be higher. The mechanism by which *S. symbiotica* bacteria protect their aphid hosts is still not well understood (4). Several studies demonstrated that the protection associated with another aphid symbiont, *Hamiltonella defensa*, is due to its interaction with APSE phages (24, 55). In our case, the genome of the cultivable strain (CWBI-2.3^T) does not contain APSE phages (F. Renoz et al., unpublished data). The mechanisms of protection are likely to be different. The endosymbionts occur in the aphid hemolymph, and parasitoid eggs are directly exposed to them (4) while cultivable bacteria are dwelling in the aphid gut. Our hypothesis is that the protection could be due to the costs associated with the presence of cultivable *S. symbiotica* that affect the optimal development of the parasitoid larvae. This raises questions about the implication of these costs associated with the presence of symbiotic bacteria (not only the cultivable *S. symbiotica* bacteria) in defensive symbiosis. In addition, other putative beneficial effects associated with cultivable *S. symbiotica* bacteria will have to be investigated to clarify their implication in the ecology and evolution of aphids. Indeed, it cannot be ruled out that they can

protect aphids against other stresses (56, 57) or have a nutritional role, given the preservation of a large repertoire of genes related to metabolism (39). Thereby, our results emphasize that cultivable *S. symbiotica* bacteria, like other symbionts in aphids, can be both parasites and mutualists, depending on the context. Future studies are required to determine whether the cultivable bacteria confer a selective advantage to their new host under natural conditions and whether the immediate benefits outweigh the associated costs. If so, it could promote the establishment of a stable and persistent mutualistic interaction. It should also be kept in mind that the observed costs and benefits may be dose dependent. Further studies using aphids naturally infected and/or a range of doses are also needed to clarify whether the results depend on the bacterial concentration.

Vertical symbiont transmission from mother to offspring is one of the most important processes for the establishment and maintenance of stable and intimate host-symbiont associations (58), although stable symbioses without vertical transfer are also found in nature (45, 59). Generally, the transmission of gut bacteria lacks a reliable pathway (60, 61). Here, the transmission rate of cultivable *S. symbiotica* strains over three aphid generations remained high, although it was lower than the rates of vertical transmission observed for facultative endosymbionts of aphids (4). Our results indicate that nymphs acquire cultivable *S. symbiotica* strain S1+ (CWBI-2.3^T) after birth and that the transmission requires infected mothers on the same plants as nymphs. The bacterium is thus not transmitted through the same mechanism used by maternally transmitted symbionts that live within the body cavity of aphids. Moreover, cultivable *S. symbiotica* was detected in honeydew samples of aphids artificially infected in the gut, as previously reported in the case of aphid endosymbionts (62). This suggests that contact with infected honeydew may be responsible for the transfer of cultivable *S. symbiotica*. However, it is also possible that aphids pick them up through the circulation of bacteria in the host plant. This transmission mechanism through the phloem sap of the plant has already been observed in some insect/symbiont systems, such as *Rickettsia* with *Bemisia tabaci* (17) and *Cardinium* with leafhoppers (63). Further investigations are thus needed to analyze this tripartite interaction to determine if the host plant could be a way of cultivable *S. symbiotica* transfer. The possible transmission of cultivable bacteria suggests a potential durability of the novel association, but further tests, such as tests to measure the impact of cultivable bacteria following natural transmission, will have to be carried out to complete our knowledge.

The wide diversity of *S. symbiotica* strains varying in their degree of reliance on hosts suggests that this symbiont species is embedded in diverse evolutionary paths (40). Our phylogenetic investigation supports this hypothesis. The cultivable *S. symbiotica* strain CWBI-2.3^T has intermediate genome characteristics which place it between a free-living bacterium and a facultative endosymbiont (33), suggesting that cultivable *S. symbiotica* bacteria are involved in a nascent stage of symbiosis with aphids and would have diverged little from their free-living counterparts. Their tissue tropism and their potential independence with respect to their hosts propose that direct acquisition from the environment is likely. Through phylogenetic and evolutionary analyses, it has been established that endosymbionts have evolved from free-living ancestors residing in the environment but that they have been gradually domesticated by their hosts (18, 64). Moreover, we detected *S. symbiotica* in field-collected plants colonized by infected aphids (I. Pons, N. Scieur, and T. Hance, unpublished data). Taken together, these observations support the hypothesis that *S. symbiotica* endosymbionts with more reduced genomes evolved from free-living precursors via the oral entry route and that the environment could thus represent a reservoir for the acquisition of a new microbial partner by aphids. This has already been shown with strain HS of the bacterium *Sodalis* (a human pathogen), whose ancestral relatives would have served as progenitors for the descent of *Sodalis* endosymbionts found in insect hosts (18). There is the question of the ability of these cultivable *S. symbiotica* strains to cross the epithelial barrier of the midgut to reach the host's hemolymph through a transcytosis mechanism (65). We did not observe any red fluorescence in the hemolymph of aphids, suggesting that

cultivable *S. symbiotica* was not immediately capable of crossing the aphid gut epithelium. Alternatively, these strains could be strictly adapted to the gut of aphids and represent a new kind of symbiont. The bacteria could be preadapted to develop in the aphid gut and form a stable symbiotic association, or they may not be on an evolutionary trajectory toward greater intimacy.

In conclusion, the identification of strains with a free-living capability is very fascinating because it allows the association to be experimentally traced and may contribute to our understanding of the mechanisms that shape symbiosis in insects. We showed that cultivable *S. symbiotica* bacteria have the ability to rapidly infect the aphid gut and seem to be transmitted over generations through an external transmission mechanism. These bacteria induced a fitness costs on their new hosts when colonizing their entire digestive tract. On the other hand, cultivable *S. symbiotica* offered immediate fitness benefits under environmental stress. If our study provides new insights into the nature of the association between aphids and *S. symbiotica*, there are still many questions that need to be answered to improve our perception of bacterial mutualism in aphids. What is the origin of these strains? Are they originally present in the environment and capable of transiting through plants? What is their prevalence in natural aphid populations? Can they have a free-living capacity outside of laboratory conditions? Are they capable of transfer across the aphid gut wall to form more intimate relationships, or are they strictly associated with the aphid gut? *S. symbiotica* is thus an exciting model to tackle issues related to the complexity and the evolution of bacterial mutualism in aphids. Further field, experimental, and genomic studies are required to refine our perception of *S. symbiotica* strains with a free-living capacity and, more generally, the nature of symbiosis in insects.

MATERIALS AND METHODS

Insects and bacterial strains. A single clone, A06-407, of *Aphis fabae* was originally collected from *Chenopodium album* in St. Margrethen, Switzerland, and provided by Christoph Vorburger (Eawag, Switzerland). This clone was found to be uninfected with any known facultative symbionts of aphids (66). Aphids were maintained through parthenogenetic reproduction on *Vicia faba* at 18°C under a long-day photoperiod (16 h of light, 8 h of dark) and 65% ± 3% of humidity. The aphid parasitoid used for the experiment, *Aphidius colemani* (Hymenoptera: Aphidiinae), was provided by Viridaxis SA, Belgium, in April 2016. It is a generalist parasitoid that naturally attacks the *A. fabae* host (67). Cultivable *S. symbiotica* strain CWBI-2.3^T (S1+), isolated from a field-collected *A. fabae* aphid (36, 39), was used in this study. In addition, we isolated two novel strains of *S. symbiotica* as described previously (36, 37): strain 24.1 (S2+) from a field-collected *A. fabae* aphid and strain Apa8 A1 (S3+) from an *Aphis passeriniana* aphid collected in Tunisia. These strains, which had a free-living capacity, were preserved in frozen stocks at −80°C and cultured at 20°C in 863 medium (1% yeast extract, 1% casein peptone, 1% glucose) as described previously (36). The culture did not contain insect cell lines or fetal bovine serum (FBS), contrary to cultures for some aphid symbionts that have already been cultured outside of aphids, which use enriched medium designed for insect cell culture with insect cell lines and/or FBS (55, 68).

Diagnostic PCR. To verify the integrity of the *A. fabae* clone before infection with bacterial strains, as well as the presence of cultivable *S. symbiotica* in aphids after the inoculation procedure, DNA from individual aphids was extracted by using a QIAamp tissue kit (Qiagen). The PCR primers used for *S. symbiotica* (three strains) detection were 16SA1 (AGAGTTTGATCMTGGCTCAG) and PASScmp (GCAATGT CTTATTAACACAT) (69). PCRs were performed in a final volume of 15 μl containing 1 μl of the template DNA lysate, 0.5 μM each primer, 200 μM deoxynucleoside triphosphates, 1× buffer, and 0.625 unit of *Taq* DNA polymerase (Roche). PCR conditions consisted of 35 cycles at 95°C for 30 s, 55°C for 1.5 min, and 72°C for 1.5 min. Moreover, before the start of the experiments, to ensure clonal integrity, the aphid clone was diagnosed by diagnostic PCR to be uninfected with known facultative endosymbionts of aphids (66).

Oral infection. Oral infection of cultivable strains of *S. symbiotica* was performed by feeding aphid hosts on an artificial medium containing the desired strain to ensure the presence of the bacteria in the digestive tract (70), to mimic what is found in nature (38). Bacterial strains were first grown to an early log phase in 863 medium (36) on a gyratory shaker (160 rpm) at 20°C. When an optical density (OD) at 600 nm of between 0.5 and 0.7 was reached during the growth phase, the bacteria were centrifuged. Symbiont cells were then washed with sterile phosphate-buffered saline (PBS; Sigma) and suspended in PBS to obtain an OD at 600 nm of 1. To standardize aphid individuals, adult females of *A. fabae* were left on young *Vicia faba* plants for 24 h to produce nymphs. After removal of the adult insects, the newborn nymphs were kept on the same plants for 4 days prior to infection experiments. Third-instar aphid nymphs were then fed on an artificial diet (71) for 24 h. One hundred microliters of bacterial solution (sterile PBS for the control) was mixed with 20 ml aphid diet (approximately 10^6 CFU/ml of diet for each strain, as found previously [42, 70]).

Histological observations. To visualize the infection process of cultivable *S. symbiotica* strain S1+, whole-mount fluorescence *in situ* hybridization (FISH) was performed, as previously described (42, 72). At

TABLE 3 Genes and primers used for sequencing[a]

Gene	Hypothetical product	Primer	Sequence (5′–3′)	Melting temp (°C)	Fragment size (bp)
accD	Carboxyl transferase, subunit β	accD.S 2F	ACACCCTACTGGATAAAGGC	60	522
		accD.S 2R	GATGTTGATGTCGCCCAGC		
murE	UDP-*N*-acetylmuramoylalanyl-D-glutamate 2,6-diaminopimelate	murES 6F	CTGTTCGCTGGGCATGATGTGG	55	774
		murE6R	GCCCGGTGCGTTAAACACTTCC		
recJ	5′ → 3′ exonuclease	recJ.S 4F	GAAGCAATCGTTAATCCC	60	840
		recJ.S 4R	TATCGAGATCGTTAGCCAGC		
gyrB	DNA gyrase, subunit B	gyrB.S 2F	TGACATTCTGGCCAAGCGCC	64	615
		gyrB.S 1R	ACTACCGCGATCAGCCCCTC		

[a]Data are from Henry et al. (74).

different times after the infection procedure (0, 2, 5, 10, 15, 20, and 30 days postingestion), sampled aphids were placed in acetone for preservation. The following oligonucleotide probes were used: Cy5-ApisP2a (CCTCTTTTGGGTAGATCC), targeting the 16S rRNA of *B. aphidicola*, and Cy3-PASSisR (CCC GACTTTATCGCTGGC), targeting the 16S rRNA of *S. symbiotica*. Samples (between 2 and 6 aphids per time point) were observed under a Zeiss LSM 710 confocal microscope. Negative controls consisted of aphids not infected with *S. symbiotica* and stained with the two probes (Fig. 2) and infected aphids with no probe staining.

Cultivable *S. symbiotica* density. The infection dynamic of the cultivable *S. symbiotica* strain S1+ was measured by a TaqMan real-time quantitative PCR on an Applied Biosystems Step One Plus machine (Applied Biosystems), as previously described (43). The development of *S. symbiotica* density relative to aphid growth was quantified in six replicates collected at different time points after bacterial administration: 0, 2, 5, 10, 15, 20, and 25 days after ingestion. To estimate *S. symbiotica* density, the copy number of the *S. symbiotica dnaK* gene was quantified with the following primers and probe (24): forward primer TGGCGGGTGATGTGAAG, reverse primer CGGGATAGTGGTGTTTTTGG, and probe ATTGAAACTATGGGCA GCGTGAT. As an index of aphid cell number, the copy number of the *A. fabae EF1*α gene was quantified using the following primers and probe (43): forward primer CAGCAGTTACATCAAGAAGATTGG, reverse primer CATGTTGTCTCCATTCCATCCAG, and probe CCCAGCCGCTGTTGCTTTCGTTCC. The probes were modified with 6-carboxyfluorescein as the 5′-terminal reporter dye and black hole quencher 1 as the 3′-terminal quencher dye. DNA extraction from a whole aphid was conducted using a Qiagen DNeasy kit. qPCRs were performed in duplicate using a total volume of 25 μl containing 5 μl of template DNA. The PCR mixture included 12.5 μl of Maxima probe–carboxy-X-rhodamine qPCR master mix, 6.7 μl of double-distilled H_2O, 0.3 μl of each primer (10 nM), and 0.2 μl of probe. The cycling conditions were 10 min of activation at 95°C, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The gene copy number was estimated from the standard curve, generated by use of a 10-fold series of dilutions of genomic DNA at concentrations of 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 copies/μl.

Life history trait measurement. To measure the potential fitness costs of infection with cultivable *S. symbiotica* strains, third-instar aphid nymphs were standardized and fed on an artificial diet (71) with bacterial solution (or sterile PBS solution for the control) for 24 h. Four different host conditions were tested: no infection (control), infection with strain S1+, infection with strain S2+, and infection with strain S3+. After oral ingestion, the aphids (wingless) were individually placed on young *V. faba* plants and six life history traits were analyzed. Each third-instar aphid nymph was observed daily until death to measure the nymph survival rate, development time (i.e., the time from birth to the onset of reproduction), total fecundity (i.e., the total number of offspring produced by each aphid until death), the duration of reproduction (i.e., the time from the first to the last offspring production), and life span. To estimate the body size, the fresh mass of a set of different adult individuals (between 9 and 18) was measured at 10 days of age using a Mettler MT5 microbalance (Mettler Toledo, Switzerland). To measure fitness for each condition, 35 replicates were performed.

Parasitism assays. To measure the potential fitness benefits of infection with cultivable *S. symbiotica* strain S1+, 18 third-instar aphids were introduced into a 4-cm-diameter glass petri dish containing a *V. faba* leaf after oral ingestion by the aphids. Two different aphid treatments were tested: no infection (control) and infection with *S. symbiotica* strain S1+. A 2- or 3-day-old single parasitoid female (mated and fed) was introduced into the petri dish. Before the experiment, each parasitoid was exposed to one third-instar aphid for oviposition experience. The aphids were removed from the petri dish once an ovipositor insertion was observed (73). After 30 min, attacked aphids were transferred onto a *V. faba* plant, and 12 days later, they were inspected to measure the parasitism rate, estimated by dividing the total number of aphids mummied (i.e., dead aphids containing a developing parasitoid) by the total number of attacked aphids. The rates of live and dead aphids were also measured. Seven days later, the mummies were inspected to measure the emergence rate of parasitoids, estimated by dividing the number of adults emerging from mummies among the total number of mummies. Each emerged parasitoid was weighed using a Mettler MT5 microbalance (Mettler Toledo, Switzerland), and the nonemerged mummies were dissected. We performed 16 experimental replicates for infected aphids (i.e., 288 aphid individuals attacked) and 15 for uninfected aphids (i.e., 270 aphid individuals attacked).

Symbiont transmission mode. (i) Transfer across host generations. Offspring (10 days old) from the first generation of adult aphids infected artificially at the gut level by cultivable *S. symbiotica* strains were collected and subjected to diagnostic PCR for all strains (strains S1+ [$n = 57$], S2+ [$n = 59$], and

TABLE 4 GenBank accession numbers of target *S. symbiotica* sequences

Insect species	*S. symbiotica* strain	GenBank accession no. of *S. symbiotica* gene:			
		accD	*gyrB*	*murE*	*recJ*
A. passeriniana	Apa8 A1	MF185191	MF185194	MF185197	MF185200
A. fabae	24.1	MF185190	MF185193	MF185196	MF185199
A. fabae	CWBI-2.3^T	MF185189	MF185192	MF185195	MF185198

S3+ [$n = 46$]). A single diagnostic PCR was used for all three strains. Offspring from the second ($n = 40$) and third ($n = 40$) generations were also collected only for strain S1+ and subjected to diagnostic PCR.

To investigate how the transmission of cultivable *S. symbiotica* strain S1+ was carried out, another experiment was performed. Five infected aphids were placed on a *V. faba* plant. Ten offspring were immediately taken off the plant when born and placed on a new plant to verify if the transmission of the bacteria was carried out after birth and required infected adults on the same plants as the nymphs. Ten other offspring were left on the plant with the infected adults. The offspring (10 days old) were then collected and subjected to diagnostic PCR. Before each DNA extraction, the aphids were rinsed with ethanol (99%), bleach, and then pure water.

(ii) Honeydew inspection. Honeydew from 13 adult aphids infected artificially at the gut level by a cultivable *S. symbiotica* strain S1+ bacterium (at 9 days postinfection) was collected and subjected to diagnostic PCR. Aphids were placed on an artificial diet for 1 day to harvest the honeydew. Separate honeydew samples were then placed on 863 medium at 20°C to observe whether the *S. symbiotica* bacteria were alive in the honeydew of the aphids. Sequencing was carried out to verify the identity of the recovered *S. symbiotica* strain.

Phylogenetic analyses. Multilocus sequence typing (MLST) was performed for the *S. symbiotica* strains using the partial sequence of four housekeeping genes, *accD*, *gyrB*, *murE*, and *recJ* (Table 3) (74). PCR amplification was realized on DNA samples from all three cultivable *S. symbiotica* strains under the following conditions: an initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60 to 64°C (depending on the primer) for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min (38). Amplicons were purified and sequenced in both directions (Macrogen Inc., Amsterdam, The Netherlands). The sequences obtained were cleaned and aligned using Geneious (v9.1.5) software (75). Phylogenetic associations were analyzed for sequences from the three cultivable *S. symbiotica* strains, sequences from *S. symbiotica* strains whose genomes are already known (32, 38, 76), as well as sequences from *Serratia marcescens* Db11 and *Serratia proteamaculans* 568, which correspond to *Serratia* species living independently of hosts (77–79), and a sequence from *Yersinia pestis* CO92 (as an outgroup) (80). All sequences are available in GenBank (Tables 4 to 6). The SeaView (v4.6.1) program was used to align the sequences, and the Gblocks (v0.91b) server (81) was used to remove poorly aligned positions and divergent regions of DNA alignments. The PartitionFinder (v1.1.0) program (82) was used to determine the evolutionary model of sequences for each gene: the K80 model for the *accD* gene, the HKY model (1, 2) and the K80 model (3) for the *gyrB* gene, the K80+G model (1, 2) and the K80 model (3) for the *murE* gene, and the K80 model (1, 2) and the HKY+G model (3) for the *recJ* gene. Phylogenetic analysis was performed by Bayesian inference methods with MrBayes (v3.2.6) software (10^6 generations) (83).

Statistical analyses. The effect of cultivable *S. symbiotica* strains on the following dependent variables was tested: nymph survival rate, development time, adult fresh mass, survival rate, total number of offspring, and duration of reproduction of aphids. Survival data were analyzed with a proportional hazards regression (84) and visualized by computing the Kaplan-Meier survival functions for the different infections (85). Aphid mass was analyzed using a general linear model framework, after verification of the normality of the data. All other variables were analyzed using generalized linear models with a binomial error structure and a logit-link function for nymph survival rate, a gamma error structure and an inverse-link function for development time and duration of reproduction, and a Poisson error structure and log-link function for the total number of offspring. Analyses of parasitism and emergence rates, as well as the rates of live and dead attacked aphids, were performed by fitting generalized linear mixed models by assuming a binomial error structure and a logit-link function, while the weight of parasitoids was analyzed using a general linear mixed model, after validation of the normality of the data, testing for the effect of cultivable *S. symbiotica* strain S1+. The parasitoid female was considered a random factor in this statistical modeling because one parasitoid female attacked several aphids. We further analyzed the transmission rate using a generalized linear model with a binomial error structure and a logit-link function, testing for the effect of different cultivable *S. symbiotica* strains and generations. Statistical

TABLE 5 Genome accession numbers of other *S. symbiotica* strains

Insect species	*S. symbiotica* strain	NCBI accession no. of reference strain genome
Cinara cedri	SCc	CP002295
Tuberolachnus salignus	STs-Pazieg	LN890288
Cinara tujafilina	SCt-VLC	GCA_900002265.1
Acyrthosiphon pisum	Tucson	GCA_000186485.2

TABLE 6 Genome accession numbers of other bacterial strains

Insect species	Bacterial species	Strain	GenBank accession no. of reference strain genome
Drosophila melanogaster	*S. marcescens*	Db11	NZ_HG326223.1
Populus trichocarpa	*S. proteamaculans*	568	NC_009832
	Yersinia pestis	CO92	AL590842.1

analyses were performed using the software R (v3.0.1; R Development Core Team, 2014) and the *survival* package for survival analyses (86), the *lme4* package for linear mixed models, and the *GrapheR* package for graphics. Some graphics were also performed using GraphPad Prism (v5.01) for Windows.

Data availability. DNA sequences generated during this study have been submitted to GenBank, and the accession numbers are available in Tables 4 to 6.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02445-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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