THE ROYAL SOCIETY

Received 16 April 2003
Accepted 23 July 2003
Published online 29 October 2003

Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid

Ryuichi Koga[1], Tsutomu Tsuchida[1,2] and Takema Fukatsu[1*]

[1]*Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan*
[2]*Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Tokyo 153-8902, Japan*

Almost all aphids harbour an endosymbiotic bacterium, *Buchnera aphidicola*, in bacteriocytes. *Buchnera* synthesizes essential nutrients and supports growth and reproduction of the host. Over the long history of endosymbiosis, many essential genes have been lost from the *Buchnera* genome, resulting in drastic genome reduction and the inability to live outside the host cells. In turn, when deprived of *Buchnera*, the host aphid suffers retarded growth and sterility. *Buchnera* and the host aphid are often referred to as highly integrated almost inseparable mutualistic partners. However, we discovered that, even after complete elimination of *Buchnera*, infection with a facultative endosymbiotic γ-proteobacterium called pea aphid secondary symbiont (PASS) enabled survival and reproduction of the pea aphid. In the *Buchnera*-free aphid, PASS infected the cytoplasms of bacteriocytes that normally harbour *Buchnera*, establishing a novel endosymbiotic system. These results indicate that PASS can compensate for the essential role of *Buchnera* by physiologically and cytologically taking over the symbiotic niche. By contrast, PASS negatively affected the growth and reproduction of normal host aphids by suppressing the essential symbiont *Buchnera*. These findings illuminate complex symbiont–symbiont and host–symbiont interactions in an endosymbiotic system, and suggest a possible evolutionary route to novel obligate endosymbiosis by way of facultative endosymbiotic associations.

Keywords: pea aphid; *Buchnera*; pea aphid secondary symbiont; endosymbiotic evolution; host–symbiont interactions; symbiont–symbiont interactions

1. INTRODUCTION

Multiple endosymbiotic bacteria are often housed in the same host organisms, but the significance of such 'supersymbiotic systems' is poorly understood (Buchner 1965; Dubilier *et al.* 2001). Many insects harbour an obligate endosymbiotic bacterium, called the 'primary symbiont', in bacteriocytes, which is essential for the survival and reproduction of the host, and is therefore fixed in the host populations. Frequently, these insects also possess a facultative endosymbiotic bacterium, called the 'secondary symbiont', in different types of cells and tissues, which is non-essential for the host and usually shows partial infection in the host populations. Endosymbiotic associations comprising an obligate primary symbiont and one or several optional secondary symbiont(s) are commonly found in various insect groups such as aphids, psyllids, whiteflies, coccids, planthoppers, tsetse flies, beetles and others (Buchner 1965).

Almost all aphids possess the primary symbiont *Buchnera aphidicola* in the cytoplasms of primary bacteriocytes in the abdomen. Because *Buchnera* provides the host with essential amino acids and other nutrients (Douglas 1998), aphids suffer sterility or death when deprived of the symbiont (Houk & Griffiths 1980). Through over 100 Myr of the endosymbiotic association (Moran *et al.* 1993), the genome of *Buchnera* has lost many genes needed for independent life, resulting in drastic genome reduction and the inability to survive outside host cells (Shigenobu *et al.* 2000). Therefore, it is widely accepted that aphids and their *Buchnera* symbionts are inseparable mutualistic partners.

In natural populations of the pea aphid *Acyrthosiphon pisum*, a secondary symbiotic γ-proteobacterium, called pea aphid secondary symbiont (PASS) or R-type symbiont, has been frequently detected in addition to *Buchnera* (Unterman *et al.* 1989; Chen & Purcell 1997; Chen *et al.* 2000; Fukatsu *et al.* 2000; Sandström *et al.* 2001; Montllor *et al.* 2002; Tsuchida *et al.* 2002). Over 80% of insects in California and *ca.* 35% of insects in Japan was double-infected with PASS and *Buchnera* (Chen & Purcell 1997; Tsuchida *et al.* 2002). However, the mechanism that maintains the high infection frequency of PASS is obscure. Previous studies have indicated that PASS infection generally has negative effects on host fitness, while the intensity of the effects is dependent on environmental factors such as temperature and host-plant species (Chen & Purcell 1997; Chen *et al.* 2000). However, it was reported that PASS infection confers resistances to high temperature (Montllor *et al.* 2002) and parasitoid wasps (Oliver *et al.* 2003) on the host aphids. Being confined in the same host body must encourage various interactions between the essential symbiont *Buchnera* and the facultative symbiont PASS. For instance, they may be in competition for resources and available space in the host body, or they may cooperate in providing the host with essential

* Author for correspondence (t-fukatsu@aist.go.jp).

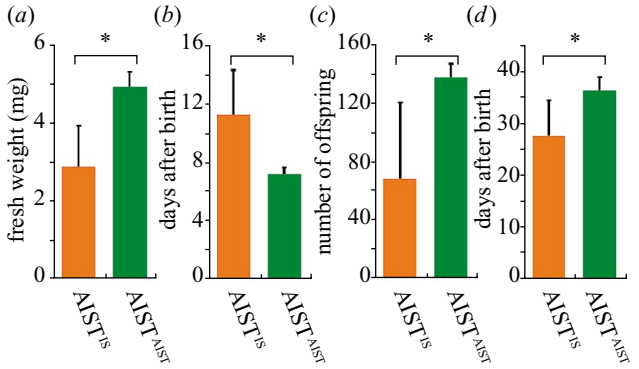

Figure 1. Effect of PASS infection on fitness parameters of the host aphid: (*a*) body weight (13 days old); (*b*) time to reproduction; (*c*) total number of offspring; and (*d*) longevity. Orange bars, PASS-infected strain $AIST^{IS}$ generated by haemolymph transfer; green bars, PASS-free control strain $AIST^{AIST}$ of identical genetic background. Means and s.d.s are shown ($n = 11$ for $AIST^{IS}$ and $n = 12$ for $AIST^{AIST}$). Asterisks, statistically significant difference (Mann–Whitney *U*-test; $p < 0.01$).

nutrients. Biological interactions in such super-symbiotic systems are to date very poorly understood.

Here, we report a series of experimental investigations aimed at understanding the complex biological interplay between PASS, *Buchnera* and the host aphid. In normal aphids with *Buchnera*, PASS infection has negative or nearly neutral effects on the growth and reproduction of the host. However, surprisingly, PASS infection restored the survival and reproduction of *Buchnera*-free aphids, which should be sterile in the absence of the essential symbiont. In the light of the *Buchnera*-dependent fitness consequences of PASS infection, we discuss the biological function of the facultative symbiont PASS, mechanisms for maintaining PASS in host populations, and, more generally, the evolutionary significance of such super-symbiotic systems.

2. METHODS

(a) *Materials*

The strains of *A. pisum* used in this study (Fukatsu *et al.* 2000) were reared on seedlings of the broad bean, *Vicia faba*, at 20 °C in a long-day regimen (16 L : 8 D).

(b) *Cloning and sequencing of PASS genes*

Whole DNA of PASS-infected aphids was subjected to PCR using primers GroE-1F (5′-GGCAGCWAAAGACGTAA-3′) and GroE-1R (5′-GAAGTAMGGDGAYAGGTA-3′) for *groEL*, and DnaK-2F (5′-TACTTYAAYGAYGCDCARCG TCA-3′) and DnaK-2R (5′-TGGTTRTCTTCHGCNGT WGAGAA-3′) for *dnaK*. The PCR products were cloned and sequenced as described previously (Fukatsu *et al.* 2000). The DNA sequences were deposited in the DNA Data Bank of Japan (accession nos. AB063612 and AB063613).

(c) *Specific PCR detection*

Buchnera was detected using primers Buch16S1F and Buch16S1R for 16S rDNA (Tsuchida *et al.* 2002), and BuchDnaK-AF1 (5′-ACAGAATTTAAAAAAGAACAAGGA ATAGATT-3′) and BuchDnaK-AR1 (5′-ATTTTTGCT TTTTCCGCAGATT-3′) for *dnaK*. PASS was detected using primers 16SA1 and PASS5′cmp for 16S rDNA (Fukatsu *et al.* 2000), and PASSGroE-AF1 and PASSGroE-AR1 for *groEL* (Tsuchida *et al.* 2002). Two primer sets were used for each symbiont to confirm the detection. As a control, the insect elongation factor 1α (*ef1α*) gene was amplified using primers ApisEF-AF1 (5′-CTGGAGAATTCGAAGCTGGTATTT-3′) and ApisEF-AR1 (5′-CACCCAAGGTGAAAGCCAATAG-3′). The PCR temperature profile was 40 cycles at 95 °C for 30 s and 60 °C for 1 min.

(d) *Haemolymph transfer*

PASS-introduced strains were generated using a micro-injection technique as described previously (Fukatsu *et al.* 2001). Briefly, haemolymph of PASS-infected donor insects was injected into 3-day-old (second or third instar) nymphs of recipient insects. Progeny of the injected insects were examined for stable and heritable PASS infection by specific PCR detection.

(e) *Antibiotic treatment*

In this study, we developed novel techniques for the selective elimination of endosymbionts. To eliminate PASS or *Buchnera* selectively, 10-day-old adult aphids were treated with ampicillin or rifampicin, respectively, using a microinjection technique. Doses of ampicillin and rifampicin were 1 μg mg^{-1} body weight and 20 ng mg^{-1} body weight, respectively. The injected insects were reared individually and allowed to deposit nymphs from 24 h to 48 h after injection. The nymphs were defined as G1 of each isofemale line. Four insects from each line per generation were subjected to specific PCR detection to confirm elimination of the symbiont.

(f) *Fitness measurement*

Adult insects (10 days old) were allowed to deposit nymphs for 12 h. The newborn nymphs were defined as 0 days old, and were reared on broad beans at 20 °C in the long-day regimen. Fresh body weight, production of offspring and survival of the insects were monitored every 2 or 3 days. To eliminate possible side effects of antibiotic treatment and haemolymph injection, aphids were used for the experiments at least 10 generations after the treatments.

(g) *Quantitative PCR*

DNA extraction from single insects was conducted using a QIAamp tissue mini kit (QIAGEN). The copy number of the symbiont genes in the DNA samples was measured by quantitative PCR using a TaqMan PCR core reagent kit and ABI7700 system (Applied Biosystems) essentially as described previously (Kondo *et al.* 2002). PASS was quantified in terms of *groEL* gene copies using primers PASSGroE-AF1 and PASSGroE-AR1, and a probe PASSGroE-TP1 (5′-AAGCAGTTGTTG CGGCGGTTGAA-3′). *Buchnera* was quantified in terms of *dnaK* gene copies using primers BuchDnaK-12F (5′-TATTGGTATTGACTTGGGAA-3′) and BuchDnaK-162R (5′-AGCAGGTTGTCCTACTAAAAC-3′), and a probe BuchDnaK-77T (5′-TTTTAGAGAATGCGGAAGGTG-3′).

(h) In situ *hybridization*

Ovaries were dissected from adult aphids, fixed in alcoholic formalin, processed into paraffin tissue sections and subjected to fluorescent *in situ* hybridization of 16S rRNA as described previously (Fukatsu *et al.* 1998). Probe ApisP2a (5′-CCTCTTTTGGGTAGATCC-3′) targeted *Buchnera*, while

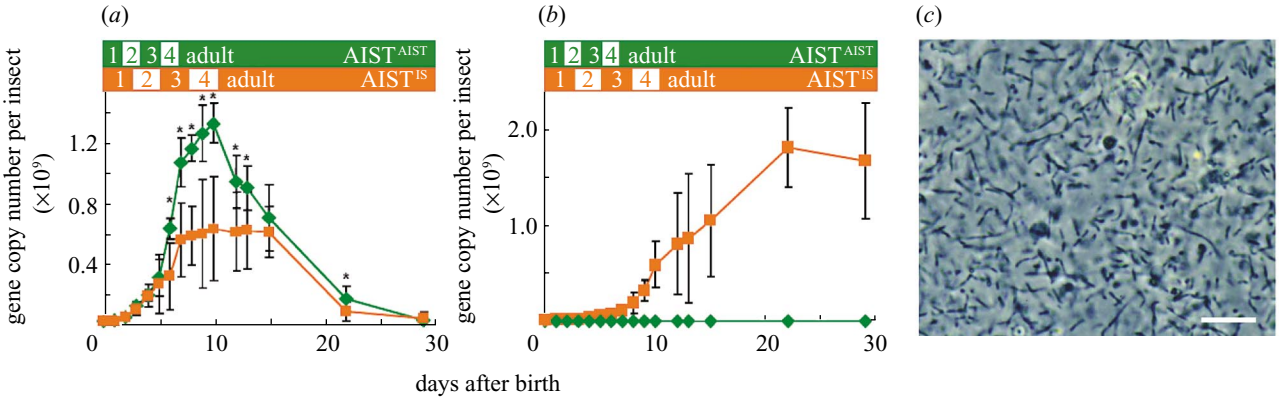

Figure 2. (*a*,*b*) Population dynamics of (*a*) *Buchnera* and (*b*) PASS, in PASS-infected and PASS-free aphids throughout the developmental course. Bacterial titres of PASS and *Buchnera* were measured by quantitative PCR in terms of *groE* and *dnaK* gene copies per host individual, respectively. Note that the gene copy numbers may not reflect the real numbers of endosymbiont cells owing to genome amplification (Komaki & Ishikawa 1999). Orange line, PASS-infected strain AIST^{IS}; green line, PASS-free strain AIST^{AIST}. Means and s.d.s are shown ($n = 10$). Asterisks, statistically significant difference (Mann–Whitney *U*-test; $p < 0.01$). (*c*) Phase contrast microscopy of haemolymph from 30-day-old AIST^{IS} aphids. Bar, 20 µm.

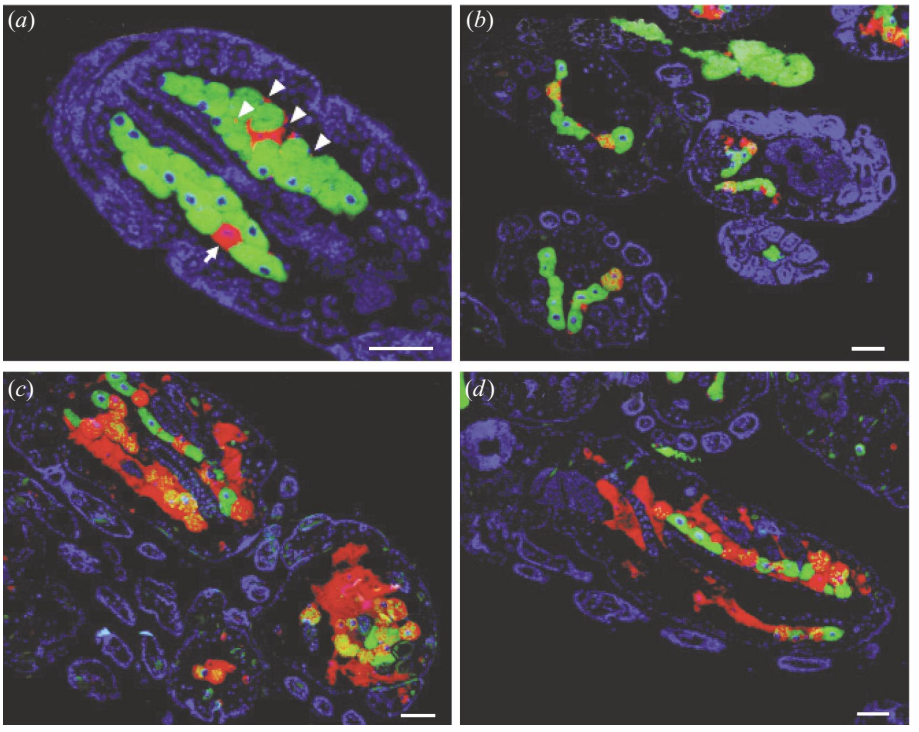

Figure 3. *In situ* hybridization of PASS (red) and *Buchnera* (green) on tissue sections of aphid embryos. (*a*) Naturally PASS-infected strain IS, showing specific localization of PASS in secondary bacteriocytes (arrows) and sheath cells (arrowheads). (*b*)–(*d*) PASS-injected strain AIST^{IS}, exhibiting disordered localization and proliferation of PASS. Localization of PASS is ordered in (*a*), partially disordered in (*b*) and disordered in (*c*) and (*d*). Bars, 50 µm. The categories of PASS localization were defined as follows. Ordered: neither invasion of PASS in the primary bacteriocytes nor massive proliferation of PASS in the haemocoel was observed. Partially disordered: co-localization of PASS and *Buchnera* in the same primary bacteriocytes was found, but no massive proliferation of PASS in the haemocoel was observed. Disordered: massive proliferation of PASS in the haemocoel was observed.

probe PASSisR (5′-CCCGACTTTATCGCTGGC-3′) targeted PASS. A mixture of ApisP2a and PASSisR, whose 5′-terminal nucleotides were labelled with fluorescein isothiocyanate and carboxytetramethylrhodamine, respectively, was used for hybridization. To confirm the specific detection, control experiments were conducted as follows: no probe control, RNase digestion control and competitive suppression control with excess unlabelled probe (Fukatsu *et al.* 2000). Nuclei of the host cells were counter-stained with 4′,6-diamino-2-phenylindole.

3. RESULTS AND DISCUSSION

(a) *Establishment of PASS-positive and PASS-negative aphids by haemolymph transfer*

To estimate the biological effects of PASS on the host aphid, it is essential to investigate aphid strains that are genetically identical and differ only in PASS-infection status. We generated PASS-positive and PASS-negative aphid strains of uniform genetic background by haemo-

lymph transfer. A PASS-free monosymbiotic strain AIST was injected with haemolymph from a disymbiotic strain IS, whereby a disymbiotic strain AIST^{IS} was established. A control monosymbiotic strain AIST^{AIST} was generated by injecting haemolymph from the same strain AIST.

(b) *PASS negatively affected fitness of the host aphid*

At the 25th generation following the injection, we compared the performances of the PASS-infected aphid strain AIST^{IS} and the PASS-free aphid strain AIST^{AIST}. The PASS-infected strain exhibited significantly reduced body weight, delayed onset of reproduction, shortened longevity and reduced number of offspring (figure 1). These results clearly indicate that PASS infection negatively affects the host aphid.

(c) *Population dynamics of* Buchnera *and PASS throughout host development*

What mechanisms underlie the negative effects of PASS infection on the host aphid? Are there any interactions between the facultative symbiont PASS and the essential symbiont *Buchnera*? In an attempt to address these questions, we investigated the population dynamics of PASS and *Buchnera* throughout host development using a quantitative PCR technique.

The population of *Buchnera* increased during nymphal growth, reaching a peak in actively reproducing young adults, and declined in older insects (figure 2*a*). The principal role of *Buchnera* for the host aphid is the provision of essential amino acids and other nutrients, particularly to support the rapid development of a large number of embryos (Douglas 1998). The population dynamics of *Buchnera*, which reflect the reproductive activity of the host aphid, are coincident with the biological function of *Buchnera*. The finding also suggests that the host aphid has sophisticated control mechanisms over the proliferation of *Buchnera*, which must have evolved during the long history of the host–symbiont association (Moran *et al.* 1993).

The population of PASS increased monotonously throughout the life of the host aphid, reaching a plateau in old insects that had ceased reproduction (figure 2*b*). The simple logistic growth pattern may suggest a lack of strict control over PASS proliferation. This idea was supported by microscopic examination of haemolymph from the old insects, which contained a surprising density of bacterial cells (figure 2*c*). The old insect was, as it were, a living sac full of PASS. The plateau in PASS proliferation is probably caused by the limited environmental capacity of its ecological niche, i.e. the body of the host aphid.

(d) *PASS infection suppressed* Buchnera *population*

Notably, the population of the essential symbiont *Buchnera* showed a striking difference between PASS-free and PASS-infected aphids (figure 2*a*). In nymphs and old insects, the titres of *Buchnera* were at similar levels in PASS-free and PASS-infected populations. However, in young adults of 7 to 10 days old, when the aphids most actively reproduce, the titres of *Buchnera* in the presence of PASS were significantly lower, at *ca*. 50% of those in the absence of PASS. These results indicate that PASS infection suppresses the *Buchnera* population at the specific developmental stage important for host reproduction.

(e) *Direct competition between PASS and* Buchnera*: histological evidence*

Two hypotheses are conceivable to explain how PASS infection negatively affects both the *Buchnera* population and host fitness: (i) PASS negatively affects the host aphid and, as a result, the population of *Buchnera* is indirectly suppressed; and (ii) PASS suppresses the essential symbiont *Buchnera*, and this negatively affects performance of the host aphid. These hypotheses are not mutually exclusive, but our *in situ* hybridization data strongly favour the latter hypothesis.

In naturally PASS-infected aphids of the strain IS, PASS was harboured in secondary bacteriocytes and sheath cells, and spatially separated from *Buchnera* harboured in primary bacteriocytes (figure 3*a*), indicating the controlled and orderly localization of PASS (Fukatsu *et al.* 2000). However, in PASS-introduced aphids of the strain AIST^{IS}, localization of PASS was often strikingly disordered, suggesting uncontrolled or virulent behaviour (figure 3*b–d*). In embryos of these insects, primary bacteriocytes for *Buchnera* were frequently invaded by PASS. In some bacteriocytes *Buchnera* and PASS coexisted (figure 3*b*) and in others *Buchnera* was replaced by PASS (figure 3*c*,*d*). In severe cases, most bacteriocytes were occupied by PASS, and massive proliferation of PASS was observed in the haemocoel (figure 3*c*,*d*). We examined 64 embryos from the strain AIST^{IS}: localization of PASS was ordered in 15 embryos (23.4%), partially disordered in 21 (32.8%) and disordered in 28 (43.8%). By contrast, in 29 embryos from the strain IS, localization of PASS was ordered in 24 (82.8%) and partially disordered in five (17.2%); no disordered localization was observed. From these results, we conclude that PASS infection has negative fitness effects on the host aphid, probably by suppressing the essential symbiont *Buchnera*.

(f) *Continuous association attenuated negative fitness effects caused by PASS*

In natural populations of the pea aphid, the infection frequency of PASS is generally high, over 80% in California and *ca*. 35% in Japan (Chen & Purcell 1997; Tsuchida *et al.* 2002), which poses a problem in interpreting the significant level of negative fitness effects caused by PASS. How can PASS be maintained in host populations in spite of the negative effects? An important point is that these fitness data were from comparisons between the aphid strains AIST^{IS} and AIST^{AIST} generated by haemolymph injection, and the AIST insects were originally free of PASS. Therefore, transfer of PASS into the strain AIST supposedly established a novel endosymbiotic association; there was no previous experience of coadaptation. If the negative fitness effects of PASS infection on the host aphid are caused by the lack of host–symbiont coadaptation, it is expected that continuous association between them might lead to attenuation of the negative effects. Indeed, it has been reported that, in the *Drosophila–Wolbachia* endosymbiotic system, transfer of *Wolbachia* into a novel host initially caused a reduction in reproductive fitness, but the

fitness costs declined with the number of generations after the transfection (McGraw *et al.* 2002).

To test this idea, about eight months after the initial measurements (equivalent to 50 generations after the injection), we conducted the same fitness measurements using the PASS-infected strain $AIST^{IS}$ and the PASS-free strain $AIST^{AIST}$ (figure 4). Notably, PASS-infected $AIST^{IS}$ insects at generation 50 exhibited a significant recovery in body weight, onset of reproduction and number of offspring. The levels of the fitness parameters were almost comparable to those of PASS-free $AIST^{AIST}$ insects. Only lifespan did not show such improvement. *In situ* hybridization revealed that the disordered localization of PASS was less frequently observed in the $AIST^{IS}$ insects at generation 50 than in those at generation 25 (data not shown), suggesting a degree of recovery in the endosymbiotic system. These results suggest that, as in the case of the *Drosophila–Wolbachia* system (McGraw *et al.* 2002), negative effects on the host aphid caused by PASS can be attenuated through continuous association between them.

Conceivably, there are two possible mechanisms underlying such attenuation: an evolutionary process with genetic adaptation and a stabilizing process with physiological adjustment. To evaluate which of these mechanisms is mainly involved, further experiments such as repeated injection and elimination of PASS should be performed.

(g) *Selective elimination of PASS*

If continuous association between PASS and the host aphid attenuates the negative effects, it is expected that naturally PASS-infected aphids must have already accomplished such attenuation. This expectation could be tested by depriving a naturally disymbiotic aphid strain of PASS, although no technique for selective elimination of the secondary symbiont without affecting *Buchnera* has been available.

The cell wall of *Buchnera* is reduced (Hinde 1971), and the genome of *Buchnera* lacks some of the genes for the biosynthetic pathway of the cell wall (Shigenobu *et al.* 2000). Therefore, we had the idea that a drug that inhibits cell-wall synthesis would selectively act on PASS without affecting *Buchnera*. As expected, by injecting ampicillin into a disymbiotic strain IS, PASS was selectively eliminated from offspring of the injected insects. After 10 generations of maintenance, PASS infection did not recover whereas *Buchnera* infection was not affected (figure 5*a*). In this way, we successfully established a PASS-free monosymbiotic strain IS^{amp}. A control disymbiotic strain IS^{dw} was generated by injecting distilled water instead of the antibiotic.

(h) *PASS elimination improved only the longevity of the host aphid*

When performance of the host insects was compared between the PASS-free strain IS^{amp} and the PASS-infected strain IS^{dw}, longevity was significantly improved in IS^{amp}, whereas body weight, time to reproduction and total number of offspring were not affected (figure 5*b*). These results indicate that PASS infection certainly has a negative effect on the lifespan of the naturally disymbiotic host aphid but scarcely affects the other fitness parameters mentioned above. Notably, these results are complementary to the pattern of the attenuation data (figure 4), reinforcing the idea that continuous association between PASS and the host aphid can lead to attenuation of the negative fitness effects caused by the symbiont.

(i) *Mechanism and consequences of PASS infection in the presence of* Buchnera

On the basis of these results, we propose the following hypothetical mechanism and consequences of PASS infection in the pea aphid: (i) PASS infection suppresses the essential symbiont *Buchnera* through competition for resources and space in the host body; (ii) the suppression of *Buchnera* results in deterioration of its biological functions such as the supply of essential nutrients; (iii) consequently, the host aphid suffers reduced growth and reproduction; (iv) however, the negative effects are attenuated through continuous PASS–host association; and (v) in natural populations, therefore, PASS infection scarcely impedes survival and reproduction of the host.

However, the possibility that PASS also acts directly on the host aphid to cause the negative effects is not yet excluded. To examine this possibility, the effects of PASS infection on the host aphid must be investigated in the absence of *Buchnera*.

(j) *Selective elimination of* Buchnera

It has been reported that rifampicin treatment effectively eliminates *Buchnera* infection and results in retarded growth and sterility of the cured host aphids (Ishikawa & Yamaji 1985). In this study, we developed a technique for selective elimination of *Buchnera* using moderate rifampicin treatment (figure 6*a*). By injecting an appropriate dose of rifampicin into aphids of the disymbiotic strain $AIST^{IS}$, we successfully generated *Buchnera*-free PASS-infected offspring named $AIST^{IS/rif}$. For a control, the monosymbiotic strain $AIST^{dw}$ was treated in the same way to obtain *Buchnera*-free PASS-free offspring named $AIST^{dw/rif}$.

(k) *PASS infection restored reproduction of* Buchnera*-free aphid*

The aposymbiotic $AIST^{dw/rif}$ aphids were, as expected, sterile. However, the PASS-infected $AIST^{IS/rif}$ aphids were, unexpectedly, able to produce fertile offspring. Out of 12 $AIST^{IS/rif}$ lines generated, one line continued for eight generations on the host plant without *Buchnera* infection (figure 6*b*). These results indicate that, in the absence of *Buchnera*, PASS infection has positive effects on the survival and reproduction of the host aphid.

The size of these aphids (1.74 ± 0.30 mg per adult insect; $n = 12$; G3 of the strain $AIST^{IS/rif}$) was smaller than that of normal aphids (2.90 ± 1.05 mg per adult insect; $n = 12$; the strain $AIST^{IS}$). The fecundity of these aphids (2.02 ± 4.40 insects per mother; $n = 42$) was also smaller than that of normal aphids (69.0 ± 52.3 insects per mother; $n = 12$). These differences were both statistically significant (Mann–Whitney *U*-test; $p < 0.01$). Only 10 out of 42 aphids of the strain $AIST^{IS/rif}$ at G3 produced offspring (8.50 ± 5.19 insects per mother; $n = 10$); the remaining 32 aphids were sterile. These results indicate that PASS is certainly able to compensate for the lack of *Buchnera* but only partially, and that the novel PASS–aphid endosymbiotic system is unstable, probably owing to the lack of intimate coevolution between them.

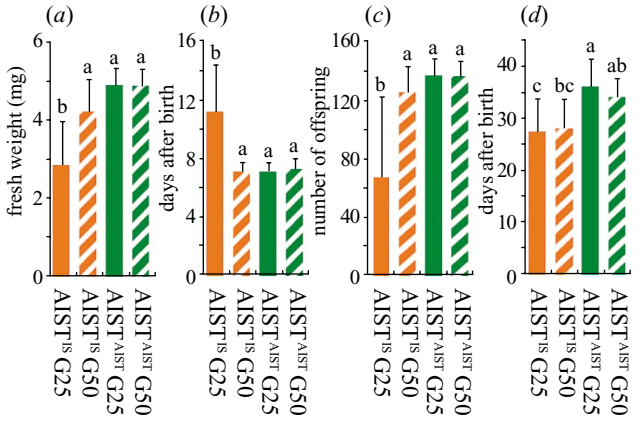

Figure 4. Attenuation of negative fitness effects of PASS infection on the host aphid after continuous association: (*a*) body weight (13 days old); (*b*) time to reproduction; (*c*) total number of offspring; and (*d*) longevity. Orange bars, PASS-infected strain $AIST^{IS}$; green bars, PASS-free strain $AIST^{AIST}$. Filled bars, 25 generations after haemolymph injection; striped bars, 50 generations after haemolymph injection. Means and s.d.s are shown ($n = 11$ and 19 for $AIST^{IS}$ G25 and G50, respectively, and $n = 12$ and 18 for $AIST^{AIST}$ G25 and G50, respectively). Statistically significant differences (Kruskal–Wallis ANOVA; $p < 0.05$) between different characters.

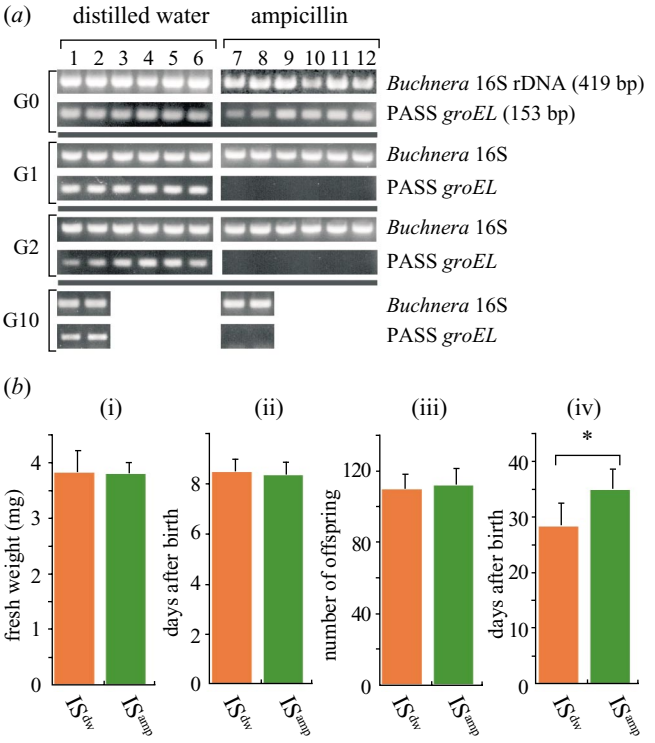

Figure 5. Selective elimination of PASS by ampicillin treatment. (*a*) Diagnostic PCR analysis to confirm selective elimination of PASS. Lanes 1–6, control aphid lines injected with distilled water. Lanes 7–12, treated aphid lines injected with ampicillin. G0, injected generation; G1, offspring of G0 etc. (*b*) Effect of PASS elimination on fitness parameters of the host aphid: (i) body weight (14 days old); (ii) time to reproduction; (iii) total number of offspring; and (iv) longevity. Orange bars, PASS-infected control strain IS^{dw}; green bars, PASS-free strain IS^{amp} generated by ampicillin treatment. Means and s.d.s are shown ($n = 20$ for IS^{dw} and $n = 27$ for IS^{amp}). Asterisks, statistically significant difference (Mann–Whitney *U*-test; $p < 0.01$).

The discovery of the survival and reproduction of aphids lacking *Buchnera* was so surprising that we carefully repeated the same experiment using the IS host background. Similarly, $IS^{amp/rif}$ aphids lacking both *Buchnera* and PASS produced no offspring, whereas $IS^{dw/rif}$ aphids infected with PASS were able to reproduce. With careful handling, we successfully maintained an $IS^{dw/rif}$ line for 24 generations in the complete absence of *Buchnera* (data not shown). On the basis of these results, it was concluded that the facultative symbiont PASS has the potential to compensate for the removal of the essential symbiont *Buchnera*.

(l) *PASS established a novel endocellular localization in* Buchnera*-free aphids*

How is the endosymbiotic system organized in the *Buchnera*-free PASS-infected fertile insects? *In situ* hybridization revealed that, in addition to infecting the haemolymph, PASS was found intracellularly in the uninucleated primary bacteriocytes that normally harbour *Buchnera* (figure 6*c*). In these aphids, surprisingly, PASS has taken over the symbiotic niche of *Buchnera* not only physiologically but also cytologically, and has established a novel endosymbiotic system.

(m) *Biological role of PASS for the host aphid*

We experimentally showed that PASS benefits the host aphid in the absence of *Buchnera*. In natural conditions, however, all aphid individuals possess the essential symbiont *Buchnera*. Thus the question arises: does the positive effect of PASS make sense in the context of aphid ecology and life history?

Buchnera is vulnerable to high temperatures. Temperatures above 30 °C result in no reproduction of host aphids, reduced numbers of bacteriocytes and elimination of *Buchnera* (Ohtaka & Ishikawa 1991; Montllor *et al.* 2002). Notably, it was recently reported that PASS infection improves host reproduction under heat stress (Montllor *et al.* 2002). In the light of our results, the fitness improvement can be interpreted as PASS-dependent compensation for heat-induced *Buchnera* deficiency. The idea that the biological function of PASS is to improve the survival of the host aphid during the hot summer season appears plausible, but requires further confirmation.

(n) Buchnera*-dependent positive and negative effects of PASS on host fitness*

From all these results taken together, we conclude that PASS has *Buchnera*-dependent fitness consequences on the host aphid. In the presence of *Buchnera*, PASS has slightly negative effects on host fitness. However, in the absence or malfunction of *Buchnera*, PASS positively affects the survival and reproduction of the host in place of the essential symbiont. This study provides a striking example of a facultative endosymbiont drastically changing its biological effects on the host organism according to the interactions in the internal endosymbiotic ecosystem.

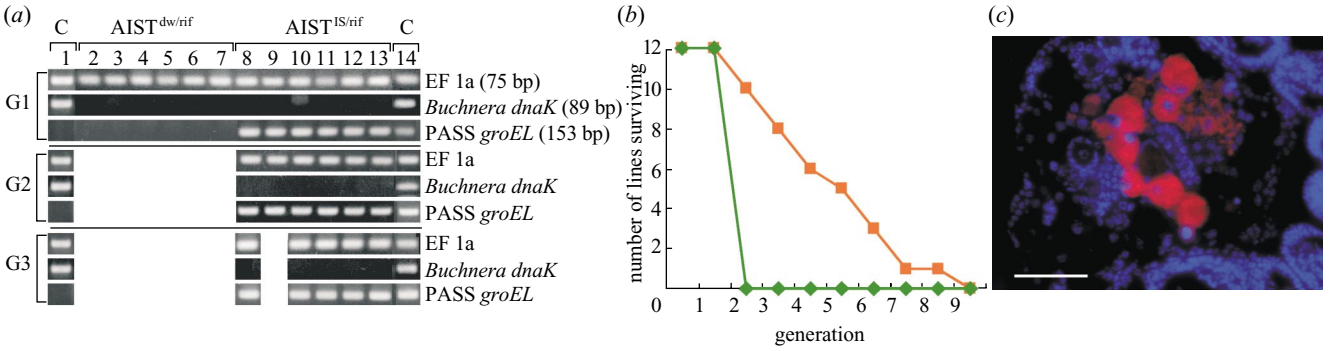

Figure 6. Selective elimination of *Buchnera* by rifampicin treatment. (*a*) Diagnostic PCR analysis to confirm selective elimination of *Buchnera*. Lane 1, a control PASS-negative aphid line injected with distilled water. Lanes 2–7, PASS-negative aphid lines injected with rifampicin. Lanes 8–13, PASS-positive aphid lines injected with rifampicin. Lane 14, a control PASS-positive aphid line injected with distilled water. G1, offspring of injected insects; G2, offspring of G1; G3, offspring of G2. (*b*) Restored reproduction of *Buchnera*-free aphid in the presence of PASS. Orange line, *Buchnera*-free and PASS-infected lines AIST$^{IS/rif}$; green line, *Buchnera*-free and PASS-free lines AIST$^{dw/rif}$. (*c*) Endosymbiotic system in an embryo of a *Buchnera*-free PASS-infected aphid AIST$^{IS/rif}$. Tissue sections of G4 insects were hybridized with a PASS-specific probe PASSisR. Bar, 50 μm.

(o) *How is PASS maintained in host populations?*

Why PASS infection is neither fixed nor lost in host populations is an intriguing problem. Provided that PASS infection affects the host fitness in a temperature-dependent manner (Montllor *et al.* 2002), retarded fitness at lower temperatures could be cancelled out by improved fitness at higher temperatures in different seasons, environments and geographical areas, which might result in moderate and fluctuating infection frequencies of PASS in host populations, as has been described (Tsuchida *et al.* 2002). The resistance to parasitoid wasps conferred by PASS infection may also be involved in the process (Oliver *et al.* 2003). To confirm this idea, however, further laboratory and field studies are needed to clarify the interrelationships between PASS infection, environmental factors and fitness parameters of the host aphid.

(p) *Physiological contribution of PASS to the host aphid*

The mechanism whereby PASS restores survival and reproduction of the *Buchnera*-free aphids is intriguing but totally unknown. Considering the known biological functions of *Buchnera* (Douglas 1998), it appears likely that PASS supplies, though less efficiently, the host with essential amino acids and other nutrients as *Buchnera* does. Detailed experimental analyses of the disymbiotic, monosymbiotic and aposymbiotic aphid strains using defined synthetic diet systems would provide further insights into the physiological interplay between PASS, *Buchnera* and the host aphid.

(q) *Evolutionary implications*

During over 100 Myr of the association, the aphid and *Buchnera* have established an intimate endosymbiotic relationship (Moran *et al.* 1993; Shigenobu *et al.* 2000). Acceptance of the notion that they have been integrated into an almost inseparable biological entity appears tempting. However, our experiments showed that other microorganisms may potentially be able to take over the roles of *Buchnera* that are essential for the life and reproduction of the host. The revised notion, that even the essential symbiont is replaceable, is concordant with the remarkable diversity of primary symbionts among closely related insects. For example, although aphids, coccids, whiteflies and psyllids constitute a well-defined clade in the Homoptera, their primary symbionts, which are conserved within each taxon and harboured in bacteriocytes with similar cytological traits, belong to distinct bacterial lineages (Unterman *et al.* 1989; Munson *et al.* 1992; Clark *et al.* 1992; Fukatsu & Nikoh 1998). Considering that homopteran insects possess endocellular symbionts in general, the diversity of the primary symbionts is best explained by repeated symbiont replacements at early evolutionary stages of these insect taxa. In a small aphid group called the Cerataphidini, *Buchnera* and bacteriocytes have been completely lost and replaced by a yeast-like ascomycetous symbiont (Fukatsu & Ishikawa 1996). Certainly endosymbiosis with *Buchnera* has been a successful system for aphids, but we would like to point out that the system is not the sole solution for aphids and is potentially open for renewal and improvement over evolutionary time.

(r) *General significance of super-symbiotic system*

In many insects, it is commonly found that several types of cells specialized for endosymbiosis, such as primary bacteriocytes, secondary bacteriocytes and sheath cells, harbour specific endosymbiotic micro-organisms (Buchner 1965; Fukatsu *et al.* 2000). This system will enhance acquisition and upkeep of secondary symbionts, which may provide various biological roles supplementary to those of the essential primary symbionts. Moreover, the system would facilitate the evolution of novel essential endosymbiotic associations. It is believed that gene duplication has enabled the exploitation and evolution of novel gene functions, because the new gene copy is freed from the functional constraints of the original gene copy (Ohno 1970). Similarly, the super-symbiotic system would allow exploitation of new symbionts with more efficient or novel biological functions, while the original primary symbionts are maintained. Endosymbiosis is an important source of evolutionary innovation (Margulis & Fester 1991). Facultative endosymbionts such as PASS might provide a pool of raw materials for such innovative evolution.

We thank A. Sugimura and K. Sato for technical assistance, and T. Wilkinson and N. Lo for helpful comments on the manuscript. This research was supported by the Program for Promotion of Basic Research Activities for Innovation Biosciences (ProBRAIN) of the Bio-Oriented Technology Research Advancement Institution.

REFERENCES

Buchner, P. 1965 *Endosymbiosis of animals with plant microorganisms*. New York: Interscience.

Chen, D. Q. & Purcell, A. H. 1997 Occurrence and transmission of facultative endosymbionts in aphids. *Curr. Microbiol.* **34**, 220–225.

Chen, D. Q., Montllor, C. B. & Purcell, A. H. 2000 Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. *Entomol. Exp. Appl.* **95**, 315–323.

Clark, M. A., Baumann, L., Munson, M. A., Baumann, P., Campbell, B. C., Duffus, J. E., Osborne, L. S. & Moran, N. A. 1992 The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. *Curr. Microbiol.* **25**, 119–123.

Douglas, A. E. 1998 Nutritional interactions in insect–microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *A. Rev. Entomol.* **43**, 17–37.

Dubilier, N. (and 11 others) 2001 Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature* **411**, 298–302.

Fukatsu, T. & Ishikawa, H. 1996 Phylogenetic position of yeast-like symbiont of *Hamiltonaphis styraci* (Homoptera, Aphididae) based on 18S rDNA sequence. *Insect Biochem. Mol. Biol.* **26**, 383–388.

Fukatsu, T. & Nikoh, N. 1998 Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). *Appl. Environ. Microbiol.* **64**, 3599–3606.

Fukatsu, T., Watanabe, K. & Sekiguchi, Y. 1998 Specific detection of intracellular symbiotic bacteria of aphids by oligonucleotide-probed *in situ* hybridization. *Appl. Entomol. Zool.* **33**, 461–472.

Fukatsu, T., Nikoh, N., Kawai, R. & Koga, R. 2000 The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **66**, 2748–2758.

Fukatsu, T., Tsuchida, T., Nikoh, N. & Koga, R. 2001 *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* **67**, 1284–1291.

Hinde, R. 1971 The control of the mycetome symbiotes of the aphids *Brevicoryne brassicae*, *Myzus persicae* and *Macrosiphum rosae*. *J. Insect Physiol.* **17**, 2035–2050.

Houk, E. J. & Griffiths, G. W. 1980 Intracellular symbiotes of Homoptera. *A. Rev. Entomol.* **25**, 161–187.

Ishikawa, H. & Yamaji, M. 1985 Symbionin, an aphid endosymbiont-specific protein I. Production of insects deficient in symbiont. *Insect Biochem.* **15**, 165–174.

Komaki, K. & Ishikawa, H. 1999 Intracellular bacterial symbionts of aphids possess many genomic copies per bacterium. *J. Mol. Evol.* **48**, 717–722.

Kondo, N., Ijichi, N., Shimada, M. & Fukatsu, T. 2002 Prevailing triple infection with *Wolbachia* in *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Mol. Ecol.* **11**, 167–180.

McGraw, E. A., Merritt, D. J., Droller, J. N. & O'Neill, S. L. 2002 *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc. Natl Acad. Sci. USA* **99**, 2918–2923.

Margulis, L. & Fester, R. 1991 *Symbiosis as a source of evolutionary innovation*. Cambridge, MA: MIT Press.

Montllor, C. B., Maxman, A. & Purcell, A. H. 2002 Independent origins and horizontal transfer of bacterial symbionts of aphids. *Ecol. Entomol.* **27**, 189–195.

Moran, N. A., Munson, M. A., Baumann, P. & Ishikawa, H. 1993 A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proc. R. Soc. Lond.* B **253**, 167–171.

Munson, M. A., Baumann, P. & Moran, N. A. 1992 Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera: Pseudococcidae) based on 16S rDNA sequences. *Mol. Phylogenet. Evol.* **1**, 26–30.

Ohno, S. 1970 *Evolution by gene duplication*. New York: Springer.

Ohtaka, C. & Ishikawa, H. 1991 Effects of heat treatment on the symbiotic system of an aphid mycetocyte. *Symbiosis* **11**, 19–30.

Oliver, K. M., Russell, J. A., Moran, N. A. & Hunter, M. S. 2003 Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl Acad. Sci. USA* **100**, 1803–1807.

Sandström, J. P., Russell, J. A., White, J. P. & Moran, N. A. 2001 Independent origins and horizontal transfer of bacterial symbionts of aphids. *Mol. Ecol.* **10**, 217–228.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. & Ishikawa, H. 2000 Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407**, 81–86.

Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T. & Fukatsu, T. 2002 Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. *Mol. Ecol.* **11**, 2123–2135.

Unterman, B. M., Baumann, P. & McLean, D. L. 1989 Pea aphid symbiont relationships established by analysis of 16S rRNAs. *J. Bacteriol.* **171**, 2970–2974.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.