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ORIGINAL PAPER

Fitness costs of the cultivable symbiont *Serratia symbiotica* and its phenotypic consequences to aphids in presence of environmental stressors

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Abstract
Associations between symbiotic microorganisms and animals are ubiquitous and hosts may benefit from hosting microbial communities through enhanced protection to environmental stresses or resource exploitation. Like many insects, aphids are hosts of a wide diversity of heritable symbionts that can be important drivers of their evolutionary ecology. *Serratia symbiotica* is one of the most common symbiont associated with aphids and includes a great variety of strains whose degree of interdependence on hosts varies significantly. Among these strains, some are gut-associated and have been isolated from aphids and cultivated. One of these strains (CWBI-2.3^T) confers immediate protection against parasitoids. Here, we investigated additional associated phenotypes to elucidate the implication of cultivable *S. symbiotica* in the aphid evolutionary ecology. We show that under benign conditions, the aphids tended to suffer from reduced survival and fecundity when harboring the symbiont. We also demonstrate that gut infection with cultivable *S. symbiotica* does not protect aphids from the fungal pathogen *Zoophtora occidentalis* and from the lethal pathogen *Serratia marcescens*. However, while the bacterium is costly for aphids, this effect is no longer observed in the presence of the fungus, suggesting a negative effect of *S. symbiotica* on the latter. Our results further demonstrate that the cultivable *S. symbiotica* strain does not confer benefits to its hosts after the aphids were heat-stressed. These findings exposed that cultivable *S. symbiotica* does not have the same fitness effects on aphids as endosymbiotic strains, highlighting the significance of considering intraspecific variation of symbionts when studying their associated extended phenotypes.

Keywords Bacterial mutualism · Facultative symbiosis · *Aphis fabae* · Ecological benefits · Extended phenotype

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Introduction

Insects are considered as the most diverse animal group and during their long evolution have colonized every imaginable ecological land niche (Engel 2015). However, in many cases, the evolutionary radiation has been made possible through their associations with symbiotic partners. For instance, the success of insects that feed on a nutritionally limited diet is partly due to their association with a large number of symbionts providing them with essential nutrients (Douglas 2009). These obligate symbionts are thus vital for the survival and reproduction of their host (Moran et al. 2008). Each partner becomes a permanent element of the environment of the other and when the evolutionary influence is reciprocal, co-evolutionary process occurs (Selosse 2001). Insects can also host facultative symbionts, whose presence is not indispensable for the host but may have implications for many of their traits (Sachs et al. 2011; White et al. 2013). In the case of facultative mutualisms, not all individuals in the same insect populations harbor such symbionts, and these relationships have evolved depending on environmental pressures explaining prevalence level and their persistence in the host populations (Sicard et al. 2014).

The spread, fixation, and evolution of symbioses depend to a large extent on the reliability of the transmission mechanisms (Bright and Bulgheresi 2010). Facultative symbionts are usually vertically transmitted from mother to offspring, although horizontal transfers also occur at lower frequencies (Gehrer and Vorburger 2012; Henry et al. 2013; Pons et al. 2019a). One of the strategies used by facultative symbionts to maximize their vertical transmission is to manipulate the reproduction of their insect hosts. This is the case of the symbiont *Wolbachia* that has been described as a master reproductive parasite inducing a diversity of sex manipulations to promote its spread in host populations (Werren et al. 2008; Sicard et al. 2014). An alternative strategy that fosters the propagation of facultative symbionts in insect populations is the ability of these microorganisms to confer beneficial effects to their hosts (Oliver et al. 2014; Zug and Hammerstein 2015). Many symbiont species can affect a wide range of life-history and ecologically important traits of their hosts, playing a significant role in their ecology and evolution (Duron and Hurst 2013; Oliver et al. 2014).

Aphids represent a valuable model to investigate the mechanisms of mutualist associations in insects. Almost all aphid species harbor an ancient obligate symbiont *Buchnera aphidicola* for delivering essential amino acids that are deficient in its host diet (Douglas 1998). In addition, aphids can also harbor a wide variety of facultative symbionts that are involved in more recent associations (Oliver et al. 2010). Unlike obligate symbionts that are hosted in specialized cells called bacteriocytes, facultative partners can inhabit sheath cells, hemolymph, secondary bacteriocytes and gut (Buchner 1965; Moran et al. 2005; Renoz et al. 2018). Facultative symbionts have been generally studied in the pea aphid *Acyrthosiphon pisum* model, which is known to host at least nine different symbionts (Guo et al. 2017). In this aphid species, they can confer an array of extended phenotypes having the potential to increase its fitness in certain ecological context, such as their resistance to parasites (Oliver et al. 2003; Vorburger et al. 2010), a capacity to withstand heat shock (Burke et al. 2009; Russell and Moran 2006), their performance on different host plants (Tsuchida et al. 2004; Wagner et al. 2015), the frequency of sexual reproduction (Simon et al. 2011) and body coloration (Tsuchida et al. 2010). However, these symbionts can also impose fitness costs on their hosts that make them parasitic (Oliver et al. 2008; Vorburger et al. 2013; Polin et al. 2014; Pons et al. 2019b).

S. symbiotica is of great interest to study the role of facultative symbionts in aphid ecology and evolution, because it comprises a variety of strains associated with very distinct biological characteristics and localizations (Renoz et al. 2018). Studies already highlighted that the endosymbiont *S. symbiotica* can have a nutritional role (Lamelas et al. 2011), but also confer protection for their hosts against parasitoids (Oliver et al. 2003), as well as under heat shock (Burke et al. 2009; Heyworth and Ferrari 2015). Some strains are also gut-associated (Renoz et al. 2018) and have recently been isolated from aphids in the *Aphis* genus and cultivated freely on a pure artificial medium (Sabri et al. 2011; Grigorescu et al. 2017). It has been suggested that they are in the early stages of symbiotic life with aphids (Manzano-Marín et al. 2016; Pons et al. 2019b) and in previous studies, we showed that in laboratory conditions cultivable *S. symbiotica* is potentially extracellularly transmitted, via contamination with honeydew (Pons et al. 2019b) and through the plant phloem (Pons et al. 2019a). We also demonstrated that artificial gut colonization by cultivable *S. symbiotica* induces fitness costs to their hosts in the absence of stresses, but can offer an indirect immediate protection against parasitoids (Pons et al. 2019b). Despite its costs, the presence of cultivable *S. symbiotica* in natural aphid populations (Renoz et al. 2018) could be due to the diverse benefits it brings to its host, allowing to be selected in different ecological scenarios (Heyworth and Ferrari 2015; Pons et al. 2019b). Understanding the additional beneficial effects of cultivable *S. symbiotica* could thus be important 1) to explain why this gut-associated bacterium spread in aphid populations despite its costs and 2) to clarify its biological role and implication in the aphid evolutionary ecology. Here, we hypothesize that the aphid will be protected by cultivable *S. symbiotica* during heat stress, as found for other endosymbiotic strains (Russell and Moran 2006; Burke et al. 2009), and the existence of antimicrobial effectors in the cultivable strain (CWBI-2.3^{T}) genome (Foray et al. 2014; Renoz et al. 2017) suggests a probiotic effect. Alternatively, in view of its costs (Pons et al. 2019b), an additional stress could be more detrimental for aphid hosts if cultivable *S. symbiotica* does not bring benefits.

In the present work, we investigated experimentally if the cultivable *S. symbiotica* can provide multiple ecological benefits to the aphid host, in addition to the protection against parasitoids already shown (Pons et al. 2019b). We examined whether cultivable *S. symbiotica* infection results in increased aphid protection against a natural enemy, the fungal pathogen *Zoophthora occidentalis* and resistance against a lethal bacterial pathogen *Serratia marcescens*. Moreover, we tested whether cultivable *S. symbiotica* infection offers tolerance to its host under thermal stress. Indirectly, we also analyzed the constitutive cost of harboring cultivable *S. symbiotica*. In doing so, we have contributed an account of cultivable *S. symbiotica* induced effects on the aphid phenotype in an attempt to determine its propagation strategy in nature.

Materials and methods

Aphids and cultivable *S. symbiotica* symbiont

The clone A06–407 of *Aphis fabae* used in this study was originally collected from *Chenopodium album* in Switzerland and provided by Dr. Christophe Vorburger (Eawag, Switzerland). This clone was found to be uninfected with any known facultative symbionts of aphids (Vorburger et al. 2009). Aphids were reared through parthenogenetic reproduction on seedlings of *Vicia faba* at 18 ± 1 °C under a long-day regimen (16 h light, 8 h dark) and

65 ± 3% of humidity. The cultivable *S. symbiotica* strain CWBI-2.3^T, isolated from a field-collected *A. fabae* (Sabri et al. 2011; Foray et al. 2014), was used in this study. This strain was preserved in frozen stocks at − 80 °C and cultured at 20 °C with 863 medium (1% yeast extract, 1% casein peptone, 1% glucose) as described in Sabri et al. (2011).

Pathogens

For the fungal pathogen infection, the entomopathogenic fungus *Zoophtora occidentalis* was used. The fungus isolate was obtained from the USDA ARS Collection of Entomopathogenic Fungal Cultures (New York) as ARSEF 3073. We cultured the fungal pathogen on plates of SDAEY for 2 weeks at 20 °C (Parker et al. 2013; Barribeau et al. 2014). For the bacterial pathogen infection, the entomopathogenic *S. marcescens* strain Db11 was used. This strain is a spontaneous streptomycin-resistant mutant of Db10 originally isolated from a moribund fly (Flyg et al. 1980). *S. marcescens* strain Db11 was preserved in frozen stocks at − 80 °C and cultured at 37 °C with LB medium with streptomycin (100 µg/ml).

Aphid oral infection

To check the integrity of our population of *A. fabae* before administration of bacteria and the presence of *S. symbiotica* in aphids after inoculation procedure, DNA from individual aphids was extracted by using the QIAamp tissue kit (Qiagen). A diagnostic PCR was then performed as described in Fukatsu et al. (2000), Pons et al. (2019b).

Oral infection of cultivable *S. symbiotica* was performed by feeding aphid hosts on an artificial medium containing the bacterium to ensure its presence in the aphid digestive tract (Altincicek et al. 2011). In a first time, the bacterium was grown to an early log phase on 863 medium (without antibiotic) (Sabri et al. 2011) on a gyratory shaker (160 rpm) at 20 °C. When reaching an optical density (OD) between 0.5 and 0.7 at 600 nm during the logarithmic growth phase, bacteria were centrifuged. Symbiont cells were then washed with sterile PBS (Sigma) and suspended in the buffer to obtain an OD of 1 at 600 nm. To standardize aphid individuals, reproductive mature females of *A. fabae* were left on young *V. 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 (only 2 days for the heat shock experiment). Third-instar aphid nymphs were then fed on an artificial diet (Cambier et al. 2001) containing a solution with the bacterium (or sterile PBS for the control) for 24 h.

Performance after a heat shock

The capacity of *S. symbiotica* to improve the heat stress tolerance of its aphid host was tested according to the infection status. Two-day-old aphid nymphs were standardized and fed on an artificial diet (20 ml) (Cambier et al. 2001) with a solution containing the cultivable *S. symbiotica* (100 µl) (only sterile PBS solution for the control) for 24 h (approximately 10^6 CFU/ml of diet). This step yielded two aphid populations with different symbiotic status: one uninfected population (control) and one population infected with cultivable *S. symbiotica*. Each population was divided into two treatment groups: one group exposed to 18 °C (control treatment) and the other group exposed to a heat shock stress as carried out in Montllor et al. (2002), Russell and Moran (2006), Heyworth and Ferrari 2015). After the oral ingestion, 6 to

10 three-day-old aphids (reared at 18 °C) were immediately transferred on a *V. faba* seedling with a vented 2 L cage formed by plastic bag covering the pot in climatic chambers. During the heat shock treatment, the temperature rose steadily from 18 to 38 °C over a period of 2 h. The temperature was maintained for 4 h and then decreased to 18 °C for another 2 h. After the treatment, the plants were changed to ensure consistent plant quality. The proportion of surviving aphids was counted 7 days after the treatment and 5 surviving wingless aphids per pot were then individually placed on a novel plant and number of offspring per female was recorded every 5 days during 25 days. Six repetitions per modality were performed (corresponding to 56 aphids for survival rate and 30 aphids for fecundity).

Resistance to an entomopathogenic fungus

To investigate its potential antifungal role, the impact of the entomopathogenic fungus *Z. occidentalis* was evaluated on fitness of aphids with cultivable *S. symbiotica* versus without the symbiont (control) (Parker et al. 2013; Heyworth and Ferrari 2015). Third-instar aphid nymphs were standardized and fed for 24 h on an artificial diet (20 ml) (Cambier et al. 2001) with a solution containing the cultivable *S. symbiotica* (100 µl) (or sterile PBS solution for the control; approximately 10^6 CFU/ml of diet). Two infectious statuses were tested: without cultivable *S. symbiotica* (control) and with cultivable *S. symbiotica*. These two populations were divided into two treatments: one group exposed to the fungus and one group not exposed to the fungus (control). Before fungal infection, a small piece of fungal mycelium (5 mm^2) was cut with a sterile scissor and placed onto 1.5% tap water agar overnight at 20 °C and at high humidity to provoke the sporulation of the fungus. At the infection time, 3 to 5 aphids with or without cultivable *S. symbiotica* were placed at the bottom of a hollow tube and the agar plate was inverted over the tube for 90 min. To ensure that each aphid group was exposed to an equal dose of fungal spores, the different plates were rotated during the treatment. After exposure, aphids were then placed on *V. faba* plants during 10 days and the survival of each aphid was recorded. Every 2 days, the different groups of aphids were checked and fungus-killed aphids were removed. Sixteen repetitions were performed for the aphids exposed to fungal treatment and twelve repetitions were performed for the aphids exposed to control treatment.

Resistance to a pathogenic bacterium

To investigate the existence of probiotic effects associated with the cultivable *S. symbiotica*, the impact of the pathogenic bacterium *S. marcescens* was estimated on fitness of aphids with cultivable *S. symbiotica* versus without the symbiont (control). Two experiments were conducted. In the first experiment, third standardized instar aphid nymphs were fed for 24 h on an artificial diet (Cambier et al. 2001) containing either the cultivable *S. symbiotica* and the pathogenic bacterium *S. marcescens* or only the pathogen. Different concentrations of *S. marcescens* were administered. One hundred µl of solution with the cultivable *S. symbiotica* bacterium and 10 µl or 1 µl of solution with the *S. marcescens* bacterium were mixed with 20 ml aphid diet (approximately 10^6 CFU/ml of diet for *S. symbiotica* and *S. marcescens* (10 µl) and 10^5 CFU/ml of diet for *S. marcescens* (1 µl)). For the control treatment, 100 µl of sterile PBS solution and 10 µl or 1 µl of solution with the *S. marcescens* bacterium were mixed with 20 ml aphid diet. After the oral infection, aphids were placed on a *V. faba* plant and the proportion of survivors was counted during 3 days because aphids do not usually

survive after a 3-day infection with the pathogenic bacterium *S. marcescens*. Seven repetitions per modality were performed with 5 aphids per repetitions.

In the second experiment, a delay of 6 days was observed between *S. symbiotica* and *S. marcescens* infection to allow the symbiotic bacterium to establish in the aphid gut before the pathogen infection. Indeed, studies already showed that cultivable *S. symbiotica* is capable to colonize the aphid gut in a few days and persists throughout the life of aphid hosts (Renoz et al. 2015; Pons et al. 2019b). Third instar standardized aphid nymphs were fed on an artificial diet (Cambier et al. 2001) with a solution with cultivable *S. symbiotica* or without the symbiont. To achieve this task, 100 µl of solution with the cultivable bacterium or PBS (control) were mixed with 20 ml aphid diet (approximately 10^6 CFU/ml of diet). During the 6-day wait period, aphids were transferred from the artificial diet to plants and after this delay, the aphids were fed on the artificial diet containing 10 µl of solution with the *S. marcescens* bacterium mixed in 20 ml aphid diet (approximately 10^6 CFU/ml of diet). After the oral infection, aphids were placed on a *V. faba* plant and the proportion of survivors was also counted during 3 days. Six repetitions per modality were performed with 5 aphids per repetitions.

Statistical analysis

Aphid survival was analyzed using generalized linear models (GLM) with a binomial error structure and a logit-link function. The number of offspring was analyzed using a general linear model (LM) framework, after verification of normality of the data. The presence of the cultivable *S. symbiotica*, the stress treatment and their interactions were the explanatory variables for the heat-shock and the fungal exposure experiments. The presence of the cultivable *S. symbiotica*, the concentration of *S. marcescens*, the initial installation of cultivable *S. symbiotica* and their interactions were the explanatory variables for the pathogenic ingestion experiment. For the fungal exposure experiment, the post hoc Tukey HSD test was performed using "glht" function. Statistical analyses were performed using the software R version 3.5.3 (R Core team 2018), using *multcomp* package for post hoc Tukey test (Hothorn 2007) and *GrapheR* package for graphics (Hervé 2011).

Results

Performance after a heat shock

Heat shock significantly affected survival of aphids, whether infected or not by the cultivable *S. symbiotica* (GLM, $df = 1$, $\chi^2 = -2.74$, $P = 0.0062$). A reduction of about 20% of survival of uninfected aphids and about 10% of survival of infected aphids were observed when exposed to heat stress compared to survival of aphids without stress (Fig. 1a). However, cultivable *S. symbiotica* did not have a significant effect on aphid survival under both treatments (heat shock and control) (GLM, $df = 1$, $\chi^2 = -0.17$, $P = 0.87$, Fig. 1a).

Heat shock had a significant effect on the aphid fecundity, whether or not infected by cultivable *S. symbiotica* (LM, $df = 1$, $F = 36.01$, $P < 0.001$). Fecundity was lowered by a little less than an half for aphids exposed to heat stress compared to aphids not exposed to stress under both conditions (infected or uninfected by the bacterium; Fig. 1b). Moreover, cultivable *S. symbiotica* had a significant negative effect on aphid fecundity under both

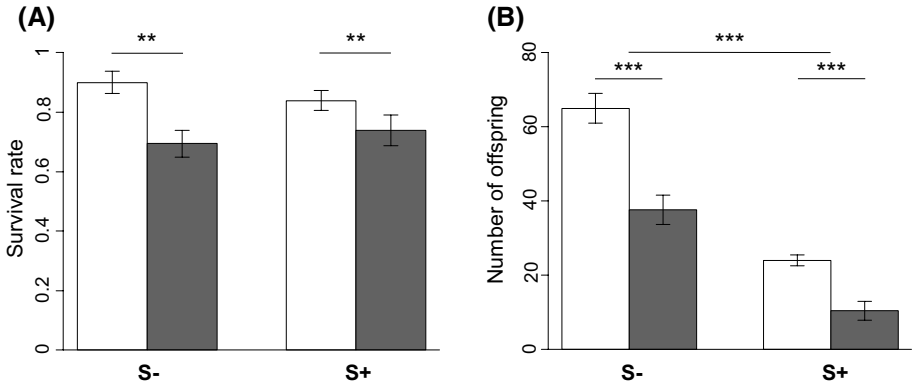

Fig. 1 Effect of cultivable *S. symbiotica* on **a** survival rate at 7 days after heat shock (6 to 10 aphids per repetition and 6 repetitions per modality) and **b** total number of offspring at 25 days after heat shock (5 aphids per repetition and 6 repetitions per modality) of *A. fabae* aphids. Two symbiotic statuses were used: uninfected aphids (S−) and aphids infected by cultivable *S. symbiotica* (CWBI-2.3^T; S+). Two conditions were used: aphids exposed to heat-shock treatment (dark grey) and aphids exposed to benign treatment (white). Error bars depict the standard error. Significant differences are shown (**$P<0.01$; ***$P<0.001$)

treatments (LM, $df=1$, F=99.76, $P<0.001$, Fig. 1b). Fecundity was lowered by more than an half for infected aphids compared to uninfected aphids (Fig. 1b).

Resistance to *Zoophtora occidentalis*

The interaction between aphid symbiotic status and *Z. occidentalis* infestation had a significant effect on aphid survival (GLM, $df=1$, $\chi^2=2.88$, $P=0.0034$). Aphids with cultivable *S. symbiotica* had a significantly lower survival than aphids without the symbiont in the control condition (GLM, $df=1$, $\chi^2=-3.2$, $P=0.0075$), with a reduction of about 30% of survival of aphids with the symbiont (Fig. 2). When exposed to *Z. occidentalis*, the survival of aphids without cultivable *S. symbiotica* was significantly lower than that of aphids without the symbiont in the control condition (GLM, $df=1$, $\chi^2=-2.68$, $P=0.037$), with a reduction of about 20% of aphid survival exposed to the fungus (Fig. 2). This suggests that the fungus had a negative effect on aphids. However, when aphids were exposed to the fungus, no significant difference was observed between aphids with cultivable *S. symbiotica* and aphids without cultivable *S. symbiotica* (GLM, $df=1$, $\chi^2=0.61$, $P=0.93$; Fig. 2). Moreover, survival of aphids with the symbiont was not significantly different between aphids exposed to the fungus and aphids exposed to the control condition (GLM, $df=1$, $\chi^2=1.36$, $P=0.53$, Fig. 2). These results do not show a clear protection of the cultivable *S. symbiotica* to its host against the fungus but still suggest that cultivable *S. symbiotica* may interfere with fungal proliferation, such that aphids infected by the fungus suffer no additional mortality in the presence of the symbiont.

Resistance to *Serratia marcescens*

The mortality rate of aphids having ingested *S. marcescens* was over 80% in the 3 days following infection (Fig. 3), suggesting that this pathogenic bacterium is highly

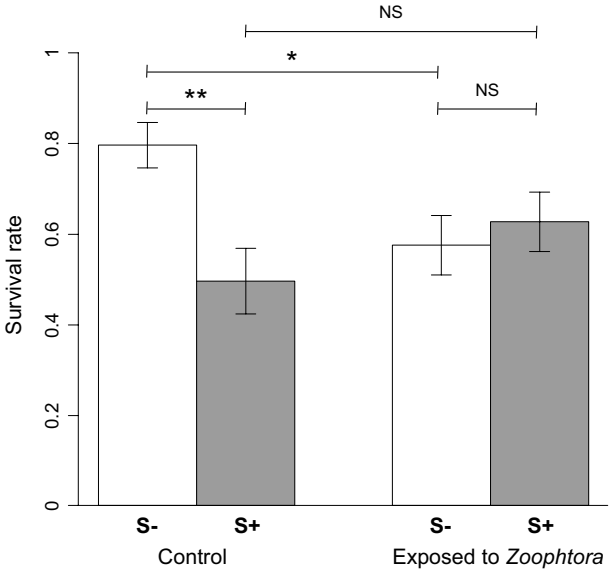

Fig. 2 Effect of cultivable *S. symbiotica* on survival rate of *A. fabae* aphids exposed to the entomopathogenic fungus *Zoophthora occidentalis* after 10 days. Two symbiotic statuses were used: uninfected aphids (S−) and aphids infected by cultivable *S. symbiotica* (CWBI-2.3^T; S+) (3 to 5 aphids per repetition and 16 repetitions for the fungal treatment per modality/12 repetitions for the control treatment per modality). Error bars depict the standard error. Significant differences are shown (*NS* not significant; *$P<0.05$; **$P<0.01$)

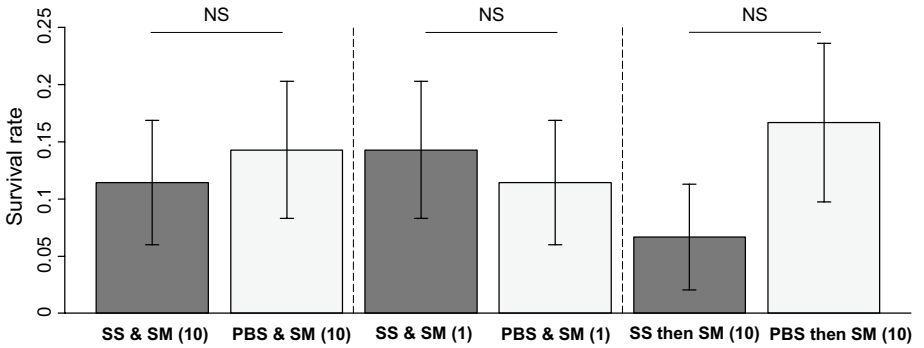

Fig. 3 Effect of cultivable *S. symbiotica* (CWBI-2.3^T) on survival rate of *A. fabae* aphids exposed to the pathogenic bacterium *S. marcescens*, after a 3-day infection. Several conditions were tested. Firstly, aphids having ingested a solution containing both bacteria at the same time (SS & SM) and aphids having ingested a solution containing only the pathogen and PBS (PBS & SM, control) (7 repetitions per modality with 5 aphids per repetition). Secondly, aphids having first ingested a solution containing *S. symbiotica* and then S. marcescens (SS then SM) and aphids having first ingested a solution containing PBS and then *S. marcescens* (PBS then SM, control) (6 repetitions per modality with 5 aphids per repetition). Different concentration of the pathogen *S. marcescens* were administered: 10 µl (10) or 1 µl (1). Error bars depict the standard error and *NS* indicates not difference

virulent in *A. fabae* aphids. The cultivable *S. symbiotica* strain did not have a significant effect on the survival of aphids exposed to the pathogen *S. marcescens* (GLM, $df=1$, $\chi^2=0.39$, $P=0.70$), meaning that the cultivable strain does not have a beneficial effect against this virulent bacterium. The survival rate did not exceed 14%, whether or not the aphids were infected by the cultivable *S. symbiotica* (Fig. 3). Moreover, the survival rate of aphids with or without cultivable *S. symbiotica* was not significantly different when

taking into account the different concentrations of the pathogenic bacterium (GLM, $df = 1$, $\chi^2 = -0.50$, $P = 0.61$; Fig. 3), as well as the presence of the cultivable *S. symbiotica* in the aphid guts before *S. marcescens* infection (GLM, $df = 1$, $\chi^2 = -0.68$, $P = 0.50$; Fig. 3).

Discussion

It has already been demonstrated that the cultivable *S. symbiotica* strain CWBI-2.3^T can offer an indirect immediate protection against parasitoids (Pons et al. 2019b). However, some studies showed that one symbiont strain can simultaneously confer multiple ecological benefits to its aphid host (Heyworth and Ferrari 2015). In this study, we thus investigated some phenotypic effects possibly associated with the presence of this cultivable *S. symbiotica* strain colonizing the aphid gut to explain the bacterium distribution in natural populations and clarify its impact on the aphid evolutionary ecology. Moreover, understanding the associated phenotypes of this cultivable symbiont could be an important step in understanding mechanisms that drive the evolutionary transition in which free-living bacteria evolve to form durable endosymbiotic associations with hosts.

The niche-specific benefits conferred by facultative symbionts to their aphid hosts can clarify why they are found at high frequencies in some populations (Heyworth and Ferrari 2015; Guo et al. 2017). Indeed, despite costs, facultative symbionts can be selected for in different ecological scenarios (Oliver et al. 2014). Here, we can confirm that under non-stringent conditions, cultivable *S. symbiotica* is costly in terms of fitness for its aphid host (Pons et al. 2019b). Generally, costs of this magnitude have previously been described for artificial host–symbiont associations (Oliver et al. 2008; Vorburger et al. 2013; Polin et al. 2014; Cayetano et al. 2015), which can be the consequence of incompatibilities between particular genotypes. In fact, incompatibilities between host and symbiont genotypes may be a force limiting the spread of symbionts among insect populations (Oliver et al. 2014; Leclair et al. 2016). These high costs can thus outweigh certain of the benefits delivered by the cultivable bacterium. Nevertheless, this pathogenicity can be a transient effect, and if the bacterium has the potential to damage its host, it is most probable to happen directly after being acquired. For instance, the detrimental effects associated with the establishment of certain *H. defensa* strains in new aphid host have been observed to decline in subsequent generations (Niepoth et al. 2018). Moreover, in a novel host species of *Drosophila*, *Spiroplasma* had also fitness costs that were reduced some generations after infection (Nakayama et al. 2015).

Even if the symbiotic bacterium affects aphid fitness traits, under fungal pathogen *Z. occidentalis* exposition, the effect of the pathogen is not added to the negative effect of the cultivable *S. symbiotica*. One explanation is that the potential protection provided by the cultivable *S. symbiotica* is not observed because its fitness cost is too much for its host and/or the symbiotic bacterium has a negative effect on the fungus, preventing its proliferation. Indeed, the genome and proteome analyses of the cultivable strain (CWBI-2.3^T) showed the existence of antimicrobial effectors such as chitinases (Foray et al. 2014; Renoz et al. 2017), which is known to attack the fungal cell wall composed in part of chitin (Adams 2004), suggesting that cultivable *S. symbiotica* could have a fungicide role. To confirm the assumption, fungal antagonistic assays will have to be realized (Li et al. 2015) and other experiments should be performed when the association is maintained and thus less costly for aphids and/or with other fungi and host aphids. Other facultative symbionts are also

known to protect aphid hosts against entomopathogenic fungi (Parker et al. 2013; Heyworth and Ferrari 2015; Guo et al. 2017). For instance, some studies showed that the symbiont *Regiella insecticola* can provide protection to aphids against fungal entomopathogens *Z. occidentalis* and *Pandora neoaphidis* (Ferrari et al. 2004; Scarborough et al. 2005; Parker et al. 2013). However, in this case, the symbiont is present in the host hemolymph and not in the aphid gut like in our case.

Our results showed that cultivable *S. symbiotica* did not offer any protection to the aphid host in case of heat stress and pathogen bacteria presence under standard lab conditions. Some studies already described that the endosymbiont *S. symbiotica* can increase host survival and/or reproduction after a heat shock (Montllor et al. 2002; Russell and Moran 2006; Burke et al. 2009), but the protection mechanisms are not completely explained (Renoz et al. 2019). The hypothesis that has been advanced is that *S. symbiotica* increase host tolerance after a heat shock by releasing metabolites as a result of cell lysis, thus preserving the integrity of the bacteriocyte of the obligate symbiont (Burke et al. 2009). In our case, we showed that cultivable *S. symbiotica* does not provide protection to its host against thermal stress. These results probably reflect the severe fitness costs associated with the cultivable bacterium and that this protection effect can vary depending on the strain but also the nature of the association. The results are therefore consistent with the protection hypothesis as previously discussed. In fact, the difference of protection between cultivable strains and uncultivable *S. symbiotica* strains may be due to the presence of the uncultivable bacteria in aphid hemolymph and around bacteriocytes of the obligate symbiont (Oliver et al. 2014), while cultivable *S. symbiotica* are dwelling in aphid gut.

Colonization of the gut with mutualistic bacteria can increase the resistance of the insect hosts against parasite and/or pathogen invasions (Engel and Moran 2013), particularly when the pathogen acquisition is done through nutrition. The genome analyses of the cultivable *S. symbiotica* strain (CWBI-2.3^{T}) showed the existence of antibiotics (Foray et al. 2014; Renoz et al. 2017), suggesting a probiotic effect. Here, we showed that the pathogenic bacterium *S. marcescens* is extremely virulent for the aphids as showed in Renoz et al. (2015) and that the cultivable *S. symbiotica* does not provide protection to its host against *S. marcescens*. This virulent pathogen is known to be able to quickly pass the multiple physical and immune barriers protecting the gut and penetrate the body cavity, involving proteases and chitinases (Flyg et al. 1980; Nehme et al. 2007). Our hypothesis is that *S. marcescens* kills and/or inhibits the development of *S. symbiotica* in aphids, killing the hosts in few days as in aphids not infected with cultivable *S. symbiotica*. Quantitative PCR study could confirm this hypothesis. However, although few studies have been conducted in insects, it has been shown that the insect gut microbiota can provide a buffering action to help prevent the proliferation of bacterial pathogens (Dillon and Dillon 2004). We could thus test if the protection is improved when cultivable *S. symbiotica* bacterium settles longer in the aphid gut. In this study, we showed that cultivable *S. symbiotica* does not protect aphids from both pathogens *S. marcescens* and *Z. occidentalis*. However, further studies are needed to test if the cultivable bacterium is able to protect its host against other pathogenic strains because individual strains can provide narrow spectrum protection against specific fungal and bacterial pathogens.

The cultivable *S. symbiotica* is gut-associated and uses extracellular transmission routes (Pons et al. 2019a, b), suggesting that the bacterium is not reliably present in the aphid through generations (Salem et al. 2015). A low reliance on symbiotic associates is thus less likely to evolve and could explain these fewer benefits. However, so that the association is maintained in natural aphid population, the cultivable *S. symbiotica* could also provide some untested benefits, such as nutritional role and modification

of host dietary range (Henry et al. 2015). Indeed, given its localization, it cannot be ruled out that the cultivable bacterium has a nutritional function, as suggested in Skaljac et al. (2019). For the time being, the nutritional role of *S. symbiotica* has been considered in the co-obligate association context (Lamelas et al. 2011; Manzano-Marín et al. 2016), but the gut-associated strain brings new perspectives. In fact, gut symbionts are often regarded as important contributors to the nutrition of their hosts (Dillon and Dillon 2004; Engel and Moran 2013). Further experiments are thus necessary to clarify its nutritional function, especially as, in comparison to the more tremendously reduced *S. symbiotica* genomes, cultivable *S. symbiotica* has preserved a large repertoire of genes related to the synthesis of most essential amino acids and vitamins (Manzano-Marín et al. 2016).

To conclude, we examined phenotypic traits possibly conferred by cultivable *S. symbiotica* to explain its presence and distribution in aphid populations, which contributes to our understanding of the mechanisms that shape symbiosis in aphids. This symbiont offers a unique opportunity to compare and contrast aphid symbiont strains that are free-living and having horizontal transmission to maternally-transmitted strains living in hemolymph and intracellularly reported in the literature. It is therefore a relevant model to better understand how symbiotic and more particularly endosymbiotic associations appear and evolve in insects. We confirmed that under benign conditions, the aphids tended to suffer from reduced survival and fecundity when harboring cultivable *S. symbiotica*. We also revealed that gut infection with cultivable *S. symbiotica* does not offer any real benefits to the aphid host for the phenotypes tested, although the bacterium could have a negative effect on the fungus. Due to the localization of cultivable *S. symbiotica* in the aphid gut, other associated phenotypes and/or additional fitness indices should be studied to better explain the spread strategy of the bacterium in natural aphid populations. Indeed, although the gut microorganisms have been studied in few insects, studies clearly showed that gut bacteria are crucial for the nutrition, physiology, pathogen protection and immune responses of many insect species (Engel and Moran 2013). Moreover, it would be interesting to test the effect of cultivable *S. symbiotica* with other aphid clones because studies have already shown that the efficiency of the infection and the associated effects seem to depend on the combination of aphid genetic background and symbiont strains (Russell and Moran 2005; Vorburger et al. 2009; Oliver et al. 2010; Leclair et al. 2016; Niepoth et al. 2018), but the underlying mechanisms are not yet known. The different phenotypic effects observed in this study could also be derived from the association between cultivable *S. symbiotica* and bacteria from plants in the aphid gut. The study highlights the significance of considering intraspecific variation of symbionts when studying their associated extended phenotypes and shows that further studies are required to improve the perception of cultivable *S. symbiotica* and more particularly, the nature of aphid symbiosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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